

Original Article

The effect of Migri-Heal® on nitric oxide production in an in vitro inflammatory model of primary microglial cells

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Received: 23 May, 2016, Accepted: 6 September, 2016

Abstract

Background: Recently, much attention has been directed towards considering activated microglial cells as putative targets for treatment of neurological disorders. MigriHeal® as a novel herbal remedy was introduced for the treatment of migraine headaches. The previous researches has shown that MigriHeal® extracts can decrease NO in an in vitro inflammatory model. The aim of this study was to investigate the effect of MigriHeal® on NO generation from LPS- stimulated microglia cells. **Materials and Methods:** Neonatal rat primary microglial cells were isolated from the mixed glial cultures and the purity of the cultures was determined by immunocytochemistry. Microglial cells were pretreated with Migri-Heal® and activated by 1µg/ml LPS. Subsequently, NO levels in the culture supernatants were measured by a griess reaction. Our results showed that Migri-Heal® 50µg/ml significantly reduced NO level in inflamed microglia in a dose-dependent manner. **Results:** The results showed that different concentrations of Migri-Heal® had no prominent effect on cell viability in presence of LPS as compared with the control group. In addition, the pretreatment of microglia cells with Migri-Heal® can prevent from a morphological changes of the cells into the round and phagocytic shape. **Conclusion:** Our study demonstrated that MigriHeal® might have NO scavenging properties. Integrative studies are warranted to uncover the novel pharmacological insights of this herbal remedy as an putative therapeutic approach against diseases - associated with inflammation.

Keywords: inflammation, microglia, Migri-Heal®, nitric oxide

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Please cite this article as: Hassani M, Sabouni F, Emamgholipour S, Rafiee M H, Fallah M S, Abbasi Sh-S, Ansari M . The effect of Migri-Heal® on nitric oxide production in an in vitro inflammatory model of primary microglial cells . Arch Med Lab Sci. 2016;2(2): 54-61.

Introduction

Microglial cells - the smallest glial cells that constitute approximately 15% of the population of brain cells - are primary immune cells that regulate the innate immunity and participate in adaptive

immune responses in the neural tissue (1). These cells also are considered as specific macrophages which could phagocytose and protect neurons in the central nervous system (CNS).

Microglial cells , as a result of exposure to inflammatory stimuli and several pathological

conditions (such as injury and ischemia) can rapidly activate ,thereby amplifying, immigrating to injured site, phagocytizing cell cell debris and producing neurotoxic and neurotropic agents(2).

. Furthermore, it has been shown that proinflammatory and cytotoxic factors produced by microglial cells can induce neurodegeneration.

When neurons die, microglial cells become more active and change to phagocyte. The subsequent biological changes in active microglial cells can provide a complicated network of interaction between immune responses in CNS (3).

For example, microglial cells through the secretion of cytokines and prostaglandins can be considered as a signal for enhancement of inflammatory pathways is astrocytes. Furthermore, microglia activation could lead to release of inflammatory mediators ,thereby recruiting more number of microglial cells to the CNS (4).

Moreover, it has been demonstrated that chronic activation of microglia has been associated with various neurodegenerative diseases (5, 6).

For these reasons, much attention has been directed towards considering these cells as putative targets for treatment of neurological disorders.

Recently, based on anecdotal evidence found in traditional Iranian medicine MigriHeal® as a novel herbal remedy was introduced in order to treat migraine headaches. This drug has been patented by the Invention and Patent Registration Office of I.R. of Iran .Notably, thus far, there e is no report on adverse effects of MigriHeal® in investigation performed on animal models and human.

The previous researches in our laboratory have shown that MigriHeal® extracts can decrease NO in some inflammatory models (7, 8). Moreover, studies performed by our group demonstrated that some constituents of MigriHeal® decreased NO levels in endothelioma cell culture (9).

Against the background of these findings, investigating the effect of MigriHeal® on NO generation from microglia cells stimulated with LPS for the first time may be useful to illustrate other therapeutic aspects of this herbal remedy in the neuroinflammatory diseases.

Methods

Reagents

Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM) and Griess reagent were purchased from Gibco BRL (Grand Island, NY, USA). Bacterial LPS (E5:055), and MTT kit (M2128 500MG), Antibiotics including streptomycin and penicillin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). FITC conjugated anti-OX42 antibody to rat CD11b/c was from CALTAG (Sketty, Swansea). Dimethyl sulfoxide (DMSO) (1.02952.1000) was purchased from Merck and Migri-Heal® was prepared by Dr Mohammad Ansari.

Cell Culture

Primary microglial cells were isolated from fetal brain of a 1-3 day old, Wistar rat, as previously described (10). Briefly, the cells were cultured in DMEM, supplemented with 100 UI/ml penicillin G, 100 µg/ml streptomycin, and 20% FBS. Cells were seeded on polystyrene culture dishes (Nunc), and incubated in a humidified atmosphere with 5% CO₂ at 37°C. In order to obtain primary microglia-rich mixed cultures, all media and the isolated tissues were re-fed with fresh medium supplemented with 10% FBS.

Isolation of Microglia

After reaching confluence at 12-14 days , the culture flasks were gently shaken in order to isolate microglial cells (figure2). Then, the isolated microglia were divided into 24-wells plates (70.000 cells per well) and incubated under the previous conditions for 24 hours. In order to measure the purity of the cells, the Immunocytochemistry (ICC) was carried out by using the OX-42 antibody against the CD11b protein.

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Briefly, the cells fixed with PBS containing 4% paraformaldehyde, became permeable with PBS containing 0.2% Triton x-100, and a blocker solution of PBS containing 3% BSA was added. Finally, the antibody which is conjugated with FITC (diluted with blocking solution) to be added to the cells. After one hour in room temperature, the cells were seen with fluorescence microscope with green filter (figure 4).

Migri-Heal® Extracts

One hundred grams of Migri-Heal® powder were dissolved in 250 ml water, boiled for 10 minutes, and subsequently filtered using a filter paper. Finally, Extract solutions was dried using Freeze Dryer system.

One mg/ml of the dried Migri-Heal® extract was dissolved in DMEM and filtered by 0.22µm filter. This procedure was applied to prepare fresh Migri-Heal® extract for each time experiment.

Extraction of the Essential Oil

The essential oil was obtained from one hundred grams of Migri-Heal® powder using hydro distillation (4 hours) in a Clevenger-type apparatus. The essential oil yield was about 0.90% (v/w). The oil was dried with anhydrous sodium sulphate and stored at -20°C until use.

Gas chromatography mass spectrometer (GC-MS)

Essential oil from Migri-Heal® was analyzed by a Thermoquest Trace gas chromatography (GC) (USA) connected to a Thermolinnigan Trace mass spectrometer (MS), Ionization mode EI, mass range 37-457 m/z and interface and ion source temperatures 265 and 200°C, respectively. The device equipped with a 30 m × 0.25 mm × 0.25 µm DB-5MS (Agilent J&W Scientific). The oven temperature was programmed from 50°C, held 2 min, raised to 265°C at 2.5°C/min, and held for 20 min. The injector and detector temperatures were 265°C and 280°C, respectively. The carrier gas was helium at 1.5 ml/min and the split ratio was 1:100. The flow rate of the carrier gas (He) was 1.5 mL per min and the split ratio was 1:10. For the injection, 1 µl of essential oil diluted solution (1/100, v/v, in ethyl acetate) was injected manually in the split mode. Identification of the compounds was performed by comparison of their mass spectra with those of the Wiley libraries.

Cell Treatment with Migri-Heal®

The cells were treated with different doses of Migri-Heal® (25, 75, 100, 150 and 200 µg/ml) in fresh medium containing 1% fetal bovine serum (FBS) for one hour, and then stimulated with 1µg/ml LPS for 48 h. The control sample did not receive any treatment.

Nitrite Measurement

NO production from cultured glia was measured using the Griess nitrite assay (1, 11). Briefly, the 50 µl of cell-free supernatant from each sample and equal amount of freshly prepared Griess reagent were added in duplicate to 96-well flat-bottom microtiter plates. Then the samples were incubated for 15 min at room temperature.

The amount of nitrite was assessed by measuring its absorbance at 540 nm using a microplate reader (Labsystems Multiskan MS).

Cell Viability Assay

For the cell viability assay, MTT (3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyl-tetrazolium bromide) solution (1 mg/ml) was added in a volume equal to 10% of DMEM and incubated for 4 hours at 37°C in dark. Next, the solution was discarded and the formazan crystals formed within the cells were dissolved using DMSO (dimethylsulphoxide). The absorbance was measured at 540 nm using a microplate reader and the percentage survival of the cells, relative to untreated control cells, calculated. The experiments were run in triplicate and were repeated twice. It should be noted that, all condition was similar to the main experiments in current study.

Statistical Analysis

Statistical analysis of the study was performed by SPSS 16 software. One way ANOVA followed by the LSD test was used to determine the statistical differences among various cell groups. Values of P<0.05 were considered as significant.

Results

Growth of Primary Glial Cells

Proceeding growth of microscopic glial cells during 2 weeks was considered, the results of this work are as follows (figure 1). The primary mixed glial cells were cultured after 12-14 days.

Isolated microglial cell culture

After the cells reached an appropriate confluency (at 12-14 days), the flasks were gently shaken in order to remove the microglial cells.

Microglia cells were plated onto 24 well tissue culture plates (80000 cells/well) with the aim of confirming purity of microglia in the plates. The microglial cells were isolated by gentle shaking (figure 2). The adherent microglia cells were cultured

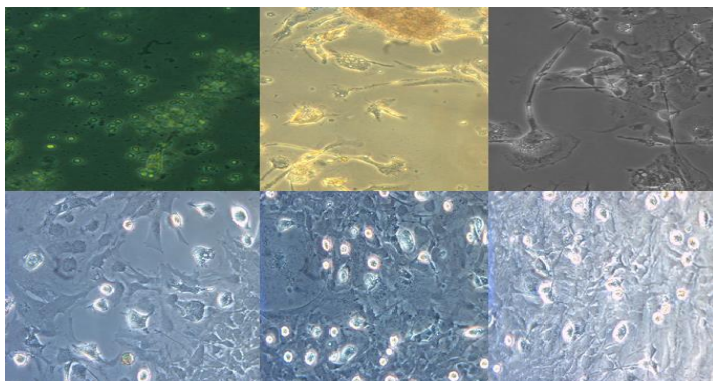


Fig. 1. Growth of glial cells using 2 weeks (A) 1st day, (B) 3rd Day, (C) 5th day, (D) 7th Day, (E) 10th day, (F) 14th day

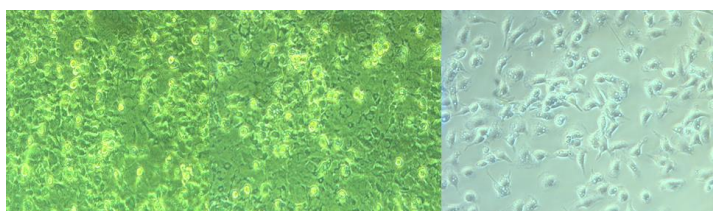


Fig. 2. Isolation of microglial cells. (A), (B) Microglial cells in a mixed glial and neuron culture after 2 weeks (arrows), (C) Purified microglial cells.

for 24 h, and the purity of the cultures was more than 95%, as judged with immunostaining with ICC method by using the OX-42 antibody against CD11b protein (figure 3).

Microscopic investigations of microglial cells exposed to LPS and Migri-Heal®

Using phase contrast microscope, we investigated the effects of LPS and Migri-Heal® on the cell morphology changes.

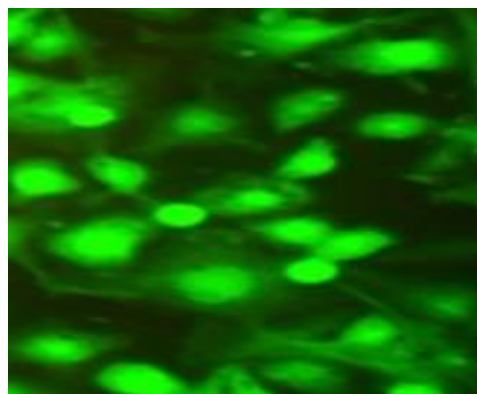


Fig. 3. Isolated microglia cells, immunostained with OX42 antibody against CD11b showed purity greater than 95%.

Accordingly, it was observed that microglia cells activated with LPS had amoeboid morphology (figure.4b) as compared with ramified shape in untreated cells (Figure.4a) as control group. Also, the morphology of the cells pretreated with Migri Heal® (figure.4c) had high similarity with control group. According to the figure 4c, effective concentration of Migri-Heal® on microglia cells is 50µg/ml (figure 4c).

Effect of MigriHeal® on lipopolysaccharide-induced NO production

As represented in figure 5, the cells pretreated with MigriHeal® following stimulation with LPS after 1h has decreased level of culture media nitrite in comparison with cells treated with LPS in dose - dependent manner.

Our results demonstrated that in primary rat microglia activated by LPS, 50µg/ml of Migri-Heal® has a strong inhibitory effect on NO production from cells (P<0.05) (Figure 5).

Analyses of Migri-Heal® by GC/MC

The results of the analysis with GC/MC on The essential oils obtained from the Migri-Heal® Shows that, this mixture, rich of monoterpenoids such as thymol and α 1,8-Cineole, that occupying a dominant part of the Migri-Heal®. Our results Indicates that the majority of the Migri-Heal® is 21/16%, α 1, 8-Cineole and 13/1%, thymol.

Evaluation of toxicity of Migri-Heal® concentrations

We performed MTT assay in order to investigate that possibility of the cytotoxic action of Migri-Heal® on decrease in production of NO.The results showed that different concentration of

Migri-Heal® had no prominent effect on cell viability in presence of LPS as compared with the control group (Figure 6).

Discussion

Inflammation is a phenomenon which has an important role in the immune system. Within the brain, inflammatory pathways are initiated by microglia cells, which function as the resident macrophages of the central nervous system (CNS) (12). Under normal conditions, microglia cells, are effective in the maintenance and performance of the neurons. These cells rapidly activate and similar to

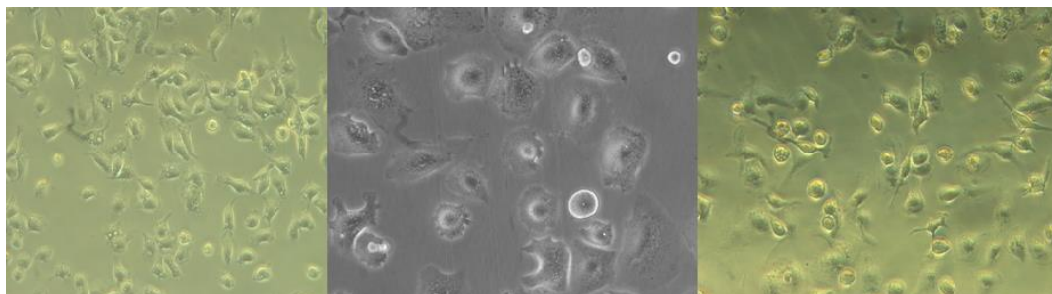


Fig. 4. Morphological changes of primary microglial cells. (A) Untreated microglial cells (negative control) which was ramified in comparison with LPS-treated microglial cells (B) LPS-treated microglial cells (positive control) which had amoeboid activated morphology (C) pretreated by 50 µg/ml Migri Heal®.(A,C Phase contrast [200×])(B Phase contrast [320×]).

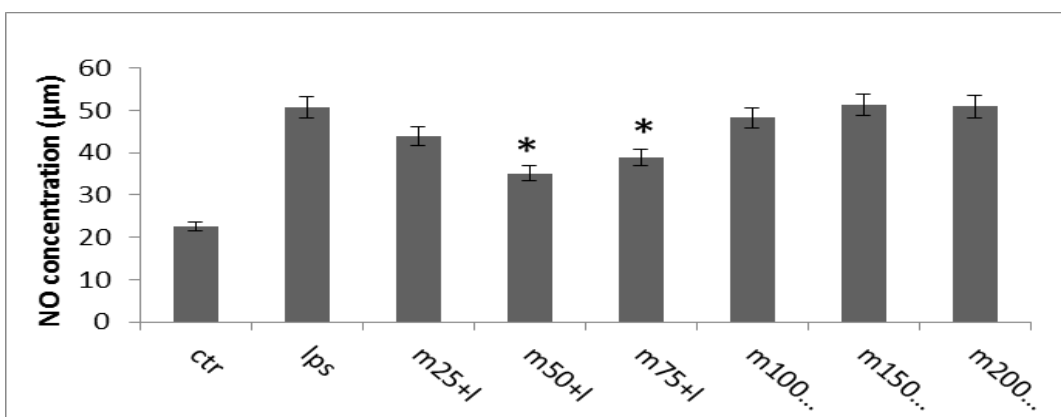


Fig. 5. Effect of Migri-Heal® in NO production in activated microglial cells. Microglia cells were pretreated with different doses of Migri-Heal®, after 1 h microglial cells were stimulated with LPS. After 48 hours, nitrite concentration (µM) in culture media was assessed by Griess reaction. Amount of 50µg/ml Migri-Heal® has been shown maximum effects of anti-inflammatory. * Means p <0.05 compared with the groups treated with LPS 1µg/ml

macrophage functions could phagocytose, secret pro-inflammatory cytokines and present antigens to immune system thus can be considered as functional cells in response to pathological conditions (3, 12, 13).

Activated microglia cells with production of various proinflammatory cytokines and free radicals such as NO, play a pivotal role in the process of neuroinflammatory diseases. The dysregulation of inflammatory responses has a central role in pathogenesis of multiple sclerosis, Parkinson’s disease, migraine, Alzheimer’s disease and even cerebral ischemia and traumatic brain injuries (for a review, see Ref. (14)]. The high production of NO from the activated microglia has been demonstrated in several lines of evidence (15, 16). Also, it has been shown that excessive amount of NO could lead to neurodegenerative disorders (5).

In accordance with the involvement of NO in migraine pathophysiology, Migri-Heal® reduces NO in patients who have decreased levels of melatonin (17-19). Interestingly, consistent with our finding that Migri-Heal® reduced NO levels in LPS-induced primary microglial cells, other studies performed by our group demonstrated that some constituents of MigriHeal® decreased NO levels in endothelioma cell culture (7, 8).

Since increasing evidence is highlighting the emerging role of dysregulation of function of endothelium system and microglia cell in pathogenesis of neurological disorders, particularly migraine, neuroinflammatory diseases, stroke and neurodegenerative diseases, it can be deduced that Migri-Heal® through the regulation of these cells might be considered as a potential therapeutic approach, although further in vivo and in vitro studies

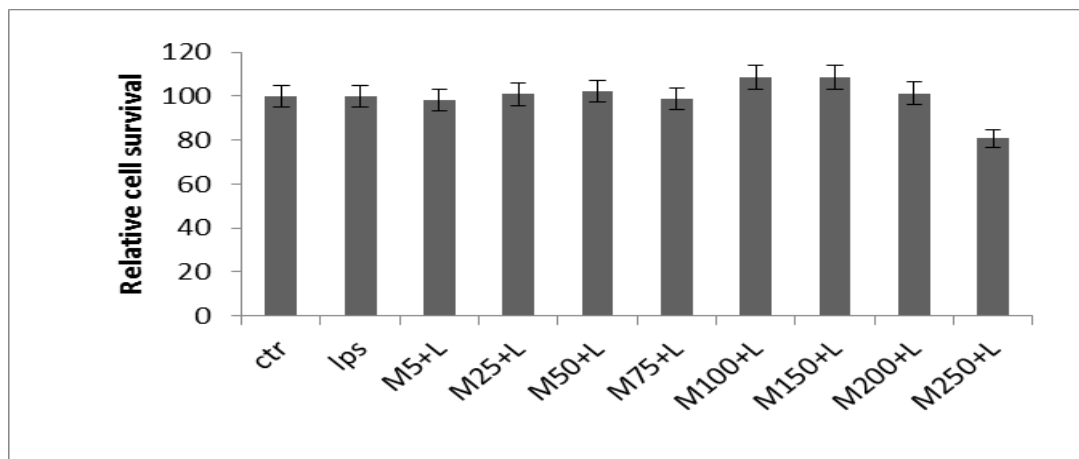


Fig. 6. Effect of Migri-Heal® on cell viability viability after 48 hours. different concentration of Migri-Heal® had no prominent effect on cell viability in presence of LPS as compared with the control group. Means $P < 0.05$ compared with the group treated with LPS $1 \mu\text{g/ml}$. M: melatonin, L:LPS

are needed in this regard.

Additionally, our result regarding the effect of the effects of MigriHeal® on basal and lipopolysaccharide (LPS)-induced NO levels are also consistent with recent study from our group ,which showed that hot water extract and essential oil of MigriHeal® can be suppressed the LPS induced NO in a dose-dependent manner in Raw 264.7 cells (9).

However, in contrast to Rafiee Kh et al. (9), the highest inhibition of NO generation by MigriHeal® treated samples vs. LPS alone occurred in $50 \mu\text{g/mL}$ concentration during 48 h.

The analysis of Migri-Heal® by GC/MS showed that ®, 1,8-cineole and thymol are constitute, two major and principal compounds in Migri-Heal® .It has been illustrated that 1,8-cineole can inhibit antigen confliction - induced airway inflammatory symptoms in guinea pigs (20). It also suppressed arachidonic acid metabolism and reduced secretion of $\text{TNF-}\alpha$, $\text{IL-1}\beta$, LTB_4 and TXB_2 from human blood monocytes (21, 22).Furthermore, Juergens et al. reported this element could decrease cytokine production from LPS-stimulated monocytes and lymphocytes (23).

Recently,it has been demonstrated that thymol suppressed expression of inducible nitric oxide synthase (iNOS) in a dose –dependent manner in mouse mammary epithelial cells (24).

Antioxidant and anti-inflammatory properties of thymol have been reported in human neutrophils

and cell-free systems (25).

In view of the low level of nocturnal melatonin in migraine patients during night in comparison to those in those in healthy subjects (18),on the other hand ,and the putative function of melatonin as a scavenger of free radicals such as NO from biological environment (26), on one hand ,it is plausible that the effectiveness of MigriHeal® therapy may be associated with high concentration of melatonin in some medicinal plants used for preparation of MigriHeal® (27).

Also, it should be noted that the usefulness of MigriHeal® can be related to the combined action of secondary plant compounds.

Although, the findings from the current study, together with previous investigations, may open novel therapeutic avenue for diseases that associated with disturbed nitric oxide homeostasis ,more investigations are required to clarify exact mechanism.

Also, the results of this study as well as previous reports showed that LPS stimulation of microglia leads to a morphological change of the cells into the round and phagocytic shape, and increase the production of NO (28, 29). The current study pointed up the pretreatment of microglia cells with Migri-Heal® can prevent from a morphological change of the cells into the round and phagocytic shape.

Collectively, our study demonstrated that MigriHeal® may has NO scavenging properties. Integrative studies are warranted to uncover the novel

pharmacological insights of this herbal remedy as a putative therapeutic approach against diseases - associated with inflammation.

Conflicts of Interest

There is no conflict of interest.

Acknowledgment

Thanks and appreciation from the National Institute of Genetic Engineering and Biotechnology, which funded the project [Grant No. 398].

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