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Kombucha tea ameliorates experimental autoimmune encephalomyelitis in mouse model of multiple sclerosis

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Experimental autoimmune encephalomyelitis (EAE) is a mouse model for multiple sclerosis (MS), in which an inflammatory demyelination and axonal damage occurs. Kombucha tea is a fermented beverage made from kombucha mushroom, brewed tea, and sugar. In recent years kombucha tea has attracted interest due to its pharmacological properties like antioxidant effects. The aim of the present research was to test the therapeutic effect of kombucha tea in EAE. We induced EAE model in 18 female C57BL/6 mice by inoculation of myelin oligodendrocyte glycoprotein-35-55 (MOG_{35-55}) in complete Freund's adjuvant emulsion. Then, in order to ameliorate EAE symptoms, we used kombucha tea. During the course of study clinical evaluation was assessed, and on the day 21 post-immunization, for evaluation of nitric oxide (NO), total antioxidants capacity and tumor necrosis factor-alpha (TNF-α), blood samples were taken from the heart of mice. The mice were sacrificed and brains and cerebellums of mice were removed for histological analysis. Our findings demonstrated that kombucha tea had beneficial effects on EAE by lower incidence, attenuation in the severity, and also a delay in the onset of disease. Histological analysis showed that inflammatory criteria including the number of infiltrated immune cells and plaques as well as demyelination in kombucha tea dosed mice were significantly lower than the control group. Also, in comparison with control mice, the serum levels of NO and TNF-α in kombucha tea-treated mice were significantly decreased. Kombucha tea with its potential therapeutic effects and immunomodulatory properties might be proposed, after additional necessary tests and trials, for treatment of MS.

Keywords: experimental autoimmune encephalomyelitis; kombucha tea; multiple sclerosis; nitric oxide; tumor necrosis factor-alpha

Introduction

Multiple sclerosis (MS) is an autoimmune inflammatory disease of the central nervous system (CNS) which afflicts higher than 2.5 million people worldwide. MS disease is characterized by the destruction of the myelin sheath that surrounds neuronal axons in the CNS, a process that results in neurodegeneration and consequently the formation of sclerotic

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plaques in the brain (Steinman, 2001). Experimental autoimmune encephalomyelitis (EAE) is currently the most commonly used animal model for the study of MS. This model causes brain inflammation and demyelination that is similar to the human MS manifestations (Peiris, Monteith, Roberts-Thomson, & Cabot, 2007). Activated innate and adaptive immune cells infiltrate into the CNS where they act synergistically in inducing and perpetuating local inflammation and demyelination (Huitinga et al., 1995). In EAE, myelin oligodendrocyte glycoprotein (MOG) is an autoantigen which is recognized by autoreactive T cells. Migration of these encephalitogenic T cells into the CNS plays a key role in the development of EAE. Recent findings suggest that both Th1 and Th17 cells which secrete interleukine (IL)-17 and interferon (IFN)-γ infiltrate in the CNS prior to the onset of clinical symptoms of EAE. These cells may mediate the CNS inflammation, in part, through microglial activation (Murphy, Lalor, Lynch, & Mills, 2010). Both the activated microglia and the released mediators are detrimental to oligodendrocyte. It has been suggested that through these mechanisms, innate immunity proceeds demyelination in EAE (Sriram, 2011).

Another type of innate immune cell that plays a pivotal role in EAE pathogenesis is the macrophage. It is demonstrated that, about 50% of the mononuclear cells in the perivascular lesions in the CNS of rats suffering from EAE are the blood-borne macrophages (Huitinga et al., 1995). Macrophages drive demyelination via secretion an array of pro-inflammatory mediators, including tumor necrosis factor-alpha (TNF-α) cytokine, which exert neurotoxic and chemoattractant effects in the CNS and eventually lead to immune cell infiltration and inflammation in the CNS. The probable role of TNF-α in the demyelinating process is the prolongation of macrophage and microglia activation (Taupin et al., 1997).

Oxidative stress could also induce pathogenesis of MS. It is believed that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are mainly produced by activated macrophages and microglia in the CNS context of MS patients. These mediators could be responsible for demyelinization and axons disruption (Lu et al., 2000; Miller, Wachowicz, & Majsterek, 2013). Circumstantial evidence suggests that nitric oxide (NO) has a role in several features of the MS disease, such as disruption of the blood-brain barrier, oligodendrocyte injury, demyelination, and axonal degeneration. It may lead to the loss of neuron function by impairment of axonal conduction (Smith & Lassmann, 2002).

Antioxidants reduce the expression of inflammatory molecules such as induced nitric oxidase syntase (iNOS) and nitrotyrosine, a marker of peroxynitrite reactivity in the CNS of EAE mice (Moriya et al., 2008). Antioxidant dietary molecules are well known for their antioxidant activity, but recent findings indicate that they may have additional properties independent of their roles as antioxidants and radical scavengers (Riccio, Rossano, & Liuzzi, 2010). Antioxidants are present in a variety of plants and they promote health by removing damaging free radicals from the cellular environment and inhibiting the synthesis of inflammatory mediators. Recent studies showed that antioxidants have a significant therapeutic potential for the treatment of inflammatory disorders (Lu et al., 2009).

Kombucha is a traditional drink used in various parts of the world, largely in Asian countries. Kombucha tea is sugared black tea fermented with a symbiotic culture of acetic acid bacteria and yeasts. It is claimed that Kombucha tea may have multiple useful effects on human health (Murugesan et al., 2009). Moreover, the efficacy and antioxidant activity of kombucha tea have been demonstrated against oxidative stress-induced nephrotoxicity and hepatotoxicity, as well as in diabetic rats and gastric ulceration in mice

(Banerjee et al., 2010; Bhattacharya, Gachhui, & Sil, 2011, 2013; Gharib, 2009). In the present research, our aim was to test the therapeutic efficacy of kombucha tea in experimental model of MS.

Material and methods

Preparation and fermentation of kombucha tea

The 100 g of sugar was added to 1 L of distilled water and then boiled for 15 min in a glass jars. Black tea was added to the glass jars (12 g/L), which was then allowed to infuse and cool down at room temperature for 60 min. By using a sterile nylon mesh, the preparation was filtered and then filtrate was used as black tea (Murugesan et al., 2009). The 200 ml cooled black tea was poured in a glass jar, which were sterilized beforehand, and inoculated with 3% (w/v) of freshly grown kombucha mat that had been grown and maintained in the same medium (Hesseltine, 1965). The fermentation, kept under aseptic conditions (this means that the sterile jar was covered with a clean cheese cloth and fastened tightly with rubber bands), was carried out by incubating the kombucha culture at 28 ± 1°C for 12 days. Subsequently, the medium (brew) was centrifuged aseptically at 1500 × *g* for 30 min and stored in polypropylene vials at –20°C for further use (Sai Ram et al., 2000).

Animal selection and grouping

Eighteen female C57BL/6 mice (10 weeks old), weighing 18–20 g, were obtained from the Experimental Animal Center of Pasteur Institute of Iran. Mice were randomly separated into three groups; normal, control, and KT (kombucha tea), with six mice in each group. All mice were housed in cages under 12-h light–dark cycle and free access to food and water. All procedures involving animals were performed according to the guidelines of the Animal Ethics approved by Tehran University of Medical Science.

EAE induction and therapeutic protocol

We performed EAE induction as most commonly used animal model for studying the pathogenesis and treatment of MS by Hooke Kit (Hooke Laboratories, Inc., USA). The kit consisted of two components: antigen (MOG_{35-55}) in an emulsion with complete Freund's adjuvant in two pre-filled syringes, and a vial of lyophilized pertussis toxin (PTX). The lyophilized PTX was dissolved in phosphate-buffered saline on day 0. The mice were injected subcutaneously on upper back and lower back with 0.1 ml of emulsion, respectively. Approximately 2 h of injection of the emulsion, the first dose of PTX (0.1 ml/mouse) was injected intraperitoneally (IP). This was repeated 24 h later as second dose of PTX (0.1 ml/mouse). The mice in control and KT groups were administered orally with vehicle (sterile water) and kombucha tea, respectively (6 ml per kg of body weight) once daily, for 3 weeks, from day 0 post-immunization through animal feeding needles. Mice were monitored daily and assessed by clinical score. Clinical score of EAE mice was defined as follows: 0, no clinical sign; 1, limp tail; 2, limp tail and weakness of hind legs; 3, limp tail and complete paralysis of hind legs (most common) or limp tail with paralysis of one front and one hind leg; 4, limp tail, complete hind legs and partial front legs paralysis; 5, mouse is spontaneously rolling in the cage or mouse is found dead due to paralysis.

Histopathology

On the day 21 following immunization, all mice were anesthetized and blood samples were taken from their hearts for evaluating the total antioxidant capacity, NO, and cytokine levels. Mice were sacrificed following anesthetization and then brains and cerebellums from normal, control, and kombucha tea-treated mice were removed and fixed in neutral 10% formalin, embedded in paraffin, sectioned (8 μm thick) and then stained with Hematoxylin–Eosin (H&E) for meningeal and parenchymal inflammatory foci counting and Luxol fast blue (LFB) to distinguish demyelination (Azizi et al., 2014). All stained slides were analyzed in a blinded manner by an expert pathologist.

Nitrite assay

NO in the serum samples was assessed by Griess method based on the assessment of the end product of nitrite. Griess method uses a colorimetric reaction for measuring the NO_2– nitrite level in aqua's solution. Griess reagent was prepared by solving 1g sulfanilamide in 100 ml phosphoric acid (5%) mixed with 0.1g naphthyl ethylenediamine–HCl in 100 ml distilled water. Serum sample (50 μl) was mixed with 50 μl of Griess reagent at room temperature for 10 min. Absorbance was measured using enzyme-linked immunosorbent assay (ELISA) reader instrument at 540 nm. Concentration of nitrite was determined by standard curve of 0.1 M sodium nitrite in distilled water.

FRAP test

Total antioxidant capacity was measured according to the ferric reducing ability of plasma (FRAP) assay test. In this method, antioxidants in the serum sample reduce ferric tripyridyltriazine complex (Fe3+–TPTZ), to a blue colored ferrous form (Fe^{2+}), with an increased absorbance at 593 nm. The working FRAP reagent was provided by mixing 10 volumes of 300 mM acetate buffer, pH 3.6, with 1 volume of 10 mM TPTZ (2, 4, 6 Tris (pyridyl)-s-triazine) in 40 mM HCl and with 1 volume of 20 mM $FeCl_3$ prepared in deionized water. The working FRAP reagent was then warmed to 37°C and its absorbance was measured at 593 nm against distilled water (reagent blank). Subsequently, 30 μl of serum sample was added to 970 μl of FRAP reagent and the absorbance was monitored within 4 min. The results were represented as μmol of ferric antioxidant capacity (the FRAP value) and were compared with the standard curve prepared using $FeSO_4$, $7H_2O$ 1 mM in a range of concentration from 125 to 1000 μM.

Assessment of TNF-α

The levels of pro-inflammatory cytokine TNF-α were determined in the serum of normal, control, and kombucha tea-treated mice by a sandwich ELISA technique. To evaluate TNF-α, we used R&D Kit (R&D Systems, Inc. Minneapolis, MN, USA). All assays were performed according to the manufacturer's instructions. Absorbance was read at 450 nm in a 96-well microplate ELISA reader.

Statistical analysis

Data were expressed as mean ± SD, except for histological scores, which were calculated as mean ± SEM. Statistical analysis was performed with Mann–Whitney *U* test

for nonparametric data and Student's *t*-test for parametric data. A *P*-value < 0.05 was considered statistically significant.

Results

Clinical findings

We induced EAE in C57BL/6 mice by immunizing them with ready to use Hooke kit. Mice were dosed orally with kombucha tea in KT group, or vehicle in control group. The clinical course and severity of the disease differed consistently between KT and control groups. The mean severity score of disease was higher in control mice than in KT group (Figure 1). Also EAE incidence was lower, and EAE onset was delayed in kombucha tea-treated mice compared to control mice (Figure 2). These effects were led to significant clinical improvement and delayed disease progression during 21 days of observation, indicating that kombucha mushroom can inhibit the progression of EAE.

Histology findings

We examined whether it could be a correlation between the clinical symptoms of EAE with histopathology of CNS in control and kombucha tea-treated mice. Histopathological analysis was performed by LFB and H&E staining on brains and cerebellums in EAE mice receiving kombucha tea or vehicle. All sections were scored by an expert pathologist blinded to the study by light microscopic examination. Representative images of H&E and LFB-stained tissue sections from all groups demonstrated that inflammation criteria and demyelination in EAE mice treated by kombucha tea were significantly less than control mice (Figure 3). The results illustrated in Table 1 indicate that the severity of

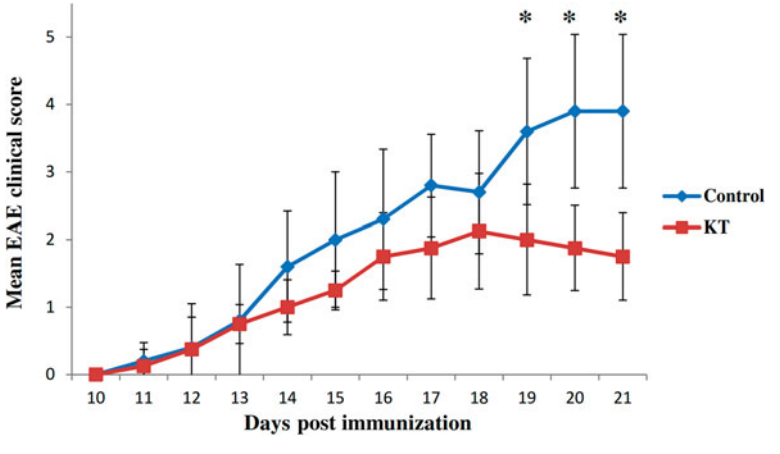

Figure 1. Effect of kombucha tea on clinical score of EAE.
Note: Female C57BL/6 mice (n = 6) were administered orally with 6 ml/kg/day of kombucha tea from day 0 until day 20 post-immunization in kombucha tea (KT)-treated group. Disease severity was assessed by a visual cumulative scoring system. Cumulative scores from day 10 until day 21 are given as mean ± SEM.*P < 0.05 at each data point by Mann–Whitney U test with comparing KT group versus control was considered.

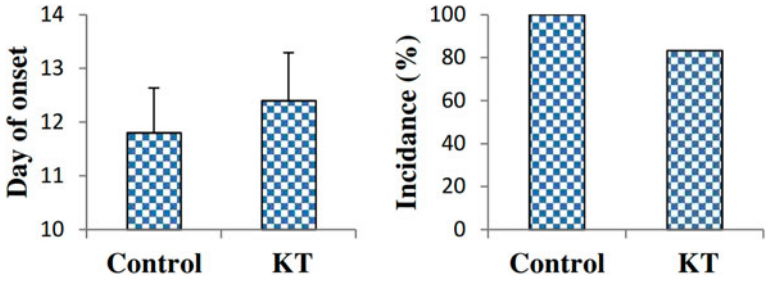

Figure 2. Effect of kombucha tea on EAE onset and incidence.
Note: After immunization, C57BL/6J mice ($n = 6$) were dosed orally with kombucha tea. In the KT group, kombucha tea showed a delay at onset and lower incidence of disease compared to vehicle-treated mice.

inflammation observed in histopathology of CNS is correlated with the clinical severity of EAE in kombucha tea-treated and control mice.

NO production findings

All mice were sacrificed 21 days after EAE induction and blood samples were taken from their hearts. Griess reaction was performed on serum sample. As shown in Figure 4, NO production was significantly reduced in KT (8.38 ± 1.97 μM/dL, $P < 0.001$) group, compared to control mice (13.00 ± 1.00 μM/dL). In the normal group NO production was 5.85 ± 0.53 μM. Therefore, kombucha tea therapy showed a decrease in NO concentration in serum and this was in agreement with clinical findings.

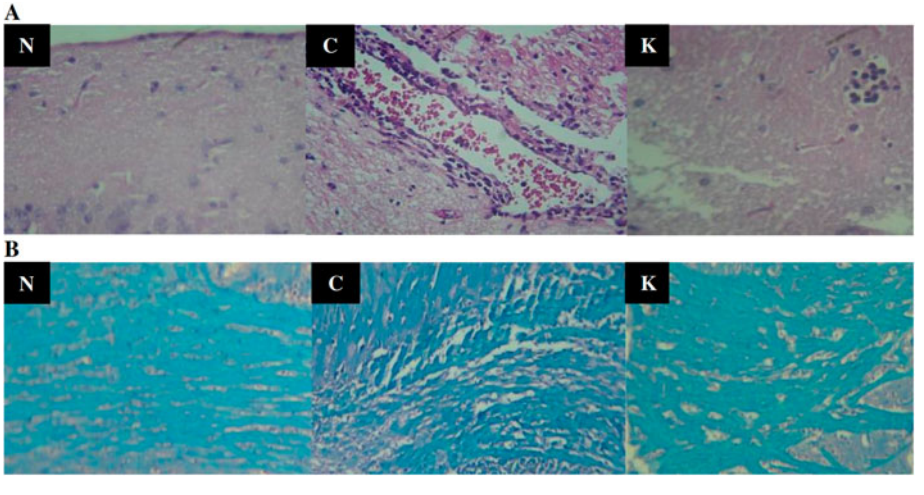

Figure 3. Representative light microscopic view of histopathological slides of CNS in different groups. (A) H&E staining of brains sections showed that kombucha tea therapy could suppress the progression of inflammation significantly by restricting leukocyte infiltration. (B) LFB staining showed lower demyelination sites in kombucha tea-dosed mice compared to control group. N, normal; C, control; K, kombucha tea treated.

Table 1. Comparison of inflammation criteria in histopathology examination in the CNS of EAE mice.

Inflammation criteria	Control (Mean ± SD)	KT (Mean ± SD)*
Demyelination	2.62 ± 0.32	0.87 ± 0.13
Neuronal degeneration	2.68 ± 0.25	0.91 ± 0.12
Infiltration of inflammatory cells in leptomeninges	1.84 ± 0.39	0.75 ± 0.22
Meningeal vessels hyperemia	2.34 ± 0.37	0.75 ± 0.22
Leukocyte margination	2.56 ± 0.47	0.95 ± 0.10
Perivascular cuffing	2.56 ± 0.41	0.66 ± 0.20
Perivascular edema	1.84 ± 0.42	0.66 ± 0.20
Hypercellularity	2.53 ± 0.41	0.62 ± 0.13
Layer necrosis	2.65 ± 0.29	0.66 ± 0.12
Hypertrophy	2.21 ± 0.41	0.95 ± 0.10
Spongy-like	2.12 ± 0.61	0.79 ± 0.18

Source: Histopathology examination. 1 = mild, 2 = moderate, 3 = severe.
*All the differences were statistically significant ($P < 0.05$).

FRAP evaluation

There was no significant difference between control mice compared with KT group (data not shown).

TNF-α quantification

The effect of kombucha tea on TNF-α cytokine concentration in serum of mice was assessed. The analysis was performed using an ELISA cytokine assay. It was observed that treatment with kombucha tea significantly reduced TNF-α production (Figure 5).

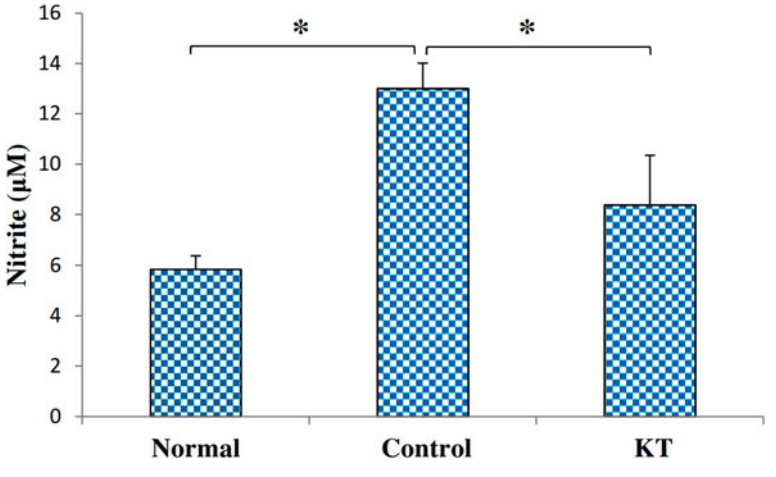

Figure 4. Effect of kombucha tea on serum NO in EAE mice.
Note: The NO production was measured by the Griess reagent. Data are presented as the mean ± SD of duplicate samples from five mice in each group. *$P < 0.001$.

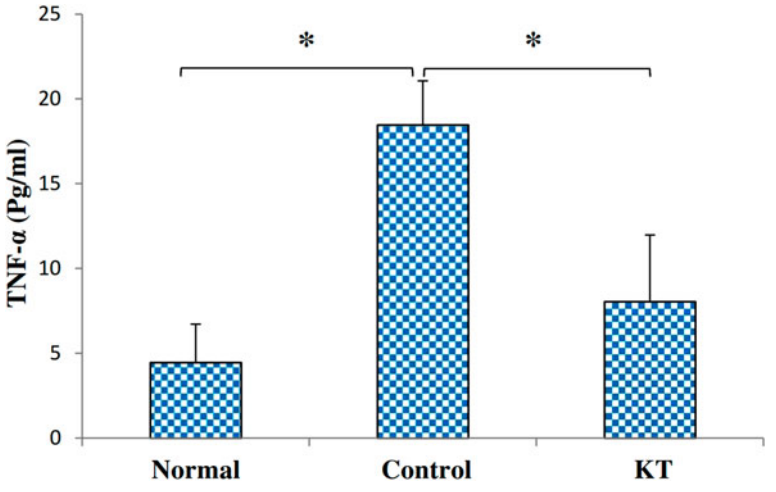

Figure 5. Inhibitory effects of kombucha tea on TNF-α cytokine level.
Note: The TNF-α cytokine analysis was performed in normal, control, and kombucha tea-treated mice. It is observed that treatment with kombucha tea significantly reduced TNF-α production. Error bars, mean ± SD statistics were calculated using *t*-test and $P < 0.05$ was considered significant (*$P < 0.001$).

Discussion

EAE as experimental model of MS is an autoimmune disease of CNS mediated by $CD4^+$ T lymphocytes specific for autoantigens of the myelin sheath, including myelin basic protein, proteolipid protein, and MOG peptide (Greer, Kuchroo, Sobel, & Lees, 1992; Mendel, Kerlero de Rosbo, & Ben-Nun, 1995). $CD4^+$ T cells (Th1 and Th17) and their pro-inflammatory cytokines including IL-17, IFN-γ, and TNF-α along with myelin-specific $CD8^+$ T cells and infiltrated macrophage within the CNS are assumed to be central in the immunoinflammatory-mediated demyelination of MS (Beck et al., 1988; Murphy et al., 2010). Immunomodulatory agents could be reasonably effective in the treatment of MS and EAE, and they can also postpone the time of progression in disabling stages (Lopez-Diego & Weiner, 2008). In the present study, we evaluated the efficacy of kombucha tea in animal model of MS and we observed that this anti-inflammatory agent (Barati et al., 2013; Bhattacharya et al., 2013) can treat the EAE by reducing severity, lower incidence and delayed onset of EAE in C57BL/6 mice. Our findings suggest that kombucha mushroom is capable of suppressing a pre-activated immune response in the EAE model. The histopathological studies showed that the severity of inflammation indices such as demyelination, neuronal degeneration, infiltration of inflammatory cells, and perivascular cuffing in the brain and cerebellum of orally kombucha tea-treated EAE mice were significantly lower than vehicle one.

NO may be involved in the pathogenesis of MS, the hallmark of which is the demyelinated plaque with reactive glial scar formation (Smith & Lassmann, 2002). Downregulation of myelin gene expression in human oligodendrocytes by NO has been reported in a study by Jana and Pahan in 2013. This study illustrated a novel biological role of NO in downregulation of myelin genes preceding the death of oligodendrocytes (Jana & Pahan, 2013). Also, Shin et al. (1998) showed that the inhibition of iNOS

expression and NO production prevented development of EAE. Several studies revealed that kombucha tea could be useful as protecting agent against diseases associated with oxidative stress (Bhattacharya et al., 2013; Gharib, 2009). In animal models, it is demonstrated that the anti-inflammatory and antioxidant effects provided by the kombucha tea can ameliorate the inflammation and oxidative stress in the pancreatic, hepatic, renal, and cardiac tissues of diabetic animals (Aloulou et al., 2012; Bhattacharya et al., 2013). In 2011, Banerjee et al. suggested that the healing capacities of the kombucha tea against indomethacin-induced gastric ulceration in mice could be attributed to their antioxidant activity (Banerjee et al., 2010). In an in vitro study, Bhattacharya et al. (2011) showed that kombucha tea reduced the ROS induced apoptosis in murine hepatocytes probably due to its antioxidant activity. However, in present study there was no significant difference in serum total antioxidant capacity between kombucha tea-dosed mice compared with control mice. It could be concluded that only the anti-inflammatory property of kombucha tea is responsible for its therapeutic efficacy in experimental model of MS. In addition, our study showed that kombucha tea modulates EAE, at least in part, by suppressing NO production, so that, the level of serum NO in kombucha tea-treated mice was significantly less than control group. The results of our examination confirmed data presented by Gharib (2009), which had showed that kombucha tea administration significantly reduced NO contents in kidney, and also improved lipid peroxidation and oxidative stress induced nephrotoxicity in rats (Gharib, 2009).

TNF-α as a pro-inflammatory cytokine has been associated in the pathology of MS and EAE. It is demonstrated that in MS individuals, TNF-α is elevated in serum, CSF and also at the site of active lesions, and this is linked to the severity of the disease (Beck et al., 1988). Our findings showed that treatment by kombucha tea could reduce the level of serum TNF-α in EAE mice and this was in agreement with clinical and histopathological findings as well as along with reduction of NO levels in serum. It is showed that high NO levels are produced after high-level expression of iNOS gene. Recent investigations have shown that inflammatory cytokines such as TNF-α could increase iNOS gene expression and consequently they could make an increased NO production as a cytotoxic effector molecule (Fereidoni, Sabouni, Moghimi, & Hosseini, 2013; Fonseca et al., 2003; Murphy, 2000). These data are in parallel with our findings which kombucha tea can reduce serum level of NO and TNF-α probably under the controlled regulation of signaling pathway.

In short, our study demonstrates that the kombucha tea ameliorates the severity of EAE and attenuates the inflammatory cells infiltration into the CNS. In addition, we observed kombucha tea decreases production of NO probably by suppressing the expression of iNOS and TNF-α. On the other hand, it should be noted that at administered doses no side effects or adverse events have been revealed in EAE mice treated with kombucha tea. However, a few case reports raise doubts about the safety of kombucha tea. They include suspect adverse effects, such as stomach upset, allergic reactions, and infections in kombucha tea drinkers (Ernst, 2003). It is reported that kombucha tea is consumed in many countries as a health beverage and is a combination of kombucha mushroom, tea, sugar, water, and starter culture. It is suggested that kombucha tea may efficiently act in health prophylaxis and recovery due to antioxidation, detoxification, and energizing potencies properties. In addition, the recent experimental studies on the use of kombucha tea recommend that it might be suitable for prevention against wide-spectrum infective and metabolic disorders (Nummer, 2013; Vina, Semjonovs, Linde, & Denina, 2014). These properties along with reported

anti-inflammatory effects make kombucha tea attractive as an applicable fermented functional beverage for alternative therapy in MS.

Disclosure statement

The authors declare that they have no conflict of interest.

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