Original Article

Effects of Lavandula angustifolia essential oil on Interleukin 23 and Brain-Derived neurotrophic factor gene expression in peripheral blood mononuclear cells of multiple sclerosis patients

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Abstract

Background: We aimed to determine the effect of lavandula angustifolia essential oil (LEO) on IL-23 and brain-derived neurotrophic factor (BDNF) gene expressions in peripheral blood mononuclear cells (PBMCs) of relapse-remitting MS (RRMS) patients. **Materials and Methods:** LEO was prepared using the hydrodistilation method on the plants aerial parts. 8 female RRMS patients and 8 healthy sex and age matched controls were entered into this study. PBMC cells were separated using Ficoll method and were treated with a concentration of 225 µg/ml LEO which and then the mRNAs were used for determining the effects of LEO on IL-23 and BDNF gene expressions using Quantitative Real Time PCR technique. Moreover in order to determine the anti-inflammatory effects of LEO, we measured the gene expression of IL-6 and IL-23 in stimulated healthy PBMC cells treated with LEO. **Results:** Results showed that there is no significant difference between PBMC of patients compared to healthy controls in case of IL-23 gene expression. Moreover, LEO has no significant effect on gene expression of IL-23 in PBMC of neither patients nor control. Also the results showed that BDNF gene expression is reduced to 41% compared to healthy controls and LEO can increase the BDNF gene expression by 81% in patients PBMCs. Moreover we observed that LEO can significantly reduce the LPS stimulated IL-6 gene expression in healthy PBMCs but had no significant effect on IL-23 gene expression. **Conclusion:** The present study demonstrated that L.angustifolia essential oil may have a protective effect against neuron damage via increasing the gene expression of BDNF in PBMCs from RRMS patients. However, further studies are necessary to confirm our results.

Keywords: *Lavandula Angustifolia*, PBMC, RRMS, IL-23, BDNF, Quantitative Real Time PCR

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Introduction

Multiple Sclerosis (MS) is the most common neurological disability in youth populations around the world. MS has been recognized as a well-established autoimmune disease. In spite of numerous efforts to find therapeutically approaches for alleviating the disease activity and progression, there is no known cure for it yet (1).

In most cases (80%), MS begins in Relapse-Remitting (RRMS) form which presents with periods of relapse accompanied with clinical symptoms that may take days or weeks followed by periods of remission and recovery (2).

Precise mechanism underlying MS is still a matter of discuss but current theory involves

inflammatory sites called lesions or plaques. Structurally, lesions contain multiple immune cells (T cells , B cells , macrophages , plasma cells , etc.) which present an autoimmune response to neuronal myelin sheets and subsequently result in demyelination and axonal loss over the course of the disease (3).

Numerous factors contribute to MS pathogenesis as a complex disease. In recent years pro-inflammatory T-helper cell called TH17 has been identified as the key players in pathomechanism of autoimmune diseases (4). Among many molecules that are necessary for producing functional Th17 cells, IL-6 , IL-21, IL-23 and TGF-β are the most important factors (5).

IL-23 is a heterodimer cytokine composing of two subunits; p19 which is exclusive to IL-23 and p40 which is shared with IL-12 (6).

It has been shown that IL-23 is necessary for experimental autoimmune encephalomyelitis (EAE) pathogenesis and it is also critical for Th17 polarization and the acquisition of encephalitogenicity during EAE(7, 8). IL-23 level is elevated in both serum and CSF of MS patients and it is also a validated biomarker for MS (9-12).

Brain-Derived Neurotrophic Factor (BDNF) is a member of neurotrophin family which is primarily produced not only by neurons but also by immune cells (13). There are two functional forms of BDNF; pro-BDNF and mature BDNF. Pro-BDNF binds to p75 receptor and induces neuronal apoptosis, but mature BDNF binds to tyrosine receptor kinase B (TrkB) and is associated with neuronal survival and differentiation and synaptic plasticity (14-17).

There is also evidence that BDNF is associated with a number of neurological diseases such as Huntington's disease , Alzheimer's disease , Parkinson's disease and also psychiatric diseases (18).

Studies have shown that BDNF production is reduced in MS lesions and are instead being produced by activated immune cells in the area (19). Studies have shown differences in BDNF levels of MS patients with healthy population including decreased BDNF levels in SPMS and RRMS patients in remission and also increased BDNF levels in RRMS patients in relapse period which may be related to ameliorating the CNS damage caused by MS attacks (18).

Lavender has long been used as a therapeutic herb in traditional medicine and has been used as an antibacterial, antifungal, analgesia, anti-depressant, antispasmodic, sedative, anti-inflammatory agent (20). Lavender essential oil (LEO) has more than 100 constituents but the most prominent components of LEO are Linalool and linalyl acetate which are considered to have the prominent role in LEO's biological effects (21).

It has been shown that LEO and linalool can modulate the CNS neurotransmission (22) and there is also evidence of LEO neuroprotective properties in cerebral injury models (23).

The mechanism of action of most of the well-known old and new drugs that are used to manage the disease progression, is based on modulating the immune system. Drugs such as IFN beta, Glatiramer acetate, Fingolimod, Daclizumab, Alemtuzumab, Natalizumab and Ocrelizumab all change the disease progression through either reducing the immune cell count or reducing the pro-inflammatory cytokine production and efficacy (24). There is accumulating evidence addressing the beneficial anti-inflammatory effects of LEO and its constituents as they have been shown to modulate different inflammatory factors such as IL-6, TNF-α, MIP-2, IL-1β, TRL4, NF-κB, TNF-α and MCP-1 (25-27), however there is no study evaluating the LEO effects on regulation of inflammatory pathways in peripheral blood monocular cells. Hence, we were encouraged to determine the effects of LEO on PBMC gene expression of IL-23 and BDNF in MS.

Methods

Study Groups .The patients group was consisted of 8 women with relapse remitting multiple sclerosis (RR-MS) that were diagnosed at Firoozgar Hospital affiliated by Iran University of Medical Sciences,Tehran,Iran. Patients didn't have any other disease prior to the diagnosis of MS. Blood samples were collected before administration of drug. The control group was consisted of 8 healthy age-matched women. Controls didn't have any history of autoimmune disease and didn't receive any immunomodulatory drugs. Patients were clinically

Table 1. Real-Time PCR primer sequences for β-Actin, IL-6, IL-23 and BDNF

	Forward	Reverse
IL-23	5´AGAGGGAGATGAAGAGACTAC ´3	5´ GCAAGCAGAACTGACTGT 3´
IL-6	5´ ACTCACCTCTTCAGAACGAATTG 3´	5´ CCATCTTTGGAAGGTTCAGGTTG 3´
BDNF	5´ CCCATTCAGCCCATTTCA 3´	5´ GCTTATCCCTCACCCTACTA 3´
B-Actin	5´ GCAAGCAGGAGTATGACGAG 3´	5´ CAAATAAAGCCATGCCAATC 3´

diagnosed according to McDonald criteria (2) with Expanded Disability Status Scale (EDSS) between 0-2. The study protocol was approved by the Ethics Committee of Tehran University of Medical Sciences, and written informed consent was obtained from all subjects.

Isolation of PBMC and cell culture .Blood samples were collected in collecting tubes containing sodium heparin as an anti-coagulant. Isolation of peripheral blood mononuclear cells (PBMCs) were done by Lympholyte® Cell Separation Media (CEDARLANE , Canada) and the obtained cells were washed two times with phosphate buffered saline (PBS).Then total number of the isolated cells were counted and PBMCs at density of 4×10^6 cells/well were seeded in 6 well plates (SPL Life Sciences . Korea). Culture medium used in this experiment was RPMI 1640 (Containing L-glutamine and HEPES) (Caisson Labs. USA) containing 10% heat inactivated fetal bovine serum (GIBCO, United Kingdom) and 1% penicillin/streptomycin solution.

In order to cell stabilization, plates were incubated for 12h before treatment. Cells were treated with LEO at 225 µg/ml (equal to 0.025% concentration of LEO) for 24h.

Essential oil preparation. Lavandula angustifolia dried aerial parts were purchased from a renowned local provider in Tehran. Plant was identified by prof. Narges Yasa, department of pharmacognosy in faculty of pharmacy, Tehran University of Medical Sciences. Essential oil was obtained via hydrodistiliation using a clevenger-type apparatus for 4h. Then it was dried with anhydrous sodium sulfate and was filtered with 0.22 µm syringe filter (Jet Bio-Filtration Co, China) and stored in the dark at 4 °C.

MTT Assay. LEO was diluted in dimethyl sulfoxide (DMSO) in a way that the highest concentration contained 0.4% DMSO. Concentration of LEO was in following order: 450 µg/ml, 225 µg/ml, 112.5 µg/ml, 56.25 µg/ml, 28.12 µg/ml, 14.06 µg/ml, 7.03 µg/ml and 3.51 µg/ml.

Assay was done in triplicate and 250×10^3 Cells were treated with LEO and incubated for 24, 48 and 72 h in 37 °C with 5% CO2.

MTT (Sigma- Aldrich. USA) assay was done in 96 well plates (SPL Life Sciences. Korea) with 4h incubation time. Toxicity of LEO on PBMC was determined with measuring the absorption of color produced with MTT using BioTek EON Microplate Reader (BioTek Instruments, Inc . USA) at 570nm wave length.

Real Time PCR. Then cells were harvested with a scrapper and were immediately used for RNA extraction.

Hybrid-R Blood RNA kit (GeneAll Biotechnology co. Seoul. Korea) was used for RNA extraction procedure. After the extraction, concentration and quality of RNA was measured with NanoDrop 1000 Spectrophotometer (Thermo Scientific. USA) and then the integrity of the extracted RNA was determined by 1.5% agarose gel electrophoresis.

The cDNA synthesis was done with RevertAid First Strand cDNA Synthesis Kit (Theromo scientific. USA). Total concentration of RNA that was used for this procedure was 500ng/µl.

Real-Time PCR was performed with SYBR Premix Ex Taq II (TaKaRa. Japan) using a Rotor-Gene Q Real-time PCR Cycler (QIAGEN Hilden. Germany). Primers used for IL-23, IL-6 and BDNF are provided in Table 1.

Determining the anti-inflammatory effects of the LEO on IL-6 and IL-23 gene expression. In a complementary assay for determining the anti-inflammatory effects of the LEO, healthy PBMCs were isolated as described.

Briefly, isolated PBMCs were pre-treated with

225 µg/ml of LEO for 12h. Then cells were treated with 100 ng/ml concentration of Lipopolysaccharide (LPS) (Sigma- Aldrich. USA) for 3h (LPS concentration was borrowed from previous work of our colleagues on PBMC (28)) and subsequently were collected to determine the effects of LEO on IL-23 and IL-6 gene expression. It should be noted that the final concentration of DMSO in all wells was 0.2%.

Results

Study Groups. A total of 8 female RRMS patients and 8 healthy females were selected for this study. All the patients were in remission period with mean disease duration of 2.625 ± 0.73 years (mean ± SEM). All the Participant in the control group were at the same age group as the patients and there was no statistically significant difference between healthy and patient group in this regard (RRMS 33.25 ± 2.05 years vs healthy controls 29.25 ± 0.99 years , mean ± SEM, p = 0.1).

MTT assay. LEO toxicity was determined by MTT assay. We observed no significant change in cell viability after exposure of cells to LEO at concentrations of 225 µg/ml and lower for 24h, 48h and 72h in comparison with untreated PBMCs and the cell viability was more than 90% under treatment with these concentrations. However, the exposure of PBMCs with LEO at 450 µg/ml for 24h,48h and 72 h significantly reduced the cell viability to 85% (p <0.01) , 81% (p <0.05) and 84% respectively (p <0.05) (figure 1).

Effect of LEO on IL-23 Gene expression. Gene expression of IL-23 was measured using quantitative real-time PCR method. Results shown no significant difference between PBMCs of RRMS patients and controls in case of IL-23 gene expression. Also LEO has shown no significant effect on IL-23 gene expression both in RRMS patients and controls group (figure 2).

Effect of LEO on BDNF Gene expression. Results of BDNF gene expression showed a significant decrease in BDNF expression level in PBMCs of RRMS patients compared to control group.

BDNF expression in RRMS patients significantly decreased to 41% of control group (P<0.0001). LEO treatment for 24h resulted in significant increase of BDNF expression by 81% in in PBMC of RRMS patients (P=0.013). It was also shown that LEO treatment reduced BDNF gene expression by 34% in PBMC of control group (P<0.01) (figure 3).

Effect of LEO pre-treatment on LPS induced IL-6 gene expression IL-23 Gene expression. Results of this test repeated our previous result in the case of IL-23. There was no significant difference in term of IL-23 gene expressions in LPS-treated healthy PBMCs compared to pre-treated group. There was also no significant difference between LEO –treated PBMCs and untreated cells regarding IL-23 gene expression. Results of IL-6 gene expression showed a significant

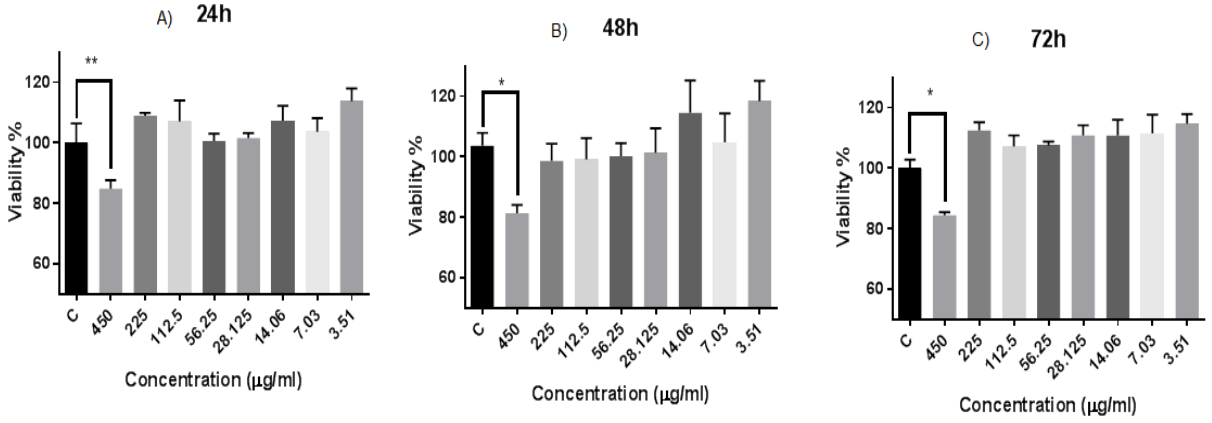

Figure 1. Assessment of PBMCs viability in response to LEO treatment. Cells were incubated with different concentrations of LEO (from 450µg/ml to 3.51µg/ml) for A) 24 h, B) 48 h and C) 72h and cell viability was determined by MTT assay. The MTT assay was performed in triplicate and data are reported from the mean of triplicates. Data are expressed as means ± SEM.

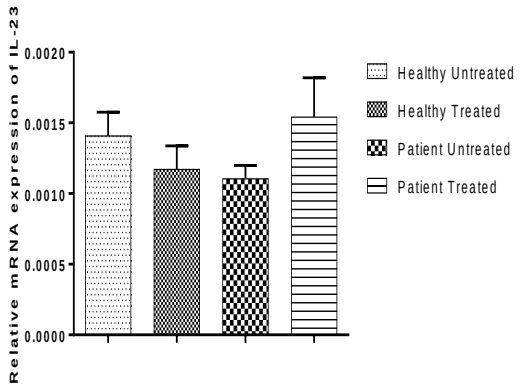

Figure 2. Effect of LEO on IL-23 mRNA expression in PBMCs from RRMS patients and healthy subjects. PBMCs were treated with LEO (225 µg/ml) for 24 h. All untreated cells were treated with 0.4% % of DMSO as a control. Data are expressed as mean ± SEM...

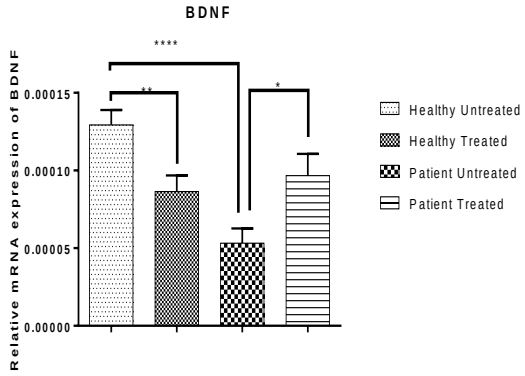

Figure 3. Effect of LEO on BDNF mRNA expression in PBMCs from RRMS patients and healthy subjects. PBMCs were treated with LEO (225 µg/ml) for 24 h. All untreated cells were treated with 0.4 % of DMSO as a control. Data are expressed as mean ± SEM.(* = P<0.5, ** = P<0.01, **** = P<0.0001)

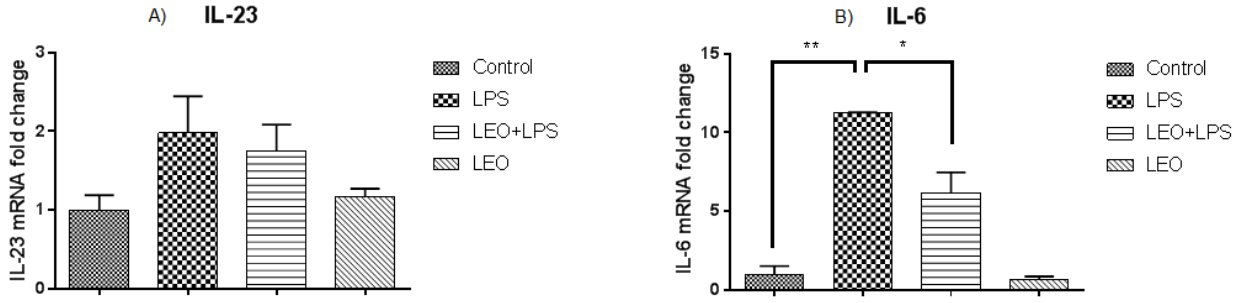

Figure 4. Effect of LEO, LPS, and LEO plus LPS on mRNA expression of (A) IL-23 and (B) IL-6 in PBMCs from healthy subjects. PBMCs were treated as follows: untreated (only with 0.2% DMSO for 15 h); LEO (225µg/ml) for 15 h; LPS (100 ng/ml) for 3 h; and LEO plus LPS group receiving LPS (100 ng/ml) for 3 h following 12 h pretreatment with LEO (225µg/ml). All untreated cells were treated with 0.2% of DMSO as a control. Data are expressed as mean ± SEM. (* = P<0.05, ** = P<0.01)

increase in PBMC IL-6 gene expression in LPS-only group compared to control (11.2 fold increase, P<0.01). It was also shown that LEO pre-treatment can significantly reduce the LPS- induced IL-6 gene expression to 6 fold of baseline level, P<0.05). There was also no significant difference between LEO-only group and control regarding IL-6 gene expression (figure 4).

Discussion

As a T-cell related autoimmune disease, immune cells play a pivotal role in MS pathology. In MS, Th17 cells can cross the blood-brain barrier and secrete inflammatory cytokines like IL-17, IL-22, Granulocyte-macrophage colony-stimulating factor (GM-CSF) and granzyme-B. Th-17 cells can facilitate the infiltration of other immune cells and also mediate inflammatory responses through neuron, astrocyte and microglia (29, 30).

A number of cytokines mediate the differentiation of Th17 from naïve $CD4^+$ T cells. TGF-β, IL-6 and IL-21 play a key role in emerging of Th17 from naïve T cells but it is the IL-23 which mediates the final differentiation and expansion process and production of a fully functioning Th17 cells (31). Thus IL-23 has been an interesting candidate in MS researches and it even has been considered as a therapeutic target (32).

There is a number of studies that investigated the level of IL-23 in MS patients. Shajarian et al.

measured the IL-23 serum levels of RRMS patients in remission period of the disease. Results showed a six fold increase in patients compared to controls (11) also wen et al . demonstrated an increased IL-23, IL-17 and TNF-α in Chinese population with RRMS in remission period (12). There is also evidence of IL-23 presence in MS related brain lesions as Li et al's study on MS brain lesion specimens showed an increased expression and production of IL-23p19 subunit in microglia/macrophage and dendritic cells in the specimens (33).

In present study we didn't observed any significant difference between PBMC gene expression of IL-23 in patient and control group.

Although our result is in contrast with the majority of studies regarding the connection between IL-23 and MS, there is also evidence supportive of our findings. Hasheminia et al. studied the inflammatory and anti-inflammatory cytokines in newly diagnosed RRMS patients that were in relapse stage of the disease and didn't find any statistically significant difference between PBMC gene expression and serum level of IL-23 between patient and controls (34).

Contradiction between the results of Hasheminia et al and our work with other studies that showed increased IL-23 production in MS patients might be due to differences in disease duration in patient group among these studies. As in Hasheminia et al's study only newly diagnosed patients were enrolled and in our study the disease duration was just 2.6 years while patients enrolled in other studies had a well stablished disease with several years of duration.

After LEO treatment we didn't see any significant difference between PBMC IL-23 gene expression neither in patient nor in control group.

This was a disappointing result as the anti-inflammatory effects of lavender is scientifically stablished by several studies. Algieri et al investigated the anti-inflammatory effects of Lavandula stoechas and Lavandula dentate in murine epithelial cells and also BMDM cells and results shown a significant reduction in IL-6, TNF-α and MIP-2 gene expression (25). Also Huang et al showed a significant reduction in IL-1β and TRL4 production and NF-κB phosphorylation in THP-1 cells treated with Lavender essential oil (26). Linalool, one of the most prominent constituents of LEO, also has the potential to reduce the production of IL-6, IL-1β, and TNF-α and MCP-1 and inhibition of NF-ќB activation (27).

To confirm our LEO's anti-inflammatory effects we measured the gene expression levels of IL-23 and IL-6 in LEO pre-treated PBMCs that were activated with LPS. Previous results were repeated and no significant effect was seen on IL-23 gene expression but LEO pre-treatment significantly reduced the IL-6 gene expression induced by LPS. This finding is in line with previous studies that seen an inhibitory effect on IL-6 production as a result of Lavender treatment (25, 27). This results confirms the anti-inflammatory effects of LEO but it also suggests that LEO mechanism of action may not affect the IL-23 gene expression.

BDNF is also an interesting candidate in multiple sclerosis researches. BDNF is a neurotrophic factor which plays an essential role in neuronal development, regeneration and plasticity (18). Neurons are the main producers of BDNF in CNS but In MS-related lesions, the BDNF production capacity is significantly reduced. Instead it seems that residing activated immune cells in the lesions can produce BDNF and may have a role in ameliorating the damage caused by MS attacks (13).

There are multiple studies regarding the BDNF levels in MS patients. Azoulay et al and Urshansky et al have shown reduced production of BDNF in PBMCs of RRMS patients in remission period of the disease (35, 36). Also Sarchielli et al showed increased levels of BDNF in RRMS patients in relapse period compared to patients in remission period suggesting the potential neuroprotective roles of BDNF after the MS attacks (37). There is also studies regarding the effects of MS treatments on BDNF levels. Azoulay et al. showed augmentation of BDNF to normal levels in RRMS patients treated with Glatiramer acetate (GA) (38). Mehrpour et al also showed similar results in RRMS, Secondary progressive MS (SPMS), Primary progressive MS (PPMS) and Progressive relapsing MS (PRMS) patients with active disease treated with Interferon-β (39).

In present study we have observed a significant decrease in PBMC BDNF gene expression levels in RRMS patients in remission period compared to control group which is in line with previous studies and suggesting a reduced capacity of BDNF production in MS patients.

Effect of LEO on BDNF production is not very well stablished. To our knowledge there was only one study regarding the effects of LEO on BDNF production. Xu et al investigated the effects of Linalool and LEO in Alzheimer disease Murine model which the resulted in increased gene expression of BDNF and TrkB (40).

Taken together, the present study showed a significant increase in PBMC BDNF gene expression of RRMS patients after LEO treatment which may suggest a possible neuroprotective effect of LEO through increasing the BDNF levels in RRMS patients. However, more investigations are needed to reach a conclusive view regarding the effects of LEO on BDNF production.

Conclusion

Based on our results it seems that L. angustifolia essential oil may have the potential to increase BDNF gene expression in PBMCs of RRMS patients.

It also may have anti-inflammatory effects through reducing the gene expression of inflammatory cytokines like IL-6 although it was shown that it may not be effective on IL-23 gene expression.

In future with the help of further studies, L.angustifolia may have the potential to alleviate MS CNS damage as a supplementary treatment.

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Conflict of interest:

There is no conflict of interest among authors.

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