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Original Research

Evaluating nitric oxide produced by rat inflamed microglial cell Lline, CHME-5, under the effect of IMOD

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Abstract: Nitric oxide (NO), as a free radical, is produced by inflamed microglia cells and is one of the destructive factors of the immune system and a factor in myelin degradation. Therefore, inhibition of microglia activity is a chief strategy in reducing neurotoxic damage to the central nervous system. In this study, an herbal Immunomodulatory Drug (IMOD) was used to evaluate the effects of this drug in controlling the amount of nitric oxide. Nitric oxide induction was performed by bacterial lipopolysaccharide (LPS) in rat inflamed microglial cell line, CHME-5. ELISA test was used to measure the produced nitric oxide at 24, 48, and 72 hours. The results showed that the high concentrations of IMOD (1.2, and 4% V/V) had anti-inflammatory effects on microglial cells and were able to reduce the amount of nitric oxide in these cells but the effective dose of IMOD was in the range of 1.2% V/V. Therefore, the safest dose and the best time for the effect of IMOD on inflammatory cell groups are 1.2% V/V and 72h, respectively. Hence, with further studies, IMOD can be considered as an herbal anti-inflammatory drug that is effective in controlling neurodegenerative diseases.

Key words: Central nervous system; IMOD; Microglia cell; Nitric Oxide.

Introduction

Throughout human history, medicinal plants have always had numerous uses in the treatment of diseases (1-3). Herbs have always been common to reduce inflammation caused by various complications, and recently, they have been considered as a solution to reduce the inflammatory symptoms of some inflammatory brain diseases such as Parkinson's, Alzheimer's, prion, and multiple sclerosis (4). The herbal drug IMOD has been able to attract the attention of many researchers in this field. IMOD is a mixture of *Urtica dioica*, *Tanacetum vulgare,* and *Rosa canina* with selenium and urea (5). It can reduce tumor necrosis factor activity, improve T helper lymphocytes in HIV-positive patients, and decrease oxidative stress. This drug is also effective in experimental models of immunoinflammatory-based diseases and reduces patient mortality rate in the Intensive Care Unit (ICU) without any adverse effects (6-8). Inflammation of the microglial cells is one of the most common diseases based on inflammation of the immune system (9).

Microglia is an immune cell located in the central nervous system, and it is responsible for regulating natural immunity and interfering with central nervous system immune responses. In the healthy adult brain, microglia is at resting form with low expression of surface antigens. They become active phenotypes in response to brain damage, ischemia, and inflammatory stimuli such as microbial signals, pathological proteins, extracellular ATP, and serum factors. Under these conditions, they can proliferate, synthesize nitric oxide (NO), migrate to the damaged site, phagocytose cell debris, and secrete diffuse factors such as cytokines, chemokines, trophic factors, and inflammatory mediators of E2, fatty acid E2, and fatty acid E2 (10, 11). Many microglia cell stimuli, a wide range of neurological damage and disease, are associated with iNOS induction and NO production (12). The induction of iNOS in microglial cells by LPS endotoxin derived from the wall of gram-negative bacteria has been suggested as the first potent stimulant of microglia cells in scientific papers. It is induced particular cytokines, such as interferon-gamma (INF-γ), tumor necrosis factor-alpha (TNF-α), or interleukin-one-alpha (IL-1α), which act through specific receptors on plasma membranes (13). Like other myeloid cells, microglia respond to LPS by releasing large amounts of cytokines, chemokines, Reactive Oxygen Species (ROS), proteases, and nitric oxide (NO). These effects are mediated by TLR4 receptors, members of the TLR family. The iNOS enzyme, which is responsible for NO synthesis in microglia cells, appears to be regulated by the NF-Kβ transcription factor during inflammatory responses (14). The binding of LPS to its receptor leads to destruction and immediate release of the inhibitor of this factor, IκB. The transfer of NF-Kβ into the nucleus regulates the expression of many genes involved in the inflammatory process, including iNOS. The synthesized nitric oxide as a result of this enzyme activity promotes inflammatory events (15). Nitric oxide, while acting as

a vasodilator and a neurotransmitter, is effective in regulating the inflammatory process at higher concentrations by forming peroxynitrite and nitrosylation of cellular signal messengers (16). Therefore, while NO has a physiological role in the neuronal cellular signal, its excessive synthesis causes neurodegenerative diseases (17).

In this study, the effects of different IMOD concentrations were investigated in 3 different periods on rat microglial cells. Therefore, reducing the inflammatory factor of nitric oxide by IMOD to achieve the most effective concentration was the most important goal of this study.

Materials and Methods

Microglial cell culture

In the present study, the CHME-5 microglial cell line of the rat was used as an inflammation model in the brain. The culture medium containing DMEM was prepared with 10% FBS, 100mg/ml streptomycin, and, 100u/ml penicillin. Cells were purchased from the AcceGen company (U.S) containing 5×10^6 cells per milliliter with a 96% survival rate. Also, to prepare 50ml of culture medium, 5.44ml DMEM, 5ml FBS, and 0.5ml Pen-Strep were used.

Cell counting

20 µl of the cell was taken and mixed with 20µl of trypan blue dye. Then, 20µl of the mixture was transferred to a Neubauer cell counting chamber, and the cells were carefully counted under Optika IM-5 inverted microscope.

Treatment of cells with LPS and IMOD drug

LPS solution (prepared by Sigma Pharmaceutical Company) with a concentration of 1µg/ml in DMEM medium was used to treat the cells. After preparing the required concentrations of IMOD drug (0.2, 0.4, 1.2, and 4% V/V), LPS was used to activate the cells and then they were treated with IMOD drug. Then the culture and adjacent process was performed for 24 to 72 hours.

Measurement of nitric oxide

ELISA test was used to determine the amount of nitric oxide in this study. A problem with this type of test is the presence of laboratory contaminants that affects the test results. Therefore, the control group without LPS and IMOD was also used to evaluate the presence of contamination. To measure the amount of nitric oxide, the ELISA reader (Microplate reader labsystem multiscan) was used at a wavelength of 580nm (18). The linear regression equation was measured based on the light absorption of the samples and the concentration of the samples was calculated. The accuracy of the Bioassay Technology kit was 0.55µM for measuring the amount of nitric oxide and the measuring range of the kit was 1 to 400 µM.

Statistical analysis

Analysis of variance (ANOVA) for the data was performed by SPSS (version 18) as a factorial experiment design with two factors of time (3 levels) and concentration (4 levels) in a completely randomized design. Tukey Multiple Comparison Test was used and the comparison between the levels of significant factors was done at a 95% probability level. Values with $P<0.05$ were considered significant.

Results

The aim of this study was to select the safest dose and the best time for the effect of IMOD on inflammatory cell groups, so by performing intragroup and intergroup analyzes, cell responses were examined over time. Statistical analysis showed that the amount of inflammatory factor (nitric oxide) decreases with increasing the concentration of IMOD. Also, based on multiple comparisons between treatments by Tukey test, a significant relationship was observed between the groups over time, however, for more than 48 hours of inflammation, the concentration of IMOD should be increased. The above results were observed again by multiple comparisons of the effect of treatment and time.

On the other hand, minimal amounts of nitric oxide produced in the control group without LPS and IMOD, indicate the absence of secondary laboratory contamination and no effect on test results. According to the data obtained from the ELISA test, the response of inflamed microglia cells affected by IMOD was recorded with 4 different concentrations of 0.2, 0.4, 1.2 and 4% V/V. First, descriptive statistics were obtained from the total data of time groups in 24, 48 and 72 hours, and four concentrations of IMOD drug were measured by ELISA kit and then other statistical analyzes were calculated by the software.

As can be seen in Figure 1, in three-time groups (based on the effect of IMOD concentrations), the highest mean for nitric oxide was related to the concentration of 0.2% V/V of IMOD and the lowest was related to the concentration of 1.2% V/V and 4% V/V. Also in Figure 2, the lowest metabolic activity of microglia cells belonged to those cells that received 0.2% V/V of IMOD and the highest metabolic activity of microglia cells belonged to cells treated with 1.2% V/V of IMOD. Therefore, according to Figure 2, it seems that at low concentrations of IMOD (0.2, 0.4% V/V), the cell survival rate is the lowest and has a significant difference with the control C+ group. With increasing drug concentration (2, 4% V/V), an upward trend in cell survival was observed and the amount of metabolic activity of cells was almost similar to the control group. Also, over

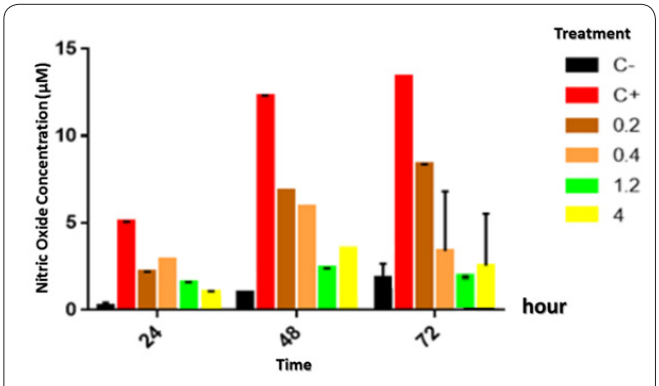

Figure 1. Bar chart of average and standard deviation for nitric oxide in different groups at 24, 48, and 72 hours, based on increasing concentration of IMOD ($p<0.05$).

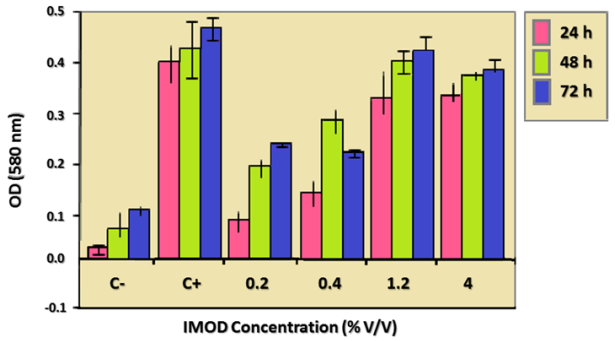

Figure 2. Metabolic activity of microglial cells treated with LPS and IMOD at three times: 24, 48 and 72 hours ($p<0.05$).

time, from 24 hours to 72 hours, the metabolic activity of cells has increased, which confirms the effect of time on better performance of the drug on cells.

In this regard, the experimental groups are as follows:

Group C- (healthy CHME-5 cells without LPS stimulation), Group C+ (cell CHME-5 stimulated with LPS), Group 0.2 (LPS-stimulated CHME-5 cells treated with 0.2% V/V IMOD), Group: 0.4 (LPS-stimulated CHME-5 cells treated with 0.4% V/V IMOD), Group 1.2 (LPS-stimulated CHME-5 cells treated with 1.2% V/V IMOD) and Group 4 (LPS-stimulated CHME-5 cells treated with 4% V/V IMOD).

Discussion

In this study, IMOD is proposed as an inhibitor for the iNOS gene. Because according to the results, this drug had anti-inflammatory properties in higher concentrations (1.2, 4% V/V) and reduced the amount of nitric oxide produced by inflamed microglia cells compared to the control group (Fig 2). Examination of the metabolic activity of cells at higher concentrations also indicates that the cells were able to maintain their survival so that at higher concentrations, the survival rate of cells is almost similar to the control group. On the other hand, each drug has a specific effective dose, so it seems that the effective dose of IMOD is in the range of 1.2% V/V to reduce the amount of nitric oxide. The other important factor in the metabolic activity of microglial cells is time treatment. The cell's activity has increased from 24h to 72h, which confirms the effect of time on better performance of IMOD on inflamed cells.

Various studies have shown that bacterial and viral products can stimulate microglial cells and can lead these cells from a resting state to an active state (19). This result was also demonstrated by bacterial lipopolysaccharide (LPS) in this project. Studies have shown that one of the most important factors involved in inflammatory responses is the nuclear factor NF-κB, which is involved in regulating the expression of the iNOS gene, and many microglial cell stimulators such as LPS trigger inflammatory responses by triggering the MAPKs signal cascade NF-Kβs (20, 21).

The hypothesis in this study is that the IMOD drug may inhibit the expression of the iNOS gene by inhibiting this signal pathway, especially the NF-Kβ factor, and thus inhibiting this signal pathway inhibits the production of nitric oxide.

Due to the increasing use of synthetic anti-inflammatory drugs and the numerous side effects of their use, replacing herbal medicines that can reduce or minimize inflammation will be the goal of effective treatment with minimal side effects. Many researches show that IMOD can be used as an herbal anti-inflammatory drug in both healthy people and people with the HIV virus (7, 22). Therefore, according to the results of other researchers and the current project, it seems that the use of IMOD as an herbal anti-inflammatory drug can be effective in controlling neurodegenerative diseases.

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