Change in microRNAs Expression Profile of Glioblastoma Multiforme (GBM) Cell Lines Following Treatment By Ascorbic Acid

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Abstract

Whereas several clinical trials are ongoing or have been completed examining the benefits of Ascorbic acid-based therapy of cancer patients, its efficacy at GBM treatment has not been sufficiently investigated. In the present study, the influence of L-Ascorbic acid (Vitamin C, VC) on two GBM cell lines (U87 and U251) was evaluated in terms of cytotoxicity, induction of cell cycle arrest, reactive oxygen species (ROS) production, and alteration in the level of GBM related microRNAs. The half maximal inhibitory concentration (IC_{50}) of VC was obtained by Crystal Violet assay as 2 mM and 1.8 mM for U87 cell line following 24 and 48 h treatment, respectively. These values were obtained in a similar way for U251 cell line as 3.2 mM and 2.9 mM. Propidium iodide (PI) staining of the cells revealed that ascorbic acid caused cell cycle arrest in G2/M phase in both of studied GBM cell lines. Moreover, RT-qPCR results indicated that VC-treatment of GBM cell lines causes downregulation of Bcl-2 alongside increase in BAK-1 and BAX expressions. Flowcytometry-based DCFH assay confirmed drastic increase in reactive oxygen species (ROS) within U87 and U251 cells following VC-treatment. Eventually, study on microRNAs expression profile implied significant increase in four tumor suppressor miRNAs including miR-7, miR-34a, miR-128, and miR-182 in both of U87 and U251 cell lines after treatment with ascorbic acid. Besides, the expression levels of three onco-miRs (i.e., miR-10b in both of cell lines, miR-222 in U87 and miR-93 in U251) were significantly diminished.

1. Introduction

Glioblastoma multiforme (GBM) is the most pernicious and aggressive brain tumor with the highest mortality and sorely poor prognosis which has been turned to a dilemma due to its unpredictable clinical behavior. Accordingly, GBM is considered as a fatal cancer with the median survival rate of 12–15 months even with aggressive treatments. Besides, GBM usually comes up with recurrence of treatment-resistant tumor growth with a median overall survival of 9 months [1,2]. Structurally, GBM tumors are highly heterogeneous with not only stem-like glioma-initiating cells (GICs), but also intermingled non-neoplastic parenchymal cells including microglia, peripheral immune cells, vascular cells and neural precursor cell (NPC)s [3,4]. microRNAs (miRNAs, miRs) are the small single-stranded non-coding RNA molecules well characterized as the momentous players during embryogenesis, cell proliferation and differentiation through a process of post-transcriptional modification [5,6]. Expression profile of some miRNAs is also associated with heterogeneity of cancers and histopathological grades. These miRNAs target either tumor-associated or tumor suppressor genes, named as tumor suppressor miRNAs and onco-miRs, respectively. It is well documented that microRNAs are involved in the pathogenesis, tumorigenesis, angiogenesis, invasion and apoptosis of GBM, hence they can serve as the promising biological markers with a possible diagnostic and prognostic potential [7,8]. Ascorbic acid (Asch₂, vitamin C) is a water-soluble micronutrient with the ketolactone structure having two ionizable hydroxyl groups. According to its pK_a values ($pK_a1 = 4.2$, $pK_a2 = 11.6$), the Ascorbate monoanion, Asch⁻, is the dominant form under physiological pH conditions (~99.9%) [9]. Several clinical trials are ongoing or have been accomplished investigating the benefits of ascorbate therapy of cancer patients. It is demonstrated

that intravenous administration of ascorbic acid attains high plasma concentrations needed to act as chemotherapeutics unachievable through oral consumption [10]. Some possible mechanisms are introduced for anti-tumor effect of ascorbic acid. One of the main proposed mechanisms is the excessive H_2O_2 accumulation within the target cells due to extracellular generation by ascorbic acid function followed by diffusion across the plasma membrane. Paralyzing reductive ability of Glutathione (GSH) through its oxidation to Glutathione disulfide (GSSG) also stimulated by ascorbic acid is an ancillary process helping intracellular retention of H_2O_2 [11-13]. H_2O_2 is naturally a kind of ROS (reactive oxygen species) with the ability to generate other downstream ones by the catalytic action of transition metal ions such as Fe^{2+} and Fe^{3+} in Fenton chemistry [14]. It has been confirmed that increase in the ROS level over the cytotoxic threshold, disrupts the redox homeostasis specially in cancerous cells, triggering their death by the various events [13]. Nevertheless, the performance of ascorbic acid as a potential ROS producer in GBM treatment is not sufficiently evaluated. In the present study, the influence of ascorbic acid on cell viability, cell cycle, ROS generation, and expression level of ten microRNAs including four onco-miRs (i.e., miR-10b, 21, 93 and 222) and six tumor suppressors (i.e., miR-7, 34a, 128, 182, 124 and 137) as well as anti-apoptotic (Bcl-2) and apoptotic (BAK-1 and BAX) genes in two GBM cell lines were investigated.

2. Materials And Methods

2.1. Stereochemical analysis

10 mg of L-Ascorbic acid (> 99% purity) was dissolved in 1 mL of double distilled water (DDW) at room temperature ($24\pm1^\circ C$) to obtain a colorless solution. The optical activity (i.e., specific optical rotation) of this freshly prepared solution was then determined using a polarimeter (PerkinElmer).

2.2. Cytotoxicity measurement by Crystal violet assay

Crystal violet assay was performed as described previously [15] with some modifications. Briefly, Crystal Violet (CV) solution (0.5 wt%) was prepared by dissolving CV powder in 20% V/V ethanol. U87 and U251 GBM cell lines were provided from Stem Cell Technology Research Center (Tehran, Iran). After preliminary culture of cells in T25 flasks (DMEM, supplemented by 10% FBS), the cell seeding in 96 well plates (at a density about 7.0×10^4 /well) was carried out in quintuple and incubation of the cells at $37^\circ C$ in 5% CO_2 with daily medium exchange was performed. After either 24 or 47 h, the media were completely removed and replaced by 50 mL CV solution mentioned above. Following 20 min incubation at room temperature with a mild shaking, the solution was meticulously removed. Then, the microwells were washed four times (300 mL/well each time) by the phosphate-buffered saline (PBS, 10mM). The plates were air-dried overnight at room temperature. Afterwards, 200 mL absolute methanol was added to each microwell followed by 20 min incubation at room temperature with a gentle shaking. Finally, the absorbance at 570 nm was measured with an ELISA plate reader (Biotek).

2.3. Cell cycle analysis

The cells (U87 and U251 cell lines, separately) were treated 24 h with the half maximal inhibitory concentration (IC₅₀) of ascorbic acid obtained by the Crystal Violet assay as aforementioned. Untreated cells were employed as the control. Afterwards, the media was completely removed, and the cells were washed gently by the PBS (pH 7.4, 10mM). After harvesting the cells, fixing process was carried out by dropwise adding of cold 70% ethanol while vortexing. This step was accomplished by 2 h incubation at 4 °C. Then, the fixed cells were centrifuged (900 g), washed, and resuspended in 500 µL PBS containing ribonuclease (100 µg/mL) for 30 minutes to eliminate RNAs from the sample. Eventually, cells were stained by 200 µL propidium iodide (PI) in dark and analyzed by the FACSCalibur flow cytometer (BD Biosciences, San Jose, California). The results were evaluated using FlowJo v7.6.1 software.

2.4. RT-qPCR analysis of apoptotic and anti-apoptotic genes

The expression levels of Bcl-2 (as a key proto-oncogene/anti-apoptotic gene) as well as BAK-1 and BAX (as two key factors involving in intrinsic/ mitochondrial apoptosis pathway) were investigated by RT-qPCR. Briefly, total RNA was extracted from the cells treated by half maximal inhibitory concentration (IC₅₀) of ascorbic acid as well as untreated cells using total RNA extraction kit (iNtRON Biotechnology, Korea). cDNA was synthesized by PrimeScript RT reagent Kit (Takara Bio, Japan) according to the manufacturer's instructions. Then, RT-qPCR was carried out using SYBRPremix Ex Taq II (Takara Bio, Shiga, Japan) in Applied Biosystems StepOne instrument (Applied Biosystems, Foster City). The specific primers were obtained from Stem Cell Technology Research Center (Tehran, Iran), which are listed in Table 1. The expression level of each gene was normalized relative to 18S rRNA gene. The expression fold change of each gene was obtained using the $2^{-\Delta\Delta C_t}$ method.

2.5. Probing ROS production

Reactive oxygen species (ROS) production in GBM cell lines treated by ascorbic acid was investigated by DCFH assay and flowcytometry as previously described [16,17]. Briefly, cells were treated by IC₅₀ of ascorbic acid for 24 h, then removed from the culture flask and washed twice with PBS (10 mM). After 10 min incubation in pre-warmed PBS, 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) freshly prepared solution in ethanol absolute was added to the cells suspended in PBS (2 µM DCF-DA in the final mixture). Following 30 min incubation, the mixture was analyzed by flowcytometer to determine obtained 2', 7'-dichlorofluorescein (DCF) emitting green fluorescence. Untreated cells and H₂O₂-treated cells (10% for 30 min) were employed as the negative and positive controls, respectively.

2.6. miRNA expression analysis by RT-qPCR

Change in miRNA level in ascorbic acid-treated GBM cells was studied by RT-qPCR technique as previously described [18]. Briefly, total RNA extraction from untreated and VC-treated cells was performed as described in the previous section. cDNA was synthesized using high sensitivity BON-miR miRNA first-Strand cDNA synthesis Kit (Stem cell Technology Research Center, Tehran, Iran) as described by the manufacturer; First, poly (A) polymerase was used at 37°C for 30 minutes to add poly (A) tail to miRNAs. RNA poly (A) tail was mixed with BON-RT adaptor (primer, 10 µM) and incubation was performed at 75°C for 5 minutes for specific primer ligation. Then, RT enzyme, dNTPs, and RT buffer were added. cDNA

synthesis was performed at 25°C for 10 minutes, 42°C for 60 minutes, and 85°C for 5 minutes. Reverse transcription-qPCR (RT-qPCR) was carried out with SYBRPremix Ex Taq II (Takara Bio, Shiga, Japan) in Applied Biosystems StepOne instrument (Applied Biosystems) based on the following program: 95°C for 30 S, followed by 40 cycles at 95°C for 30 S and 60°C for 30 S. The expression levels of microRNAs were normalized to that of SNORD-47 (U47), as an internal control. The specific primers were purchased from Stem Cell Technology Research Center (Tehran, Iran) which are listed in Table 1. All reactions were performed in triplicate. The expression level of each miRNA was evaluated using $2^{-\Delta\Delta C_t}$ method.

2.7. Statistical analysis

All data are expressed as the average $\pm$ standard deviation (SD). Statistical comparisons were performed by GraphPad Prism 8 using unpaired t-test or one-way ANOVA (followed by Tukey's post-test) as required.

3. Results

3.1. Stereochemical analysis

Specific optical rotation $[\alpha]$ of L-Ascorbic acid (10 mg/mL) at room temperature ($24\pm 1^\circ\text{C}$) was determined as +28, confirming its stereochemical purity [19].

3.2. Cytotoxicity assay

According to the results obtained by Crystal Violet viability assay (Fig. 1), cytotoxicity of ascorbic acid in both of U87 and U251 GBM cell lines showed a dose-dependent manner in the range of our study (i.e., 1.5-9 mM). The half maximal inhibitory concentration (IC_{50}) of ascorbic acid was estimated using GraphPad Prism 9 software as 2 mM and 1.8 mM when U87 cells were VC-treated for 24 and 48 h, respectively. Likewise, these values were obtained for U251 cells as 3.2 mM and 2.9 mM, respectively.

3.3. Cell cycle arrest

To further investigate the effect of ascorbic acid on GBM cell lines, cell cycle analysis of VC-treated U87 and U251 cell lines was performed by flowcytometry. To prevent the false reduction of phases population compared to the untreated (control) cells, the supernatant containing dead cells with highly elevated sub-G1 fraction was discarded and the analysis was just carried out on adherent cells. The results (Fig. 2) indicated that VC in its half maximal inhibitory concentration (IC_{50}) caused cell cycle arrest in G2/M phase in both of studied cell lines ($p<0.05$ and $p<0.01$ in U87 and U251 cell lines respectively). Furthermore, significant increase ($p<0.01$ for both cell lines) in sub-G1 fraction compared to the untreated cells was also observed in VC-treated cells implying population of already adherent cells involved in apoptosis stage.

3.4. Change in expression of apoptotic and anti-apoptotic genes

The RT-qPCR analysis (Fig. 3) revealed that Bcl-2 as an anti-apoptotic protein promoting cellular survival and inhibiting the actions of pro-apoptotic proteins, was significantly decreased ($p<0.05$) in transcript-level in both of examined GBM cell lines after treatment by ascorbic acid. On the other hand, there was an increment in the amounts of BAK-1 and BAX transcripts as the apoptotic markers in these cells following the ascorbic acid -treatment.

3.5. ROS production analysis

According to the results (Fig. 4), intracellular mean fluorescence intensity (MFI) in untreated U87 cells (i.e., negative control) was about 58.4, indicating DCF generation through oxidation of DCF-DA by the reactive oxygen species naturally produced within these cells. Treatment of U87 cells by ascorbic acid raised the green fluorescence intensity to 87.3, representing increase in intracellular level of ROS. Likewise, MFI was about 93.5 in U251 untreated cells which was elevated to 149 following treatments by ascorbic acid. The positive controls (H_2O_2 -treated cells) showed fluorescence intensities equal to 239 and 265 in the case of U87 and U251 cell lines, respectively.

3.6. Ascorbic acid influence on microRNA signature

Since the aberrant expression of microRNAs in GBM is well documented, the impact of VC on the level of ten GBM-attributed microRNAs (including oncomiRs and tumor suppressor microRNAs) were investigated in U87 and U251 cell lines. According to the results (Fig. 5), VC in its half maximal inhibitory concentration caused a significant decrease in the level of oncomiR-10b ($p<0.05$) and oncomiR-222 ($p<0.05$) in U87 cell line, however its effect on oncomiRs-21&93 was not detectable. In this regard, treatment of U251 with VC in its IC_{50} showed a significant decrease in the expression level of oncomiR-10b ($p<0.001$) and oncomiR-93 ($p<0.001$). Nevertheless, the change in expression of oncomiRs-21&222 was not statistically significant. On the other hand, VC showed an increasing effect on the level of tumor suppressor microRNAs including miR-7 ($p<0.01$), miR-34a ($p<0.05$), miR-128 ($p<0.05$), and miR-182 ($p<0.05$) in U87 cell line, however the expression of two other tumor suppressor microRNAs, miR-124 and miR-137, remained statistically in a same level. Like what observed in U87 cell line, VC treatment of U251 cells resulted in overexpression of miR-7 ($p<0.05$), miR-34a ($p<0.05$), miR-128 ($p<0.05$), and miR-182 ($p<0.01$). The change in the level of miR-124 and miR-137 was also insignificant in VC-treated U251 cells as observed in U87 ones.

Table 1
Sequence of Primers used in RT-qPCR

Primer	Sequence (5'-3')
miR-7 (Forward)	TTGCGTGGAAGACTATTGAT
miR-34a (Forward)	ATGGTGGCAGTGTCTTAGC
miR-124 (Forward)	AGGCACGAGGTGAATGC
miR-128 (Forward)	AGTCACAGTGAAGTGGTCTCTT
miR-137 (Forward)	CGTTATTGGTTAAGAATACGC
miR-182 (Forward)	TTTGGCAATGGTAGAACTC
miR-10b (Forward)	TGACTGTAGAACCGCATTTG
miR-21 (Forward)	ACGTGTTAGCTTATCAGACTGA
miR-93 (Forward)	ACCAAAGTGCTGTTCGTG
SNORD-47 (Foreword)	ATCACTGTAAAACCGTTCCA
miR-222 (Forward)	ACCGAGCTACATCTGG
All studied miRs (Reverse)	GAGCAGGGTCCGAGGT
Bcl-2 (Forward)	GATAACGGAGGCTGGGATG
Bcl-2 (Reverse)	CAGGAGAAATCAAACAGAGGC
BAK-1 (Forward)	CTTCTGAGGAGCAGGTAGC
BAK-1 (Reverse)	AGTCATAGCGTCGGTTGAT
BAX (Forward)	CAAAGTGGTGCTCAAGGC
BAX (Reverse)	CACAAAGATGGTCACGGTC
18S rRNA (Forward)	AGGAATTCCCAGTAAGTGC
18S rRNA (Reverse)	GCCTCACTAAACCATCCA

4. Discussion

Numerous studies have shown that appropriate concentrations of ascorbic acid selectively kill cancerous cells by induction of either apoptosis or autophagy as well as cell cycle arrest, indicating its potential efficacy in cancer treatment [20-22]. In this regards, it is demonstrated that chemotherapeutic function of ascorbic acid emerges in high concentrations, which can only be achieved by intravenous infusion [23,10]. Nevertheless, only limited research programs have been launched to evaluate efficiency of vitamin C-based therapy of Glioblastoma. In the present study, first, U251 (formerly known as U-373 MG) and U87 were selected as the typical human-derived GBM cell lines and treated by 1.5-9 mM VC for either

24 or 48 h. Since the reducing property of ascorbic acid interfere with the cell viability tests which are based on activity of oxidoreductase enzymes (e.g., MTT, XTT, etc. assays), crystal violet assay was employed instead herein. Given that the IC_{50} values of cytotoxic agents greatly depends on the nature of cell lines utilized, the difference between those calculated for U87 and U251 cell lines were well justified. The IC_{50} obtained for U87 (i.e., 2 and 1.8 mM for 24 and 48 h continuous treatments, respectively) was much less than that reported previously by Chen et al (i.e., > 20 mM) [24]. Regarding the fact that in their study, only 2 h cell exposure to ascorbate was carried out, this variation between the results was reasonable. Induction of G2M arrest by ascorbic acid agreed with some previous reports [20,25], although it was contrary to what reported by Herst et al, in which ascorbate not only showed no cell cycle arrest in GBM cells (including LN-18 cell line and GBM primary cells) but inhibited 6 Gy radiation-induced G2M arrest [26]. This discrepancy can be explained by the difference in type of cells used in these studies. Elevated percentage of sub-G1 population detected by flowcytometry indicated occurring VC-induced apoptosis in both of GBM cell lines as was observed in other types of cancer cells after treatment with ascorbate [27,28]. Bcl-2 is well recognized as an anti-apoptotic agent maintaining cellular survival. Moreover, a higher expression of Bcl-2 in the glioblastoma samples compared to normal brain specimens is reported imputing Bcl-2 as an inhibitor of programmed cell death in glioblastomas [29]. On the other hand, BAK and BAX are renowned proapoptotic proteins involved in mitochondrial mediated apoptosis [30]. In the present study, downregulation of Bcl-2 expression alongside increase in expression of BAK-1 and BAX was observed in both of U87 and U251 cell lines post treatment with ascorbic acid. This finding was consistent with those reported by Chen XY et al in which Vitamin C induced overexpression of BAX protein whereas reduced the expression level of Bcl-2 in human melanoma A375 cells [31]. Since the intracellular ROS-induced oxidative damage is one of the known therapeutic effects of anti-cancer medicines [32], DCFH assay was performed to investigate increase in ROS level within the cells after being treated by ascorbic acid. It was well documented that due to multiple reasons such as high metabolic activity and mitochondrial dysfunction, over-proliferative cancerous cells show high reactive oxygen species levels [33] as observed in the present study. Flowcytometry-based results demonstrated that treatment of GBM cells with the half maximal inhibitory concentration (IC_{50}) of ascorbic acid leads to increase in ROS levels as expected regarding the previous reports [34,35]. microRNAs (miRNAs, miRs) are a class of short non-coding RNAs that modulate cell homeostasis through impeding translation or promoting degradation of target genes mRNAs. Since the product of some target genes are involved in incidence of cancer, the relevant microRNAs belong to either tumor suppressor miRNAs or onco-miRs. In this study, first the influence of treatment with ascorbic acid on cellular level of six tumor suppressor miRNAs in GBM cell lines was investigated. miR-7 is considered as a tumor suppressor one which inhibits glioblastoma cells proliferation via reducing the expression of PI3K, phosphorylated Akt, Raf-1, phosphorylated MEK1/2 and cyclin D1 [36]. Moreover, it is reported that this microRNA is aberrantly downregulated in glioblastoma tissues and cell lines and its enforced overexpression markedly decreases cell invasion and migration by targeting special AT rich sequence binding protein 1 (SATB1) [37]. In the present study, treatment of U87 and U251 cells with ascorbic acid favorably led to a significant increase in miR-7 level in both of GBM cell lines. miR-34a was another tumor suppressor miRNA studied in this work. Analysis of human specimens has demonstrated that miR-34a is downregulated in glioblastoma

tissues compared to normal human brain [38]. Multiple targets have been identified for miR-34a among the oncogenes including Notch-1, Notch-2, CDK6, and c-Met [38]. It is also documented that forced expression of miR-34a diminishes the migrating ability of GBM-derived cell lines and lower the levels of cyclins -A1, -B1, -D1, and -D3 [39]. In the present study we found that both of studied GBM cell lines show a significant increase in miR-34a level after treatment with ascorbic acid. In this regard, ascorbate-induced ROS production can be considered as a key event probably involved in miR-34a upregulation as reported by Cheleschi et al, in which H₂O₂ upregulated the expression level of miR-34a in human osteoarthritic chondrocytes [40]. One another of tumor suppressor miRNAs studied herein was miR-128 known to be downregulated in glioma [41,42]. Various direct and indirect targets such as Bmi-1, p70S6K1, NPTX1, mTOR, insulin-like growth factor 1 (IGF-1), and PIK3R1 have been identified for this microRNA justifying its anti-proliferative effect in glioma cells [41,43,44,42]. Significant increase in the level of miR-128 expression was also observed after treatment by ascorbic acid in GBM cell lines. The last investigated tumor suppressor miRNA showing upregulation in GBM cells by vitamin C treatment was miR-182 introduced as an anti-tumor agent through suppressing Bcl2-like12 (Bcl2L12), cMet, and hypoxia-inducible factor 2 α (HIF2A) [4]. Among 470 miRNAs profiled by The Cancer Genome Atlas (TCGA) program, miR-182 was found to be the single one miRNA associated with the all predefined criteria including patient survival, neurodevelopmental context, susceptibility to temozolomide-induced apoptosis, and being overexpressed in the least aggressive oligoneural subclass of GBM [45]. The change in expression level of miR-124 and miR-137 as two other tumor suppressor miRNAs with the proven anti-proliferative effect in glioblastoma multiform cell lines [46] was also surveyed after ascorbate-treatment and no statistically significant difference was observed. In the next step, expression level of four onco-miRs was studied in VC-treated GBM cell lines. As can be deduced from the heatmap (color-coded) graph, the mean of onco-miR levels studied herein was lower than that of tumor suppressor miRNAs in pristine GBM cell lines. It is revealed that overexpress of miR-10b enhances GBM cells proliferation, migration, and epithelial-mesenchymal transition (EMT) [47,48]. Furthermore, some tumor-associated genes such as E-cadherin, Apaf-1 and PTEN are identified as the target genes of miR-10b [47]. In the present study, ascorbic acid-induced downregulation of miR-10b was achieved in both of U87 and U251 cell lines. However, this finding was contrary to what formerly observed in vascular smooth muscle cells (VSMCs) [49] and PC12 cells [50] after treatment with H₂O₂. Cell type-dependent action of H₂O₂ as well as H₂O₂-independent performance of ascorbic acid in promoting expression of miR-10b in GBM cells can be proposed. Surveys such as that conducted by Fang et al [51] sowed that miR-93 upregulation promotes angiogenesis and tumor growth by suppressing integrin- β 8 expression. In the present study, ascorbic acid-induced downregulation of miR-93 eventuated in U251 cells and not in U87 ones. The influence of Vitamin C on miR-93 expression was already investigated in human breast neoplastic cell line T47D by Singh B et al. They reported that Vitamin C reduces the expression level of miR-93; however, statistically nonsignificant. Nevertheless, it increased the expression of NRF2 as a target of miR-93 resulting inhibited carcinogenesis [52]. Previous studied have specified miR-222 as an onco-miR participating in glioblastoma invasiveness and cell survival [53,54]. Herein, decrease in miR-222 expression level was observed in U87 cells post treatment by ascorbic acid, whereas no significant change was detected in U251 cell line. The expression level of miR-21 as another recognized onco-miR involved in GBM

tumorigenesis [55] was not modulated as well in both of studied GBM cell lines after treatment by Ascorbic acid.

5. Conclusion

Herein, the effect of L-Ascorbic acid on two prevalent GBM cell lines including U87 and U251 was evaluated. Collectively, our results including ascorbic acid cytotoxicity, its increasing effect on the level of intracellular ROS, capability to induce cell cycle arrest, and specially change in microRNAs expression profile demonstrated that ascorbic acid can be considered as a promising therapeutic agent against glioblastoma multiform.

Declarations

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Figures

Figure 1

Effect of VC (Ascorbic acid)-treatment on viability of GBM cell lines studied by Crystal Violet assay.

Figure 2

Cell cycle analysis by PI staining and flowcytometry. (b) Comparing percentage of cells in different cell cycle phases between untreated and VC-treated cells. ns: non-significant ($p > 0.05$), *: $p < 0.05$, **: $p < 0.01$.

Figure 3

Change in transcript-level expression of Bcl-2, BAK-1, and BAX proteins after treatment with Ascorbic acid in (a) U87 and (b) U251 cell lines. *: $p < 0.05$, **: $p < 0.01$

Figure 4

DCFH assay indicating increase in intracellular ROS after Ascorbic acid-treatment of GBM cell lines. (a) Intracellular mean fluorescence intensity (MFI) determined using flowcytometry was 58.4 in untreated,

87.3 in VC-treated, and 239 in H2O2-treated (positive control) U87 cells. (b) Intracellular mean fluorescence intensity (MFI) was 93.5 in untreated, 149 in VC-treated, and 265 in H2O2-treated (positive control) U251 cells.

Figure 5 Change in expression level of studied microRNAs after treatment with Ascorbic acid. (a) Change in relative expression of tumor-suppressor microRNAs in U87 cell line. (b) Change in relative expression of

onco-miRs in U87 cell line. (c) Change in relative expression of tumor-suppressor microRNAs in U251 cell line. (d) Change in relative expression of onco-miRs in U251 cell line. ns: non-significant ($p>0.05$), *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. (e) Heatmap (color-coded) of microRNAs levels in U87 and U251 cell lines before and after treatment by Ascorbic acid. The used data were logarithm of the raw results normalized to SNORD-47 (U47) as the internal control.