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Effect of β-D-Mannuronic Acid (M2000) on Oxidative Stress Enzymes’ Gene Using Healthy Donor Peripheral Blood Mononuclear Cells for Evaluating the Anti-Aging Property

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Abstract: Objective: This research aimed to study the anti-aging and anti-inflammatory effects of low and high doses of the β-D-mannuronic (M2000) on gene expression of enzymes involved in oxidative stress (including SOD2, GST, GPX1, CAT, iNOS, and MPO) in peripheral blood mononuclear cells (PBMCs) of healthy donors under in vitro conditions.

Methods: The PBMCs were separated and the RNAs were then extracted and the cDNAs synthesized, and expression levels of the mentioned genes were detected by qRT-PCR.

Results: Our results indicated that the high dose of this drug could significantly reduce the expression level of the SOD2 gene compared to the lipopolysaccharide (LPS) group (p < 0.0001). Moreover, it was found that the high dose of this drug could significantly decrease the expression level of the GST gene compared to the LPS group (p < 0.0001). However, no significant reductions were observed in expression levels of the CAT and GPX1 genes compared to the LPS group. Furthermore, our data revealed that the level of iNOS and MPO gene expression was significantly reduced, in both doses of M2000, respectively, compared to the LPS group (p < 0.0001).

Conclusion: This research showed that M2000 as a novel NSAID with immunosuppressive properties could modify oxidative stress through lowering expression levels of the SOD2, GST, iNOS, and MPO genes compared to the healthy expression levels, with a probable reduction of the risk of developing inflammatory diseases related to age and aging.

Keywords: Mannuronic acid, M2000, Anti-aging, Oxidative stress, NSAID, Aging.

1. INTRODUCTION

The production of free radicals and reactive oxygen species are unavoidable in biological systems, and the human body tries to neutralize their harmful effects by running the antioxidant defense mechanisms [1, 2]. In other words, the oxidative stress is caused due to an imbalance between the production of free radicals and of pro-oxidants and the antioxidant defense system [3, 4]. The majority of free radicals derived from oxygen during aerobic metabolism are called reactive oxygen species (ROS) and reactive nitrogen species (RNS). Free radicals damage biological molecules of tissues including lipids, proteins, and DNA [5]. The human body decreases the oxidative stress by producing antioxidants [1]. Antioxidants can prevent or repair damages caused by the activities of ROS and RNS and therefore, they are able to enhance immune defense and anti-aging process activity and reduce the risk of diseases incident [6-8]. The antioxidant systems are divided into two groups: enzymatic and non-enzymatic phenomenon. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and...
also glutathione s-transferase (GST) which are some of the most important enzymatic antioxidants [9, 10]. The SOD can act as the first line of defense against oxidative stress under physiological and pathological conditions [11]. Based on tissue distribution and differences in metal cofactor, four groups of SOD have been identified. Superoxide dismutase 2, mitochondrial (SOD2), also known as manganese-dependent superoxide dismutase (MnSOD), which is encoded by the SOD2 gene on chromosome 6q25.3 [11, 12]. The GST enzyme is induced in many organisms that are exposed to different stresses, including oxidative stress [13]. Cytosolic GST has a wide range of genetic polymorphisms in the human population. The GSTM1 in human is located on chromosome 1p13.3 [13]. In a study conducted for evaluating the effect of aging on the muscular skeleton of men and women, Fano found the role of aging effects of only one of the glutathione (GSH) isoenzymes entitled GST [14].

2. MATERIAL AND METHODS

2.1. Blood Collection and Isolation of PBMCs

The venous blood of twenty healthy volunteers (between 25-45 years, which were recruited at Blood Transfusion Center of Rafsanjan-Iran, after getting the written informed consent), collected on sodium heparin as an anticoagulant. The healthy volunteers were from no smoking group and without using illicit drugs or suffering from diabetes, hypertension or metabolic disease and they had no history of the chronic or autoimmune disease. After getting a blood sample, the peripheral blood mononuclear cells (PBMCs) were isolated through Ficoll-Hypaque (Meditech Cellgro) density gradient centrifugation fromuffy coats. The PBMCs were then resuspended in RPMI-1640 supplemented with 5% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, 2mM L-glutamine and 1 mM sodium pyruvate (all reagents provided by Gibco, USA). The cell viability was determined by trypan blue 0.4% (Sigma-Aldrich) with (1 part of cells: 4 parts of dye). The samples were only used when viability was > 95%. This experiment was approved by Ethics Committee of Rafsanjan University of Medical Sciences (RUMS).

2.2. Drug Preparation

Small molecule β-D-Mannuronic acid (M2000), patented (DE-102016113018.4), a novel non-steroidal anti-inflammatory drug with the immunosuppressive property was prepared in immunology section of pathobiology department of Tehran University of Medical Sciences (TUMS) by the modified method of Mirshafiey et al.

2.3. Treatment of PBMCs with M2000 and LPS

PBMCs were cultured in 4-well of culture plate with RPMI-1640 supplemented with 5% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, 2mM L-glutamine and 1 mM sodium pyruvate (all reagents provided by Gibco, USA). In this way, the first well contains 2×10^6 cells in the absence of LPS and M2000 (negative control) and second well contains 2×10^6 cells in the presence of LPS-EBS from E. coli O111: B4 (1 μg/ml) in order to stimulate PBMCs and absence of M2000 (positive control). The third and fourth wells of culture plate contain 2×10^6 cells and LPS-EBS (1 μg/ml) to which after 4 hours incubation at 37°C, d 5 μg/well of M2000 (the low dosage of the test well) and 25 μg/well of M2000 (the high dosage of the test well) were added. Cells were incubated for 18 hours at 37°C in humidified 5% carbon dioxide (CO2) incubator.

2.4. RNA Extraction

RNA was extracted from control and treated cells using Hybrid-R™ Mini kit (GeneAll, Republic of Korea) according to the manufacturer’s guidelines. The extracted RNA quality was identified by electrophoresis on the GelRed™ (Biotium, USA) contained agarose gel and measured its absorption on A260/280 nm by NanoDrop® ND1000 spectrophotometer (Isogen Life Science, the Netherlands).
2.5. CDNA Synthesis and Quantitative Real-Time PCR (RT-qPCR)

Total RNA from control and treated cells was reverse transcribed using HyperScript™ First-strand Synthesis Kit (GeneAll, Republic of Korea) based on the manufacturer’s instructions. The CDNA synthesis reactions included 10 µl total RNA, 1 µl Random hexamer, 1 µl dNTPs, 2µl Nuclease-free water, 2 µl RT reaction buffer (10X), 2 µl MDTT (0.1), 2 µl HyperScript Reverse Transcriptase (200 U/µl), 1 µl ZymAll™ RNase Inhibitor. The total reaction volume was 15 µl. The cDNA synthesis reactions included 10 µl total RNA, 1 µl Random hexamer, 1 µl dNTPs, 2µl Nuclease-free water, 2 µl RT reaction buffer (10X), 2 µl MDTT (0.1), 2 µl HyperScript Reverse Transcriptase (200 U/µl), 1 µl ZymAll™ RNase Inhibitor. The total reaction volume was 15 µl. Then, the cDNA was denatured at 50°C, 5 min at 85°C, and held at 4°C. Real-time PCR was performed using SYBR® Premix Ex Taq™ II (Dalian, Takara Co., Ltd), produced cDNA and appropriate primers (Table 1). Twenty microliters of real-time PCR reactions included 1 µl genomic template DNA, 10 µl SYBR® Premix Ex Taq™ II, 0.4 µl Rox, 7.6 µl nuclease-free water, 0.5 µl forward primer, 0.5 µl reverse primer. The following program was run on the ABI StepOne Plus real-time PCR system (Applied Biosystems Company, USA): one cycle of 95°C for the 30s, 70°C for 15s. Real-time PCR was carried out in optical 8-cap strips (MicroAmp® Applied Biosystems, Singapore) and the β-actin as housekeeping gene was used for normalizing the amplification. The relative amounts of PCR product were calculated using the 2-ΔΔCt method. The quality of graphs, melting curves and quantitative analyses of the data were performed using StepOne Software Version 2.2.2 (Applied Biosystems, USA).

2.6. Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software Version 6.02 (GraphPad Software, San Diego California USA; www.graphpad.com). Repeated measures one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons post hoc tests were utilized to analyze of genes expression between treated and untreated (control) group. The results were expressed as the mean ± standard deviation (SD). A p-value < 0.05 was considered to be a statistically significant difference.

3. RESULTS

3.1. Effects of M2000 on mRNA Expression of SOD2, GST, CAT, and GPX1

Our data demonstrated that the gene expression of SOD2 in PBMCs of healthy donors was decreased 62% ± 27 and 198% ± 5 by M2000 at a low dose + LPS and high dose + LPS respectively, in comparison to LPS group (340% ± 24) with p = 0.09 and p < 0.0001 (Fig. 1). The results also showed that the mRNA expression of GST in PBMCs of healthy donors was decreased 36% ± 10 and 124% ± 7 by M2000 at a low dose + LPS and high dose + LPS respectively, in comparison to LPS group (225% ± 17) with p = 0.08 and p < 0.0001 (Fig. 2). Our results indicated that the gene expression of CAT in PBMCs of healthy donors was decreased 60% ± 20 and 67% ± 14 by M2000 at a low dose + LPS and high dose + LPS respectively, in comparison to LPS group (329% ± 22) with p = 0.10 and p = 0.06 (Fig. 3). The results also illustrated that the mRNA expression of GPX1 in PBMCs of healthy donors was decreased 28% ± 23 and 41% ± 16 by M2000 at a low dose + LPS and high dose + LPS respectively, in comparison to LPS group (363% ± 20) with p = 0.64 and p = 0.33 (Fig. 4).

3.2. Effects of M2000 on mRNA Expression of iNOS and MPO

Our finding showed that the mRNA expression of iNOS in PBMCs of healthy donors was decreased 230% ± 16 and 233% ± 17 by M2000 at a low dose + LPS and high dose + LPS respectively, in comparison to LPS group (353% ± 29) with p < 0.0001 (Fig. 5). The results also indicated that the gene expression of MPO in PBMCs of healthy donors was decreased 63% ± 15 and 224% ± 9 by M2000 at a low dose + LPS and high dose + LPS respectively, in comparison to LPS group (363% ± 19) with p < 0.01 and p < 0.0001 (Fig. 6).

4. DISCUSSION

It has been proven that the oxidative stress, or elevated intracellular levels of ROS, plays an important role in the aging process. The oxidative stress theory of aging states that the ROS, as the primary result of normal mitochondrial metabolism, causes a progressive damage that is referred to as “aging” [26, 27]. In the aging process, the damage to
DNA, proteins, and lipids in human, mouse, and Drosophila melanogaster has been observed [27, 28]. The reduced antioxidant defense or increased oxidative stress shortens the lifespan [29]. The chronic oxidative stress and inflammation can be a starting point for many age-dependent diseases such as atherosclerosis, arthritis, Alzheimer’s, and Parkinson’s [30].

Fig. (1). The effects of M2000 on the level of SOD2 mRNA in PBMCs of healthy volunteers. The groups were untreated control, LPS-treated (1 µg/ml) and M2000 with two doses, low (5 µg/well) and high (25 µg/well). The SOD2 mRNA expression was measured by qRT-PCR using total RNA extracted from control and treated cells. Values were normalized to the β-actin content of the samples. The results were expressed as the target/reference ratio of the treated samples divided into the target/reference ratio of the untreated control sample and expressed as mean ± SD. **p < 0.0001 vs. LPS-treated group and ####p < 0.0001 vs. untreated control group.

Fig. (2). The effects of M2000 on the level of GST mRNA in PBMCs of healthy volunteers. The groups were untreated control, LPS-treated (1 µg/ml) and M2000 with two doses, low (5 µg/well) and high (25 µg/well). The GST mRNA expression was measured by qRT-PCR using total RNA extracted from control and treated cells. Values were normalized to the β-actin content of the samples. The results were expressed as the target/reference ratio of the treated samples divided into the target/reference ratio of the untreated control sample and expressed as mean ± SD. ****p < 0.0001 vs. LPS-treated group and ####p < 0.0001 vs. untreated control group.

Fig. (3). The effects of M2000 on the level of GPX1 mRNA in PBMCs of healthy volunteers. The groups were untreated control, LPS-treated (1 µg/ml) and M2000 with two doses, low (5 µg/well) and high (25 µg/well). The GPX1 mRNA expression was measured by qRT-PCR using total RNA extracted from control and treated cells. Values were normalized to the β-actin content of the samples. The results were expressed as the target/reference ratio of the treated samples divided into the target/reference ratio of the untreated control sample and expressed as mean ± SD. ****p < 0.0001 vs. LPS-treated group and ####p < 0.0001 vs. untreated control group.

Fig. (4). The effects of M2000 on the level of CAT mRNA in PBMCs of healthy volunteers. The groups were untreated control, LPS-treated (1 µg/ml) and M2000 with two doses, low (5 µg/well) and high (25 µg/well). The CAT mRNA expression was measured by qRT-PCR using total RNA extracted from control and treated cells. Values were normalized to the β-actin content of the samples. The results were expressed as the target/reference ratio of the treated samples divided into the target/reference ratio of the untreated control sample and expressed as mean ± SD. ****p < 0.0001 vs. LPS-treated group and ####p < 0.0001 vs. untreated control group.

The studies conducted in peripheral blood cells have shown that MnSOD gene expression in patients with rheumatoid arthritis (RA) is higher compared to those with inflammatory bowel disease (IBD). Therefore, MnSOD could be a notable gene involved in the development of RA. These results suggest that overexpression of MnSOD mRNA may be an RA-specific inflammatory response [31]. Several studies in animal models have reported the role of GST function in the prevention of chemical-driven hepatotoxicity.
GSTM1 null polymorphism is associated with chronic pancreatitis, leukemia, cancer, rheumatoid arthritis, and asthma. Bekris et al showed that environmental toxic agents and oxidative stress are of special importance in type 1 diabetes (autoimmune) and GSTM1 isoform may also be involved in this disease, as it has been proven that the loss of GSTM1 function is a benefit for type 1 diabetes because it reduces inflammatory responses [33]. The reduced CAT performance and increased levels of hydrogen peroxide have been observed in the epidermis of patients with vitiligo, a condition that leads to epidermal oxidative stress and ultimately causes damage to pigment cells [34]. Another study shows that CAT and GST activities reduce when they are exposed to RNS in vitro [35, 36]. Tanaka proved that the high glucose concentration in diabetes can cause intracellular accumulation of ROS in islets of Langerhans and thereby increase GPX function in the beta cells [37]. In a study conducted by Jang et al., the diabetic rats, under the influence of streptozotocin, presented a reduced GPX activity in the liver mitochondria and increased expression in pancreatic and renal mitochondria [38]. It has been revealed that, in the PBMCs of RA patients, we can show an increased gene expression of iNOS and an increased the formation of nitric oxide (NO), which is associated with the disease activity [39]. In addition, the overproduction of NO by iNOS is a mediator for apoptosis in RA joints [40] as well as a key regulator of Th1/Th2 balance in autoimmune diseases [41]. The MPO acts as the main catalyst of lipid oxidation on inflamed sites. This agent basically stimulates the process of formation and spread of plaque in atherosclerosis [42]. Choi (2005) and Green (2004) showed that MPO gene expression significantly increases in patients with Parkinson’s and Alzheimer’s, respectively [43, 44]. Thus, it can be more confidently claimed that this enzyme helps the improvement of oxidative damage in multiple chronic neurological disorders [43].

Based on our findings and existing data, the present research conducted to study the anti-aging and anti-inflammatory effects of low (5 µg/well) and high (25 µg/well) doses of M2000 on the gene expression of the enzymes involved in oxidative stress (including SOD2, GST, GPX1, CAT, iNOS, and MPO) in PBMCs of healthy volunteers under in vitro conditions. The results showed that the high dose of this drug can significantly reduce the SOD2 gene expression compared to the control sample and expressed as mean ± SD. "****p < 0.0001 vs. untreated control group.

M2000 has been tested as an anti-inflammatory and a novel immunosuppressive agent in various experimental models, such as animal models of multiple sclerosis, nephrotic syndrome, rheumatoid arthritis and immune complex glomerulonephritis [45-49]. The molecular mechanism of therapeutic efficacy of this novel patented (DE-102016113018.4) drug is based on its inhibitory effects on matrix metalloproteinase 2 and 9 activity, diminishing of immune cells infiltration in inflammatory foci, reduction of the level of inflammatory cytokine IL-6 and TNF-α a decrease in antibody production, and induction of apoptosis using fibrosarcoma cell line [50-54]. Based on our research on an extensive scale, mannuronic acid is a very safe anti-inflammatory and immunosuppressive drug and also, it has the lowest molecular weight with no gastro-nephrotoxicity, in comparison with conventional NSAIDs such as diclofenac [46, 49]. Moreover, the anti-aging efficacy of this novel drug was tested in an animal model, based on gene expression of enzymatic and non-enzymatic oxidative stress [55].

Based on our findings and existing data, the present research conducted to study the anti-aging and anti-inflammatory effects of low (5 µg/well) and high (25 µg/well) doses of M2000 on the gene expression of the enzymes involved in oxidative stress (including SOD2, GST, GPX1, CAT, iNOS, and MPO) in PBMCs of healthy volunteers under in vitro conditions. The results showed that the high dose of this drug can significantly reduce the SOD2 gene expression compared to the control-treated with LPS (Fig. 1). It was also found that the high dose of this drug can also considerably reduce the level of GST gene expression. However, no significant reduction was observed in CAT and GPX1 compared to the LPS group (Figs. 2-4). In addition, the study findings revealed that the level of iNOS and MPO gene expression was significantly reduced following the
treatment with M2000, compared to the control-treated by LPS (Figs. 5 and 6).

CONCLUSION

Many researchers, taking into account the enzymes involved in oxidative stress as the medicinal targets, have tried to improve the symptoms of age-related diseases and the control of the risk of progression of inflammatory diseases and aging using antioxidant-based drugs. The present study showed that M2000, as a novel NSAID with immunosuppressive properties, could modify the oxidative stress through lowering expression levels of the SOD2, GST, iNOS, and MPO genes to the healthy expression levels, with a probable in the reduction of the risk of developing inflammatory diseases related to age and aging.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This experiment was approved by Ethics Committee of Rafsanjan University of Medical Sciences (RUMS).

HUMAN AND ANIMAL RIGHTS

No animals were used in the study. The study was conducted under guidelines established by Helsinki manifest and it's later amendments or comparable ethical standards.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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