

Citric Acid Inhibits Clonogenic Power and Anchorage-Independent Growth of Glioma Cells

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ABSTRACT

Background: Glioblastoma multiforme (GBM) is the most fatal glioma with poor prognosis. C6 glioma is an experimental model to simulate GBM growth and biology. GBM benefits from Warburg effect (aerobic glycolysis ending in lactate formation even in the presence of oxygen) in tumor growth, invasion and metastasis. Citric acid is an antioxidant and inhibitor of glycolysis pathway (phosphofructokinase inhibitor) that constitutes the major source of energy supply to aggressive cancer cells. Citrate is a promising inhibitor of Warburg effect through blocking glycolysis upstream of lactate formation step. **Methods:** Ability of citric acid to inhibit experimental GBM colony formation and anchorage-independent growth were investigated.

Results: Citrate induced a dose-dependent inhibition of growth and proliferation of glioma colonies (attached to substratum). High citrate dose (9 mM) inhibited initial formation of glioma colonies. A similar picture was observed where citrate induced a dose-dependent inhibition of growth of glioma colonies in soft agar (i.e. not attached to a substratum). High citrate dose (9 mM) inhibited initial formation of glioma colonies. In conclusion, citrate inhibited 3D tumor models of GBM. Citric acid inhibited clonogenic power of glioma cells.

Conclusion: Citric acid may be a promising therapeutic modality for glioma and glioblastoma. That is quite promising in treating GBM tumors and can be generalized for research in different tumors.

INTRODUCTION

Glioma tumors are driven by glycolysis^[1] and exhibits Warburg effect (increased glycolysis to produce ATP with metabolic shift from oxidative phosphorylation to glycolysis even in the presence of oxygen). Glioblastoma multiforme (GBM) is the most fatal type of glioma with poor prognosis. C6 glioma is an experimental model to simulate GBM growth and biology. Ability of GBM cells to form colonies can be investigated versus anticancer treatments using *in vitro* models. GBM benefits from Warburg effect in tumor growth, invasion and metastasis^[2-3].

Citrate is safe, available in citrus fruits, and proved to be effective in many therapeutic uses. Recently, high dose of citrate (4-6 grams/day) was reported in the treatment of medullary thyroid carcinoma in a child with no report of metabolic acidosis^[4]. Also, citrate was reported to have anticancer effects in treating mesothelioma^[5] and gastric cancer^[6]. Low citrate (citric acid) level (< 1 mM) was reported to be inhibitory of phosphofructokinase-1 (PFK-1), a key glycolytic enzyme^[7]. Inhibition of PFK by citrate disturbs Warburg effect (metabolic alteration of cancer cells in which glycolysis ends with formation of lactate even in the presence of oxygen) (Figure 1).

Citrate was reported to have many diagnostic uses in oncology e.g. citrate is an *in vivo* marker to discriminate prostate cancer from benign prostatic hyperplasia^[8] to facilitate diagnosis of prostate cancer^[9]. Citrate concentrations in human seminal fluid outperforms prostate specific antigen in prostate cancer detection^[10]. Citrate level is further reduced in metastatic prostate disease^[8]. Citrate concentrations declined significantly

with time in progressing brain stem glioma^[11]. ⁵²Fe-citrate and positron emission tomography can measure iron uptake in brain tumors^[12]. Moreover, citrate was reported to show a specific, dose-dependent lympholytic activity in neoplastic cell lines^[13].

Citrate was reported to have a lot of therapeutic uses as well e.g. in pediatrics, citrate is efficient in the correction of diarrhea-induced metabolic acidosis as sodium citrate equals sodium bicarbonate in oral rehydration therapy for treating childhood diarrhea^[14].

In nephrology, citrate inhibits formation and retention of crystals in the kidneys making citrate commonly prescribed for treating kidney stone disease^[15], urinary calculi^[16] in addition to treatment of hyperuricosuric calcium oxalate nephrolithiasis^[17].

Interestingly, citrate helps the treatment of antibiotic-resistant postoperative wounds in cancer patients^[18] and facilitates sperm motility^[19].

Little research is done regarding the role of citrate as an anticancer agent. Citric acid is not suspected of being a carcinogen or a teratogenic agent. This may make citrate a suitable anti-neoplastic drug targeting glycolysis and Warburg effect, a common metabolic alteration in most cancer cells in which cancer cells use glycolysis as a major energy source even in the presence of oxygen (Figure 1)^[20].

In this study, dose-dependent citrate-induced inhibition of glioma colonies was investigated using both the clonogenic power and the anchorage-independent growth assay.

MATERIALS AND METHODS

Reagents

Citrate and agar were purchased from El-Nasr Company (Cairo, Egypt). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St. Louis, MO, USA). DMEM/F12 and penicillin-streptomycin antibiotic mixture were from Invitrogen Life Technologies (Carlsbad, CA, USA).

Cell culture

C6 rat glioblastoma cell line was maintained in DMEM containing 10% (v/v) FBS and 1% penicillin-streptomycin at 37°C under a humidified atmosphere containing 5% CO₂. **C6 cell culture and passage**

C6 glioma cells (1 x 10⁴ cells/well) were seeded into plastic culture dishes for 48 h until cells reaching 80% confluency. Medium aspiration and stimulating medium (DMEM/F12 containing 1% (v/v) FBS) addition was done. Cells are then trypsinized followed by medium addition. C6 cells are then plated into new culture dishes till reaching confluency. Biology of C6 cells confirms that they are rapidly growing cells simulating GBM tumors.

Colony formation assay

Under complete aseptic conditions, C6 cells were seeded in 6 cm plates in nutrient medium (DMEM/F12 containing 15% (v/v) horse serum, 2.5% (v/v) FBS, and 1% penicillin-streptomycin). Seeding density was 1 x 10³ cells/plate. Plates were shaken gently for even distribution. Plates were incubated in CO₂ incubator for 10 h. Cells received treatment in the form of serial doses of citrate (3, 5 and 9 mM) treatment. Cells were incubated in CO₂ incubator. Daily follow up using Nikon phase contrast light microscopy was done till colonies in control plates form 50 cells or more per colony. Aspiration of medium was done followed by careful gentle washing of plates using 1X PBS. Cells were fixed with 100% methanol for 15 min. Methanol was aspirated and plates were stained with 0.5% crystal violet (in 2% ethanol) for 30 min at room temperature. Crystal violet was removed carefully and plates were rinsed with tap water carefully and air-dried at room temperature. Plates were photographed by digital camera.

Soft agar assay

Using 6 well plates, basal agar layer (0.5% agar) was prepared. Top agarose layer (0.3% agarose) containing C6 cells (2.5 x 10³ cells/well) was prepared by mixing 0.6% agarose with an equal volume of nutrient medium

(DMEM/F12 containing 15% (v/v) horse serum, 2.5% (v/v) FBS, and 1% penicillin-streptomycin) containing C6 cells. Plates were incubated at 37°C in a humidified incubator for 10 h. Treatment in the form of serial doses of citrate (3, 5 and 9 mM) was added. Fresh media containing treatment was added every other day. Daily follow up of colony growth in agarose top layer was done using Nikon phase contrast light microscopy. Anchorage-independent growth power was calculated by comparing percentage ratio of treated wells versus control wells as regard number and size of colonies as previously reported^[21].

Statistics

Results shown are (Mean ± S.E.M) of the values obtained from the indicated number of experiments. Differences from control (untreated cells) were analyzed by Student's t test. Significant differences at p < 0.05, p < 0.01, and p < 0.001 versus control are indicated by *, ** and ***, respectively.

RESULTS

Citrate significantly reduced colony number in glioma cells

As clonogenic power (colony numbers) reflects strength of cancer cells to initiate new metastatic tumors^[22], investigation of citrate effect on clonogenic power of C6 glioma cells was done in a colony formation assay. C6 (1 X 10³ cells/ plate) were seeded. Plating efficiency (Number of colonies X 100 /Number of seeded cells) was estimated to be 34% in C6 cells versus 26% in C6 cells. Serial doses of citrate at 3, 5 and 9 mM significantly reduced clonogenic power in C6 cells (p<0.05, p<0.001 and p<0.001), respectively. At 9 mM citrate, almost total absence of clonogenic power was observed (Figure 2A-B).

Citrate significantly decreased glioma growth in soft agar

As rapid growth in soft agar (anchorage-independent growth) is a powerful characteristic of cancer cells which differentiates them from normal cells. We investigated citrate effects on soft agar colonies regarding colony number and colony size of C6 glioma cells. Serial doses of citrate at 3, 5 and 9 mM significantly reduced colony number of anchorage-independent growth in C6 cells (p<0.05, p<0.001 and p<0.001), respectively (Figures 3A-C). Near total absence of colonies was observed at 9 mM citrate (Figures 3A-C).

There was a dose-dependent decrease in colony size with serial doses of citrate treatment e.g. colony size decreased significantly at 3, 5 and 9 mM citrate (p<0.05, p<0.001 and p<0.001) (Figures 2A-B), respectively.

Figure 1

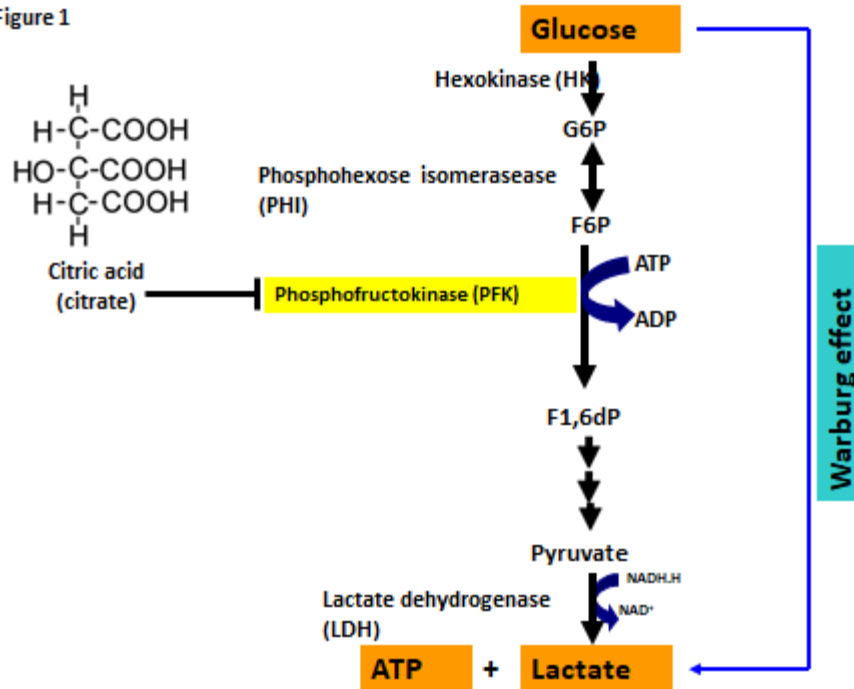
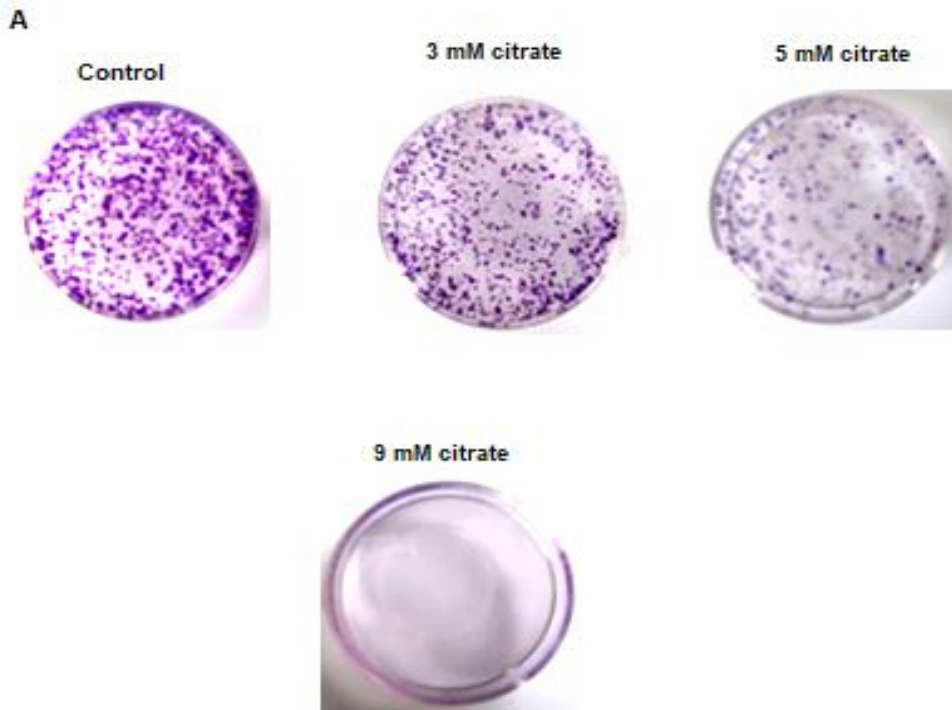


Figure 1 – Glycolysis inhibition using citrate is promising in treating glioma tumors via targeting the Warburg effect. This figure is from own understanding of Warburg effect biology and effects on cancer cells.

Figure 2



B

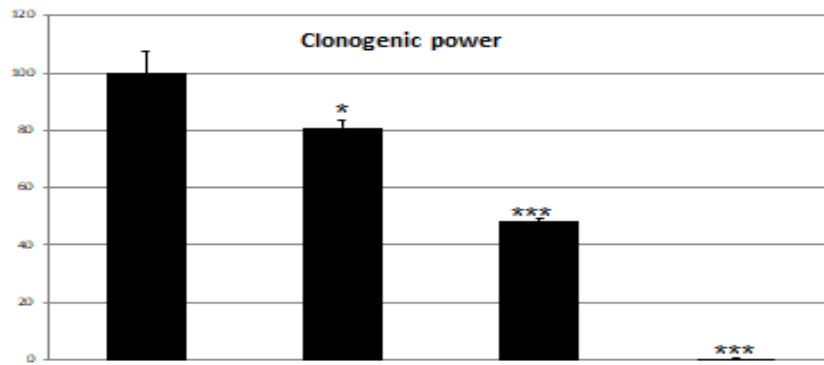
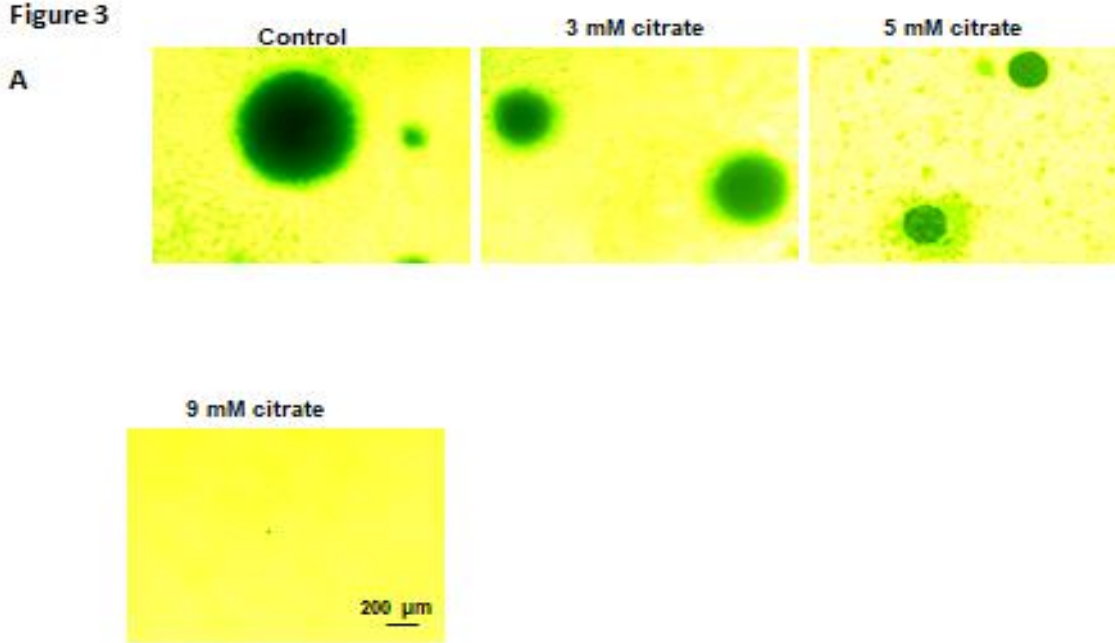


Figure 2 - Citrate decreased clonogenic power of glioma cells significantly in a colony formation assay. (A) C6 glioma colonies. B) Effect of citrate in decreasing clonogenic power of glioma cells. Data are (Mean \pm SEM) of the percentages of the control values of three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate significance between control and different treatment conditions

Figure 3



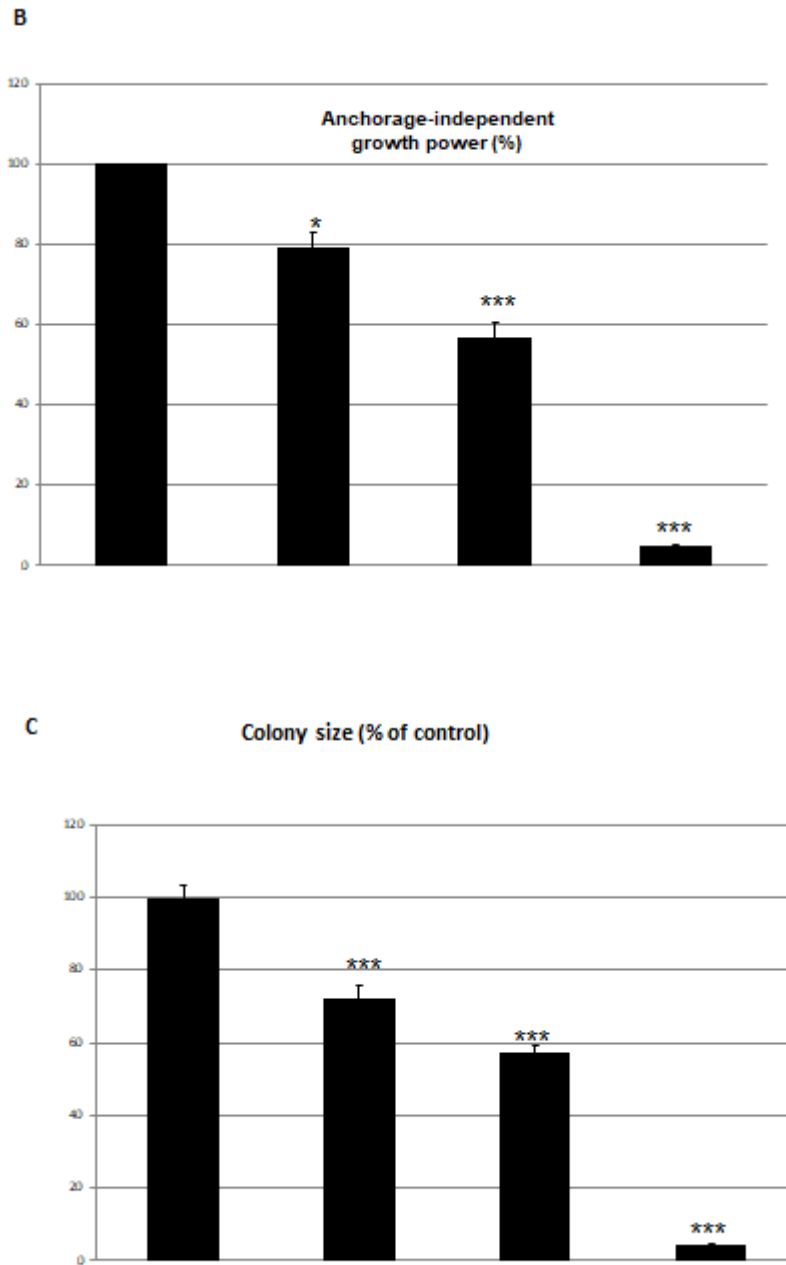


Figure 3 - Citrate decreased significantly anchorage-independent growth of glioma cells in a soft agar assay. (A) C6 anchorage-independent growth of glioma colonies in agarose top layer. (B) Effect of citrate in decreasing anchorage-independent growth power of glioma colonies. (C) Effect of citrate in decreasing size of anchorage-independent growth of glioma colonies. Data are (Mean \pm SEM) of the percentages of the control values of three independent experiments. * p <0.05, ** p <0.01 and *** p <0.001 indicate significance between control and different treatment conditions.

DISCUSSION

Glioma cells are driven by glycolysis. Phosphofruktokinase (PFK) is a key enzyme of glycolysis and Warburg effect (Glucose oxidation with production of lactate) in GBM cells. PFK catalyzes the irreversible phosphorylation step of fructose-6-phosphate (F6P) to fructose 1 and 6 diphosphate (F1, 6 dP). Cytotoxicity of glioma cells may increase with citrate-induced energy depletion due to PFK inhibition. Glioblastoma multiform (GBM, grade IV astrocytoma) is refractory to conventional surgical and chemotherapeutic measures^[23]. This aggressive type of astrocytoma continues to have a poor prognosis in spite of aggressive therapeutic efforts. GBM displays the Warburg effect (aerobic glycolysis ending in lactate formation even in the presence of oxygen). Chemotherapy designed to kill dividing neoplastic cells remains ineffective for non-dividing invading cells^[24]. Gliomas are driven by glycolysis as gliomas have a very low content of normally functioning mitochondria. That causes gliomas to shift their energy metabolism towards a high level of glycolysis to generate their cellular ATP supply (Warburg effect)^[2-3].

C6 glioma was reported to be an experimental model for glioblastoma growth and invasion^[25]. Rat C6 glioma cell line is rapidly proliferating and is morphologically similar to GBM when injected into the brain of neonatal rats where tumoral cells show an undifferentiated morphology. C6 glioma tumors (formed by injecting C6 cells into the brain of neonatal rats) have several characteristics of malignant glioma including nuclear pleomorphism, high mitotic index, foci of tumor necrosis, intratumoral hemorrhage and parenchymal invasion. Pallisading cells delineate the foci of necrosis and lymphocytic infiltration with the occasional formation of proteinaceous eosinophilic edema fluid^[26].

Multimodality therapy in cancer is recommended to get benefit of many drugs acting by different mechanisms of action and to minimize side effects. In this study, antiglycolysis therapy for glioma using citrate was introduced.

Citric acid is an antioxidant and inhibitor of glycolysis pathway (PFK inhibitor) that constitutes the major source of energy supply to aggressive cancer cells. Citrate is a promising inhibitor of Warburg effect through blocking glycolysis upstream of lactate formation step. Natural products treatment attracts attention of physicians, nutritionists and oncologists for its safety, tissue-protection and anticancer potential. Citric acid has many applications in hematology research e.g. sodium citrate is an anticoagulant.

In this study, ability of citric acid to inhibit experimental GBM colony formation (attached to substratum) and anchorage-independent growth (colonies not attached to substratum) were investigated. Citrate induced a dose-dependent inhibition of growth and proliferation of glioma colonies. High citrate dose (9 mM) inhibited the initial formation of glioma colonies (Figures 2A-B). A similar picture was observed where citrate induced a dose-dependent inhibition of growth of glioma colonies in soft agar (i.e. not attached to a substratum). High citrate dose (9 mM) inhibited initial formation of glioma colonies. In conclusion, citrate inhibited 3D tumor models of GBM. Citric acid inhibited clonogenic power of glioma cells. Citric acid may be a promising therapeutic modality for glioma and glioblastoma. That is quite promising in treating GBM tumors and can be generalized for research in different tumors.

Citrate treatment seems to be safe for normal cells. This may be explained by the fact that normal cells with normal mitochondria are usually less sensitive to inhibition of glycolysis as their intact mitochondria enable them to use alternative energy sources such as fatty acids and amino acids to produce ATP^[27]. The metabolic energy pattern differs in normal cells from cancer cells (glioma cells) as a major part of normal cells energetics (ATP) comes from respiration (oxidative phosphorylation) which has high energy yield^[28].

In agreement with the report that citrate is an inhibitor of PFK, this study data agrees with the report by Zhang *et al.* (2009) who reported that the anti-tumor effect of citrate includes depletion of ATP and diminution of the expression of the anti-apoptotic proteins^[5].

Citrate significantly decreased clonogenic power of glioma cells in a dose-dependent manner. Almost no glioma colonies were seen with 9 mM citrate (Figures 2A-B).

Citrate significantly decreased anchorage-independent growth power of glioma cells in a dose-dependent manner (Figures 3A-C). Citrate therapy induced a significant reduction in size of glioma colonies which was dose-dependent on citrate concentration (Figure 3C). In glioma, the activities of mitochondrial key enzymes were significantly decreased when compared with enzyme activities of normal cortex tissue while activities of glycolytic enzymes were increased^[29].

The levels of hexokinase and PFK in the low grade glioma-derived cell lines were not significantly different from those of normal astrocyte cultures. However, the activities of hexokinase and PFK were consistently and significantly increased in the high grade glioma-derived

cell lines. Citrate inhibits glioma and normal astrocytic PFK, but the magnitude of the inhibition is much less in astrocytes than in glioma-derived lines^[30].

As cancer cells depend mainly on glycolysis for supplying energy demands, cancer cells suffer when glycolysis is impaired. Tumors exhibit an acidic extracellular pH (pHe = 6.2-6.9)^[31], which makes tumor cells resistant to basic chemotherapeutic agents as mitoxantrone, while activity of acidic chemotherapeutic agents as 5-fluorouracil is enhanced by this low pHe^[32]. As citrate is acidic in nature, activity of citrate as an anticancer agent may be enhanced by the acidic pHe in tumors. Citrate causes a decrease in pH of the culture medium (DMEM) *in vitro* in a dose-dependent manner. Chan et al. reported that 0.1 % citrate (4.76 mM) keeps DMEM at an alkaline pH (pH: 7.2), while 0.25 % citrate (10.71 mM) reduces pH of DMEM to 6.22^[33]. High dose of citrate e.g. 15 % citrate (0.7 M) for 7 days moderately decreased viability of macrophages^[34] Similar to that is the report by *Malheiros et al.* who reported that growth of NIH3T3 fibroblasts was progressive and continuous at 0.1 % citrate treatment for 7 days, while moderate cytotoxic effects of citrate treatment occurred at 0.5 % (23.8 mM)^[35].

CONCLUSION

In conclusion, citrate-induced acidosis *in vitro* may enhance cytotoxicity of high doses of citrate. Citrate inhibited 3D tumor models of GBM. Citric acid inhibited clonogenic power of glioma cells. Citric acid may be a promising therapeutic modality for glioma and glioblastoma. That is quite promising in treating GBM tumors and can be generalized for research in different tumors.

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