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Effects of gallic acid on expressions of MMP-2 and MMP-9 through the pathway of p38/JNK in C6 glioma cells

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Abstract: In our study, the effects of gallic acid (GA), a natural therapeutic agent, on oxidative stress biomarkers and MMP-2 and MMP-9 expressions via the p38/JNK pathway in C6 glioma cells were investigated. The toxicity of GA was determined by the WST-1 method. JNK, p38 and MMP-2/-9 mRNA expressions in the cell line were detected by RT-qPCR. JNK/SAPK, Grap-2/p38 and MMP-2/-9 protein levels were analyzed by using ELISA methods. Biochemical markers were analyzed. GA reduced the cell viability of C6 glioma cells after 24, 48 and 72h of treatment. The expression levels of MMP-2 and MMP-9 mRNA decreased in C6 glioma cells treated with 150µg/ml GA for 24 and 48h compared to the control cells. Unlike SOD activity, GA treatment significantly increased PCO and MDA levels in the cells treated with 150µg/ml GA for 24 and 48h compared to the non-treated cells. According to our results, GA inhibited the proliferation of C6 glioma cells. Also, it reduced MMP-2 and MMP-9 expressions and increase oxidative stress. Therefore, GA may have preventive effects on gliomas progression and/or invasion.

Keywords: Matrix metalloproteinase, oxidative damage, gallic acid, glioma, toxicity

INTRODUCTION

Glioma is the type of the most extensive aggressive and malignant primary brain tumor. Moreover, this tumor type, which accounts for the majority of brain cancer related deaths, is resistant to treatment and has poor survival (less than 2 years on average) (Tan *et al*., 2020). Gliomas are derived from glial cells and vary in type depending on the cells from which they originate (Oh *et al*., 2019). C6 glioma cell line has been mostly used in recent studies (Giakoumettis *et al*., 2018; Ersoz *et al*., 2020). Although advances have been made in the treatment of glioblastoma, no specific successful results have been observed.

Natural antioxidants have many biological activities. In nature, gallic acid (GA) is widely isolated from various plants, nuts and fruits (Bai *et al*., 2021). Recently, it has been reported that GA exhibits pharmacological properties. Studies have shown that GA may prevent neuroinflammation, oxidative stress and neuropathic pain in neurodegenerative diseases (Liu *et al.*, 2020; Kaur *et al.,* 2019). Besides, GA has protective effects against many types of cancer, such as the stomach, colon, prostate and breast cancer by inducing apoptosis, inhibiting cell proliferation and migration (Jiang *et al.,* 2021; Rezai-Seresht *et al*., 2019; Liao *et al*., 2018).

It is known that jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) signaling are associated with cancer. These signaling pathways affect the survival, proliferation, differentiation and migration of cancer cells (Wagner *et al*., 2009). Additionally, studies have reported that MAPK signal pathways including JNK and p38 are responsible for the regulation of matrix metalloproteinase (MMP)-2 and -9 (Aroui *et al*., 2016; Kang *et al*., 2019). These two MMPs are involved in the breakdown of the extracellular matrix (ECM) and thus cause invasion and metastasis in cancer diseases (Alrehaili *et al*., 2020). To date, although studies are showing the effect of GA on different brain tumor cells, there is no evidence of a relationship with JNK and p38 kinase regulated under cellular stress conditions.

Therefore, we aimed to investigate the effect of GA on C6 glioma cells. In this way, it was determined whether GA (i) had a cytotoxic effect on C6 glioma cells, (ii) contributed to the regulations of the JNK, p38, MMP-2 and -9 expressions and (iii) oxidative stress.

MATERIALS AND METHODS

Cell culture

C6 glioma cells (CCL-107, American Type Culture Collection, ATCC Rockville, Maryland, USA) were gifted from Demiroglu Bilim University. The cell line was cultured in Dulbecco's Modified Eagles Medium/ F12 medium supplemented with 5% fetal bovine serum (Sigma Aldrich, St. Louis, MO, USA) at 37°C in an incubator containing 5% CO_2.

Cell toxicity assay

The cells were counted and seeded at a density of $1x10^4$ cells/ml per well in a microplate. GA solved in culture medium was added at different concentrations and incubated for 24, 48 and 72h. Following the incubation,

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cell viability and cytotoxic effects induced by GA were detected by a WST-1 assay (Roche, Germany). According to this method, WST-1 reagent was added to cells and kept for 2 h in a humidified atmosphere. The absorbance of samples was measured using a spectrophotometer (BioTek, Winooski, VT, USA) at an absorbance of 450nm.

Expression of mRNA by quantitative reverse transcription PCR (RT-qPCR)

The C6 glioma cells at a density of $1x10^5$ were seeded in a 6-well plate and GA at 75, 100 and 150µg/ml concentrations were added on them for 24 and 48h. GA was not applied to the control cells. The obtained cell lysates were used in RNA extraction using a total RNA isolation kit (Hibrigen, Turkey) according to the protocol of the manufacturer. cDNA was synthesized from 250ng RNA using the high-capacity cDNA reverse transcription kit according to the instruction provided by the manufacturer (Applied Biosystems, CA, USA). An SYBR green real-time PCR kit (Hybrigen, Turkey) was used to quantify the expression levels of JNK, p38, MMP-2 and MMP-9 mRNAs in a CFX96 Touch Real-Time PCR detection systems (Bio-rad Laboratories Inc., CA, USA). Beta actin (β-actin) was used as a normalization control. Primers of JNK were 5'-GCAGCCGTCTCCTTTAGGT-3' (forward) and 5'-CATTGACAGACGGCGAAGA-3' (reverse), the primers of p38 were 5'-GAGCTTG TCCTG TCTTCTGG A-3' (forward) and 5'-CAGCGTAGG TGCGTGGATA-3' (reverse), the primers of MMP-2 were 5'-CA CCACCGA GGATTATGACC-3' (forward) and 5'-CACCCACAGTGGACATAGCA-3' (reverse), the primers of MMP-9 were 5'-CCTCTGCATGAAGA CGA CATAA-3' (forward) and 5'-GGTCAGGTTTAGAG CCACGA-3' (reverse) and the primers of β-actin were 5'-CTAAGGCCAACCGTGAAA AG-3' (forward) and 5'-TCTGGC GAGTCCATCACAAT -3' (reverse).

Enzyme-linked immunosorbent assay (ELISA)

To examine protein concentrations of JNK/SAPK, Grap-2/p38, MMP-2 and MMP-9, ELISA kits (Bioassay Technology Laboratory, Chine) were used according to the manufacturer's protocol. The absorbance of samples was read with a microplate reader at 450 nm (BioTek, Winooski, VT, USA). A standard curve was used for the calculation of sample concentrations.

Biochemical analysis

The cells at a density of $2x10^5$ were seeded in eight different flasks and GA at 75, 100 and 150µg/ml concentrations were applied to the cells for 24 and 48h, but not control cells. Cell lysates obtained as a result of sonication were used in the biochemical analysis. To estimate the levels of total oxidant status (TOS) and total antioxidant status (TAS) in C6 glioma cells, we used TAS and TOS Assay Kits (Rel Assay Kit Diagnostics, Turkey). The absorbance of samples was measured using a microplate reader at 450 nm (BioTek, Winooski, VT, USA). Oxidative stress index (OSI) was calculated by dividing the TOS level by the TAS level and expressed in %. Glutathione (GSH) level was determined colorimetrically based on the reaction with 5,5-dithiobis 2-nitrobenzoic acid (DTNB). Lipid peroxidation was assessed by measuring malondialdehyde (MDA) according to the thiobarbituric acid reactive substance (TBARS) method. Protein carbonyl (PCO) level in cell lysates was detected with Reznick and Packer's method (Reznick and Packer, 1994). Superoxide dismutase (SOD) activity was detected by the xanthine-oxidase method. The total protein content of the cells was analyzed using the Bradford protein assay (Bradford, 1976).

STATISTICAL ANALYSIS

GraphPad Prism software version 5 (La Jolla, CA, USA) was used for statistical analysis. The experiments were repeated≥3 times. For multiple comparisons of analysis, the non-parametric Mann-Whitney and Kruskal-Wallis tests were used. $p<0.05$ was considered as significant.

RESULTS

Gallic acid (GA) inhibited the proliferation of C6 glioma cells

Morphological changes were detected in C6 glioma cells after GA treatment with a phase-contrast microscope (fig. 1A-D). As shown in fig. 1E, due to the increase in GA concentration, cell viability decreased significantly after treatment with GA for 24, 48 and 72h, respectively. GA reduced cell viability by approximately 89%, 77% and 49% in 24h at concentrations of 10, 50 and 100µg/ml, respectively. Especially, with GA at a concentration of 50µg/ml and above, the cell viability was significantly decreased for 24, 48 and 72h ($p<0.05$ for all). However, the 48 and 72h incubation results were found to be similar in terms of cell viability. Because of this similarity in cell viability, the study was continued with 24 and 48h incubations.

Changes in the mRNA expressions of JNK, p38, MMP-2 and MMP-9

According to the findings of cell viability analysis, JNK and p38, MMP-2 and MMP-9 mRNA levels were examined in cell lysates obtained by applying GA at concentrations of 75, 100 and 150µg/ml to C6 glioma cells for 24 and 48h (fig. 2). While the JNK mRNA expression level in C6 glioma cells increased significantly with the administration of GA at a concentration of 75µg/ml for 48h compared to the control group, it decreased significantly with the administration of GA at a concentration of 150µg/ml ($p<0.05$ for all). The 24 and 48h incubation of GA at a concentration of 75µg/ml did not show a significant change in the p38 mRNA expression levels of C6 glioma cells compared to the control cells ($p>0.05$). However, with the 24 and 48h treatment of GA at a concentration of 150µg/ml, the p38 mRNA expression

level of C6 glioma cells was found to be decreased compared to the control, 75 and 100µg/ml GA groups (p<0.05 for all). The 24h incubation of GA at a concentration of 75, 100 and 150µg/ml significantly decreased the MMP-2 mRNA expression levels of C6 glioma cells compared to the control group (p<0.05). While 75µg/ml of GA applied to the cells for 48h did not make a significant difference in MMP-2 mRNA level compared to the control group (p>0.05), the administration of GA at concentrations of 100 and 150µg/ml caused a significant reduction in the level of MMP-2 mRNA expression (p<0.05). It was determined that the expression level of MMP-9 mRNA was significantly decreased in the group treated with GA at a concentration of 150µg/ml for 24h compared to the control cells and C6 glioma cells treated with 75 and 100µg/ml GA (p<0.05). The administration of GA at a concentration of 75µg/ml for 48h did not differ compared to the control group, similar to the 24h incubation (p<0.05). MMP-9 mRNA expression levels of C6 glioma cells treated with GA at concentrations of 100 and 150µg/ml for 48h decreased significantly compared to the control group (p<0.05).

Changes in the levels of JNK/SAPK, Grap-2/p38, MMP-2 and MMP-9 proteins

JNK/SAPK, Grap-2/p38, MMP-2 and MMP-9 protein levels in the cells are shown in fig. 3. JNK/SAPK protein levels in C6 glioma cells treated with GA at concentrations of 75, 100 and 150µg/ml for 24 and 48h showed a significant increase compared to the control group (p<0.05 for all). Similar to the JNK/SAPK protein results, the Grap-2/p38 protein level also increased in C6 glioma cells treated with GA at concentrations of 75, 100 and 150µg/ml for both 24 and 48h. Although no significant difference was observed in the MMP-2 protein level in C6 glioma cells treated with GA for 24 and 48h, a non-significant decrease was observed in the MMP-2 protein levels of the cells treated with GA compared to the control group (p>0.05). Similar results were found at the MMP-9 protein level. It was found that cells treated with GA at concentrations of 75, 100 and 150µg/ml for the 24 and 48h incubation period showed a non-significant decrease in MMP-9 protein levels compared to the control group (p>0.05).

Effects of GA on biochemical parameters

In C6 glioma cells, TAS, TOS and OSI values are shown in fig. 4. While there was no significant change in TAS values of C6 glioma cells treated with GA at a concentration of 75µg/ml for 24h (p>0.05), GA at concentrations of 100 and 150µg/ml significantly increased TAS value in C6 glioma cells compared to the control group and cells treated with 75µg/ml GA (p<0.05 for all). As a result of the 48 h treatment of GA to C6 glioma cells, it was observed that GA at concentrations of 75 and 100µg/ml decreased TAS levels in C6 glioma cells compared to the untreated (control) cells (p<0.05 for both). On the contrary, when the group given 150µg/ml GA was compared with the 75 and 100µg/ml groups, it was found that the levels of TAS in the group given 150µg/ml GA increased significantly (p<0.05 for both). The TOS level in C6 glioma cells treated with GA at concentrations of 75 and 100µg/ml for 24h showed a non-significant increase compared to the control group (p>0.05). The administration of GA at a concentration of 150µg/ml to the cells significantly increased the TOS level compared to the control group (p<0.05). Similar to the TOS levels, the OSI value showed a significant change only in the cells treated with GA for 24h, but not 48h. The OSI value was lower in cells treated with 100 and 150µg/ml GA for 24h than the control and 75µg/ml GA cells (p<0.05 for both).

As shown in fig. 5, GSH, MDA and PCO levels showed a significant increase in the cells treated with 150µg/ml GA for 48h compared to the control cells (p<0.05). MDA and PCO levels were higher in cells treated with 150µg/ml GA for 24h than in the other groups (p<0.05 for all). Unlike the level of MDA and PCO levels, GA treatment at concentrations of 100 and 150µg/ml for 24 and 48 significantly decreased the SOD activity in the cells compared to untreated cells (p<0.05 for all).

DISCUSSION

In the present study, we revealed that GA could induce the inhibition of cell proliferation and cellular invasion in C6 glioma cells. Lu *et al*. (2010) determined that GA (10-40µg/ml) decreased cell viability in U87 and U251n cell lines after 24h of treatment by MTT assay. Similarly, Hsu *et al*. (2016) demonstrated the cytotoxic effect of GA (20-60µM) on DBTRG-05MG human glioblastoma cells and CTX TNA2 rat astrocytes using the WST-1 test.

They reported that administration of GA at the indicated doses for 24h showed a cytotoxic effect on DBTRG-05MG cells, but did not affect CTX TNA2 cells. In another study, the cytotoxic effect of GA (5-200µg/ml) on MCF-7 human breast cancer cells for 48 and 72h was detected by the MTT test. As a result of this analysis, it was determined that GA decreased MCF-7 cell viability. However, the effect of GA on MCF-7 cells at 48 and 72h has been reported to be similar to the study by Rezaei-Seresht *et al*., (2019) our findings showed that the cytotoxic effect of GA on C6 glioma cells was similar at 48 and 72h. Therefore, our findings indicated that as in other cell lines, GA could efficiently inhibit the proliferation of C6 glioma cells depending on the concentration and/or the duration of treatment.

The Jun N-terminal kinase (JNK), one of the main protein kinases, a pathway is involved in many diseases, from dysfunctions of the immune and nervous systems to cancer. Studies have shown that high levels of JNK activity have been detected in some cancer cell lines (Bubici *et al*., 2014; Das *et al*., 2011). Kim *et al*. (2009) showed in breast and

stomach cancer cell line studies that SP600125, a JNK signaling pathway inhibitor, reduced the growth of cancer cells. Also, in a study using JNK inhibitors by Konno *et al*. (2015), it was suggested that this inhibitor may have the potential of a therapeutic drug for pancreatic cancer through the induction of differentiation. p38 is one of the main protein kinases that regulate many physiological processes in the cell like JNK.

A review emphasized that the p38 MAPK pathway can have both protective and deleterious effects even in very similar systems (Cuenda and Rousseau, 2007). On the other hand, according to the findings of the study by Kim *et al*. (2014) p38 and JNK inhibitors inhibit angiogenesis, but ERK inhibitors do not. Lu *et al.* (2010) reported that gallic acid suppresses ADAM17, which contributes to cancer cell invasion via the PI3K/Akt and Ras/MAPK/Erk signal pathways in glioma cells.

In this study, the reduction in JNK and p38 expression levels due to the increase in GA concentration was consistent with the cell cytotoxic effect. JNK and p38 pathways are often activated by environmental and genotoxic stresses and are therefore commonly known as stress-activated protein kinases (SAPK) (Martínez-Limón *et al*., 2020). Zhang *et al*. (2018) showed that silibinin suppressed cell proliferation and induced apoptosis via upregulating the JNK/SAPK signaling pathway in SW19990 cells (a human pancreatic cancer cell line). Similar to this, our findings showed that GA increased the JNK/SAPK level in C6 glioma cells.

This may be related to the inhibition of C6 glioma cell proliferation. It has been suggested that the growth factor receptor-bound protein 2 (Grb2)-related adapter protein (Grap-2) can lead to the death of tumor cells by suppressing NF-kB activation (Ludwig *et al*., 2003). Grap-2 is recognized as a tumor suppressor gene by combining with the protein that interacts with cyclin D1 and has been reported to inhibit the progression of colon cancer, lung cancer, breast cancer and liver cancer through a variety of mechanisms (Chen *et al.,* 2014; Chang *et al.,* 2008). In parallel with other studies, our findings showed that GA increased the level of Grap-2/p38 protein in C6 glioma cells. This increase indicates that GA may prevent the progression of the tumor by contributing to the prevention of proliferation of C6 glioma cells.

Vasculogenic mimicry, which is the formation of vascular-like structures by invasive tumor cells, is considered one of several mechanisms responsible for the failure of anti-angiogenesis therapy in glioma patients. Also, the Akt/MMP-2/MMP-9 pathway has been reported to play a regulatory role in the formation of vasculogenic mimics in glioma (Cai *et al*., 2019). Wu *et al.* (2015) reported in their studies on human hepatocellular carcinoma cells that JNK and p38 molecules induced MMP-2 and MMP-9 molecules and gained invasion and metastasis properties. Temozolomide, which has anti-neoplastic properties used in the treatment of glioblastoma multiforme patients in the 1990s, has been shown to cause a decrease in MMP-2 and MMP-9 activities in U87MG glioblastoma cells (Mirabdaly *et al.,* 2020). Although GA has been observed to reduce the activity of MMP-2 in human bladder cancer cell TSGH-8301, it has been reported that MMP-9 cannot be detected in these cells. Thus, the researchers demonstrated that GA inhibits the invasion of TSGH-8301 cells by reducing MMP-2 activity (Liao *et al*., 2018). In another study, it has been suggested that GA treatment causes down regulation of MMP-2 and MMP-9 and may be useful in preventing metastasis in the treatment of leukemia (Chen *et al*., 2012). In our study, GA caused a concentration-dependent decrease in MMP-2 and MMP-9 expression levels of C6 glioma cells. Thus, we can say that GA applied in optimum concentration and period has the effect of reducing invasion and metastasis in C6 glioma cells.

Like other tumor types, brain tumor formation has been associated with oxidative stress, defined as an imbalance between the production of free radicals and antioxidant mechanisms (Ramírez-Expósito *et al.,* 2019) It has been emphasized that GA has antioxidant properties in studies on diseases that result from or increase oxidative stress. In a study of rats with diabetic nephropathy, GA treatment was observed to reduce oxidative stress (Garud and Kulkarni, 2018). According to another study, it was reported that GA applied to rats with the hepatotoxicity model caused by doxorubicin had a hepatoprotective effect (Omobowale *et al.,* 2018). According to our findings, it was seen that the level of TAS increased in 24h depending on the dose of GA administration, but this effect did not persist for 48h. This may be related to the half-life of GA.

GSH level in cells also supported TAS results. Therefore, time should be taken into consideration for the continuity of the antioxidant effect of GA. On the other hand, according to De Miguel and Cordero, (2022) high levels of reactive oxygen species (ROS) cause the cessation of a cell programmed death and thus contribute to the development of tumor cells. However, if ROS levels rise above levels considered to be compatible with cell survival, they may exert cytotoxic effects leading to the death of tumor cells and therefore limit the progression of cancer.

Gallic acid has been reported to increase cytotoxicity due to ROS in LNCaP human prostate cancer and HL-60RG promyelocytic leukemia cells (Inoue *et al.,* 2000; Russell *et al.,* 2012). According to our findings, GA showed an effect on the TOS level in 24h, but not in 48h. This duration is compatible with the TAS level. Similar to the TOS results, MDA and PCO levels increased and SOD activity decreased with GA treatment. Therefore, 150µg/ml concentration of GA is thought to have a cytotoxic effect by increasing TOS on C6 glioma cells.

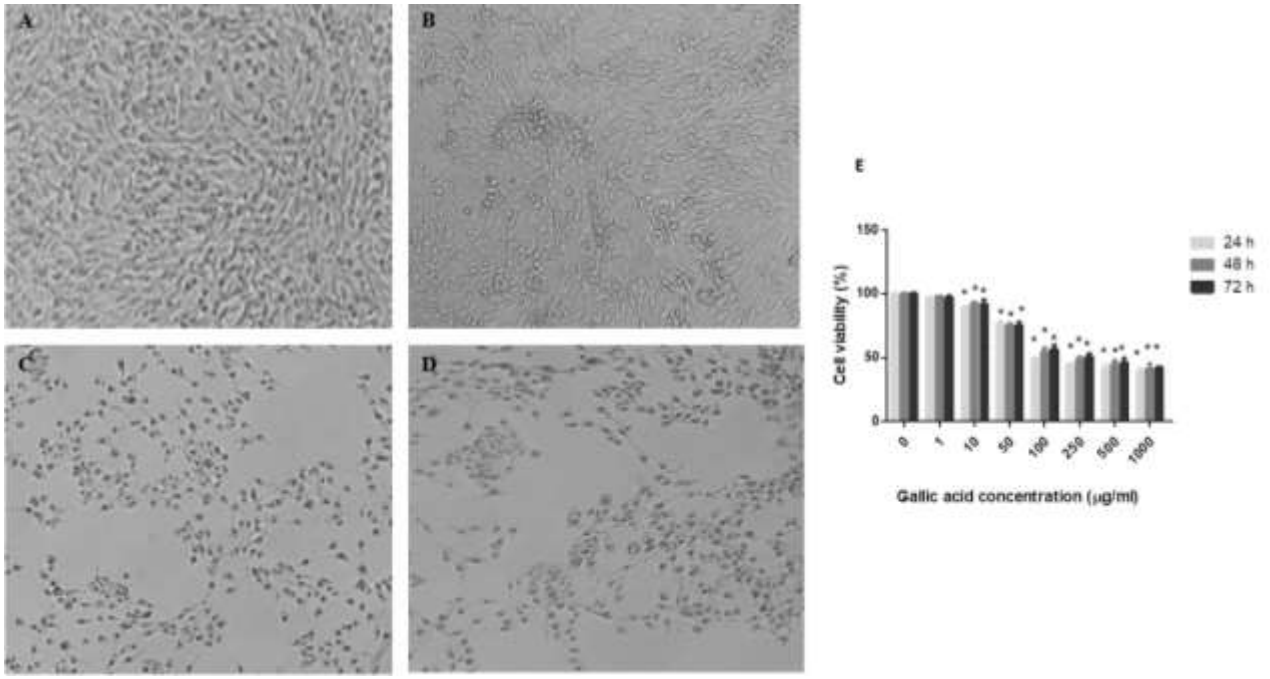

Fig. 1: Phase-contrast microscope (x10) images of C6 glioma cells. Untreated C6 glioma cells (A) and C6 glioma cells treated with gallic acid (GA) at 75µg/ml (B), 100µg/ml (C) and 150µg/ml (D) of concentrations for 24h. The graph showing cell viability (%) of C6 glioma cells against concentrations of GA for 24, 48 and 72h (E). The values are shown as the mean ±standard error of the mean. Compared to control groups, * p<0.05.

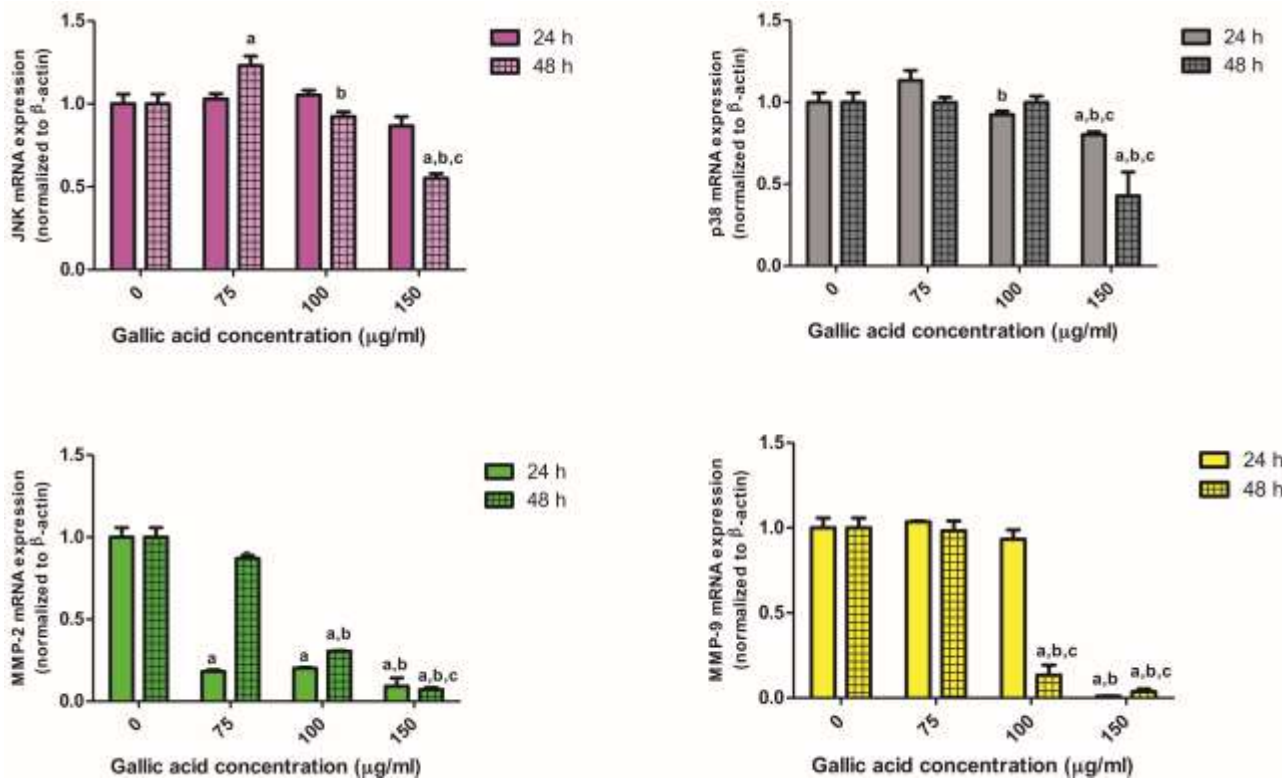

Fig. 2: JNK, p38, MMP-2 and MMP-9 mRNA expression levels in C6 glioma cells treated with gallic acid (GA) were examined by RT-qPCR. The results are shown as the mean ±standard error of the mean. [a]p<0.05 versus untreated C6 glioma cells, [b]p<0.05 versus C6 glioma cells treated with 75µg/ml GA, [c]p<0.05 versus C6 glioma cells treated with 100µg/ml GA.

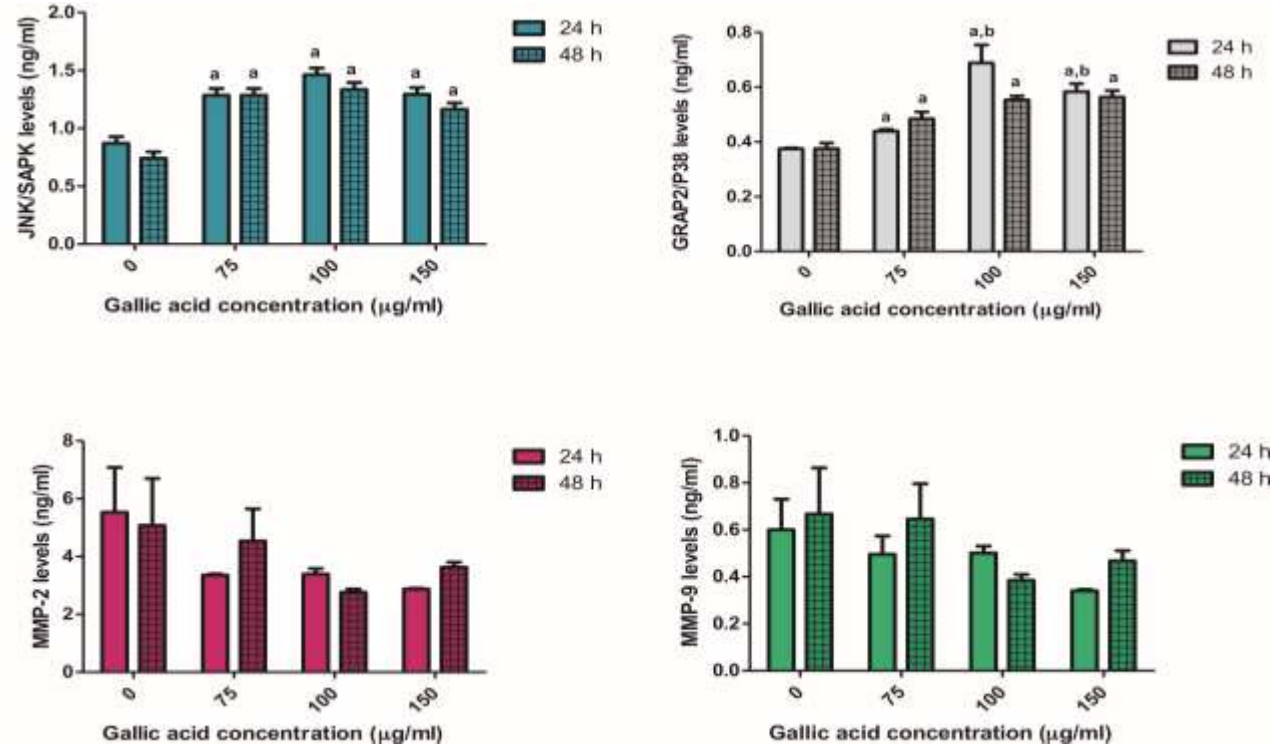

Fig. 3: Changes of JNK/SAPK, Grap-2/p38, MMP-2 and MMP-9 protein levels in C6 glioma cells treated with gallic acid (GA) were analyzed by ELISA assay. Data are expressed as the mean±standard error of the mean. [a]$p<0.05$ versus untreated C6 glioma cells, [b]$p<0.05$ versus C6 glioma cells treated with 75μg/ml GA, [c]$p<0.05$ versus C6 glioma cells treated with 100μg/ml GA.

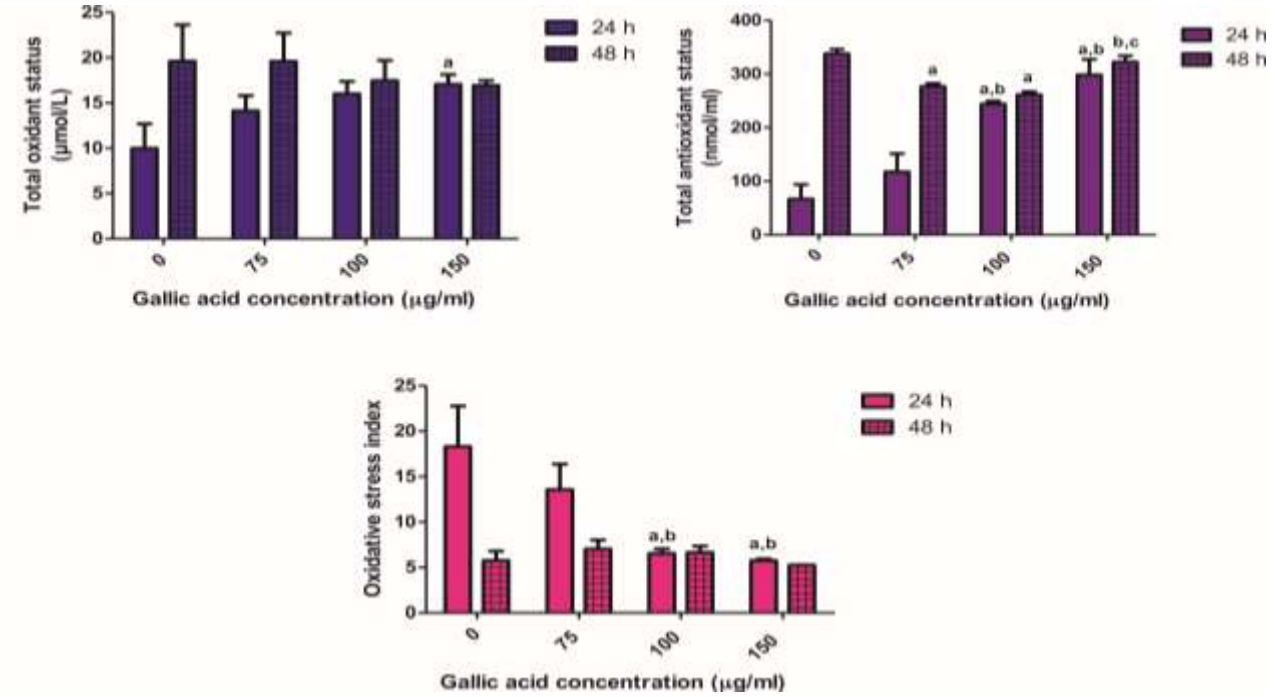

Fig. 4: The measurements of total oxidant status, total antioxidant status and oxidative stress index in C6 glioma cells treated with gallic acid (GA) for 24 and 48h. Data are expressed as the mean±standard error of the mean. [a]$p<0.05$ versus untreated C6 glioma cells, [b]$p<0.05$ versus C6 glioma cells treated with 75μg/ml GA, [c]$p<0.05$ versus C6 glioma cells treated with 100μg/ml GA.

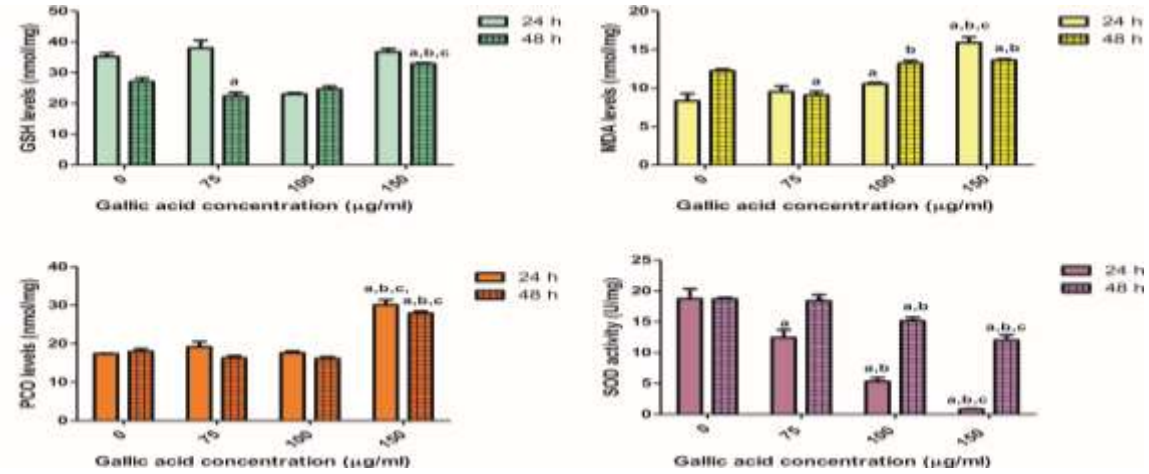

Fig. 5: Changes in the levels of glutathione (GSH), malondialdehyde (MDA), protein carbonyl (PCO) and superoxide dismutase (SOD) activity in C6 glioma cells treated with gallic acid (GA) for 24 and 48h. Data are expressed as the mean±standard error of the mean. [a]$p<0.05$ versus untreated C6 glioma cells, [b]$p<0.05$ versus C6 glioma cells treated with 75µg/ml GA, [c]$p<0.05$ versus C6 glioma cells treated with 100µg/ml GA.

CONCLUSION

As a result, we can suggest that GA may suppress the proliferation of C6 glioma cells. Also, GA may have regulatory effects on MMP-2/9 and oxidative stress, thus preventing the invasion of C6 glioma cells. Our findings highlight the important role of JNK and p38 pathways in glioma and may be a new target for glioma therapy. Future studies in this area may provide a better understanding of glioblastoma invasion and metastasis, as well as reveal new targets and effective clinical treatments.

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