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Hesperidin ameliorates immunological outcome and reduces neuroinflammation in the mouse model of multiple sclerosis

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ABSTRACT

Multiple sclerosis (MS) is the most abundant central nervous system (CNS) inflammatory disease, which is due to the reaction of auto reactive T cells with own myelin proteins, leading to physical disorder and paralysis among people suffering the disease. Hesperidin, a flavanone glycoside found abundantly in citrus fruits possesses a wide range of pharmacological properties including potential anti-inflammatory and anti-cancer effects. This study was designed to reveal the molecular and cellular mechanisms underlying the effect of hesperidin on MS alleviation. Female C57BL/6 mice were immunized with $MOG_{35–55}$. Clinical scores and other parameters were monitored daily for the 21 days. At the end of the period, brain/spinal cord histology was performed to measure lymphocyte infiltration; T-cell profiles were determined through ELISA, flow cytometry, and real-time PCR. Transcription factor expression levels in the CNS were assessed using real-time PCR; T cell differentiation was evaluated via flow cytometry. The results demonstrated that hesperidin inhibited development of EAE. Histological studies revealed limited leukocyte infiltration into the CNS. Hesperidin increased Treg cells production of interleukin IL-10 and transforming growth factor (TGF)-β, but concurrently resulted in a significant reduction in production of IL-17 and IL-6. Flow cytometry revealed there were also significant decreases in the percentages of Th17 cells, as well as significant increase in percentages of Treg cells in the spleen and lymph nodes. Real-time PCR results indicated hesperidin treatment reduced ROR-γt factor expression, but enhanced Foxp3 expression. Collectively, these results demonstrated that hesperidin could reduce the incidence and severity of disease.

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) characterized by demyelination, axonal degeneration and subsequent loss of motor function. The underlying mechanisms for disease development are not known and a complex interplay between genes and environmental factors are likely to be operating (Haghmorad et al., 2016). About 30% of the MS patients develop clinical paralysis and become wheel chair-bound for the rest of their lives. Although the etiology of MS is not known, it is generally viewed as an autoimmune disease of the CNS. The destruction of oligodendrocyte and myelin sheath in the CNS is the pathological hallmark of MS (Muthian and Bright, 2004; Nosratabadi et al., 2015). Experimental Autoimmune Encephalomyelitis (EAE) is a T cell mediated inflammatory disease of the CNS that clinical and pathological features show close similarity to human MS; therefore, it has been commonly used as an ideal animal model system to study the mechanisms of MS pathogenesis and to test the efficacy of potential therapeutic agents for the treatment of MS. It is generally accepted that auto-reactive, myelin-specific $CD4^+$ T cells are responsible for disease initiation. However, recent studies suggest that oxidative stress play a key role in the pathogenesis of EAE (Muili et al., 2012; Muthian and Bright, 2004).

The pathogenesis of CNS demyelination in EAE/MS is a complex process that involves activation of macrophage/microglial cells, differentiation of neural Ag-specific Th1 cells, and secretion of inflammatory cytokines in the CNS (Natarajan and Bright, 2002). The invasion of $CD4^+$ T cells into the CNS is thought to play a significant role in the pathology of the animal model EAE as well as the human disease MS.

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Previous studies in MS and EAE suggested that the cytokine profile of infiltrating $CD4^+$ T cells is vital in determining the extent of disease pathology (Lees et al., 2008).

It has been determined that Nitric Oxide (NO) and its various oxidative metabolites such as nitrite and nitrate are found at higher concentrations in the cerebral spinal fluid (CSF) of people with MS when compared to the CSF of healthy, non-MS controls. Although an increase in the levels of NO or metabolites and demyelination are both characteristic features of MS, it is not clear if the NO has a direct effect on the formation of these lesions or are a result of such lesions. Summarily, while much is understood about MS, the underlying causes remain unsolved (Letourneau et al., 2010).

Flavonoids are naturally occurring polyphenolic compounds that are present in the human diet. They can be found in a variety of fruits, vegetables, cereals, tea, wine, and fruit juices. Based on variations in their basic structure, flavonoids are divided into nine subclasses, of which the major three are flavones, flavonols and flavanones. Flavonoids are known for their strong anti-oxidant properties, protecting tissues against oxidative stress. Several reports have suggested that diseases associated with oxidative stress and inflammatory diseases may be beneficially influenced by flavonoids which can directly quench free radicals, inhibit enzymes of oxygen reduction pathways and sequester transient metal cations (Verbeek et al., 2005; Nones et al., 2012).

Hesperidin, a flavanone glycoside found abundantly in citrus fruits such as sweet orange and lemon, possesses a wide range of pharmacological properties including potential anti-inflammatory and anti-cancer effects. Hesperidin induces cell growth arrest and apoptosis in a large variety of cells including colon and pancreatic cancer cells (Ghorbani et al., 2012).

Recent data reveal that in vitro flavonoids inhibit antigen specific memory T cell proliferation and pro-inflammatory IFN-γ production and reduce the phagocytic activity by macrophages. As well as flavonoids could have beneficial effects during the pathogenesis of MS and EAE since some of them have a strong inhibitory effect in vitro on proliferation and IFN-γ production of auto antigen specific T cells (Verbeek et al., 2005).

Flavonoids have been described as important neuroprotective molecules in diverse neuronal insults such as ischemia, oxidative-induced damage, dopamine induced neurotoxicity and anti amyloidogenic in Alzheimer's disease. Hesperidin-primed astrocytes protect against neuronal cell death. Astroglia interactions play key roles in several events of brain development, such as the proliferation and differentiation of neuronal precursors and neuronal migration (Nones et al., 2012).

The neuroprotective efficacy of hesperidin is attributed to its ability of inhibiting Fe^{2+} induced linoleate peroxidation and auto oxidation of cerebral membranes, scavenging peroxynitrite radicals and inhibition of ROS generation, including hydroxyl radical. It protects the neurons against various types of insults associated with many neurodegenerative diseases (Raza et al., 2011). Flavonoids play an important role in platelet reactivity and in the induction of anti-inflammatory cytokines in circulating monocytes (Kim et al., 2011).

Accordingly, this paper aimed to evaluate therapeutic potentials of hespridin on the improvement of EAE induced mice and elucidate the possible mechanisms involved.

2. Material and methods

2.1. Animals

C57BL/6 mice (female, 8-week-old) were obtained from the Pasture Institute of Iran (Tehran, Iran). Mice were housed in animal facilities of the Bu-Ali Research Institute maintained at 25 °C with a 50% relative humidity and a 12 h light/dark cycle. All mice had ad libitum access to standard rodent chow and filtered tap water. All experiments were performed according to Mashhad University of Medical Science ethical guidelines.

2.2. Induction of EAE

EAE was induced by immunizing C57BL/6 mice subcutaneously in the flank with 250 μg $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) (SBS Genetech Co. Ltd., Beijing, China) emulsified in complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA) containing 4 mg/mL M. tuberculosis H37RA (Difco Laboratories, Detroit, MI, USA). On the day of immunization, and again 48 h later, mice were injected intravenously with 250 ng pertussis toxin (Sigma-Aldrich, St. Louis, MO, USA) (Huehnchen et al., 2011; Haghmorad et al., 2014a). Clinical signs of EAE were monitored and weight of the mice was measured daily until 25 days post immunization. Mice were scored for neurologic malfunction, in accordance with the following scale: 0, no clinical sign; 1, partial loss of tail tonicity; 2, complete loss of tail tonicity; 3, flabby tail and abnormal manner of walking; 4, hind leg paralysis; 5, hind leg palsy with hind body partial immobility; 6, hind and foreleg paralysis; 7, moribund or death (Haghmorad et al., 2014b; Mosayebi et al., 2010). Mice were scored daily and were assessed for incidence, onset day of disease, maximal score (at the peak day), mean score (at the last day) and Cumulative Disease Index (total disease score over experiment duration).

2.3. Hesperidin treatment

Mice were divided into four groups: 1. Control group (CTRL; n = 12), 2. Low-dose hesperidin treatment group (T1; n = 11; 50 mg/kg hesperidin), 3. Middle-dose hesperidin treatment group (T2; n = 10; 100 mg/kg hesperidin) and 4. High-dose hesperidin treatment group (T3; n = 12; 200 mg/kg hesperidin) (Trivedi et al., 2011; Shagirtha and Pari, 2011; Ahmad et al., 2012). EAE were induced in all groups and treatment groups were treated with three different dose of hesperidin to earn maximum advantage.

Hesperidin was administered in treatment groups orally simultaneous with EAE induction every day for 25 days. Control group received PBS as a solvent orally every day for 25 days.

Table 1
Sequences of primer and probes which used in study.

Genes	Forward	Reverse	Probe
Foxp3	CAGAGTTCTTCCACAACA	CATGCGAGTAAACCAATG	TGAGTGTCCTCTGCCTCTCCG
GATA3	CTGCGGACTCTACCATAA	GTGGTGGTCTGACAGTTC	CTGCTCTCCTTGCTGCCGAC
IFN-γ	CCAAGTTTGAGGTCAACA	CTGGCAGAATTATTCTTATTGG	CCGAATCAGCAGCGACTCCT
IL-4	CTGGATTCATCGATAAGC	GATGCTCTTTAGGCTTTC	TGAATGAGTCCAAGTCCACATCACT
IL-6	CCAACAGACCTGTCTATA	GCATCATCGTTGTTCATA	CACAAGTCGGAGGCTTAATTACACATG
IL-10	CAGGTGAAGACTTTCTTTC	AACCCAAGTAACCCTTAA	ACAACATACTGCTAACCGACTCCTT
IL-17	CCTCAGACTACCTCAACC	CCAGATCACAGAGGGATA	ACTCTCCACCGCAATGAAGACC
IL-23	CGGGACATATGAATCTACTAA	TGTCCTTGAGTCCTTGTG	CAACCATCTTCACACTGGATACGG
TGF-β	CGGACTACTATGCTAAAGA	CTGTGTGAGATGTCTTTG	CGTTGTTGCGGTCCACCATT
T-bet	TGTGGTCCAAGTTCAACC	CATCCTGTAATGGCTTGTG	TCATCACTAAGCAAGGACGGCG
ROR-γt	GGATGAGATTGCCCTCTA	CCTTGTCGATGAGTCTTG	CTCATCAATGCCAACCGTCCTG
B2m	CCTGTATGCTATCCAGAA	GTAGCAGTTCAGTATGTTC	TATACTCACGCCACCCACCG