Daphnetin alleviates experimental autoimmune encephalomyelitis by suppressing Th1 and Th17 cells and upregulating Th2 and regulatory T cells

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Multiple sclerosis (MS) is the most typical chronic inflammatory, autoimmune demyelinating disease of the central nervous system (CNS) which leads to physical dysfunction and paralysis in patients. A commonly used animal model for this disease is experimental autoimmune encephalomyelitis (EAE). Daphnetin (7,8-dihydroxycoumarin) has been reported to exert various pharmacological activities, such as being neuroprotective and anti-inflammatory, together with having antioxidant, anticancer, and antiviral properties. Eight-week-old C57BL/6 female mice were segregated into 3 groups, namely 1) a control group receiving PBS, 2) a low-dose treatment group receiving 2 mg/kg of daphnetin, and, 3) a high-dose treatment group receiving 8 mg/kg of daphnetin. EAE was induced with a subcutaneous injection of a combination of myelin oligodendrocyte glycoprotein (MOG) and complete Freund’s adjuvant. On the day of induction, and again two days later, mice were injected intraperitoneally with pertussis toxin. Histological studies showed low lymphocyte infiltration and demyelination in the high and low dose treated groups. The ratio of spleen Treg cells and the levels of IL-4, IL-10, TGF-β, and IL-33 enhanced significantly in the treatment group related to the control group. Furthermore, both IL-27 and IL-35 were also enhanced significantly in the treatment group compared to the control group. Moreover, the levels of IFN-γ, TNF-α, and IL-17 displayed a noticeable reduction in the daphnetin treated group. Daphnetin appears to improve the disease by increasing the expression of anti-inflammatory cytokines and transcription factors (IL-4, IL-10, IL-33, GATA3, TGF-β, FoxP3), and reducing the production of pro-inflammatory cytokines and transcription factors (IFN-γ, STAT4, T-bet, IL-17, STAT3, ROR-γt, TNF-α).

**Key words:** multiple sclerosis, experimental autoimmune encephalomyelitis, myelin oligodendrocyte glycoprotein, daphnetin, transcription factors

INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune disease characterized by abnormal responses of the immune system against the myelin and axons of the central nervous system (CNS) (Quan et al., 2019). MS is caused by molecular mimicry against environmental antigens complemented by genetic susceptibility which results in autoimmunity that can disrupt the balance of immune responses and break CNS tissue tolerance (Moorman et al., 2018).

Experimental autoimmune encephalomyelitis (EAE) is the preferential animal model of this autoimmune disease and allows an understanding of a variety of aspects of human MS. This model shares many clinical and pathological features with MS (Guo et al., 2019). There is overwhelming evidence to suggest that the main effector T cells involved in inducing
inflammatory processes in MS/EAE are pro-inflammatory subsets of IFN-γ secreting Th1 cells and IL-17 producing Th17 cells (Yang et al., 2019). Conversely, Th2/Treg subsets with anti-inflammatory or regulatory cytokines have been shown to contribute to the remission stages of the disease (Jiang et al., 2009). Protective Th2 and Treg cells, characterized by the secretion of anti-inflammatory cytokines such as IL-4, IL-5, IL-10, and TGF-β, are involved in the repression of disease symptoms (Mondal et al., 2018). In addition, Treg cells are essential for preserving self-tolerance, management of expansion, and activation of autoreactive CD4+ T effector cells (Venkatesha and Moudgil, 2019). Members of the IL-12 family, namely IL-27 and IL-10, are mainly believed to have immunomodulatory and anti-inflammatory properties (Casella et al., 2017). Administration of exogenous IL-27 to EAE mice has also been shown to reduce both CNS infiltration of Th17 cells, and their production of IL-17 (Danikowski et al., 2017). Furthermore, IL-33 was shown to be secreted by Tregs in mice and had an inhibitory effect against demyelination by suppressing the proliferation of Th1 and Th17 cells (Zandian et al., 2011; Li et al., 2014).

Immunosuppressive medications are often used to regulate the overactive or abnormal activation of T lymphocytes and the immune system related to inflammatory and autoimmune disorders (Song et al., 2014). Daphnetin (7,8-dihydroxycoumarin), extracted from Daphne koreana Nakai and Daphne odora Thunb, is an effective compound in this regard and has been reported to show various pharmacological activities such as neuroprotective and anti-inflammatory, antioxidant, anticancer, and antiviral properties (Wang et al., 2016). Furthermore, preclinical studies have reported that daphnetin is a potential therapeutic agent for the treatment of different autoimmune and inflammatory diseases, including rheumatoid arthritis (Gao et al., 2008). Daphnetin treatment significantly attenuated the clinical and pathological manifestation of arthritis through contraction of arthritis scores, repressing the infiltration of inflammatory cells and modulating the balance of Treg and Th17 cells (Yao et al., 2011). These immunosuppressive effects were tightly associated with decreasing the production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 (Liu et al., 2016), increasing expression of Foxp3 and IL-10 (Yao et al., 2011), and suppressing Th1 and Th17 cell responses (Wang et al., 2016).

These findings indicate the potential immunosuppressive role for daphnetin in the immune system. However, the underlying mechanisms are not clearly understood. In this study, we investigate the potential therapeutic effect of daphnetin in the treatment of EAE. This research aimed to characterize whether daphnetin could ameliorate EAE.

METHODS

Mice, induction of EAE and treatment

Female C57BL/6 mice (n=24) (8-10 weeks-old, 18-20g weight) were purchased from the Royan Biotechnology Institute (Isfahan, Iran). The mice were housed in a pathogen-free animal center with a 12-hour dark/light cycle with freely available food and water. The experiments were carried out according to animal care guidelines authorized by Semnan University of Medical Sciences.

EAE was induced in all mice, with subcutaneously injection of 250 μg myelin oligodendrocyte glycoprotein peptide (MOG<sub>35-55</sub>, BioBasic, Canada) emulsified with complete Freund’s adjuvant (Sigma-Aldrich, St. Louis, MO, USA) containing 4mg/ml mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI, USA) in the flanks. On the day of EAE induction, and two days later, the mice were injected intraperitoneally with 250 ng pertussis toxin (Sigma-Aldrich, St. Louis, MO, USA). Mice were randomly separated into three groups, namely the control, low-dose treatment group (LD, 2 mg/kg daphnetin) and high-dose treatment group (HD, 8 mg/kg daphnetin). Intraperitoneal administration of daphnetin was initiated on the first day of disease induction until the last day. The disease severity was scored in a blinded manner using the previously validated scoring system: 0, no clinical sign; 1, partial loss of tail tonicity; 2, complete loss of tail tonicity; 3, flaccid tail and abnormal gait; 4, hind leg paralysis; 5, hind leg paralysis with hind body paresis; 6, hind and foreleg paralysis; 7, moribund or death (Haghmorad et al., 2019).

Histological studies

Mice were anesthetized with ketamine and xylazine and perfused by intracardiac injection of PBS containing 4% paraformaldehyde at day 25, brains were collected and fixed in formaldehyde. These were embedded in paraffin and sections (5-mm thick) were prepared and stained with hematoxylin and eosin (H&E) and Luxol fast blue (LFB) for assessment of leukocyte infiltration and demyelination, respectively. The sections were then evaluated by light microscopy in a blinded manner. The evaluated inflammation scale was as follows; 0, no infiltrated cells; 1, a few diffuse infiltrated cells; 2, a configuration of infiltrated cells around blood...
vessels; and 3, considerable perivascular cuffing with expansion into nearby parenchyma, or parenchymal infiltration without distinct cuffing. Demyelination in the brains was scored as follows; 0, no demyelination; 1, infrequent region of demyelination; 2, a few regions of demyelination; and 3, large region of demyelination (Horstmann et al., 2013).

Cell culture and proliferation assay

On day 25 post-immunization, peripheral lymph nodes (inguinal and axillary) and spleens were removed from mice and single cell suspensions were prepared. Red blood cells were lysed using ammonium chloride. Cell suspensions were prepared and cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (all reagents purchased from Sigma, St. Louis, MO) in round-bottom 24-well plates (2×10^6 cells/well) and 96-well plates (4×10^5 cells/well) in the presence of 20 μg/ml MOG 35–55 (20 μg/mL). All cultures were incubated for 72 h at 37°C (under 5% CO₂). Cell proliferation was evaluated with Cell Proliferation ELISA, (colorimetric BrdU kit) (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer’s instructions. The optical density (OD) was measured at 450 nm using a microplate reader (Stat Fax 2100 Awareness, Phoenix, AZ, USA).

ELISA for cytokine detection

The supernatants from 24-well plates were collected after 72 h and the concentration of cytokines including IL-4, IL-10, IL-27, IL-35, TGF-β, IFN-γ, IL-17, and TNF-α, were assessed by ELISA according to the manufacturer’s guidelines (eBioscience, San Diego, CA, USA). In brief, capture antibodies for each cytokine were diluted in coating buffer, then ELISA plates were coated with diluted antibodies and incubated overnight at 4°C. ELISA plates were washed and blocked with ELISA diluent for one hour at room temperature. For cytokine assessment, samples and standards were placed at room temperature for two hours, then biotinylated secondary antibodies were added and incubated for one hour. Avidin-HRP (Horseradish peroxidase) was added with 30 min of incubation remaining.

Finally, tetramethyl benzidine (TMB) was added as a substrate and the reaction was stopped with the stop solution. The OD of plates was measured at 450 nm using the microplate reader. Cytokine concentrations were determined by measurements of different concentrations of recombinant cytokines.

Quantitative real-time PCR

Brain and spleen were removed at day 25 post-immunization from EAE induced mice to determine the expression profiles of cytokines and transcription factors. The brain and spleen of each mouse were separately homogenized in PBS using a nylon mesh to detach cells. For RNA extraction, the suspension was centrifuged at 3000 g for 10 min and the resulting cell pellet was suspended in TriPure Isolation Reagent (Roche Applied Science, Indianapolis, IN, USA). cDNA synthesis was performed by PrimeScript™ RT reagent Kit (Takara Bio Inc., Otsu-Shiga, Japan) according to the manufacturer’s instructions. Real-time PCR was carried out by applying SYBR Green qPCR Master Mix (Ampliqon, Odense, Denmark) with the required primers synthesized by Metabion Company (Munich, Germany) (Table 1). Reactions were conducted on the Applied Biosystems™ StepOnePlus™ (Beverly, Massachusetts, United States) to find the relative quantity of mRNA expression compared with the β2 microglobulin reference gene.

Statistical analysis

Comparison of both treatment groups vs. control mice on the development of clinical signs was conducted via two-way repeated measures analysis of variance (ANOVA). One-way ANOVA followed by Tukey multiple comparison tests was performed for analysis between groups. SPSS v. 21 was used to analyze the data. Data were presented as mean ± SEM. The confidence level of the Type I error was defined 95 percent. Statistical significance was defined as P<0.05 (*), P<0.01 (**), P<0.001 (***)

RESULTS

Daphnetin treatment ameliorates clinical severity of MOG-immunized C57BL/6 mice

Previous studies demonstrated that daphnetin has anti-inflammatory, anti-microbial, and anti-cancer properties, and this likely extends to the treatment of MS (Song et al., 2014; Venugopala et al., 2013). In this study, we investigated the effect of daphnetin in low dose (2 mg/kg) and high dose (8 mg/kg) on EAE treatment to clarify the molecular mechanisms involved in the treatment process.

Daphnetin in low and high doses was able to reduce the severity of the disease, with both groups significantly reducing the severity of disability and
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paralysis compared to the control group. Surprisingly, high-dose treated mice (HD) had greatly reduced disease symptoms.

The clinical scores, (on day 18, maximal score) of the treatment group with low dose daphnetin (LD: 2.3±0.18) and high dose daphnetin (HD: 1.9±0.14) were significantly (p<0.001) lower than the control group (CTRL: 4.7±0.19) (Fig. 1A and Table 2). The treatment groups also prevented significant weight loss in EAE mice. The mean body weight of LD and HD groups on day 18 (maximal score) were 17.8±0.25g and 18.1±0.3g respectively (p<0.05) compared to the CTRL group with 17.1±0.3g (Fig. 1B). As expected, the EAE mice with‑out any treatment developed clinical symptoms which reached a maximum score on day 18 post‑EAE induction. In contrast, EAE mice receiving daphnetin (LD and HD) showed milder symptoms of the disease.

Daphnetin administration reduce CNS inflammation and demyelination

Brain sections (5 µm thick) were prepared and stained with H&E for cell infiltration and LFB to observe demyelination. H&E staining revealed a significant reduction of infiltrating leukocytes in the CNS of the treatment groups (HD: 1.8±0.25, LD: 1.9±0.2) compared with the control group (CTRL: 4.7±0.19) (Fig. 1A and Table 2). The treatment groups also prevented significant weight loss in EAE mice. The mean body weight of LD and HD groups on day 18 (maximal score) were 17.8±0.25g and 18.1±0.3g respectively (p<0.05) compared to the CTRL group with 17.1±0.3g (Fig. 1B). As expected, the EAE mice without any treatment developed clinical symptoms which reached a maximum score on day 18 post‑EAE induction. In contrast, EAE mice receiving daphnetin (LD and HD) showed milder symptoms of the disease.

Daphnetin treatments decreased T‑cell proliferation

T cells extracted from lymph nodes and spleen were investigated by using cell proliferation ELISA and a BrdU (colorimetric) kit for T cell proliferation. Lymph node cells and splenocytes were isolated and cultured upon stimulation with MOG 35‑55 (20 μg/mL), and PHA (20 μg/mL) as a positive control, then the BrdU incorporation assay was performed.

The results indicated that mononuclear cells of both treatment groups (LD: 0.6±0.15 and HD: 0.5±0.2 groups) had considerably lower proliferative capability in comparison with the control group (1.2±0.2, ANOVA: p<0.001) (Fig. 3).
Table 2. Clinical features of EAE following the administration of daphnetin.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day of onset</th>
<th>Maximal score (Score at peak)</th>
<th>Mean score (Last day)</th>
<th>Cumulative disease index (CDI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL¹</td>
<td>9.2±0.4</td>
<td>4.7±0.19</td>
<td>3.5±0.11</td>
<td>56.5±0.77</td>
</tr>
<tr>
<td>LD²</td>
<td>10.4±0.5*</td>
<td>2.3±0.18***</td>
<td>1.3±0.14***</td>
<td>25.4±0.71***</td>
</tr>
<tr>
<td>HD³</td>
<td>10.8±0.5*</td>
<td>1.9±0.14***</td>
<td>1±0.12***</td>
<td>19.1±0.74***</td>
</tr>
</tbody>
</table>

¹ CTRL: Control group EAE induced received soybean oil. ² LD: Low dose daphnetin treatment group. ³ HD: High dose daphnetin treatment group. Data are expressed as mean ± SEM. All experiment groups compared with CTRL group. *p <0.05, **p<0.01, ***p<0.001.
Fig. 2. Comparative histopathology of spinal cords demonstrated daphnetin suppresses CNS inflammation and demyelination. Histopathological evaluation of spinal cords from treated groups (low and high dose daphnetin) and control was performed. Spinal cords from each group, collected on day 25 post immunization, fixed in paraformaldehyde and embedded in paraffin. Five μm sections from different regions of the spinal cord from each of the groups were stained with (A) H&E to enumerate infiltrating leukocytes and with (B) Luxol fast blue to assess demyelination. (C) CNS inflammatory foci and infiltrating inflammatory cells were quantified. Pathological scores including inflammation and demyelination were analyzed and shown with bar graph as mean scores of pathological inflammation or demyelination ± SEM. *p<0.05, ** p<0.01, ***p<0.001 compared with control group. Mice were divided into three groups: 1. Control group (CTRL), 2. Low dose daphnetin treatment group (LD) and 3. High dose daphnetin treatment group (HD).
Daphnetin upregulate expression of anti-inflammatory cytokines and downregulate pro-inflammatory cytokines in lymphocytes

Cytokine levels of cultured supernatants were measured by ELISA. The results demonstrated a reduction in pro-inflammatory cytokines in the treatment groups (IFN-γ, LD: 1123±71, HD: 1047±68 vs. CTRL: 3439±81, ANOVA: p<0.001, TNF-α, LD: 1418±81, HD: 1372±62 vs. CTRL: 2788±59, ANOVA: p<0.01, IL-17, LD: 1453±107, HD: 1369±77 vs. CTRL: 7647±119, ANOVA: p<0.001) as well as enhanced expression of anti-inflammatory cytokines (IL-4, LD: 133±11, HD: 153±14 vs. CTRL: 77±7, ANOVA: p<0.05, IL-10, LD: 103±8, HD: 117±12 vs. CTRL: 62±6, ANOVA: p<0.05, IL-27, LD: 121±9, HD: 137±13 vs. CTRL: 98±12, ANOVA: p<0.05, IL-33, LD: 127±14, HD: 132±13 vs. CTRL: 82±7, ANOVA: p<0.05, IL-35, LD: 137±15, HD: 148±10 vs. CTRL: 74±5, ANOVA: p<0.05, TGF-β, LD: 1644±72, HD: 1871±67 vs. CTRL: 837±82, ANOVA: p<0.01) when compared to the control group (Fig. 4). These data show that treatment with low and high dose daphnetin (LD and HD groups) significantly decreases the production of Th1 and Th17 cytokines, as well as increases the production of Th2 and Treg cytokines. As a result, changes in the balance of pro-inflammatory and anti-inflammatory cytokines in the EAE model occur during treatment with daphnetin.

Fig. 4. Effects of daphnetin on gene expression of cytokines and transcription factors in CNS lymphocyte cells

Quantitative real-time PCR was used to assess mRNA expression levels of cytokines and transcription factors secreted from infiltrating T cell to CNS, both in the brains and spinal cords. The data suggest that treatment groups exhibited decreased expression of Th1 and Th17 cytokines and transcription factors (IFN-γ, STAT4, T-bet, IL-17, STAT3, ROR-γt, TNF-α) and increased expression of Th2 and Treg cytokines and transcription factors (IL-4, IL-10, IL-33, GATA3, TGF-β, FoxP3) compared to control group (Fig. 5).

DISCUSSION

Infiltration of T cells into the CNS and subsequent demyelination are characteristic symptoms of both MS and its representative animal model EAE. At the different phases of MS, demyelination and axonal damage are prevailing, with diverse degrees of immune cell infiltration in the disease foci (Imitola et al., 2005; Rudick and Polman, 2009). In this study, the potential role of daphnetin in reducing the severity and progression of EAE in C57BL/6 mice, and the possible
mechanisms were evaluated. In previous studies, the results supported the protective effect of daphnetin in inflammatory demyelinating diseases and to date, a variety of anti-inflammatory and immune regulatory therapies have been used to prevent the progress of MS (Wang et al., 2016).

In a native immune system, there is a delicate balance between effector T cells with different functions. In particular, the balance between pro-inflammatory and anti-inflammatory factors plays a crucial role to bring about protective immune responses to pathogens without interfering with immune tolerance to self-antigens. Collapse of this balance is an important criterion in the development of autoimmune diseases. Therefore, examining new strategies that target these factors could have a potential impact on the treatment of autoimmune diseases (Wang et al., 2018).
Our data showed that daphnetin reduced symptoms in EAE-induced mice and modulated immune responses by affecting lymphocyte differentiation, gene expression, and cytokine production. These results are evident in the effect of daphnetin on suppressing T cell accumulation and the polarized Th1 and Th17 responses in the CNS (Zozulya et al., 2010).

MS is referred to as an inflammatory autoimmune disease and several studies showed that inflammatory T cells, such as the Th1 and Th17 subsets cause tissue damage in both EAE and MS (Huseby et al., 2012; Yang et al., 2015). Furthermore, it has been demonstrated that decreased function of Th2 and Treg cells vs. increased activity of Th1 and Th17 cells is involved in the pathogenesis of MS and EAE (Jamshidian et al., 2013).

Due to the role of Th1 and Th17 cells in the development of EAE, a more pronounced suppression of IFN-γ and IL-17 by daphnetin likely contributes to its en-

Fig. 5. Gene expression of cytokines and transcription factors in CNS. On day 25 post immunization, brains and spinal cords were collected and mRNA levels of cytokines and transcription factors were assessed by real time quantitative PCR. Assay was run in triplicate and fold change expression of genes was determined compared control group. (A) Th1 and Th17 related cytokines and transcription factors; IFN-γ, STAT4, T-bet, IL-17, STAT3, ROR-γt, TNF-α (B) Th2 and Treg related cytokines and transcription factors; IL-4, IL-10, IL-33, GATA3, TGF-β, FoxP3. Results were expressed as fold change compared with control group. *p<0.05, **p<0.01, ***p<0.001. Mice were divided into three groups: 1. Control group (CTRL), 2. Low dose daphnetin treatment group (LD) and 3. High dose daphnetin treatment group (HD).
hanced therapeutic effect on EAE (Vaknin-Dembinsky et al., 2006; Kroenke and Segal, 2007; Rudick and Polman, 2009). The results of this study determined that treatment with daphnetin could significantly reduce the severity and clinical disease symptoms in EAE-induced mice.

Interestingly, previous studies have shown that mice lacking essential components of the Th1 pathway containing IL-12 and IFN-γ are still sensitive to EAE (Becher et al., 2002; Ferber et al., 1996), suggesting that other essential mechanisms are responsible for these inflammatory responses. IL-23 is an important cytokine that plays a key role in the differentiation and maturation of Th17 cells and IL-17 production (Vaknin-Dembinsky et al., 2006). Adaptive immunity, especially Th1/Th17-mediated inflammation, plays a central role in the progression of MS and many other autoimmune diseases such as Rheumatoid arthritis, Systemic lupus erythematosus, and type 1 diabetes (Li et al., 2015). In addition to the effect of Th1 and Th17 lymphocyte responses on the pathogenesis of EAE, and the role of Treg cells in this disease are also critical. Treg cells play an important role in promoting immunological tolerance by suppressing the activation of effector T lymphocytes. Treg cells characterized by expression of the transcriptional factor FoxP3 and cytokine of Treg cells, respectively (Kumar and Sercarz, 1998).

FoxP3 has the ability to directly suppress the expression of IL-17 and IFN-γ cytokines (Martinez et al., 2014), and therefore, increasing FoxP3 expression has a beneficial effect in the treatment of EAE. This is then combined with the selectivity of daphnetin for pathogenic factors in MS, including Th1 and Th17 cells that differentiated through different signaling pathways and transcription factors. Daphnetin causes downregulation of STAT3 phosphorylation and ROR-γt expression in differentiating Th17 cells and mice with ROR-γt deficient T cell have attenuated EAE. Daphnetin also reduced STAT4 and STAT1 phosphorylation and T-bet expression during Th1 differentiation. T-bet, the major transcription factor in the Th1 differentiation pathway, has been demonstrated to be vital for the development of EAE, and T-bet deficient mice could not develop EAE (Yang et al., 2009; Lovett-Racke et al., 2011).

Our results indicated that treatment with daphnetin reduced T-bet and ROR-γt expression while simultaneously increased GATA3 and FoxP3 expression. The transcription factor GATA3 is required for Th2 cell differentiation and can also play a role in regulating the expression of T-bet and ROR-γt transcription factors (Gocke et al., 2009). As a result, switching of the immune responses from Th1 and Th17 cells to Th2 and Treg cells, which is due to increased expression of the GATA3 and ROR-γt transcription factors, may reduce the severity of demyelinating inflammatory diseases, including MS (Fernando et al., 2014).

**CONCLUSION**

The data presented here demonstrated the novel role for daphnetin in ameliorating the symptoms of EAE by increasing the expression of anti-inflammatory cytokines and transcription factors (IL-4, IL-10, IL-33, GATA3, TGF-β, FoxP3), reducing the production of pro-inflammatory cytokines and transcription factors (IFN-γ, STAT4, T-bet, IL-17, STAT3, ROR-γt, TNF-α) and blocking the disease process of EAE. Finally, it is proposed that these beneficial effects of daphnetin could have potential clinical applications for the prevention and reduction of T cell-mediated autoimmune diseases, which will be the subject of new investigations moving forward.

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