

The Antioxidant Glycolysis Inhibitor (Citric Acid) Induces a Dose-dependent Caspase-mediated Apoptosis and Necrosis in Glioma Cells

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Abstract Background: Glioma tumors are still a big challenge being incurable with current chemotherapy and radiotherapy treatments. Surgical treatment of glioma needs adjuvant effective targeting therapy for better glioma cell treatment. Citrate is a well-known antioxidant organic acid abundant in citrus fruits and is an inhibitor of glycolysis through targeting the glycolytic enzyme phosphofructokinase, one of the key enzymes of glycolysis. Citrate is a natural product that is formed inside mitochondria during Krebs cycle to the extent that Krebs cycle is often referred to as citric acid cycle. It was reported that glioma cells are driven by glycolysis where glioma cells upregulates the expression of glycolysis genes and enzymes. **Objectives:** This aim is to investigate effect of citrate on glioma cells viability, morphology and mode of glioma-induced cell death. **Methodology:** In this study, citrate-induced glioma cell death was investigated using MTT assay, western blot analysis and flowcytometric evaluation was done to C6 glioma cells. **Results:** Citrate induced a potent anti-glioma effect by significantly decreasing viability of C6 glioma cells in a dose-dependent manner. Flow cytometric analysis revealed that at 5 mM, citrate induced a caspase-dependent apoptotic glioma cell death. Higher doses of citrate (9 mM) induced necro-apoptotic glioma cell death. **Conclusion:** citrate may be a promising therapeutic treatment for glioma and glioblastoma. Citrate-rich fruits are strongly recommended as a nutritional treatment for glioma patients.

Keywords: citrate, phosphofructokinase, Glioblastoma, apoptosis

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1. Introduction

Brain tumors particularly glioblastoma multiforme (GBM) are the most aggressive diffusely infiltrating glioma in human. Despite intensive treatment, glioblastoma tumors regrow rapidly, invade and infiltrate normal brain tissues [1]. Cancer cell energy metabolism is characterized by a high glycolytic rate, which is maintained under aerobic conditions [2]. Malignant gliomas constitute more than 49% of all central nervous system (CNS) tumors and exhibit very poor prognosis. Two main events are involved in the progression of gliomas: the deregulation of genes by genetic events and cellular damage elicited by the formation of reactive oxygen species (ROS). In gliomas, epigenetic abnormalities implicated in the deregulation of DNA methylation, nucleosome rearrangement, and acetylation of histones have been depicted. The overproduction of ROS has been implicated in the promotion of such modifications [3].

Most cancer cells have a glycolytic phenotype. Based on that, treatments that cause glycolysis suppression e.g. citrate may be promising, selective and effective. Added to that, citrate (a member of Krebs cycle and an inhibitor of the key glycolysis enzyme PFK) is an antioxidant that confers tissue-protective effects [4]. Cellular energy metabolism is remodeled during malignant cellular transformation i.e. transition from normal to cancer cells. This promotes cell viability, proliferation, immortality and prevents cancer cells death. Otto Warburg described this phenomenon early in the 20th century where cancer cells exhibit a glycolytic phenotype ending in the production of lactate even in the presence of oxygen (Warburg effect). Glycolysis reaction and related reactions e.g. pentose phosphate pathway provide energy, nucleotides, fatty acids, and amino acids to supply the progressively increased demands of the hyperactive and dividing cancer cells. Another important phenomenon in cancer cells is the mitochondrial biogenesis with a progressive increase in oxidative stress that's mediated via continuous ROS generation. This malignant phenotype is markedly evident in gliomas e.g. GBM (the malignant adult brain tumor) [5].

Glycolysis genes e.g. phosphofructokinase (PFK) and hexokinase (HK) are overexpressed in cancer cells particularly gliomas. Genes that promote cell proliferation and inhibit apoptosis as PFK variants e.g. PFKFB1, PFKFB2, PFKFB3, PFKFB4 and HK2 are upregulated in pediatric glioma tissues. Those genes help enhancing the tumor cells viability, growth and invasion [6]. Based on that, glycolysis suppression in cancer cells may selectively reduce cancer cells proliferation and viability.

Citrate is a natural antioxidant product that is quite abundant in citrus fruits e.g. orange, grapefruits, lemon and tangerine. Citrate is an evident glycolysis inhibitor through targeting the key glycolysis enzyme (PFK). Anticancer effects of citrate act through inhibiting PFK that proved effective against many types of cancer cells e.g. against two human gastric carcinoma cell lines where citrate caused a destruction of the cell population in both cell lines. Citrate-induced apoptotic cell death occurred through the mitochondrial pathway in a dose- and time-dependent manner, associated with the reduction of the anti-apoptotic Mcl-1 protein in both lines [7].

Moreover, sodium citrate was reported to induce

apoptotic cell death in human gastric cancer cells by inhibiting glycolysis and stimulating mitochondria-induced apoptotic cellular death [8]. Same promising citrate-induced anticancer effects were observed in animal models. Sodium citrate was reported to target glycolysis, suppress survivin, and induce mitochondrial-mediated apoptosis in gastric cancer cells and inhibit gastric orthotopic transplantation tumor growth [9].

Citrate is a well-known antioxidant organic acid abundant in citrus fruits and is an inhibitor of glycolysis through targeting the glycolytic enzyme phosphofructokinase, one of the key enzymes of glycolysis. Citrate is a natural product that is formed inside mitochondria during Krebs cycle (Figure 1B) to the extent that Krebs cycle is often referred to as citric acid cycle. Citrate was reported to have many diagnostic uses in oncology e.g. citrate is an important diagnostic marker to differentiate malignant prostate tumors from benign prostatic hyperplasia [10] to facilitate diagnosis of prostate cancer [11]. Citrate concentrations in human seminal fluid outperforms prostate specific antigen in prostate cancer detection [12]. Citrate level is further reduced in metastatic prostate disease [10]. Citrate concentrations declined significantly with time in progressing brain stem glioma [13]. ⁵²Fe-citrate and positron emission tomography can measure iron uptake in brain tumors [14]. Citrate was reported to show a specific, dose-dependent lympholytic activity in neoplastic cell lines [15].

Citrate was reported to have a lot of therapeutic uses as well e.g. in pediatrics, citrate is efficient in correction of diarrhea-induced metabolic acidosis as sodium citrate equals sodium bicarbonate in oral rehydration therapy for childhood diarrhea [16]. Citrate helps cure of antibiotic-resistant wounds that occur postoperatively in cancer patients [17] and facilitates sperm motility [18].

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. Low citrate level (< 1 mM) was reported to be inhibitory of phosphofructokinase-1 (PFK-1), a key glycolytic enzyme [19]. 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 [20].

In this study, I investigated citrate-induced glioma cell death as a treatment modality for glioma.

2. Materials and Methods

2.1. Reagents and Antibodies

Citrate (Figure 1A) was purchased from Wako (Nasr pharmaceuticals, Egypt). Cell nutrient medium Dulbecco's modified Eagle's medium (DMEM), monoclonal anti- β -actin antibody, fetal bovine serum (FBS) and MTT viability assay reagent were all purchased from Sigma (St. Louis, MO, USA). Penicillin-streptomycin antibiotic mixture and DMEM/F12 were purchased from Invitrogen life technologies (Carlsbad, CA, USA). Precision plus proteinTM standard (for western blot) was purchased from Bio-Rad laboratories (Hercules, CA).

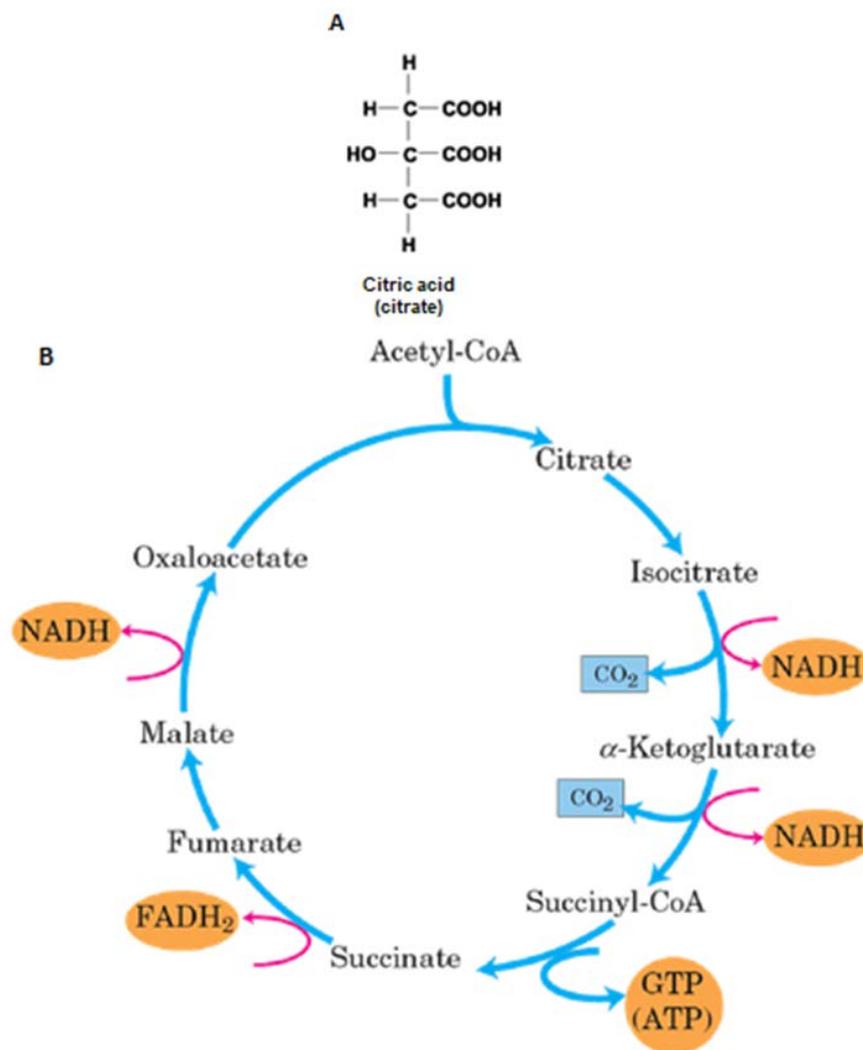


Figure 1. Citrate is an antioxidant inhibitor of glycolysis. A. Structure of citrate. B. Krebs cycle is also called citric acid cycle as citrate is an important intermediate in the cycle.

2.2. Cell Culture

C6 gliomas (also referred to as C6 glioblastomas) were grown in suitable media to act as an experimental GBM in vitro 2D cell model. C6 rat glioma cells were grown in DMEM/F12 to which nutrient and antibiotic mixture were added e.g. horse serum, FBS and penicillin-streptomycin. Cells were maintained at at 37°C in a humidified atmosphere having 5% CO₂. C6 glioma cells were grown in DMEM/F12 having with FBS, horse serum, and 1% penicillin-streptomycin.

2.3. MTT Assay

In order to investigate the effects of serial doses of citrate upon proliferation power and growth of GBM experimental cells C6 cells, serial doses of citrate (3, 5 and 9mM) were given to C6 glioma cells. C6 glioma cells (1 x 10⁴ cells/well) were plated into 96-well plates for 24 h till cells got about 80% confluency. Medium aspiration then stimulating medium in the form of DMEM/F12 containing FBS was added. Cells received treatment serial citrate treatments (1, 3, 5 and 9 mM) and were then incubated for 21 h. MTT solution was utilized in testing glioma cells viability in a dose of 50 µl (of 1 mg/ml). That was immediately followed by incubation for an additional

3 h in 96 well plates. Centrifugation, elimination of supernatant followed by dimethylsulfoxide addition (150 µl/well) were done. Dimethylsulfoxide helps at dissolution of the insoluble formazan crystals producing a color that is photometrically used to evaluate the degree of cellular viability. That was followed by measuring the absorbance at 550 nm using Infinite™ M200 microplate reader.

2.4. Microscopic Analysis

In order to investigate the morphological damage features exerted by serial doses of citrate (in millimolar range) upon C6 glioma cells, cells were photographed for investigating the citrate-induced changes in glioma morphology. Morphology of C6 cells which received citrate treatment (3, 5 and 9 mM) for 12 h was investigated using phase contrast microscopy with a held camera (Nikon). Images were taken with the help of Nikon digital sight screen.

2.5. Annexin V-propidium Iodide Flow Cytometry

In order to investigate the mode of citrate-induced glioma cell death (apoptosis or necrosis), relatively large doses of citrate were used in an Annexin V-propidium

iodide flow cytometry. Annexin V stains apoptotic cells while propidium iodide stains necrotic cells. Using 6 well plates, C6 cells (1×10^5) were seeded for 24 h and received serial citrate treatment (5 and 9 mM) for a duration of 12 h and 24 h. ApoAlert™ Annexin V Apoptosis Kit (Clontech, CA) was used according to instructions of manufacturer's. Cells were washed with binding buffer and were then centrifuged and washed again in binding buffer. Cellular staining was done using of Annexin V (5 μ l) and propidium iodide (10 μ l). Incubated of C6 cells was in the dark (at room temperature for 15 min.). 300 μ l Binding buffer was added to C6 samples to be ready for flow cytometry analysis.

2.6. Western Blot

In order to investigate the occurrence of caspase-dependent apoptotic cell death in C6 glioma cells treated with citrate, cleaved caspase protein detection using western blot was done. C6 glioma cells were collected by scraping using a scraper spoon. Cells were lysed immediately using RIPA radioimmunoprecipitation assay lysis buffer. RIP buffer was formed of Triton X-100, deoxycholate, 137 mM NaCl, 10% (v/v) glycerol, 20 mM Tris.Cl, pH 7.4, , 0.1% (w/v) SDS, 1 mM PMSF and 2 mM EDTA]. RIPA buffer was added to Complete Mini Protease Inhibitor Cocktail Tablets purchased from Roche Diagnostics (Mannheim, Germany). Cells were then immediately sonicated, extracted at 4 °C for 30 min and then centrifuged at 14,000 g for 20 min. SDS-PAGE was done. Caspase identification using western blot were done in reducing and denaturing conditions. 40 μ g of protein samples were

used per lane. Protein samples were denatured through addition to denaturing sample buffer (4 X) formed of % (v/v) 2-mercaptoethanol, 0.2% (w/v) bromophenol blue, 8% (w/v) SDS, 40% (v/v) glycerol and 200 mM Tris.Cl, pH 6.8, 8. The mixture was electrophoresed on 12.5% polyacrylamide gel containing 0.1% SDS. Proteins were immediately blotted via transfer to Immobilon-P Transfer Membrane purchased from Millipore (Bedford, MA). After that, membranes were incubated with monoclonal anti- β -actin antibody (1:5000) and rabbit polyclonal anti-caspase-3 antibody (1:200). Detection of cleaved caspase was done according to manufacturer's instructions using ECL Advance Western blotting detection systems purchased from Amersham Biosciences (Little Chalfont, UK).

2.7. Statistics

Results expressed are (Mean \pm S.E.M) of the data got from at least 3 independent experiments. Differences between groups were analyzed using Student's t test. Significance values detrmind significance values at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)

3. Results

Glioma cells are driven by glycolysis. Phosphofructokinase (PFK) is a key enzyme of glycolysis and Warburg effect (Glucose oxidation with production of lactate) in cancer cells. PFK catalyzes the irreversible phosphorylation step of fructose-6-phosphate (F6P) to fructose 1 and 6 diphosphate (F1, 6 DP) [21].

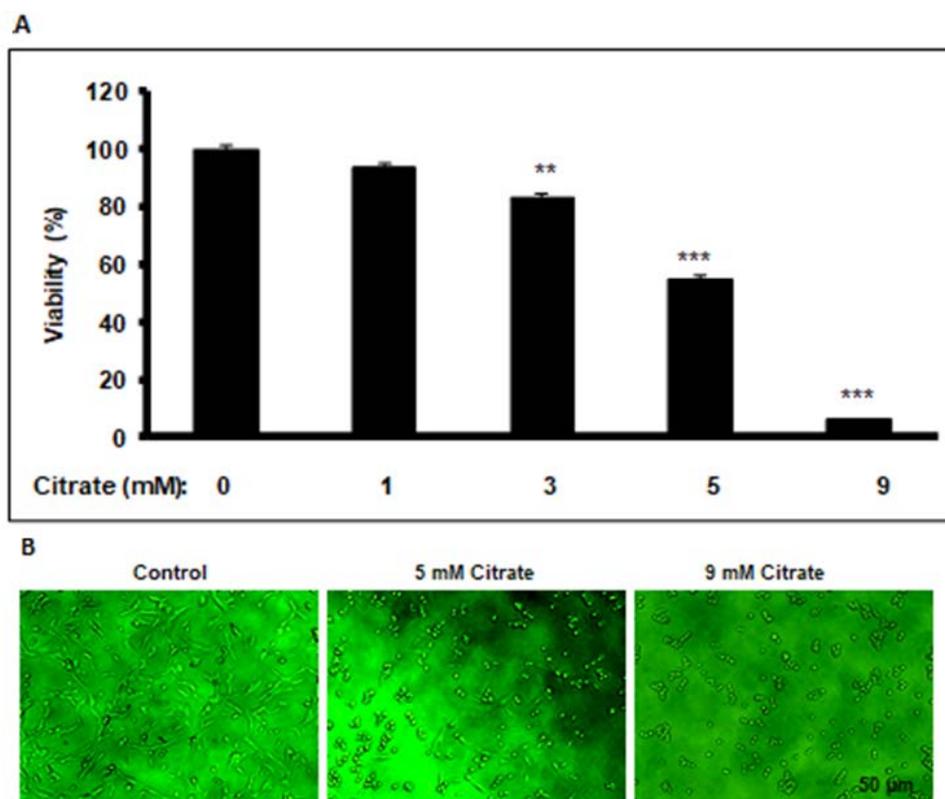


Figure 2. Citrate induces glioma cell death. (A) Effect of serial doses of citrate on C6 glioma viability. Viability levels in untreated controls were taken as 100%. Data are (Mean \pm SEM) of the percentages of the control values of three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate significance between control and different treatment conditions and also between treatment conditions within the same group. (B) Effect of citrate on morphology of C6 glioma.

3.1. Citrate Significantly Decreased Glioma Growth

C6 glioma cells exhibited a dose-dependent glioma cell death upon treatment using serial doses of citrate. Citrate significantly decreased glioma growth and viability. Dose 9 mM citrate was able to decrease viability in C6 glioma cell lines by more than 94% (Figure 2A). As C6 glioma is a rapidly proliferating cell line and is considered as a model for GBM growth and invasion [22], we investigated most of the next steps using C6 cells. Citrate-induced C6 glioma cell death was evident in affecting C6 glioma morphology. C6 glioma lost partially its fibroblast shape at 5 mM citrate and lost it totally at 9 mM citrate (Figure 2B).

3.2. Annexin V-Propidium Iodide Flow Cytometry

As citrate induces a potent glioma cell death, we

investigated the type of glioma cell death caused by citric acid. Differentiating apoptosis from necrosis in cell death was done using flow cytometric analysis using annexin V-Propidium iodide. As shown in Figure 3, C6 glioma cell death caused by citrate was dependent on both dose and time. Citrate induced apoptotic glioma cell death (Annexin V positive- Propidium iodide negative population) at 5 mM and necro-apoptotic death (Annexin V positive- Propidium iodide) positive population) at 9 mM.

3.3. Citrate Induced Caspase-3 Cleavage and Marked Reduction in Anti-apoptotic Proteins

In search of more mechanisms to explain the potential of citrate as an anti-glioma therapy, western blot was performed to investigate the effect of citrate on activation of cleaved caspase-3. Citrate induced-apoptotic C6 glioma cell death occurred via caspase pathway through cleavage of caspase-3 (Figure 4).

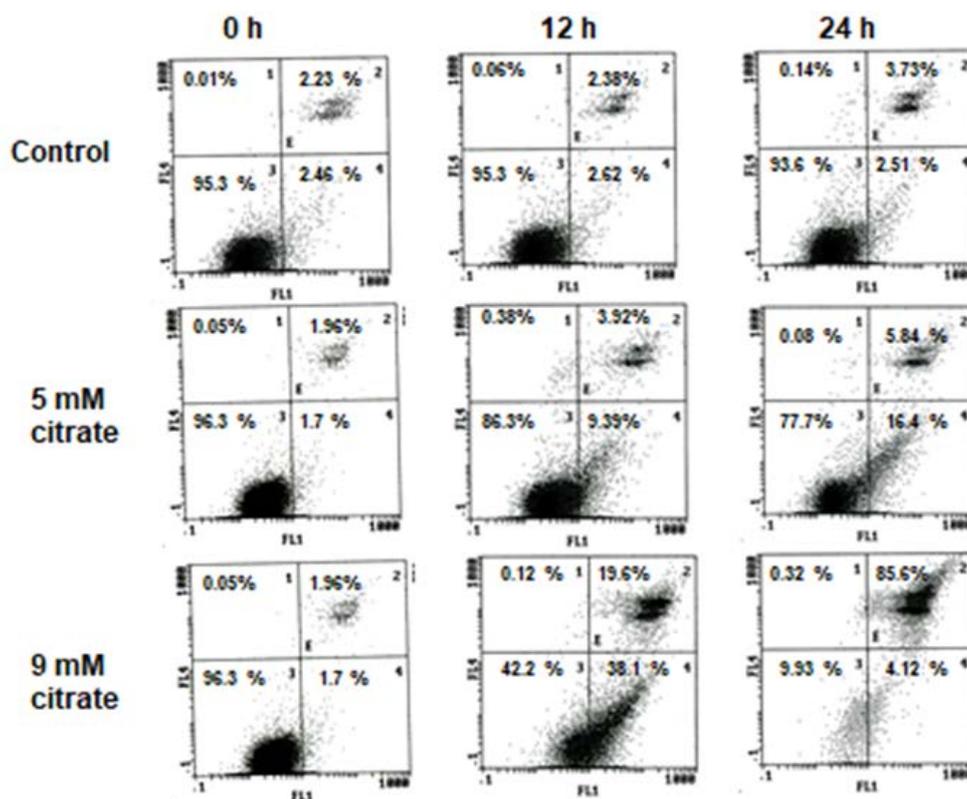


Figure 3. Annexin V-Propidium iodide flow cytometric analysis of citrate-induced C6 glioma cell death

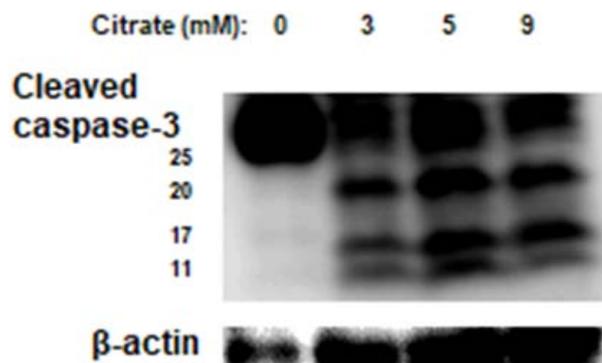


Figure 4. Western blot analysis. (A) Citrate (0, 3, 5 and 9 mM) treatment of C6 glioma cells caused cleavage of caspase-3

4. Discussion

PFK is an important therapeutic target in many cancer cell lines. The glycolysis enzymes HK and PFK levels in low-grade glioma-derived cell lines were not significantly different from those of the normal astrocyte cultures. However, the activities of HK and PFK were consistently and significantly increased in the high-grade glioma-derived lines [23,24]. PFK-1 was reported to be upregulated by 22-56 times in rat AS-30D hepatoma cells than in normal freshly isolated rat hepatocytes [25]. Importantly, there is a positive correlation between glycolysis enzymes gene expression and rate of cells proliferation. Rapidly proliferating cells exhibited increased concentrations of glycolysis intermediates e.g. glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-bisphosphate in comparison with differentiated cells. In differentiated cells under maximally activating conditions, the specific activity and Vmax of HK and PFK enzymes decreased by 3- and 28-fold. The opposite occurs in transformed and malignant cells that upregulate glycolysis genes and enzymes. This strongly suggests that both HK and PFK play important roles in cancer cellular proliferation of C6 glioma cells [26]. Moreover, glycolysis and gluconeogenesis enzymes 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3) were reported to be up-regulated in high-grade astrocytomas [27]. Upregulation of PFK1 protein during chemically-induced hypoxia was reported to be mediated by the hypoxia-responsive internal ribosome entry site element, present in its 5'untranslated region of PFK I [28].

Importantly, treatment of human gliomas and glioblastomas using anti-angiogenic agents e.g. bevacizumab was disappointing. Many gliomas rapidly established many escape mechanisms. In preclinical GBM models, it had been recently shown that bevacizumab, a blocking antibody against vascular endothelial growth factor, induces hypoxia in treated tumors, which is accompanied by increased glycolytic activity and tumor invasiveness. Genomic transcriptomic analysis of GBM cells obtained from patients in addition to GBM stem cell lines revealed a strong up-regulation of glycolysis- genes and genes favoring induction of hypoxia. Suppression of glycolytic enzymes genes expression significantly inhibited GBM growth under conditions of both normoxia and hypoxia. Using orthotopic tumor models e.g. targeting of seven glycolytic genes e.g. PFKP gene in intracranial GBM xenografts caused a significantly increased survival benefit to mice [29].

C6 glioma cells (Figure 2A-B) were reported to be a good experimental model to simulate GBM growth and invasion [22]. Rat C6 glioma cell line is rapidly proliferating and is similar to GBM in morphology when being injected into neonatal rats brains where glioma cells exhibited an undifferentiated morphology. C6 glioma tumors -formed by injecting C6 cells into the brain of neonatal rats- exhibited many criteria of malignant glioma including high mitotic figures, nuclear pleomorphism, areas of tumor necrosis, tumoral hemorrhage and brain tissue invasion [30].

Citrate (citric acid) was reported to be a potent inhibitor of PFK (one of the key enzymes of glycolysis) [31]. 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 treatment of medullary thyroid carcinoma in a child with no report of metabolic acidosis [31]. Also, citrate was reported to have anticancer effects in treating mesothelioma [32] and gastric cancer [33]. In agreement with that, in this study, serial doses of citrate were powerful in decreasing glioma viability depending upon the used doses (Figure 2A). Citrate exerted a potent anti-glioma effect (in millimolar range) as citrate significantly decreased viability of C6 glioma (Figures 2A-B).

The metabolic energy pattern differs in normal cells (astrocytes) from cancer cells (glioma cells) as a major part of astrocytic energetics (ATP) comes from respiration (oxidative phosphorylation) which has a high energy yield [34]. Moreover, citrate contributes to the formation of acetyl units at the synaptosomes of the developing rat brain [35].

It was reported that glioma is driven by glycolysis [36] and exhibits Warburg effect (increased glycolysis to produce ATP and metabolic shift from oxidative phosphorylation to glycolysis even in the presence of oxygen) [37]. Data in this study confirmed that where citrate-induced glioma cell death was both dose-dependent and time-dependent causing both apoptotic and necro-apoptotic cell death (Figure 3).

Interestingly, this study proved that citrate-induced glioma cell death was caspase-dependent in which cleavage of caspase-3 occurred (Figure 4). This is in agreement with the report by Kruspic et al. who reported that the promising anticancer effects induced by citrate was reported to be related to the activation of apical caspases-8 and -2, caspase-3 activation, poly-ADP-ribose polymerase cleavage and release of cytochrome c [4]. This is also in agreement with another report by Gomez et al. [24] where citrate proved effective in treating different cancer cell types in a similar effect to the well-known antioxidant resveratrol. Citrate shares many common properties with the antioxidant agent resveratrol that is a promising anti-tumoral drug. Either citrate or resveratrol decreased glucose catabolism and cancer cells viability. Resveratrol is a potent PFK inhibitor that decreased glioma viability, ATP levels and glucose consumption. Biochemically, resveratrol directly inhibits purified PFK and dissociated PFK enzyme from the active state (tetramers) into a less active (dimers). This mode of resveratrol-induced anticancer effects is similar to the effects induced ATP and citrate [24].

5. Conclusion

Citrate may be a promising therapeutic treatment for glioma and glioblastoma. Citrate-rich fruits are strongly recommended as a nutritional treatment for glioma patients.

Conflict of Interest

We declare that we do not have conflict of interest with anybody.

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