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Effect of Tranexamic Acid on Migration Ability and Level of Matrix Metalloproteinases-2 and -9 of T98G and HUVEC Cells in Co-Culture Conditions

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ABSTRACT

BACKGROUND AND OBJECTIVE: High infiltration of glioma cells into the surrounding brain tissue is the cause of treatment failure in glioblastoma. Glioblastoma interactions with endothelial cells increase the migration of cancer cells. The aim of this study was to investigate the effect of tranexamic acid (TXA) on reducing the migration of glioblastoma and endothelial cells and the levels of metalloproteinases-2 and -9 (MMP-2/9) in co-culture conditions.

METHODS: In this experimental study, cells prepared from Pasteur Institute were treated with different concentrations of TXA in mono-culture and co-culture. Cell survival was determined by MTT assay. The rate of migration was assessed by making grooves in the culture dishes and comparing the groove area at different time intervals in the control and treated groups with 6 and 24 mM TXA. The levels of MMP-2/9 enzymes in the control group and cells treated with 6 mM TXA were assessed by zymography.

FINDINGS: Decreased survival of T98G and HUVECs cells was observed in mono- and co-culture from 60 mM TXA and above (p=0.0001). Groove area in HUVECs group treated with 6 and 24 mM TXA were 0.27±0.05 and 0.36±0.04 of initial groove area, respectively, which was significant compared to the control group (p=0.034, p=0.005). No difference in groove size was observed in T98G cells. In co-culture group, the groove size 36 hours after the start of the study in the control group was 0.12±0.01, which was significantly lower than T98G + HUVECs groups treated with 6 (0.28±0.06) and 24 (0.39±0.07) mM TXA (p=0.01 and p=0.002). TXA significantly reduced the levels of MMP-2 (p=0.0001) and MMP-9 (p=0.0001) in co-culture conditions.

CONCLUSION: The results showed that TXA could reduce the migration of glioblastoma and endothelial cells as well as the levels of MMP-2/9 in co-culture conditions.

KEY WORDS: *Glioblastoma, Co-Culture, Tranexamic Acid, Cell Migration, Matrix Metalloproteinase-2.*

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Introduction

Cancer is a major public health problem worldwide (1). Although primary malignant tumors of the central nervous system make up about 2% of all cancers, these are of particular importance due to their high mortality rate. About 11,000 new cases of high-grade glioma are diagnosed in the United States each year, of which 9,000 are glioblastoma multiforme (GBM) (2). Gliomas are divided into two cell types (astrocytes and oligodendrocytes) and four degrees of malignancy (I to IV); the most malignant grade (IV) is glioblastoma, which originates from the progenitor or stem cells of the astrocytic lineage (3-5).

So far, no successful treatment has been found for glioblastoma. Current treatments include maximal mass removal with radiotherapy (6) and chemotherapy -using temozolomide (7-10). Tumor heterogeneity and its surroundings microenvironment have made glioblastoma as one of the most difficult cancers to treat (11). Rapid proliferation, high invasion to healthy tissue around the tumor, and high vascular density of the cancerous mass are the main features of glioblastoma (12). Glioblastoma cell migration and invasion are regulated by changes in the invasive cell and the microenvironment surrounding the tumor. Extracellular matrix integrity is one of the main regulators of cell motility and cell movement also requires readjustment and changes in cell Transcription profile/factors and cytoskeleton (5).

Metalloproteinases-2 and -9 (known as gelatinases A and B, respectively) are zinc-dependent endopeptidase enzymes that have different substrates in extracellular matrix such as collagen type IV as well as non-cellular matrix (13). While the expression of metalloproteinases in the normal brain is low (5), the overactivity of MMP-2 (Matrix Metalloproteinase) and MMP-9 in glioblastoma has been shown (14, 15). The level of MMP-2/9 is associated with tumor invasiveness and poor prognosis of glioblastoma patients (5). MMP-2/9 is involved in cell proliferation, motility, invasion and angiogenesis of glioblastoma (16).

Decreased levels of MMP-2/9 have also been shown to reduce the metastasis of glioblastoma cancer cells (17). Interaction of glioma cells with adjacent cells such as astrocytes or endothelial cells is important for the migration of glioblastoma cells and angiogenesis (5). The endothelial cells around the glioblastoma activate Notch pathway in cancer stem cells and increase the self-renewal of these cells by expressing the ligands of the notch signaling pathway (12). It has been shown that PDCD10 (Programmed Cell Death 10) is not expressed in endothelial cells of vascular wall that are associated with a glioblastoma cancer mass. Inhibition of PDCD10 expression in normal endothelial cells that have been co-cultured with glioblastoma cancer cells causes the proliferation and migration of glioblastoma cancer cells and prevents their apoptosis (18). The association between endothelial cells and tumor cells in liver and breast cancer has also been considered (19). Glioblastoma cells are migrated toward blood vessels by chemokines such as bradykinin produced by endothelial cells (5). Glioblastoma cancer cells can migrate through co-opting with cerebrovascular endothelial cells (20).

Tranexamic acid (TXA) is a synthetic drug and lysine analogue that exerts its antifibrinolytic effects by inhibiting the active site of plasmin (21, 22). Tranexamic acid is a widely used drug to control bleeding during severe injuries, childbirth, surgery, and tooth extraction (23, 24). One study found that tranexamic acid could reduce ascites from ovarian cancer and improve the effectiveness of chemotherapy drugs (25). The study by Zhu et al. showed that TXA leads to inhibition of cell proliferation, migration, invasion and tubular formation of HUVECs through neutralization of VEGFR-1 and VEGFR-2 induced by VEGF165. In other words, TXA inhibits angiogenesis by targeting VEGFR in HUVECs (26). Suojanen et al. showed that tranexamic acid could inhibit MMP-9 activation by inhibiting plasmin (27).

Considering the importance of migration in the fatality of glioblastoma disease and the association of brain cancer cells with peripheral cells including endothelial cells, the aim of this study was to evaluate the effect of TXA on preventing migration of glioblastoma multiforme (T98G) cell line co-cultured with endothelial cells (HUVECs) and evaluating the activity of metalloproteinases-2 and -9.

Methods

Culture and evaluation of cytotoxicity: This study was approved by the ethics committee of Ardabil University of Medical Sciences with the ethics code IR.ARUMS.REC.1398.390. T98G and HUVECs cell lines were purchased from the Pasteur Institute Cell Bank and cultured in DMEM medium enriched with 10% bovine fetal serum (FBS; Gibco: 10270), 1% antibiotic (Gibco: 15140-122). Cytotoxicity was assessed by MTT assay (3-(4,5-Dimethylthiazol-2-yl) -2,5-Diphenyltetrazolium Bromide) (28, 29). For this

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purpose, 3×10^3 cells from each cell line were plated onto each well of 96-well plates. To investigate the effect of the drug in co-culture condition, 1.5×10^3 of each of the T98G and HUVECs were placed in the wells of 96-well culture containers. Treatment with tranexamic acid (Cayman: 19193) was performed at concentrations of 0.06, 0.6, 6, 12, 24, 48, 60 and 120 mM for 48 hours. The culture medium was then discarded and MTT solution at a concentration of 5 mg/ml was added to each well and incubated for 4 hours. the absorbance was read by a microplate reader at 570 nm and cell survival percentage at different concentrations was calculated as the percentage of cell viability relative to control

Wound healing assay: This test was performed to assess the rate of cell migration. 7×10^4 cells of T98G and HUVECs were cultured separately and for co-culture, 35×10^3 cells from each cancer cell and endothelial cell line were placed in 24-well plate wells. The next day, the cells were cultured with 0.5% serum for 12 hours. Then, a sterile yellow sample tip was used to create a scratch in the confluent layer of the bottom of the wells. Treatment was performed in 0.5% serum medium containing concentrations of 6 and 24 mM tranexamic acid. The closure of the scratch was monitored using an inverted microscope (Olympus, CKX41) equipped with a digital camera (Olympus, DP27) at 12, 24 and 36 hours later and images were taken. The groove area was calculated using ImageJ software using MRI Wound Healing Tool plugin. The scratch size was converted and presented in different groups during the study period in relation to the scratch area at its zero hour.

Gelatin zymography: zymography method was used to evaluate the proteolytic activity of metalloproteinases-2 and -9. T98G and HUVECs cells were treated in cell mono-culture or in co-culture condition in 0.5% serum medium without tranexamic acid or containing 6 mM. After 48 hours, their conditioned medium were collected and centrifuged. Protein concentration was determined in each case and 5 µg of each sample was introduced into 10% SDS-PAGE gel wells containing 1% gelatin (Sigma: G1890). Electrophoresis was started at 90 volts and then performed at 120 volts for 90 minutes. The zymography gel was incubated for 24 hours at 37 °C in a developing buffer containing 5 mM $CaCl_2$ and 1 µM $ZnCl_2$. In the next step, the gels were stained with coomassie Blue G-250 solution for 45 minutes. Gel destaining was performed in several rounds until the bands resulting from the activity of metalloproteinases were clarified on a blue background. Finally, the scanned images were introduced to ImageJ software and densitometry technique (30, 31) was used to quantify the bands. The results were analyzed by one-way analysis of variance following post hoc Turkey's test using SPSS software version 21. $p<0.05$ was considered statistically significant.

Results

The effect of tranexamic acid on the survival of T98G, HUVECs and co-culture of two cell types: Dose-response curve analysis showed that tranexamic acid up to a concentration of 48 mM had no significant effect on the survival of cancerous, endothelial cells and co-culture group (Figure 1). Higher concentrations of tranexamic acid gradually reduced the viability of cells in different groups ($p<0.05$). From 60 mM onwards, the slope of reduction in cell survival was significant (Figure 1). At a dose of 120 mM tranexamic acid, the survival of T98G cells reached to 65.32±6.26. The viability of HUVECs cells as well as T98G+HUVECs group at 120 concentration of tranexamic acid decreased to 31.08±5.2 and 52.92±2.28, respectively ($p= 0.000$). MTT data were fitted into version 12 of SigmaPlot software and the IC50 value was calculated. Accordingly, IC50 of tranexamic acid in T98G cells, HUVECs and in co-culture conditions was 305.03, 66.02 and 179.41 mM, respectively. Considering that the therapeutic concentration of tranexamic acid in blood serum reaches 6 mM (32), this concentration is emphasized in the study.

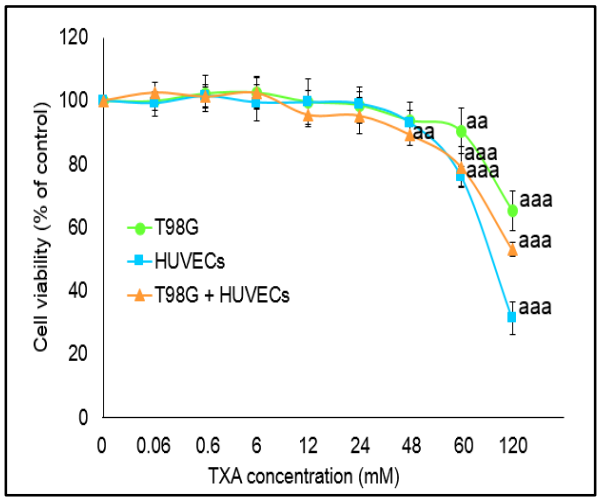

Figure 1. Comparison of the effect of different concentrations of tranexamic acid on the survival rate of T98G cells, HUVECs in mono-culture and co-culture by MTT test. **($p< 0.001$ ^{aaa} and $p< 0.01$ ^{aa} relative to control)**

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Changes in migration of T98G, HUVECs and co-culture of two cells in the presence of tranexamic acid: Scratch assay test results showed no significant difference with its control group in the treatment of T98G cells with concentrations of 6 and 24 mM tranexamic acid (Figure 2 - parts A and B). The scratch area was larger in the tranexamic acid-treated HUVECs groups compared to its control (Figure 2 - parts C and D). at 36 hours from the start of exposure, the scratch size at a concentration of 6 mM of tranexamic acid was 0.27±0.05 (p= 0.034) and at a concentration of 24 mM was equal to 0.36±0.04 (p= 0.005) as compared to wound width at zero hour of HUVECs. Almost the same trend was observed in the T98G+HUVECs group. In the co-culture group, there was a statistically significant difference between the control group and concentrations of 6 (0.28±0.06) (p= 0.01) and 24 mM of tranexamic acid (0.39±0.07) (p= 0.002) at the time interval of 12 to 36 hours after the initiation of of scratch (Figure 2 - parts E and F).

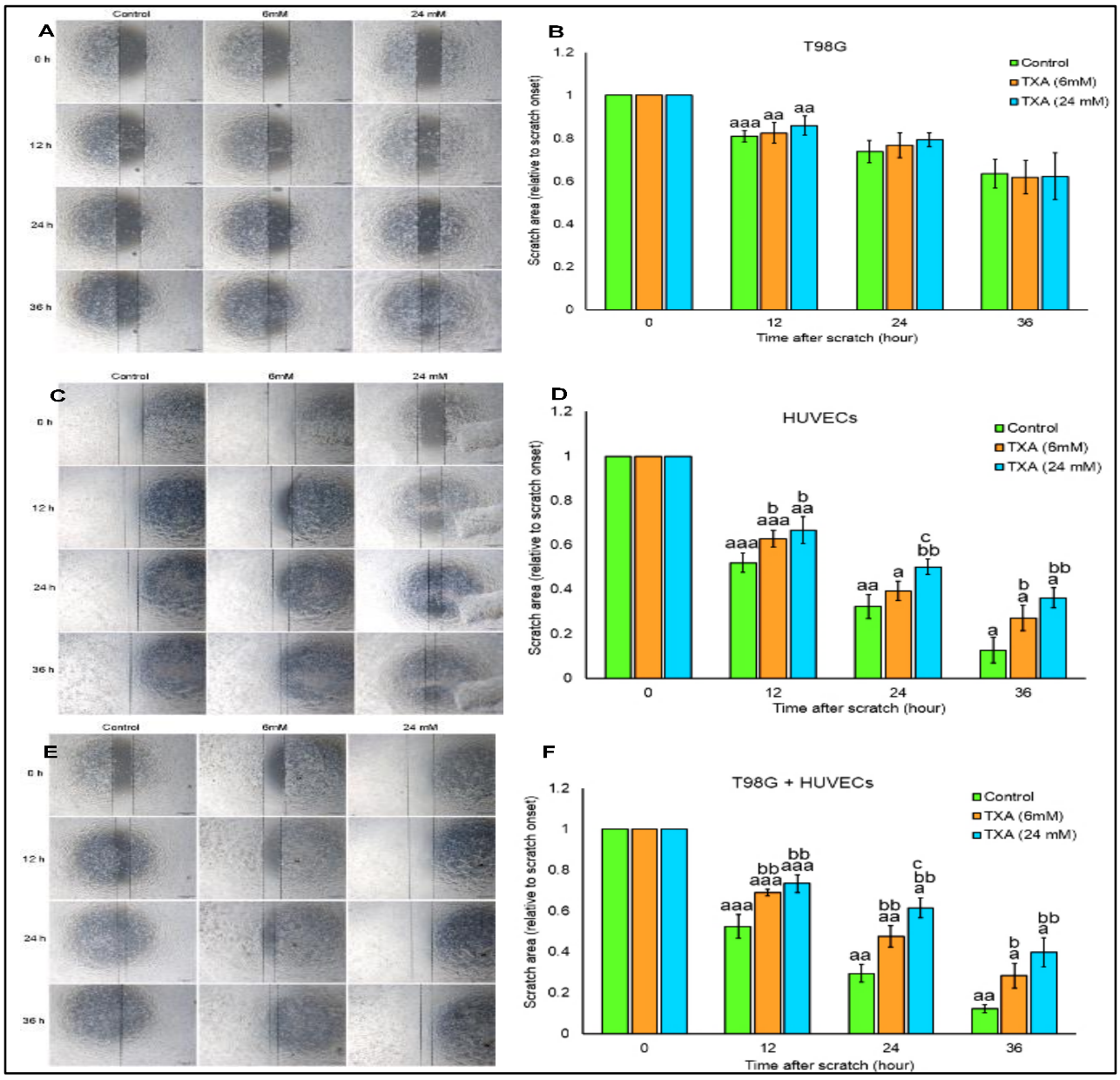

Figure 2. Scratch assay of T98G cells, HUVECs in mono-culture and co-culture of two cells. **Parts A, C, and E show the photomicrographs of T98G, HUVECs, and T98G+HUVECs cells in the control group and treated with 6 and 24 mM TXA at 0, 12, 24, and 36 h, respectively. Parts B, D and F show the quantified state of the scratch area compared to the zero hour of groove in different groups in the form of a bar graph. ($p< 0.001$ ^{aaa}, $p< 0.01$ ^{aa} and $p< 0.05$ ^{a} show comparison of each group compared to the previous time point, $p< 0.01$ ^{bb} and $p< 0.05$ ^{b} show comparison of each group relative to the control at the same time point, and $p< 0.05$ ^{c} shows comparison between concentrations of 6 and 24 mM TXA at the same time point)**

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Evaluation of gelatinase activity of T98G, HUVECs and co-culture in the presence of tranexamic acid: Gelatinase activity of metalloproteinases-2 and -9 was observed in two bands of 72 and 92 kDa, respectively. We found that MMP-2 levels were higher than MMP-9 in all groups (Figure 3 - Part A).

The highest level of MMP-2 was detected in mono-culture of T98G and the lowest level was observed in mono-culture of HUVECs (Figure 3 - Part A). Densitometry examinations did not reveal a significant difference in band strength in T98G cells in the presence or absence of tranexamic acid exposure. For HUVECs cells, however, a significant decrease in MMP-2 was observed in the tranexamic acid group compared to the control group (p= 0.01).

In the co-culture group, concentration of 6 mM of tranexamic acid was able to significantly decrease the activity of metalloproteinases 2 (0.8±0.02) (p= 0.000) and 9 (0.91±0.01) (p= 0.000) compared to the control group (Figure 3 - Part B and C).

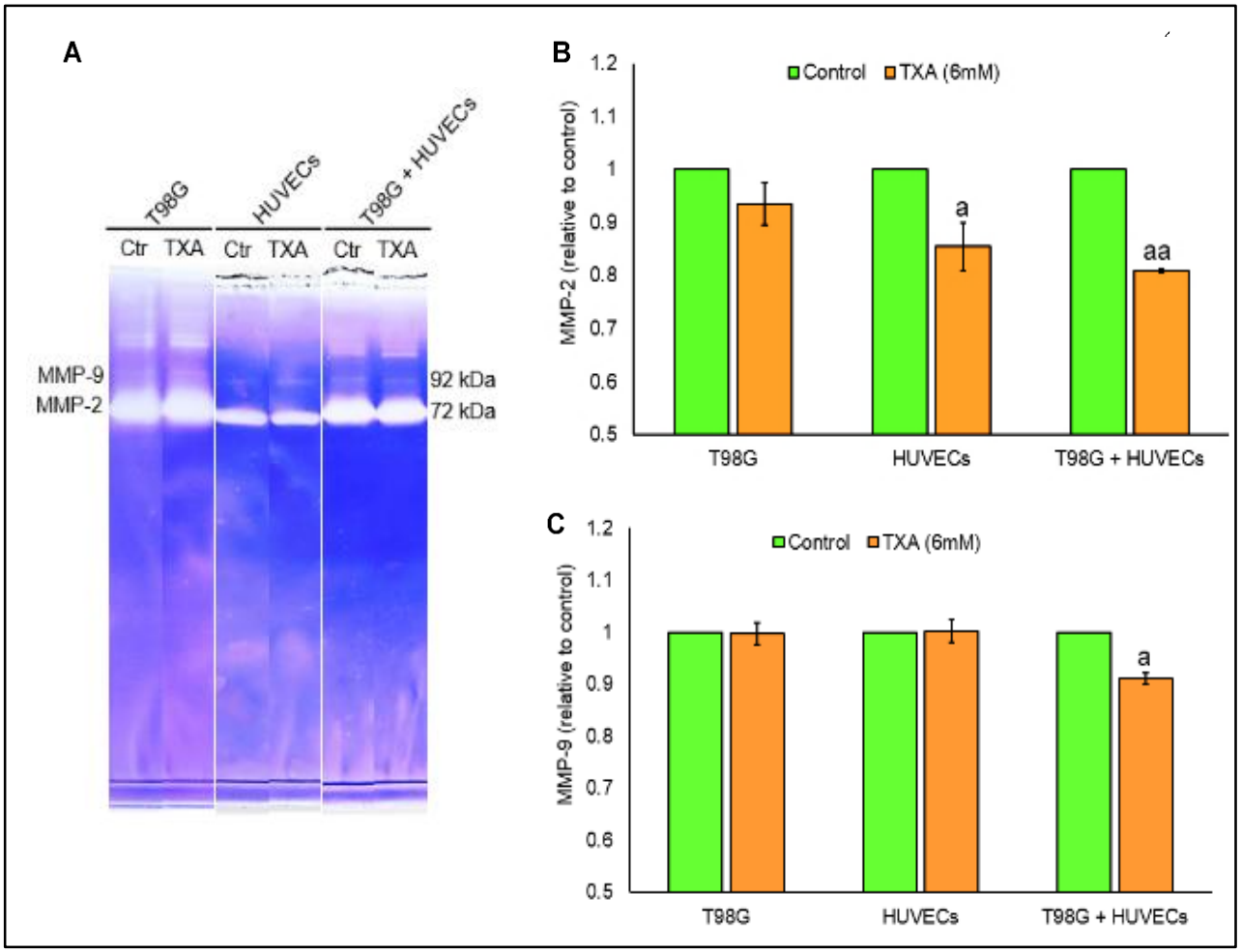

Figure 3. Zymography of T98G and cells in mono-culture and co-culture of two cells. Part A shows the pattern of gelatinase activity of MMP-2 and MMP-9 of T98G and HUVECs cells in mono-culture and co-culture of two cells. The bar graphs in parts B and C show the quantified value of MMP-2 and MMP-9 level/activity for T98G and HUVECs cells in mono-culture and co-culture of two cells, respectively (p< 0.01 [aa] and p< 0.05 [a] compared to control)

Discussion

The results of this study showed that tranexamic acid can reduce cell migration under co-culture conditions. Furthermore, the activity of metalloproteinases 2 and 9 decreases after treatment with tranexamic acid in co-culture conditions. MTT results showed that tranexamic acid at low concentrations had no effect on the survival of cancerous and endothelial cells or co-culture conditions. However, at about 60 mM and higher concentrations of tranexamic acid, a sharp decrease in cell survival was observed. This finding indicates the safety of this drug and its pharmacological application at low doses. A study by Kikuchi et al. showed that tranexamic acid at a concentration of 10 mg/ml had an inhibitory role on the

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growth of ovarian cancer cells and that its use in combination with routine chemotherapy drugs significantly reduced ascites in mice (33). The study by Cox et al. showed that tranexamic acid added to fibrin sealant clots had no effect on the initial binding strength of proliferative cells (glial cells and fibroblasts) or non-proliferative cells (nerve cells) at low drug concentrations. However, high concentrations of tranexamic acid (300-450 mM) weaken the initial attachment or separate the cells from the bottom of the coated plates; 20% and 80% reduction of fibroblast adhesion was observed at concentrations of 300 and 450 mM tranexamic acid, respectively (34).

In this study, migration of T98G and HUVECs cell was analyzed under mono-culture and co-culture of two cells by Scratch assay. Based on the results of this test, no significant difference was observed compared with control group in the treatment of T98G cells with concentrations of 6 and 24 mM tranexamic acid. In this regard, Kaphle et al. have pointed out that tranexamic acid has no effect on the migration of U87 and A172 glioblastoma cancer cells in monolayer culture (16).

The scratch width in HUVECs cells and the co-culture of T98G cells with HUVECs cells were larger in the tranexamic acid-treated group than in the control group. The relationship between glioblastoma cells and its environment, especially microglial and endothelial cells, and the importance of this relationship in increasing the invasiveness of cancer cells has been considered. Sphingosine-1 phosphate levels have been shown to increase following the co-culture of glioblastoma cells with endothelial cells. The increase in this substance is directly related to the growth of cancer cells as well as the migration of endothelial cells and angiogenesis (35).

Another study looked at the interaction between glioblastoma cells and microglia and endothelium. This study showed that the interaction of glioblastoma and microglial cells increased the expression of interleukin 6 (IL-6) and by activating the JAK / STAT3 pathway, reduced the expression of intercellular junction proteins in endothelial cells and caused blood-brain barrier permeability (36). Co-culture of U87 cells with HMEC1 endothelial cell increases the invasion rate of cancer cells. Kenig et al. found that MMP-9 secretion was increased in both cell lines. The secretion of cysteine cathepsins B and S are increased and the amount of NCAM (Neuronal cell adhesion molecule) decreased in U87 cancer cells. They also found that endothelial cells could increase the amount of MMP-9 in the microenvironment by secreting SDF1 (CXCL12) (37). The study by Suojanen et al. showed the effect of this drug on preventing the invasion of squamous cancer cells of the tongue (27). Another study showed the inhibitory role of tranexamic acid on the invasion and formation of tubular structure of HUVECs and the significant reduction in cell proliferation of HUVECs (26). The results of gelatinase activity by gelatin zymography in this study showed a higher amount of MMP-2 in all groups compared to MMP-9. T98G and HUVECs showed different behavior when exposed to tranexamic acid in mono-culture and co-culture; Exposure to a concentration of 6 mM tranexamic acid in the co-culture group was able to significantly reduce the activity of metalloproteinases 2 and 9 compared to the control group. Exposure to this drug had no effect on T98G cells, but differences in MMP-2 levels were evident after exposure of HUVECs cells.

Afsharimani et al. found that MMP-2, 9 and TIMP-1, 2 (Tissue inhibitor of metalloproteinase-1) were increased in co-culture; while at similar concentrations of tranexamic acid (6mM), the decrease in MMP-9/ TIMP-1 ratio,the decrease in MMP-2, and the increase in TIMP-1 in co-culture conditionswere significant as compared to cancer cells and endothelial cells alone (32). A study by Kaphle et al. also showed that culture conditions could play a role in the migration of U87 and A172 cancer cells. In 3D cultures, the secretion of MMP-2/9, urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA) increases compared to routine mono-culture, and therefore plasminogen inhibitors such as tranexamic acid or aprotinin in 3D cultures can be effective in preventing the migration of glioblastoma cells, whereas in conventional cultures, it could not prevent the migration of cancer cells (16). Tranexamic acid has also been shown to inhibit endothelial cell migration. Tranexamic acid targeting MMP-9 can effectively inhibit cancer cells in vitro (27).

Attempts to approximate the conditions of laboratory tests to physiological conditions of the body and the key role of interaction between cancer cells and non-cancer cells such as endothelial cells, microglia, pericytes, and macrophages in regulating the migration of tumor cells (11, 19, 38-41) necessitated the present study. Although more additional studies are needed at the cellular and molecular levels, the results of this study showed that tranexamic acid can prevent the migration of glioma and endothelial cells in

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co-culture condition by reducing the level/activity of metalloproteinases 2 and 9. One of the limitations of this study is the impossibility of detecting cells under co-culture conditions.

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