Anti-inflammatory effect of Migri-Heal[®] in an *in vitro* inflammatory model of primary mixed glial cells

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Received October 31, 2016; Accepted July 11, 2017

DOI: 10.3892/mmr.2017.8027

Abstract. Migri-Heal[®], is a novel herbal remedy that was introduced for the treatment of migraine headaches. Previous studies revealed that this drug may reduce nitric oxide (NO) in an in vitro inflammatory model. The aim of the present study was to investigate the anti-inflammatory effect of Migri-Heal® on primary mix glial cells stimulated with LPS. In the current study, neonatal rat primary mix glial cells were isolated from the mixed glial cultures via shaking, and cultured in Dulbecco's' modified Eagle's medium supplemented with 10% fetal bovine serum. Following pretreatment with Migri-Heal® (25, 75, 100, 150, 200 and 300 μ g/ml) and cells were treated with LPS (10 μ g/ml) for 1 h, and incubated for 48 h. The present study determined that 150 µg/ml Migri-Heal® significantly reduced the production of NO in rat mix glial cells stimulated with 10 μ g/ml LPS. Migri-Heal® also suppressed mRNA expression level of LPS-induced inducible nitric oxide synthase and tumor necrosis factor α , which was accompanied by inhibition of the transcription factor nuclear factor-kB. Additionally, MTT assay determined that Migri-Heal® was not cytotoxic, suggesting that the anti-inflammatory effects of Migri-Heal® observed were not due to cell death. In conclusion, the findings of the present study demonstrated that Migri-Heal® may be useful as a potential anti-inflammatory agent in

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inflammatory diseases. However, additional studies are required to confirm these findings.

Introduction

Glial cells are macrophage cells in brain that are able to perform the phagocytosis and protect neurons in the central nervous system (CNS) (1,2). They act as phagocytic cells and are the primary immune cells in CNS. Additionally, glial cells function as a debris scavengers, regulate the innate immunity and participate in the adaptive immune responses in neural tissues (3).

The pro-inflammatory responses have important functions in the pathogenesis of several CNS-associated disorders, including Alzheimer's disease, multiple sclerosis and Parkinson's disease (4-6). Activated glial cells, including both microglia and astrocytes are the primary sources for proinflammatory mediators, such as cytokines, chemokines and nitric oxide (NO) (1,7). Therefore, more attention has been directed towards considering these cells as the putative targets for treatment of inflammatory disorders.

However, over-activate glial cells may also produce excessive inflammatory substances such as NO, various cytokines and prostaglandins (1,7). Previous studies revealed that suppression of inflammatory responses from glial cells may alleviate these pathological conditions (8,9). Therefore, it is possible that anti-inflammatory agents may have neuroprotective functions. It should be noted that anti-inflammatory agents have been previously investigated for targeting particular proinflammatory mediators and selectively deactivating glial cells (8,9).

Migri-Heal[®] as a novel herbal remedy, which was introduced as potential treatment of migraine headaches based on anecdotal evidence in traditional Iranian medicine. This drug has been patented by the Invention and Patent Registration Office of I.R. of Iran (IRC1228143083). It has been previously reported that Migri-Heal[®] reduces NO levels in endothelial cell culture (10). Therefore, the evaluation of the possible anti-inflammatory effects of Migri-Heal[®] may be useful to illustrate other therapeutic aspects of this herbal remedy in neuroinflammatory diseases.

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Key words: mixed glial cells, microglia, Migri-Heal[®], inflammation, inflammatory effect

To the best of our knowledge the present study is the first to investigate the effect of Migri-Heal[®] on expression and secretion of inflammatory mediators from microglia cells stimulated with LPS for the first time.

Materials and methods

Reagents. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and Griess reagent were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Bacterial LPS (cat. no. E5:055), MTT assay kit (cat. no. M2128 500MG), antibiotics (streptomycin and penicillin) and trypsin were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) (cat. no. 1.02952.1000) was purchased from Merck Millipore and Migri-Heal[®] was prepared by Dr Mohammad Ansari (Tehran University of Medical Sciences, Tehran, Iran) (11). This drug has been patented by the Invention and Patent Registration Office of I.R. of Iran (IRC1228143083).

Migri-Heal[®] *dilution*. A total of 100 g Migri-Heal[®] powder was dissolved in 250 ml water. Following 10 min, boiling water (powder and water) extract was passed through Whatman No. 1 filter paper and dried by freeze dryer. This extract was 11% Migri-Heal[®] powder. The dried extract of Migri-Heal[®] (1 mg/ml) was dissolved in DMEM and filtered using 0.22 μ m filter (Orange Scientific, Braine-l'Alleud, Belgium). It is of note that Migri-Heal[®] should be freshly prepared.

Cell culture. The primary mixed glial cultures were prepared from whole brains of 1 to 3 days-old Wistar rats (National Institute of Genetic Engineering and Biotechnology, Tehran, Iran) (n=10; 5 males, 5 females; weight, between 4 and 5 g). Rats were housed at a constant temperature of $23\pm2^{\circ}$ C with a 12 h light/dark cycle (lights on at 7 am) and received standard rat chow and water ad libitum.

The study was reviewed and approved by the Bioethics Committee of the Health Ministry (Tehran, Iran; permit no. IR.NIGEB.EC.1395.4.1.C). Isolated cells were cultured according to previous studies with some modifications (3,12-14). Briefly, brains were excised aseptically from the skull, the meninges and blood vessels were carefully removed, and mechanically disrupted by trituration in DMEM. Then the suspended cells were transferred inside a flask prepared with DMEM supplemented with 100 UI/ml penicillin G, 100 µg/ml streptomycin and 10% of heat-inactivated FBS. Cultures were incubated at 37°C with 95% humidity and 5% CO2. The medium was replenished on day 1 after plating and every third or fourth day thereafter with medium supplemented with 10% of heat-inactivated FBS and the aforementioned antibiotics. In this study, cells after two passages were used.

Treatment with Migri-Heal[®]. Treatment with Migri-Heal[®] was done in two ways, as, in some studies (15,16), stimulation with LPS was performed prior to drug treatment, whereas in other studies (11,17), stimulation with LPS was performed following drug treatment; therefore, the two methods were investigated in the present study. The first included 10 μ g/ml

LPS was added to primary mixed glial cells, incubated in 37°C for 1 h. Subsequently, cells were treated with Migri-Heal[®] (25, 50, 100, 150, 200, 250 and 300 μ g/ml) in fresh DMEM containing 1% FBS and cells were incubated in 37°C for 48 h. The second method included primary mixed glial cell cultures were pretreated with Migri-Heal[®] (25, 50, 100, 150, 200, 250 and 300 μ g/ml) in fresh DMEM containing 1% FBS for 1 h. Subsequently, 10 μ g/ml LPS was added and cells were incubated in 37°C for 48 h.

Cell viability assay. The cell viability was evaluated by an MTT assay as previously described (18). Following various treatments, 1 mg/ml MTT solution was added 10% of medium for 3 h at 37°C. After 3 h, the medium was removed and the cells were lysed in 100 μ l of DMSO, which can release the blue product. The level of MTT formazan was determined by measuring absorbance at 580 nm using a Multiskan RC microplate reader.

Nitrate assay. The Griess nitrite assay, which may be used as an index of NO production, was used to estimate NO production in the cultured glial cells (7,14). Serial diluted NaNO₂ (Sigma-Aldrich, Merck Millipore) solutions were freshly made and served as standards (0-100 mol/l) for the assay. Briefly, samples were collected 48 h after stimulation with LPS. Following centrifugation (1,000 x g, 5 min, room temperature), 50 μ l culture medium (supernatant) was transferred to 96-well plates (BD Biosciences, Franklin Lakes, NJ, USA) and mixed with an equal volume of Griess reagent. The plate was placed in darkness for 15 min and the quantity of nitrite was calculated by measuring absorbance at 540 nm using a micro plate reader.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from treated cell cultures with Easy BLUE® (iNtRON Biotechnology, Inc., Daejeon, South Korea) according to the manufacturer's protocol. Concentration of the total RNA was determined by NanoDrop spectrophotometer at 260 nm. Identical quantity of RNA (1 µg) was reverse transcribed for 1 h at 42°C in a reaction mixture containing 20 U RNase inhibitor (Fermentas; Thermo Fisher Scientific, Inc.), 1 mM dNTP (CinaGen, Tehran, Iran), 1x reverse transcriptase buffer, and 5 U reverse transcriptase (Fermentas; Thermo Fisher Scientific, Inc.). The cDNA was amplified by PCR with using the following primers: INOS forward (F) 5'-GAC ATCGACCAGAAGCTGTC-3' and reverse (R) 5'-GGGCTC TGTTGAGGTCTAAAG-3'; TNFα F 5'-GCTCCCTCTCAT CAGTTCCA-3' and R 5'-TTGGTGGTTTGCTACGACG-3'; GAPDH F 5'-CCCCCAATGTATCCGTTGTG-3' and R 5'-TAGCCCAGGATGCCCTTTAGT-3'. PCR was conducted by using the following conditions for 33 cycles: Denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 45 sec. PCR products were separated by electrophoresis on a 2% for iNOS and 3% for TNF α agarose gels. The products were visualized by staining with ethidium bromide (MR7729; CinnaGen, Tehran, Iran) and detected under UV light. Densitometric analysis of the data was normalized to GAPDH. The intensity of bands was determined using the TotalLab version 1.1 (TotalLab, Ltd., Newcastle upon Tyne, UK).

Total protein extraction. Protein levels were determined in primary mixed glial cells 48 h after treatment. After a cold PBS wash, the cells were scraped and recovered in 100 μ l per sample of NP-40 lysis buffer (tris-HCl 50 mM, pH 8, solution with 1% Triton X-100, NaCl 150 mM). After pipetting the content of the wells was pooled and centrifuged at 9,800 x g at 4°C for 10 min and stored at -20°C. Protein content was assayed colorimetrically using the Bradford method (Abcam, Cambridge, MA, USA).

Western blotting. A total of 45 μ g denatured total protein extracts (2.5 mM DTT, 100°C for 5 min) were subjected to 12.5% SDS-PAGE and transferred to a PVDF Western Blotting membranes (Roche Applied Science, Penzberg, Germany; cat. no. 03010040001). Then, the transferred membrane was blocked with a blocking solution [1X TBS, 0.1% Tween-20 with 5% w/v dry milk (Merck Millipore; cat. no. 1153630500)] for 1 h and incubated with the following primary antibodies overnight at 4°C: NF-κB p65 (catalog no. sc-71675; dilution, 1:250; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Nurr1 (catalog no. sc-991; dilution, 1:250; Santa Cruz Biotechnology, Inc.) and monoclonal mouse anti β actin (dilution, 1:10,000; catalog no. A1978; Sigma-Aldrich; Merck Millipore), diluted in immunoblot buffer (TBS containing 0.05% Tween-20 and 5% non-fat dry milk). Then, the membranes were washed twice in 0.05% Tween-20 in TBS for 15 min and incubated with horseradish peroxidase (HRP)-labelled secondary antibodies; goat anti-mouse (1:5,000; cat. no. sc-2055; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Following extensive washes in 0.05% Tween-20 in TBS, the cells were incubated in ECL-Plus (cat. no. RPN2132; GE Healthcare, Chicago, IL, USA) for 5 min. The membranes were the exposed by X-ray film in darkroom. Densitometry analysis of the bands was performed by TotalLab version 1.10.

Statistical analysis. The statistical analysis in the present study was performed using SPSS version 16 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance followed by a Fisher's least significant difference post-hoc test was used to determine the statistical differences among groups. P<0.05 was considered to indicate a statistically significant difference compared with the LPS-treated group without Migri-Heal[®].

Results

Effect of Migri-Heal[®] on NO production in LPS-induced glial cells. To investigate the possible effect of Migri-Heal[®] in modulation of immune functions of glial cells, the present study examined the effects of 25-300 μ g/ml Migri-Heal[®] on 10 μ g/ml LPS-induced production of NO in rat primary mixed glial cell cultures. Glial cells were stimulated with 10 μ g/ml LPS and after 1 h, the cells were treated with different concentrations of Migri-Heal[®]. The findings revealed that Migri-Heal[®], had no effect on glial cells treated with LPS and there was no reduction in nitrite release of these cells (data not shown). Then, primary mix glial cells were exposed to various concentrations of Migri-Heal[®] and after 1 h were treated with 10 μ g/ml LPS. Migri-Heal[®] at 150 μ g/ml significantly reduced NO which was produced by cells in a dose-dependent manner. Migri-Heal[®] inhibited the LPS-induced production of NO with

a U-shaped concentration-response effect. As presented in Fig. 1A, the maximal inhibition of NO secretion was observed at $150 \ \mu g/ml$ of Migri-Heal[®].

Assessment of toxicity of Migri-Heal[®] on glial cell viability. In order to assess the toxicity of the Migri-Heal[®], cell viability was investigated using MTT assay. Formation of formazan crystals in treated glial cells with different concentrations of Migri-Heal[®] revealed that this compound had no cytotoxic effect on glial cells in different doses and particularly when used at a dose of 150 μ g/ml (Fig. 1B).

Effect of Migri-Heal[®] on iNOS and TNF α expression in glial cells treated with LPS. iNOS and TNF α have essential roles in the progression of inflammatory processes. The present study evaluated the effect of different concentrations of Migri-Heal[®] on the expression of the iNOS and TNF α genes in LPS-treated glial cells. The expression of iNOS and TNF α was markedly increased when compared with controls following treatment of cells with LPS. For the iNOS expression, cells were pretreated with various concentrations of Migri-Heal[®] (50, 100 and 150 µg/ml) (Fig. 2A and B) and with 50, 75, 100 and 150 µg/ml Migri-Heal[®] for evaluation of TNF α expression (Fig. 2C and D). Migri-Heal[®] 150 µg/ml was identified to have the maximum anti-inflammatory effect.

Effect of Migri-Heal[®] on LPS-induced NF- κ B in mixed glial cells. To investigate the molecular mechanism underlying the anti-inflammatory effects of Migri-Heal[®], the present study examined the its effect on NF- κ B, as a key transcription factors modulating the gene expressions of pro-inflammatory molecules, such as iNOS and TNF- α in glial cells (19). As presented in Fig. 3A, LPS treatment increased NF- κ B expression and Migri-Heal[®] decreased the NF- κ B expression. The present findings were confirmed by normalized values presented in Fig. 3B.

Expression of nuclear receptor related-1 (Nurr1) in the inflammatory process. Nurr1 exhibits different mechanisms that functions to resolve inflammatory responses. As presented in Fig. 4A, LPS treatment significantly increased Nurr1 and inhibited the NF-κB DNA binding. Therefore, the inhibitory effects of Nurr1 on NF-κB may be one of the factors contributing to the anti-inflammatory effect observed in LPS-stimulated glial cells. Migri-Heal[®] at 100 µg/ml and 150 µg/ml was able to significantly reduce Nurr1 expression in cells treated with LPS in comparison with LPS-only stimulated cells. These findings were confirmed by the normalized values Fig. 4B.

Discussion

Inflammation is a response of vascular tissues against aggressive agents such as pathogens, irritants, or damaged cells. It has been previously established that neuroinflammation in the CNS contributed to the pathogenesis and progression of several neurodegenerative diseases (4,5). Glial cells, such as astrocytes, microglia and oligodendrocytes, are more numerous than neurons in the CNS (20). Glial cells have an essential role in CNS and modulate homeostasis in the CNS. These cells were also activated by various inflammatory



Figure 1. Migri-Heal[®] inhibited production of NO in activated glial cells without toxicity. The glial cells were pretreated with different concentrations of Migri-Heal[®] and were stimulated with LPS after 1 h. After 48 h, nitrite concentration in the supernatant was assessed by (a) Griess reaction and (b) MTT cell viability assay. The LPS-stimulated glial cells exhibited increased NO levels in cell-conditioned media when compared with the control. Pretreatment of glial cells with Migri-Heal[®] (at 75-150 μ g/ml) significantly reduced NO production in the LPS-stimulated primary microglia. The MTT assay indicated that the inhibitory effect of Migri-Heal[®] on LPS-stimulated NO production was not due to cytotoxic action of Migri-Heal[®] on glial cells. The 150 μ g/ml Migri-Heal[®] treatment had the maximum anti-inflammatory effect. *P<0.05, ***P<0.001 vs. LPS 10 μ g/ml only group. Ctr, control; LPS, lipopolysaccharides.



Figure 2. Migri-Heal[®] inhibited expression of iNOS and TNF α in stimulated glial cells. Glial cells were pretreated with different concentrations of Migri-Heal[®], after 1 h glial cells were stimulated with 10 µg/ml LPS. After 48 h, total RNA was extracted from the treated cell cultures. cDNA was amplified by PCR for (a) iNOS, (c) TNF α and GAPDH. Densitometry analysis of (b) iNOS and (d) TNF α bands was performed using TotalLab software. The 150 µg/ml Migri-Heal[®] treatment had the maximum anti-inflammatory effect. ^{***}P<0.001 compared with to the group is treated with 10 µg/ml LPS. Ctr, control; LPS, lipopolysac-charides; iNOS, inducible nitric oxide synthase; TNF α , tumor necrosis factor α .

stimuli, which in turn lead to the production of cytokines and other pro-inflammatory mediators that contribute to neuronal damage (21). Hence, considerable efforts have been made to alleviate inflammation by targeting the glial cells through the introduction of novel therapeutic agents. There has been more interest in the development of novel therapeutic agents

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Figure 3. Migri-Heal[®] inhibited production levels of NF-κB in stimulated glial cells. Glial cells were pre-treated with Migri-Heal[®] and after 1 h 10 µg/ml LPS was added. (a) After 48 h incubation, the expression of NF-κB was examined using western blot analysis. (b) Densitometry analysis of the bands was performed using TotalLab. Migri-Heal[®] pretreatment significantly inhibited LPS-induced NF-κB expression. **P<0.01 and ***P<0.001 vs. LPS 10 µg/ml only group. NF-κB, nuclear factor-κB; Ctr, control; LPS, lipopolysaccharides.



Figure 4. Migri-Heal[®] inhibited production levels of Nurr1 in stimulated glial cells. Glial cells were pretreated with Migri-Heal[®] and after 1 h 10 μ g/ml LPS was added. (a) After 48 h incubation, the expression of Nurr1 was examined by western blot analysis. (b) Densitometry analysis of the bands was performed by TotalLab. Migri-Heal[®] significantly inhibited LPS-induced Nurr1 expression. **P<0.01 and ***P<0.001 vs. LPS 10 μ g/ml only group. Nurr1, nuclear receptor related-1; Ctr, control; LPS, lipopoly-saccharides.

that may selectively attenuate neuroinflammation, specifically through the inhibition of glial cell activation.

Migri-Heal[®] is an emerging drug and investigation on the underlying molecular mechanism remains to be fully elucidated; therefore, interpretation of the findings of the present study and comparison with previous studies are difficult. It is of note, that the molecular mechanisms underlying the anti-inflammatory effects of Migri-Heal[®] in glial cells are not fully understood. Therefore, the present study aimed to investigate the anti-inflammatory effect of Migri-Heal[®] on glial stimulated with LPS.

The effect of LPS stimulation suggests that the activation of the NF- κ B is a critical step in inflammatory pathways (22,23), which ultimately leads to the induction of iNOS and NO production (24,25). Increased production of NO and iNOS in monocytes during migraines occurs following the increase in NF- κ B activity. We previously reported that NO production was inhibited with Migri-Heal[®] (11,26). It was previously revealed that the aqueous extract fractions and the essence of these herbs may have a dose-dependent reduction of NO in the endothelial cell supernatant.

The essence analysis of Migri-Heal[®] by gas chromatography mass spectrometry and composition of aqueous extracts by high-performance liquid chromatography (27) indicated the chemical compounds in Migri-Heal[®]. The most abundant compounds in the essence of Migri-Heal[®] were monoterpenoids, specifically cineol and thymol. Additionally, the analysis of aqueous extracts of some plants in Migri-Heal[®] revealed that it is rich of melatonin (27). A previous study revealed 9 the role of melatonin in NO scavenging, inhibition of iNOS expression and inhibition of the NF-κB activity (28). Accordingly, the low levels of nocturnal melatonin in patients with migraines may be one of the possible reasons for the importance of melatonin in migraine therapy (29,30).

It is of note that Migri-Heal[®] significantly reduced the expression of NF- κ B, which is an important transcription factor in regulation of inflammatory pathways, iNOS and TNF- α which are two important target genes of NF- κ B in LPS-stimulated glial cells. Therefore, the present findings suggest that Migri-Heal[®] may exert beneficial effects on alleviation of inflammation.

Previous studies demonstrated that monoterpenoid compounds such as thymol and cineol inhibit expression of inflammatory factors such as NF- κ B and iNOS (31). The general effect of NO and iNOS inhibition were reported in some combinations of these herbs, including bromelain and *Nigella sativa* L. (17,32). Therefore, due to the high content of these compounds in Migri-Heal[®] they may be involved in underlying mechanism by which Migri-Heal[®] regulates inflammatory pathways. However, further studies are required to confirm these findings.

In response to over expression of NF- κ B, Nurr1 binds to the p65 subunit and blocks the activity of NF- κ B (33). There is evidence that the orphan nuclear receptor Nurr1 acts via a negative feedback mechanism, modulating NF- κ B activity and its target genes. It exerts anti-inflammatory activity by docking to NF- κ B-p65 on target inflammatory gene promoters. Subsequently, Nurr1 recruits the CoREST corepressor complex, which in turn leads to clearance of NF- κ B-p65 and transcriptional repression (33). The findings of the present study revealed that Migri-Heal[®] at concentrations of 100 and 150 μ g/ml may significantly reduce Nurr1 expression level when compared with cells stimulated with LPS.

In conclusion, it appears that Migri-Heal[®] may exert beneficial effects on inflammatory pathways and reduce cell damage; however, comprehensive studies, including *in vivo* assays with other signaling pathways, are required.

Acknowledgements

The present study was supported by the National Institute of Genetic Engineering and Biotechnology (grant no. 398).

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