Advanced Pharmaceutical Bulletin

Adv Pharm Bull, 2024, 14(2), 419-425
doi: 10.34172/apb.2024.024
https://apb.tbzmed.ac.ir

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Original Article

Methylglyoxal Affects the Expression of miR-125b, miR-107, and Oxidative Stress Pathway-associated Genes in the SH-SY5Y Cell Line

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Article info

Article History:
Received: April 29, 2023
Revised: January 3, 2024
Accepted: January 7, 2024
epublished: January 13, 2024

Keywords:
Alzheimer's disease, Methylglyoxal, miR-125b, miR-107, Oxidative stress pathway

Abstract

***Purpose*:** Alzheimer's disease (AD) is the most prevalent form of dementia globally. Research links the increase of reactive oxidative species (ROS) to the pathogenesis of AD; thus, this study investigated the impact of methylglyoxal (MGO) on the expression of miR-125b, miR-107, and genes involved in oxidative stress signaling in SH-SY5Y cells.

***Methods*:** The MTT assay assessed MGO's effects on SH-SY5Y viability. miR-125b and miR-107 expression was analyzed via real-time PCR. Additionally, the Human Oxidative Stress Pathway Plus RT2 Profiler PCR array quantified oxidative pathway gene expression.

***Results*:** MGO concentrations under 700μM did not significantly reduce SH–SY5Y viability. MiR-125b and miR-107 expression in SH-SY5Y cells increased and decreased respectively ($P<0.05$). Cells treated with 700μM MGO exhibited increased CCS, CYBB, PRDX3, SPINK1, CYGB, DHCR24 and BAG2 expression ($P<0.05$). Those treated with 1400μM MGO showed increased CCS, CYBB, PRDX3, SPINK1, DUSP1, EPHX2, EPX, FOXM1, and GPX3 expression ($P<0.05$).

***Conclusion*:** MGO alters oxidative stress pathway gene, miR-125b, and miR-107 expression in SH-SY5Y cells. Targeting MGO or miR-125b and miR-107 may provide novel AD therapeutic strategies or improve severe symptoms. Further research should elucidate the precise mechanisms.

Introduction

SH-SH5Y cells are a suitable model for exploring the mechanism of neuron cell phenotype degeneration, including Alzheimer's disease (AD), due to the expression of Aβ, tau, synaptic factors, and other neuron-specific proteins. AD is characterized by memory deficits and brain dysfunction.[1] Reactive oxidative species (ROS) are implicated in the etiology of AD. Defective mitochondria produce extreme ROS and decrease ATP production. ROS are associated with membrane damage, cytoskeletal changes, and cell death. As a result, cognitive dysfunction may be caused by increased levels of ROS, which affect synaptic activity and neurotransmission.[2] Oxidative stress is associated with the deposition of β-amyloid (Aβ) plaques and an increase in free radical activity associated with plaque formation.[3,4] A cell's response to oxidative damage is strongly influenced by changes in the expression of antioxidant enzyme genes.[5] Habib et al. showed that catalase-amyloid interactions in neurotoxic Aβ peptides stimulate oxidative stress.[6] Transcription plays an essential role in regulating the functions of antioxidant enzymes. Therefore, alterations in antioxidant gene expression may lead to oxidative damage of the central nervous system in patients with AD.[5] If oxidative stress increases in AD, one would expect to see an increase in antioxidant enzyme activity and gene expression in AD patients.[7] Postmortem studies investigating the expression of antioxidant genes in the brains of AD patients have yielded conflicting results. Non-coding RNAs are called microRNAs (miRNAs), which regulate post-transcriptional gene expression by inhibiting translation or degrading target mRNAs.[8]

The key point is that miRNAs target numerous mRNAs and can therefore regulate different genes. In addition, studies have shown that a single mRNA can be regulated by multiple miRNAs.[9,10] These processes include metabolism, neurodevelopment, neuroplasticity, and apoptosis, which are fundamental to the functioning of the nervous system.[11] Several studies have shown the correlation between miR-125b and mir-107 expression changes and oxidative stress.[12-14] The expression of miR-107 was significantly decreased in patients with AD, whereas the expression of beta-secretase 1 (BACE1) was significantly increased. BACE1 expression is regulated by miR-107 via binding to its 3'-UTR in cell culture reporter assays.[11] Several studies have suggested that miR-107 may prevent Aβ-induced neurotoxicity and blood-brain barrier dysfunction.[15,16] The expression of miR-125b was significantly increased in patients with AD. Overexpression of miR-125b in neurons

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and mice induces tau hyperphosphorylation by targeting the phosphatases dual specificity phosphatase 6 (DUSP6) and protein phosphatase 1 catalytic subunit alpha (PPP1CA), whereas inhibition of miR-125b reduces tau phosphorylation and kinase expression.[12] According to a previous study, overexpression of miR-125b can induce apoptosis and hyperphosphorylation of tau in neurons through activation of CDK5 and p35/25. Therefore, this process may be mediated by miR-125b targeting forkhead box Q1 (FOXQ1).[13]

Methylglyoxal (MGO) is a highly reactive dicarbonyl compound. It is also considered an essential precursor for the non-enzymatic glycation of proteins and DNA, leading to advanced glycation end products (AGEs). The effects of MGO and MGO-derived AGEs on organs and tissues can be detrimental. MGO has been implicated in type 2 diabetes and other age-related chronic inflammatory diseases. These include cardiovascular disease, cancer, and neurological problems. As a by-product of glycolysis, MGO is detoxified under physiological conditions, mainly by the glyoxalase system.[14] It would be helpful to understand the pathogenesis of AD by identifying the mechanisms by which MGO affects miR-125b, miR-107, and genes related to the oxidative stress pathway. Therefore, we investigated whether MGO affects the expression of miR-125b and miR-107 and genes involved in oxidative stress pathways in SH-SY5Y cells.

Materials and Methods

Cell culture and treatment

Human neuroblastoma cells (SH-SY5Y) were provided by the Stem Cell Research Center of Tabriz University of Medical Sciences. Culture flasks containing DMEM/HG (Cat. No. 41965039; Gibco) and 10% (FBS; Fetal Bovine Serum Cat. No. 11573397; Gibco) were used to seed the cells. Antimicrobial treatment was performed with penicillin-streptomycin (Cat. No: 15140148; Gibco). Cells were incubated for 24 hours in a humidified environment of 95% air and 5% CO2 at 37 °C. SH-SY5Y cells were used to investigate the effect of MGO (Sigma-Aldrich, St. Louis, MO, USA) on the expression of miR-125b, miR-107, and genes involved in oxidative stress signaling. Cells were treated with different concentrations of MGO (200, 400, 700, and 1400 μM) for 24 hours. Cells were detached in 0. 25% trypsin-EDTA solution (Sigma-Aldrich, cat. no. MFCD00130286). SH-SY5Y cells at passages 3-6 were used for the assays.

Viability assay

MGO toxicity was determined in SH-SY5Y cells using the tetrazolium microculture (MTT) assay. SH-SY5Y cells were seeded at 4×10^3 cells/well in Falcon TM 96-well plates (Becton Dickinson Labware, Franklin Lakes, NJ). To determine the sensitivity of SH-SY5Y to MGO, cell viability was measured over a wide range of MGO concentrations (200-1400 μM) for 24 h. We used 700 μM MGO for subsequent gene expression profiling because this concentration had no apparent toxicity. The viability of SH-SY5Y cells was assessed using the MTT method. After adding 20 μM MTT solution to each well containing 200 μM medium, the cells were incubated at 37 °C for 4 hours.[14] Next, 50 μL dimethyl sulfoxide (DMSO) was added to each well and incubated for 30 minutes to stop the reaction. A microplate reader was used to measure the formazan produced by the cells at 570 nm. The percentage of absorbance of the sample cells divided by that of the control cells was used to estimate cell viability.

MiRNAs extraction and cDNA synthesis

The SH-SY5Y cells were seeded in a 6-well culture plate (4×10^3 cells/well) in 2 mL culture medium and then incubated overnight. The SH-SY5Y cells were then treated with MGO (700, and 1400 μM) and incubated for 24 hours. The SH-SY5Y cells were detached and washed. Total RNA was extracted using a miRNeasy Mini Kit (QIAGEN, Hilden, Germany). The quality of the extracted RNA was assessed using the Pico Drop system (model: PICOPET01, Cambridge, UK) at 260 and 280 nm. Before real-time PCR analysis, RNAs were reverse transcribed using a cDNA synthesis kit (TaKaRa, Japan) according to the manufacturer's instructions. The sequences of miR-125b and miR-107 primers are shown in Table 1.

Real-time polymerase chain reaction

Real-time PCR was used to determine the changes in miR-125b and miR-107 expression after cDNA synthesis. TaqMan miRNA assays were used to determine miRNA expression levels. The qRT-PCR was performed in a total volume of 10 mL with the following components 0.5 μL cDNA, 5 μL master mix (Ampliqon, Denmark, Cat. No. 5000850-1250), 0. 25 mL forward and reverse primers for the candidate gene, and four microliters diethyl pyrocarbonate (DEPC) water. The MIC system was used for real-time PCR. For miR-125b and miR-107 genes, the temperature program consisted of the following: initial denaturation (1 cycle at 94 °C for 3 minutes), denaturation (40 cycles at 94 °C for 10 seconds), annealing (40 cycles at 60 °C for 25 seconds), extension (40 cycles at 72 °C for 20 seconds), and final extension (one cycle at 72 °C for 5 minutes). The Pfaffl method was used to analyze the raw data, and the results were normalized to the housekeeping gene, RNU6B.

Table 1. Primer Sequences and Characteristics.

Gene name	Primer sequence
miR-125 b	Forward: CGAGCTCCCTCTCCTACCAAGCAG Universal Reverse: GACGCGTGTCCATGGATGGTTCTG
miR-107	Forward: 5'-GCCCTGTACAATGCTGCT-3' Universal Reverse: 5'-CAGTGCAGGGTCCGAGGTAT-3'
RNU6B	Forward: AAAATTGGAACGATACAGAGA Universal Reverse: AAATATGGAACGCTTCACGAA

PCR array

The expression of oxidative stress-related genes in SH-SY5Y was measured experimentally. The expression changes produced by the doses of MGO (700, and 1400 µM) were determined by real-time polymerase chain reaction in 24 hours. For expression experiments, 4×10^3 cells/mL were plated in six-well plates at the indicated dose with dilutions appropriate for the cells. MGO was not applied to the cells used as the control group. RT2 Profiler TM PCR Array Human Oxidative Stress Pathway Plus (Cat. No.: PAHS-065Y). To obtain real-time PCR arrays, RNAs from each group were extracted using an RNA kit and transcribed into cDNA (Takara, Japan, cat. no.: 4304134). The Light Cycler 480 system II (Roche) was used. To evaluate the expression of oxidative stress genes compared with the housekeeping controls, $2^{-\Delta\Delta CT}$ values (Light Cycler 480 quantitative software) were calculated (HPRT1, ACTB, GAPDH, B2M, and RPLP0).

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's post hoc analysis were performed using GraphPad Prism 8. 4. 2 to detect significant differences between groups. *P* values < 0. 05 were considered significant.

Results and Discussion

Cytotoxicity of MGO in SH-SY5Y

Recent advances in cell biology have contributed significantly to understanding the molecular mechanisms underlying AD. It is known that AD's molecular pathogenesis is complex and involves several theories or hypotheses in which multiple factors interact. However, these postulates cannot comprehensively explain the pathology, and further investigation is needed. Synaptic destruction, tau protein phosphorylation, inflammation, oxidative stress, apoptosis, and eventual neuronal cell death are evident in AD.[17] There is evidence that elevated serum MGO levels are associated with cognitive impairment.[18-20] In a mouse model of AD, aminoguanidine-scavenging MGO restored cognitive function, suggesting the importance of MGO in cognitive impairment.[21] In addition, high MGO levels may be associated with the cognitive decline associated with AD.[21] To determine the sensitivity of SH–SY5Y to MGO, cell viability was measured over a wide range of MGO concentrations (200, 400, 700, and 1400 µM) for 24 hours (Figure 1). There was no effect on cell viability after treatment with 200 µM followed by 400 µM MGO, indicating that MGO concentrations below 700 µM are not toxic to SH–SY5Y cells. The results of our investigation showed that different concentrations have different effects on the viability of SH-SY5Y cells. Furthermore, an escalation in the concentration of MGO does not induce toxicity in SH-SY5Y cells until a certain threshold is exceeded.

miR-125b and miR-107 gene expressions

miRNAs may be involved in the pathogenesis of AD by affecting different signaling pathways. Therefore, we investigated the effects of MGO on the expression of miR-125b, miR-107 and genes related to oxidative stress signaling in SH-SY5Y cells. Based on our results, MGO increased and decreased the expression levels of miR-125b and mir-107 genes, respectively, in SH-SY5Y cells ($P < 0.05$) (Figure 2). It has been demonstrated that the expression of

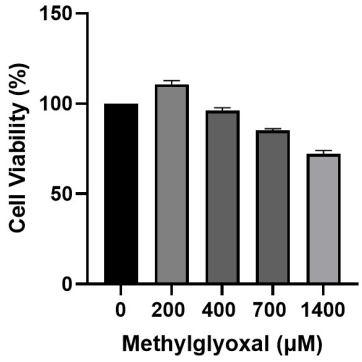

Figure 1. Effects of MGO on SH-SY5Y cell viability. MTT assay was used to determine the viability of SH-SY5Y cells after 24 h of stimulation with various concentrations of MGO. Data are presented as the mean ± SD of triplicate experiments

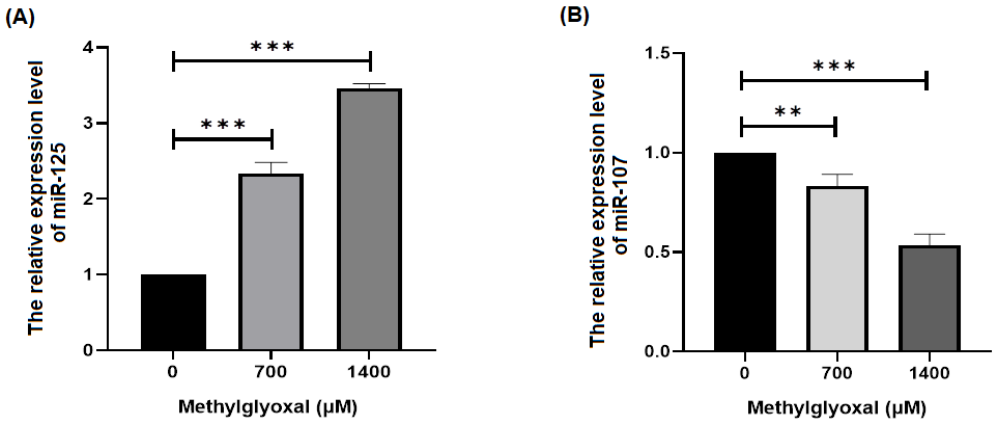

Figure 2. Differences in miR-125b and miR-107 expression between different doses of MGO in SH-SY5Y cells. This figure shows the expression of miR-125b and miR-107 in the SH-SY5Y cell line. (A) Rate of change in miR-125b expression between the groups (B). Rate of differences in miR-107 expression between the groups. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$

miR-107 is significantly decreased in patients with AD.[22] The results of our study suggest that MGO may decrease the expression of miR-107. Several miRNAs, such as miR-9, miR-124, miR-125b, and miR-132, are specifically expressed in the central nervous system.[23] Moreover, their dysregulation has been correlated with neurodegenerative diseases, including AD. Through SphK1, miR-125b regulates inflammatory factors and oxidative stress, thereby controlling neuronal growth and apoptosis.[24] miR-125b is highly expressed in AD and causes cognitive deficits[12] is associated with high levels of miR-125b expression and cognitive deficits.[13] This may increase the expression of miR-125b. It is known that the miR-125b gene plays a role in AD and can be stimulated by MGO. Therefore, analysis of miRNAs and genes associated with oxidative stress signaling pathways may contribute to a better understanding of AD pathogenesis.

MGO changed the expression of genes related to the Oxidative Stress pathway

The progression of AD has been linked to oxidative stress. ROS can modify lipids, DNA, RNA, and proteins in the brain.[25] The generation of ROS and reactive nitrogen species (RNS) can be attributed to both exogenous and endogenous sources.[26] Due to their high oxygen consumption, lipid content, and lack of antioxidant enzymes, neuronal cells are susceptible to oxidative stress.[27] Several studies have shown that oxidative damage to macromolecules and the accumulation of their products increase with time and that the relationship between ROS production and antioxidant activities (the enzymes superoxide dismutase, catalase, and glutathione peroxidase) is disturbed with age.[28-30] Unsaturated fatty acids and iron are abundant in the nervous system. The nervous system is susceptible to oxidative damage due to its high lipid and iron content. Oxidative stress is thought to be a major cause of the pathophysiology of AD.[31,32] Therefore, we investigated the changes in the expression of genes involved in oxidative stress, which may be important in AD. A PCR array was performed using SH–the SY5Y cells to investigate the effect of MGO on the expression of genes related to oxidative stress signaling. In addition, fold changes expression was determined using web-based RT2-based PCR array analysis (Figure 3). Differences in expression greater than twofold were considered acceptable limits (Table 2).

Our study showed that the expression of genes associated with the oxidative stress signaling pathway, such as CCS, CYBB, CYGB, DHCR24, PRDX3, AKR1C2, and SPINK1, was increased when SH-SY5Y cells were treated with MGO (700 μm). The expression of genes associated with oxidative stress signaling pathway such as AOX1, CCS, CYBB, DUSP1, EPHX2, EPX, FOXM1, GPX3, HSPA1A, MT3, PRDX3, PRDX6, SLC7A11, and SPINK1 increased when target cells were treated with MGO (1400 μm). In our study, MGO increased the expression of oxidative stress pathway genes in SH-SY5Y cells. The results of our study showed that the level of MGO concentration has a different effect on the expression of genes related to the oxidative stress signaling pathway. An increase in the level of MGO may have a greater effect on the expression of genes related to the oxidative stress signaling pathway. Different physiological functions are expressed by miRNAs in different brain regions, which influence the pathogenesis of AD. Whether miR-107 and miR-125b are gene regulators of oxidative stress metabolism in AD needs to be investigated. The role of other miRNAs may also be investigated. Furthermore, it is understandable that the limitations of cell lines in mimicking AD and the events that occur in AD strengthen the field for detailed investigations in animal models.

Conclusion

Our research examined the effects of MGO on SH-SY5Y neuronal cells by assessing the levels of miR-125b, miR-107 and related genes in the oxidative stress pathway. We found that MGO concentrations up to 700 μM did not adversely affect cell survival. The changes in miR-125b and miR-107 expression in the presence of MGO suggest their involvement in the cellular response to MGO. Furthermore, the expression of certain genes related to oxidative stress was modified by MGO at concentrations of 700 μM and 1400 μM, suggesting a dose-response relationship. These results highlight the importance of exploring the targeting of MGO, miR-125b, and miR-

Table 2. PCR array analysis of Oxidative Stress pathway-associated genes exposed to different concentrations of MGO compared with the control group

Gene	MGO (700 μM)		MGO (1400 μM)	
	Fold change*	*P* value	Fold change*	*P* value
AOX1	0.08	0.07	2.1	0.001
CCS	2.51	0.002	3.31	0.003
CYBB	15.14	0.0013	93.39	0.0001
CYGB	4.92	0.0027	0.80	0.012
DHCR24	2.13	0.003	0.84	0.01
DUSP1	1.40	0.003	5.45	0.003
EPHX2	1.91	0.01	2.69	0.001
EPX	1.44	0.02	2.34	0.0003
FOXM1	1.51	0.023	4.77	0.0001
GPX3	1.92	0.002	5.68	0.0001
HSPA1A	1.29	0.05	2.24	0.001
MT3	0.97	0.91	3.62	0.001
PRDX3	2.55	0.003	14.98	0.0002
PRDX6	1.04	0.64	7.54	0.0003
AKR1C2	2.66	0.001	1.28	0.02
SLC7A11	1.85	0.004	2.76	0.003
SPINK1	2.22	0.03	10.82	0.003

* A fold change of more than two was considered an acceptable value. Statistical significance was set at $P<0.05$.

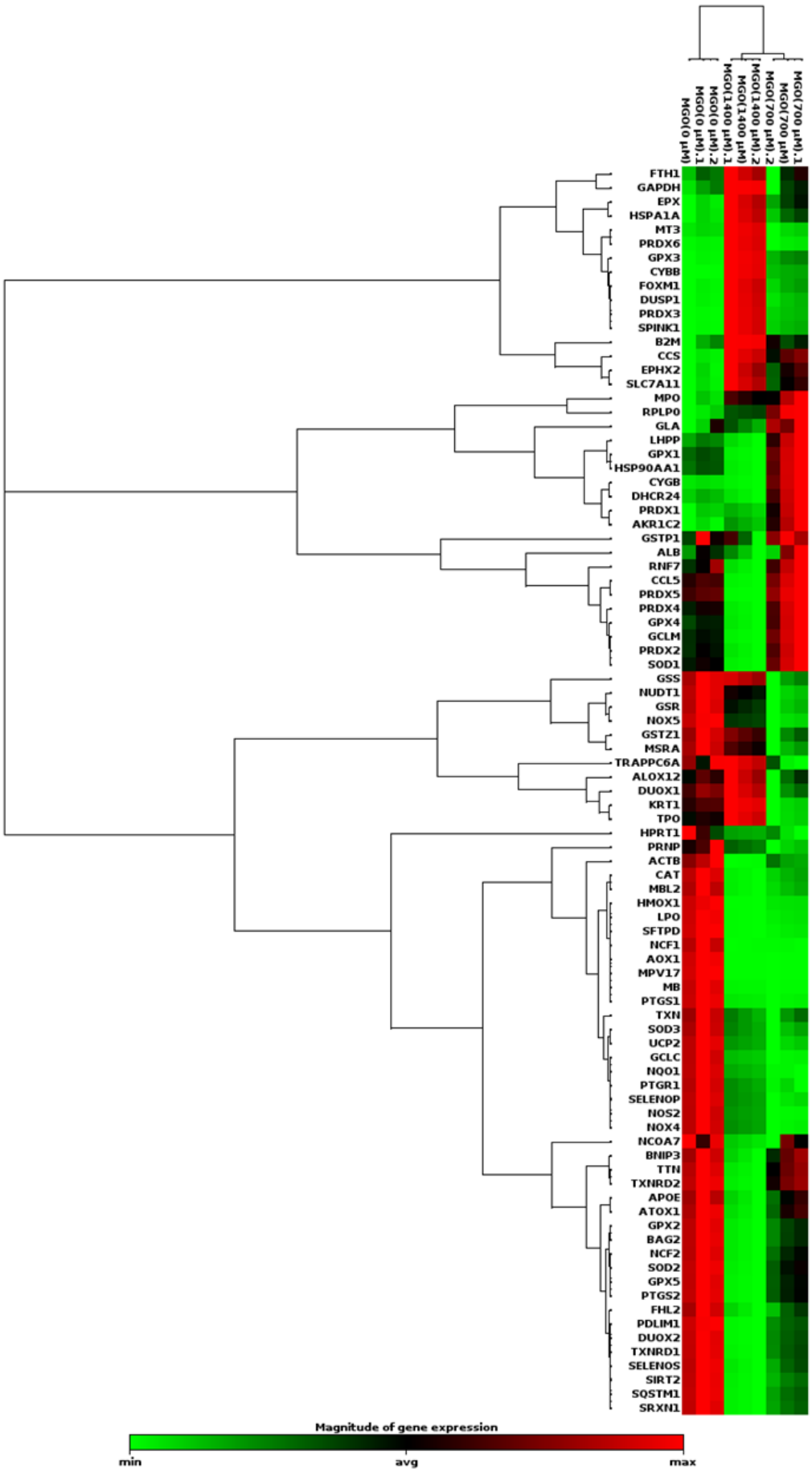

Figure 3. Clustergram analysis of oxidative stress pathway-associated genes after incubation with different concentrations of MGO in SH-SY5Y cells

107 as a potential therapeutic avenue for treating AD or alleviating its severe symptoms. Further research is needed to clarify the exact molecular interactions responsible for these observed effects and to confirm the viability of targeting MGO and miRNA regulation as a therapeutic intervention. Future research may lead to breakthroughs in the development of targeted treatments to combat oxidative stress and its role in AD.

Acknowledgments
The authors wish to thank the personnel of the Stem Cell Research Center of Tabriz University of Medical Sciences for their kindest help and guidance.

Authors' Contribution
Conceptualization: Alireza Nourazarian.
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Supervision: Alireza Nourazarian.
Visualization: Hadi Yousefi.
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Writing–review & editing: Alireza Nourazarian.

Competing Interests
The authors declare no conflict of interest.

Ethical Approval
The study protocol was approved by the Ethics Committee of Khoy University of Medical Sciences (IR.KHOY.REC.1400.011).

Funding
This study was supported by a grant from the Khoy University of Medical Sciences (IR.KHOY.REC.1400.011). Grant holder: Dr. Alireza Nourazarian.

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