Role of Epigallocatechin Gallate on *In Vitro* Model of Methylglyoxal-induced Amyloidogenesis

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Abstract

Introduction: The study aimed to investigate the potential effects of epigallocatechin gallate (EG) on the reflux of methylglyoxal (MG)-induced amyloidogenesis in human glioblastoma (U87) cells. **Materials and Methods:** The effective concentrations of MG and EG were investigated via Trypan blue test. Glyoxalase-1 (GLO-1), β-amyloid precursor protein (βAPP), and Caspase 3 (Cas 3) expression levels were determined by quantitative real-time polymerase chain reaction and intracellular glutathione (GSH) contents of cells were measured. **Results:** MG at 250 µM reduced viable cells by 33.4% as compared to control group. However, 5 µM EG pre-treatment before MG prevented 22.4% of the cell loss caused by MG ($P \leq 0.05$). MG stimulated βAPP and Caspase 3 levels by 4.13- and 3.46-fold; however, EG pre-treatment inhibited these increases by 1.76 and 3.09, respectively. In addition, EG pre-treatment increased GLO-1 levels by 3.71-fold and GSH levels by 2.30-fold according to MG group. **Conclusion:** EG demonstrated protective effect against cell death on U87 cells by suppressing amyloidogenic factors and apoptotic stimuli induced by MG.

Key words: Amyloidogenesis, epigallocatechin gallate, *in vitro*, methylglyoxal

INTRODUCTION

Alzheimer's disease (AD), one of the most common neurodegenerative disorders all around the world, is characterized by neuronal dysfunction, synaptic degeneration, senile plaques, and neurofibrillary tangles.[1] β-amyloid, formed by the proteolysis of amyloid precursor protein (APP), is the special feature of AD. Glycation is an endogenous process and leads to the formation of advanced glycation end products (AGEs). This process plays an important role in etiopathogenesis of various neurodegenerative diseases including AD. Methylglyoxal (MG) is the major precursor in the formation of AGEs and has been reported to found in high concentrations in the cerebrospinal fluid of AD patients.[2] MG and its glycation products, both stimulating extensive protein cross-linking and triggering oxidative stress, causes several violent pathological incidents, especially in nervous tissue.[3,4] Glyoxalase-1 (GLO-1) system consists of two enzymes: GLO-1 and GLO-2 and represents the most effective defense system for the detoxification of cytotoxic MG and MG-derived AGEs.[5]

Oxidative stress has been known to play an important role in the pathogenesis of various neurodegenerative diseases.[6] Normally, free radicals are produced as a result of cellular aerobic metabolism, and they are disposed of through the antioxidant system. However, overproduction of free radicals, involvement from environment, and imbalanced defense mechanism results with neurodegeneration in the brain.[7] Brain due to the lipid content of its cell membrane and low levels of antioxidant enzymes is more prone to oxidative stress.[8] Thus, in neuroscience research, approaches for the treatment of these diseases have mainly focused on the development of novel natural or synthetic antioxidants.[9] Of these antioxidants, dietary polyphenols and other natural antioxidants are the most popular compounds in clinical testing for the elimination of neurodegeneration.[10,11]

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Epigallocatechin gallate (EG) is the most important bioactive component of green tea, a plant containing catechins in vast amount. Green tea catechins and polyphenols show a strong antioxidant property by affecting the enzyme activities and a variety of transcription factors as well as their capacities of the removal of reactive oxygen species.[12] Recent studies show that green tea and its most important bioactive compound EG have also the neuroprotective effects in addition to the antioxidant capacity.[13-16] As a result, oxidative stress and neurodegenerative diseases are closely related to each other, and natural phenolic compounds with strong antioxidant capacities have a great importance for the treatment of such diseases. In the current study, we aimed to study the relieving effects of a natural polyphenolic compound, EG in MG-induced amyloidogenesis.

MATERIALS AND METHODS

Cell culture

U87 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (low glucose, Sigma) supplemented with 10% fetal bovine serum and penicillin/streptomycin 100 IU/ml and 100 mg/ml, respectively, and incubated at 37°C and 5% CO_2 in a 75 cm^2 tissue culture plates. Cells were digested with 0.25% trypsin and subcultured at 70-80% confluence. Exponentially growing U87 cells were used for all assays.

Treatments

Neuronal cells were cultured overnight at a density of 2.5×10^5/well in a sterile 12-well plate (Corning Inc., USA). To measure the concentration of exogenously applied MG and EG in the culture medium over time, medium containing 50-250 μM MG and 1-20 μM of EG (pre-treatment 1 h) were added; and the cells were incubated for 24 h. Following the incubation, medium was removed and the cells were washed twice with PBS (Ca^{2+} and Mg^{2+} free; pH 7.4), and viability was determined by MTT assay.

Trypan blue test

Cells were resuspended at amount of 1×10^5 cells/500 μl DMEM containing the H_2O_2 MG (250 μM) and EG (5 μM) incubated (24 h) in 24-well flat: Bottom plate. After the incubation time, 20 μL of cell suspension was removed and mixed with 20 μL of 0.4% Trypan blue solution (Sigma, USA) for 5 min. Live and dead cells were counted using a hemocytometer/microchamber using an automatic cell counter (EVE, South Korea).[17]

Homogenate preparation

After the incubation periods, cells were homogenized in ice-cold homogenization buffer (10 mM Tris, 1 mM EDTA, 25 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.25 M sucrose, and pH 7.4) containing complete protease inhibitor mixture (aprotinin, phenylmethylsulfonyl fluoride, leupeptin, sodium fluoride) (Sigma, Germany). Homogenates were centrifuged at 4°C, 13.000× g for 10 min and the soluble fraction was retained. The protein concentrations of cell extracts were measured by the Bradford reagent using bovine serum albumin as a standard.

Intracellular glutathione (GSH) levels

Intracellular GSH contents of glial cell homogenates were determined according to the method of Sedlak and Lindsay.[18]

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

qRT-PCR analysis was performed in a qPCR system (Bio-RAD, CFX96 Touch real-time PCR, South Korea). Total RNA from U87 cells were extracted using TRIZOL reagent (Sigma, USA) according to the manufacturer's instructions. 1 mg of total RNA was reverse transcribed in a reaction volume of 20 μl using reverse transcriptase kit (Fermentas, EU). 1 ml of each cDNA was used as templates for amplification using SYBER Green PCR amplification reagent and gene-specific primers. The human primer sets obtained from Thermo Electron Corporation (Germany): Caspase 3 forward: 5'-ACA TGG CGT CAT AAA ATA C-3', reverse: 5'-CAC AAA GCG ACT GGA TGA AC-3', β-amyloid precursor protein (βAPP) forward: 5'-TTA CTC GAG ATG CTG CCC GGT TTG GCA-3', reverse: 5'-GGA ATT CTG CAT CCA TCG CCC GAG CCG TCC AGG C-3' and GLO-1: Reverse: 5'-TGT GTC AGC TCA AGT GTA GCT TTC-3' forward: 5'-TGA GGA TAA AAA TGA CAT CCC TAA AGA-3' The amount of RNA was normalized to β-actin amplification in a separate reaction forward: 5'-CAT CGT CAC CAA CTG GGA CGA C-3', reverse: 5'-CGT GGC CAT CTC TTG CTC GAA G-3'. β-Actin was used as endogenous control, and each sample was normalized on the basis of its β-actin content.

Statistical analysis

The one-way analysis of variance and post hoc Duncan tests were performed on the data to examine the differences among groups using the SPSS statistical software package (SPSS 1 for Windows v. 20.0). The results are presented as average ± standard error. $P \leq 0.05$ was considered significant.

RESULTS

The present study performed to investigate the efficacy of EG on MG-induced amyloidogenesis. For this purpose,

human origin glioblastoma (U87) cells were used. According to Trypan blue test results as shown in Figure 1(a-c), MG at
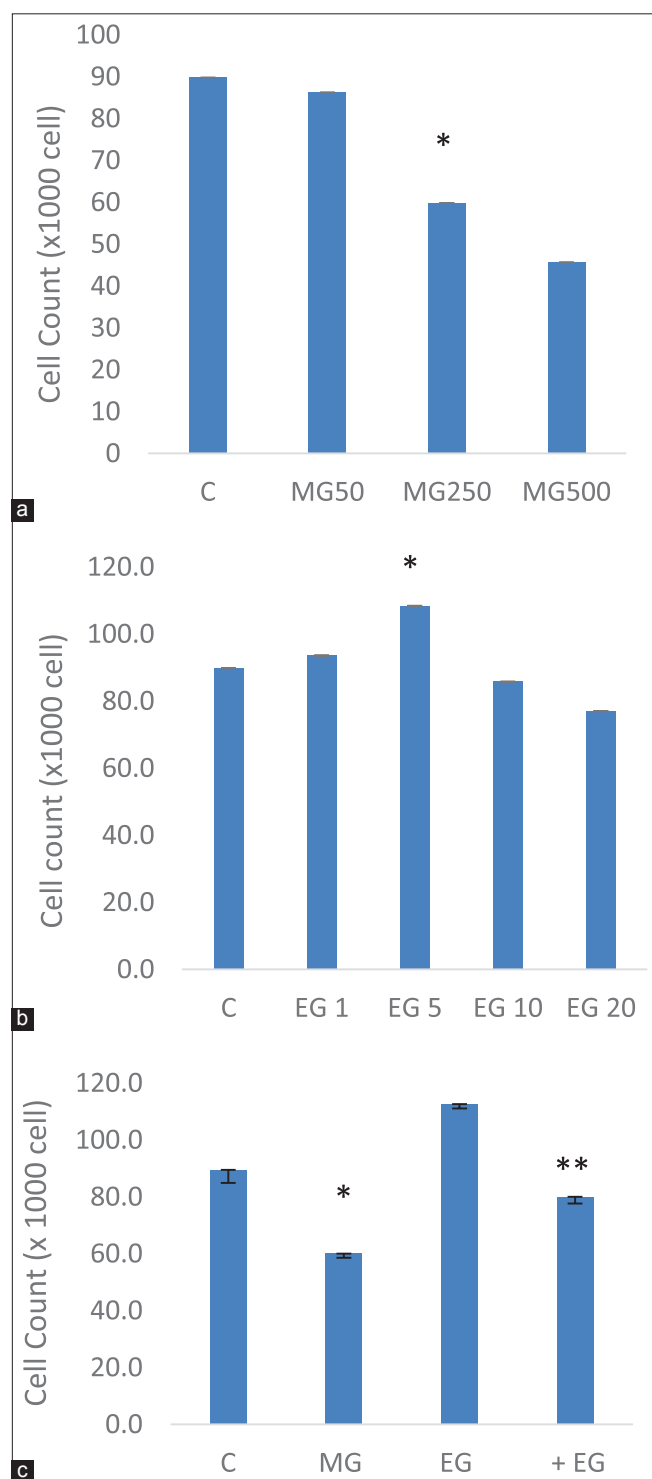
Figure 1: Results of methylglyoxal and epigallocatechin gallate on the viability of U87 cells. (a) Concentration-dependent effect of methylglyoxal on the cells. Cells were incubated for 24 h in the presence of various concentrations of methylglyoxal, (b) concentration-dependent effect of epigallocatechin gallate on the cells, (c) Effect of epigallocatechin gallate on the viability of U87 cells exposed with methylglyoxal. ($n = 6$). (*$P < 0.05$ versus control group; **$P < 0.05$ versus methylglyoxal-treated group)

different concentrations (50, 250, and 500 µM) was observed to decrease the cell viability as compared to the control group ($89.8 \pm 3.7 \times 10^3$, $P \leq 0.05$) and 250 µM of MG reduced the viable cell number by 33.4% ($59.8 \pm 5.2 \times 10^3$, $P \leq 0.05$). To investigate the effective concentration of EG on the cells, different concentrations (1, 5, 10, and 20 µM) of EG were analyzed and 5 µM of EG alone ($108.4 \pm 4.6 \times 10^3$, $P \leq 0.05$) provided 20.7% increase in the number of cells relative to control group [Figure 1b]. EG pre-treatment ($80 \pm 5.2 \times 10^3$, $P \leq 0.05$) was observed to prevent 22.4% of cell loss induced by MG [Figure 1c, $60 \pm 3.2 \times 10^3$, $P \leq 0.05$].

Expression levels of βAPP and Caspase 3 genes were determined using qRT-PCR technique. Obtained results showed that 250 µM of MG stimulated the expression levels of βAPP and Caspase 3 genes by 4.13- and 3.46- fold, respectively. However, 5 µM of EG pre-treatment was found to suppress this increase by 1.76- fold for βAPP and 3.09-fold for Caspase 3 according to MG group [Figure 2a and b]. Expression levels of GLO-1 were also searched using the qRT-PCR method. MG at 250 µM was found to decrease this gene expression by 2.38-fold as compared to control group and 5 µM of EG pre-treatment upregulated GLO-1 levels by 3.71-fold according to MG [Figure 2c]. Due to GLO-1 uses glutathione as cofactor in detoxification of MG and its end products, intracellular GSH levels were also investigated. MG treatment (1.97 ± 0.025, $P \leq 0.05$) lowered GSH levels by 2.92-fold in comparison with the control group [Figure 3, 5.76 ± 0.004, $P \leq 0.05$]. However, EG treatment (4.53 ± 0.007, $P \leq 0.05$) showed an increase of 2.30-fold according to MG group.

The results indicated that EG significantly reduced βAPP and Caspase 3 mRNA levels induced by MG [Figures 2a and b]. On the other hand, EG treatment upregulated the expressions of GLO-1 suppressed by MG [Figure 2c]. Results were obtained using qRT-PCR and expressed as a relative change of mRNA expression. Experiments shown are representative of three separate experiments with similar results.

MG group decreased GSH levels according to control group and EG pre-treatment group showed a protective effect on GSH levels in +EG group (*$P \leq 0.05$ versus control group; ** $P \leq 0.05$ versus MG-treated group).

DISCUSSION

Glycation and its end products produced endogenously in the metabolism play an important role in neurodegeneration, including AD. MG is a reactive dicarbonyl compound produced as the metabolite product of glucose. It mainly causes accumulation of AGEs that have been reported to have higher levels in samples of AD patients.[4,19] In addition, MG leads to cell damage by increasing redox parameters such as ROS and RNS and causing the formation of protein cross-linking.[20] For example, MG treatment of SH-SY5Y cells was observed to
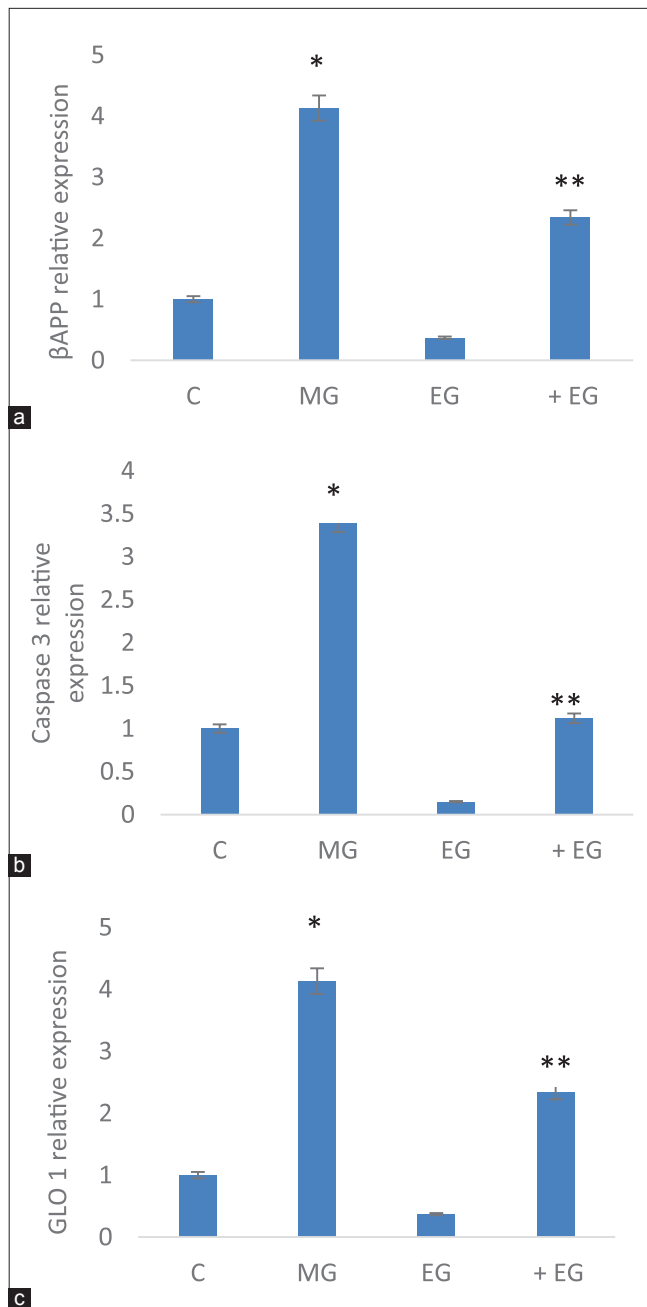
Figure 2: Quantitative real-time polymerase chain reaction analyses of β-amyloid precursor protein, Caspase 3 and Glyoxalase-1.

Figure 3: Effects of methylglyoxal and epigallocatechin gallate treatments on intracellular glutathione level of the cells. Methylglyoxal group decreased glutathione levels according to control group and epigallocatechin gallate pre-treatment group showed protective effect on glutathione levels in + epigallocatechin gallate group (*$P \leq 0.05$ versus control group; **$P \leq 0.05$ versus methylglyoxal -treated group)

Figure 4: The cell images of each group were photographed by 20-X lens after 24 h of incubation. (Scale bar 100 nm)

decrease cell viability, intracellular ATP, mitochondrial redox activity, and mitochondrial membrane potential and increase ROS production.[21] In addition, increasing concentrations of MG were reported to trigger apoptosis by decreasing Bcl2 levels and increasing proapoptotic Caspase 3 and Bax levels, and at the end lead to neurodegeneration.[22] In another study, the effects of tenuigenin were investigated on cultured hippocampal neurons. At the end of 24 h incubation time, 100 μM MG caused 49% decrease in the number of cultured hippocampal neurons, and median toxic concentration of MG was found to be 124 μM.[23] The present study aimed to investigate the potent neuroprotective effect of EG on
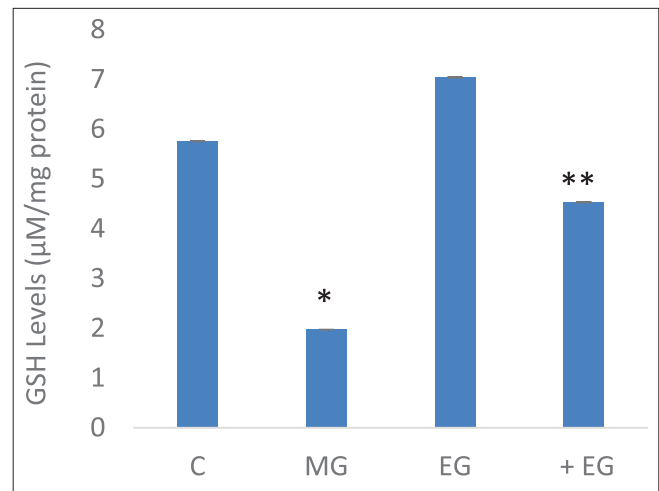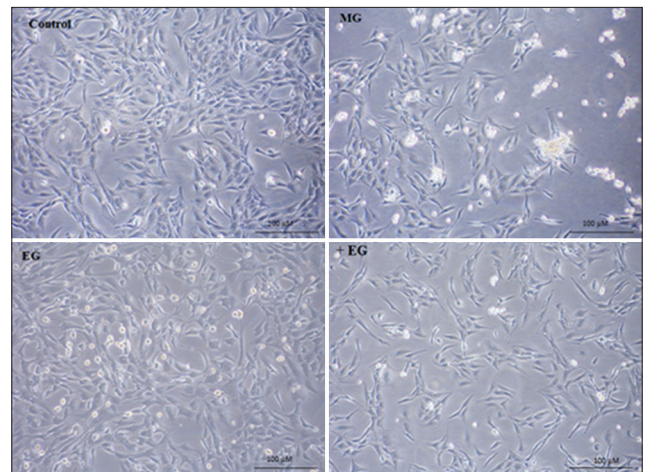
MG-induced amyloidogenesis of U87 cells, derived from human malignant gliomas. The results demonstrated that MG at different concentrations (50-500 μM) led to decrease in the number of viable cells at the end of 24 h incubation time. It was observed that 250 μM of MG significantly decreased cell viability and can be said to show sufficient neurotoxic effect on U87 cells (Figure 4).

Oxidative stress is known to be one of the main factors in neurodegenerative and age-related degenerative diseases. Various studies have shown that oxidative stress is a common feature in AD, and it contributes dysfunction or death of neuronal cells.[24,25] Therefore, a therapeutic feature of natural or synthetic antioxidants in neurodegenerative diseases has become an important issue recently. Green tea, because of its high polyphenolic content such as epicatechin (EC),

epicatechin gallate (ECG), epigallocatechin (EGC), and EG, has been tempted research attention for a varied number of disorders.[26] EG, the major component of green tea, is a flavonoid polyphenol and possesses strong antioxidant property due to hydroxyl groups on its aromatic structure. EG has been found to regenerate oxidative stress and show neuroprotective effects in a variety of studies. For instance, EG protected cultured rat cerebellar granule neurons from oxidative stress. However, the beneficial effect of EG is considerably dependent on its concentration.[27] EG at 10-50 µM concentrations significantly inhibited bupivacaine-induced apoptosis of N2a and SH-SY5Y cells and showed neuroprotective effect via suppression of ROS generation and modulation of PI3K/Akt signaling cascade.[28] In another study, the neuroprotective effect of EG on paraquat-induced apoptosis in PC-12 cells was investigated *in vitro*. EG at low concentrations significantly decreased the cell death caused by paraquat and showed protected cells by modulating mitochondrial function.[13] Similarly, in our study, we investigated the protective effect of EG at different concentrations (1-20 µM) on MG-induced cellular damage on U87 cells. 5 µM of EG was found to be the most effective concentration to protect cells from cell death induced by MG.

The cell images of each group were photographed by 20-X lens after 24 h of incubation (Scale bar 100 nm).

Most of the neurodegenerative diseases including AD are closely related with growing accumulation of protein aggregates known as amyloid plaque formation induced by oxidative and nitrosative stress in neuronal tissue. These plaques are mainly found as amyloid β (Aβ) protein fragments consisted of 36-43 amino acid residues resulting from the cleavage of APP.[29,30] MG and MG-derived AGEs are important in the etiopathogenesis of AD and known to be closely related with formation of neurofibrillary tangles and neuronal death in this disease.[31] Caspase 3 (Cas 3) is an important protein involved in the activation of intracellular apoptotic pathways. It has been shown that Caspase 3 expression increased in neurons and astrocytes of AD patients.[32]

As a strong antioxidant compound, EG has been reported to show neuroprotective effects against neuronal injuries.[33,34] Furthermore, EG has been reported to increase soluble APPα levels by increasing α-secretase activity in murine neuron-like cells.[35] In another study, EG ameliorated memory functions and brain α-secretase activity; on the other hand, it decreased β-and γ-secretase activities in mice.[16] In the present study, it was found that MG led to an increase in βAPP and Caspase 3 levels and showed neurotoxic effects on U87 cells. EG pre-treatment before MG decreased the expression levels of these genes induced by MG and indicated the protective effect on cell proliferation.

From the reactive dicarbonyl compounds, MG which is an α-oxoaldehyde produced endogenously by non-enzymatic or enzymatic degradation processes of sugars. AGEs are mainly formed by the modification of proteins with MG and known as an important marker in AD.[36] Cells possess protective systems including glutathione-dependent GLO-1 system to detoxify MG. GLO-1 system involves two Zn^{2+}-dependent enzymes (GLO-1 and GLO-2) together with the cofactor GSH and converts – oxoaldehydes to their corresponding hydroxy acids, for example, MG to d-lactate. Therefore, GLO-1 system makes the major contribution in limiting carbonyl stress-induced neurotoxicity, apoptosis, and the formation of AGEs.[4,37] Numerous studies show that green tea and its extracts are useful alternative for the moderation of oxidative stress and treatment of neurological diseases. For instance, EG has been reported to increase superoxide dismutase, catalase activities and reduce the amount of ROS, MDA, and protein carbonyls so that show the preventive effect on AGEs-induced neurotoxicity.[38] EG downregulated iNOS expression and decreased NO levels, prevented intracellular peroxynitrite accumulation and raised intracellular glutathione levels, so protected BV2 microglia cells from Aβ-induced cytotoxicity.[15] Herewith, we investigated the expression of GLO-1 in MG-induced amyloidogenesis model of U87 cells and neuroprotective effect of EG on the cells via glutathione level and GLO-1 system. In the literature, no research was found about the effect of EG on GLO-1 enzyme in amyloidogenesis. MG was observed to significantly decrease GSH levels and suppress GLO-1 expression; however, EG increased GSH levels and GLO-1 expressions in comparison with control group. Conspicuously, in +EG group, EG showed a noticeable increase in glutathione levels and significantly upregulated the expression of GLO-1. Thus, EG elevated GSH levels and replenished GLO-1 gene expression reduced by MG most probably due to its strong antioxidant capacity.

In short, the main bioactive component of green tea, EG, showed significant neuroprotective effects on MG-induced amyloidogenesis model of human origin glioblastoma cells via decreasing expression levels of βAPP and Caspase 3, increasing GSH levels and stimulating GLO-1 expression. Thus, it can be concluded that EG has preventive and therapeutic potential in amyloidogenesis. However, more extensive studies are required in this regard.

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