Life Sciences 232 (2019) 116657

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

High doses of sodium ascorbate interfere with the expansion of glioblastoma multiforme cells in vitro and in vivo

Damian Ryszawy[a,*], Maciej Pudełek[a], Jessica Catapano[a], Małgorzata Ciarach[b], Zuzanna Setkowicz[b], Ewa Konduracka[c], Zbigniew Madeja[a], Jarosław Czyż[a,*]

[a] Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland
[b] Department of Neuroanatomy, Faculty of Biology, Gronostajowa 9, 30-387 Kraków, Poland
[c] Coronary Disease Clinic, Faculty of Medicine, Jagiellonian University, Prądnicka 80, 31-202 Kraków, Poland

ARTICLE INFO

Keywords:
Ascorbic acid
Sodium ascorbate
Glioblastoma
Therapy
Vitamin C

ABSTRACT

Aims: Constant development of chemotherapeutic strategies has considerably improved the efficiency of tumor treatment. However, adverse effects of chemotherapeutics enforce premature treatment cessation, which leads to the tumor recurrence and accelerated death of oncologic patients. Recently, sodium ascorbate (ASC) has been suggested as a promising drug for the adjunctive chemotherapy of glioblastoma multiforme (GBM) and prostate cancer (PC). To estimate whether ASC can interfere with tumor recurrence between the first and second-line chemotherapy, we analyzed the effect of high ASC doses on the expansion of cells in vitro and in vivo.

Main methods: Brightfield microscopy-assisted approaches were used to estimate the effect of ASC (1–14 mM) on the morphology and invasiveness of human GBM, rat PC and normal mouse 3T3 cells, whereas cytostatic/pro-apoptotic activity of ASC was estimated with flow cytometry. These assays were complemented by the in vitro CellROX-assisted analyses of intracellular oxidative stress and in vivo estimation of GBM tumor invasion.

Key findings: ASC considerably decreased the proliferation and motility of GBM and PC cells. This effect was accompanied by intracellular ROS over-production and necrotic death of tumor cells, apparently resulting from their "autoschizis". In vivo studies demonstrated the retardation of GBM tumor growth and invasion in the rats undergone intravenous ASC administration, in the absence of detectable systemic adverse effects of ASC.

Significance: Our data support previous notions on anti-tumor activity of high ASC doses. However, autoschizis-related cell responses to ASC indicate that its application in human adjunctive tumor therapy should be considered with caution.

1. Introduction

Vitamin C (L-ascorbic acid; AA/L-ascorbate; ASC) is an essential compound that plays a vital role in the regulation of lysyl and prolyl hydroxylase activity. Thus, it is crucial for collagen synthesis, connective tissue homeostasis and scurvy prevention. At low doses, vitamin C also serves as reactive oxygen species (ROS) scavenger, which protects the cells from oxidative stress [1]. Due to mutations in the gene encoding L-gulono-γ-lactone oxidase, it is not synthesized by primates [2] and must be supplied with food. The daily intake of L-ascorbic acid recommended by U.S. Institute of Medicine (IOM) is ca. 75–90 mg [3]. At physiologic (micromolar) concentrations, L-ascorbate can prevent the progression of numerous acute and chronic diseases through interference with oxidative stress in individual cells and tissues. However, the positive effects of vitamin C have also been suggested upon its application at the quantities much higher than recommended [3,4].

At least 2 groups of mechanisms account for potential anti-cancer activity of vitamin C [5]. At low doses, vitamin C can protect the cells from oxidative stress, thus preventing the development of neoplasms [1]. By scavenging of reactive oxygen species (ROS), vitamin C prevents DNA damage and the deterioration of sub-cellular structures (proteins, lipids, DNA). It also counteracts adverse inflammatory responses through the interference with pro-oxidative activity of immune cells (deficit of catalase and superoxide dismutase) and with the depletion of antioxidants (tocopherols, ascorbate and glutathione) that results in the deterioration of sub-cellular structures (proteins, lipids, DNA). Because

Abbreviations: ASC, sodium ascorbate; AA, ascorbic acid; AnV, AnnexinV; BF, bright field; GBM, glioblastoma multiforme; kgBM, kilogram of body mass; PC, prostate cancer; PI, propidium iodide; ROS, reactive oxygen species; SSC, side scatter

* Corresponding authors.
E-mail addresses: damian.ryszawy@uj.edu.pl (D. Ryszawy), jarek.czyz@uj.edu.pl (J. Czyż).

https://doi.org/10.1016/j.lfs.2019.116657
Received 23 May 2019; Received in revised form 11 July 2019; Accepted 12 July 2019
Available online 13 July 2019

numerous links have been proposed between oxidative stress and carcinogenesis, antioxidant activity of vitamin C [5] can counteract cellular neoplastic transformation [1]. Also selective activity of ascorbic acid against cancer cells [6,7] is linked with its effects on their antioxidative systems [8,9]. Additionally, functional links between ascorbate distribution and collagen matrix synthesis, may lead to the inhibition of tumor growth and progression [10].

On the other hand, vitamin C exerts an opposite (pro-oxidative) effect, when applied at concentrations > 1 mM [7]. This activity may open perspectives for the application of vitamin C in chemotherapy. At high doses, ascorbate can induce apoptosis of cancer cells through mitochondrial-dependent pathway and by regulating Ca^{2+} influx in endoplasmic reticulum [11]. Ascorbate causes the oxidation of glutathione, thus depleting its intracellular resources [5]. It also reduces/oxidizes iron ions that are accumulated in cancer cells through overexpression of ferritins [12], leading to the formation of hydroxyl radicals (Fenton reaction and Haber-Weiss reaction; [12,13]). Destabilization of the antioxidant system deteriorates intracellular redox control systems. This leads to the oxidative stress that can cause a rapid death of cancer cells. Accordingly, in vitro studies and clinical trials [14] suggested that vitamin C can exert selective therapeutic effects on cancer cells [15–17].

Potential anti-cancer activity of vitamin C prompted speculations on its application in adjunctive chemotherapy and justifies the research on this topic [17]. Vitamin C can be used to mitigate chronic inflammations and oxidative stress which are frequent side effects observed after intensive cancer treatment [18]. However, this topic remains controversial in the light of potential side-effects of vitamin C and its limited bioaccessibility. For instance, “autoschizis” has previously been described as a mechanism of vitamin C-induced cell death [19,20]. It is specific for the cells treated with high doses of vitamin C, implies self-excision of cytoplasm and may induce dreadful systemic inflammatory reactions. Ascorbic acid also exerts disturbing effects on blood morphology. Recently, sodium ascorbate (ASC) has been suggested to exert less pronounced effects on this parameter than its acidic counterpart. To estimate the balance of therapeutic/adverse effects of ASC in the adjunctive chemotherapy, we estimated the selectivity and systemic consequences of necrotic GBM and prostate cancer cells death induced by high doses of ASC.

2. Materials and methods

2.1. Cell culture

Experiments were performed on rat AT-2 prostate cancer cells, human glioblastoma multiforme U87 and T98G cells, and Swiss 3T3 mouse fibroblasts. Cells were cultured in 25 cm^2 Falcon dishes in 37 °C/5% CO_2. AT-2 cells were grown in RPMI-1640 medium (Sigma), whereas U87 and T98G cells were cultivated in DMEM with 4500 mg/l glucose (Sigma). Swiss 3T3 fibroblast were grown in DMEM with 1000 mg/l glucose (Sigma). All media were supplemented with heat-inactivated 10% fetal bovine serum (FBS; Gibco) and 1% Antibiotic-Antimycotic solution (Sigma). Before each experiment, the cells were treated with 1 mM, 5 mM and 10 mM ASC (Sigma) in Dulbeco's Phosphate Buffered Saline (DPBS; Sigma) for 1 h, then washed with warm DPBS and covered with fresh medium. Control samples were treated with pure DPBS.

2.2. Cell motility

For cell migration analyses, the cells were seeded at the density of 15,000 cells per well in 12-well plates (Falcon), incubated for 24 h and treated with ASC as described above. Time-lapse videos were recorded for 8 h with 5 min intervals using a wide-field Leica DMI6000B system equipped with DFC360FX CCD camera, integrated modulation contrast (IMC; integrated modulation contrast) optics and temperature/CO_2 controlled chamber and a dry ×10 objective. Individual cell trajectories and average values of cell motility parameters at the population level (distance and cell displacement; N > 50 from at least 3 independent experiments) were calculated with Hiro software (written by W. Czapla [21]).

2.3. Viability tests

ImageStream® cytometry was used to estimate pro-apoptotic and pro-necrotic activity of ASC. The cells were seeded into 60 mm cell culture dishes (Eppendorf) at the density of 50,000 per dish and propagated to confluence. After ASC treatment, cells were trypsinized, washed with cold PBS, centrifuged at 400*g*, suspended in AnnexinV binding buffer (BD, Pharminogen™) supplemented with propidium iodide and FITC-AnnexinV and left on ice for 15 min. 3 independent experiments were performed for each condition using ImageStream® Imaging Flow Cytometer (Merck). In each experiment, 10,000 singlet events were analyzed with the IDEAS 6.2 software (Amnis).

2.4. Cell transduction and fluorescence microscopy

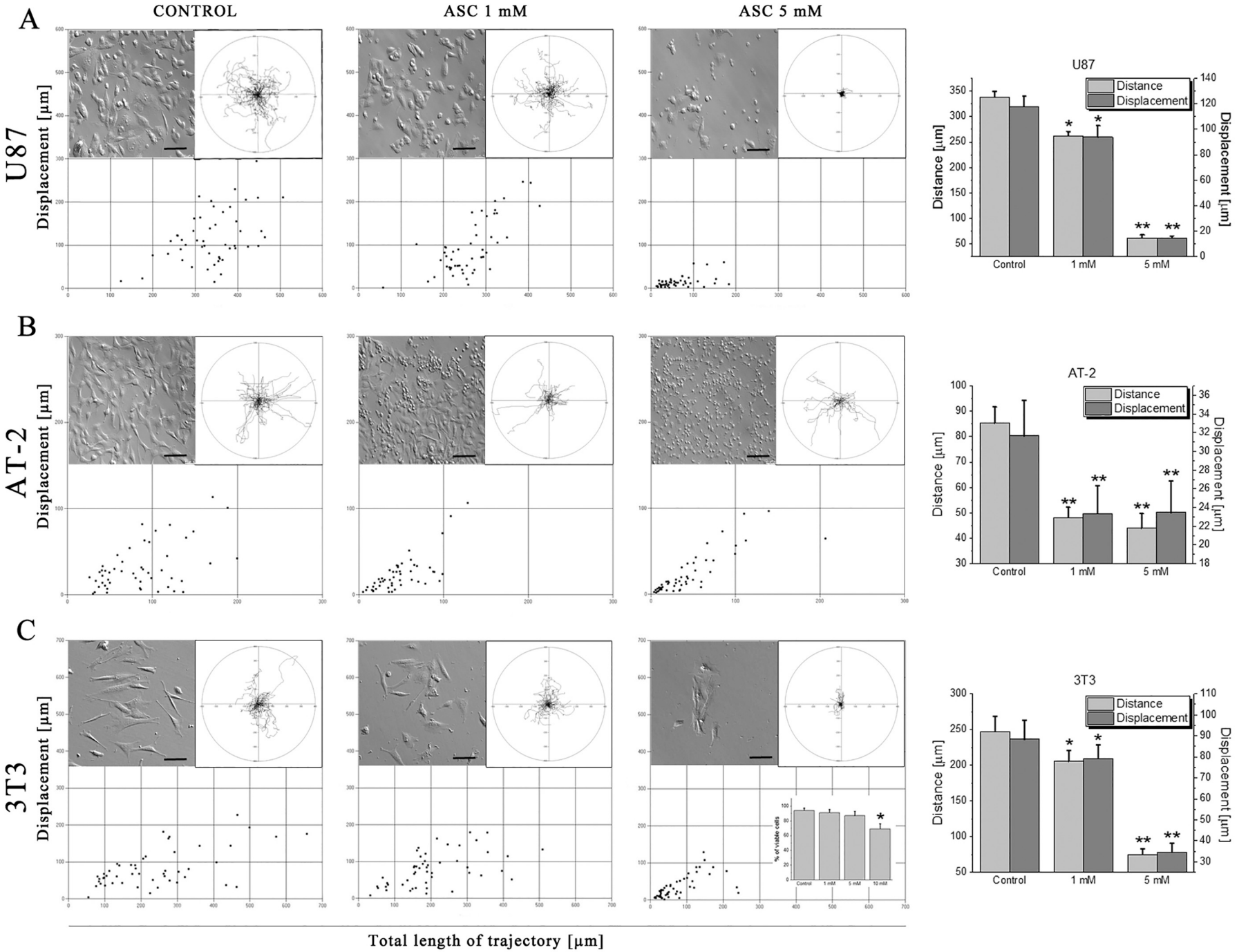
Cell Light™ Actin-GFP, Bac Mam 2.0 (Invitrogen) was used to visualize the cytoskeleton architecture of ASC-treated cells. 10,000 cells were incubated with 2.5 µl of baculovirus solution for 24 h. Afterwards, the medium was replaced for FluoroBrite DMEM (Gibco; supplemented with 10% FBS and 4 mM GlutaMax). 3 independent experiments were performed for each condition using Leica DMI6000B wide-field fluorescence microscope (duration: 1 h; ×40; 1,45 NA immersion objective; excitation: 488 nm). At least 40 cells were analyzed for each sample.

2.5. Oxidative stress measurements

Cells were seeded on 24-well imaging black plate (Eppendorf) at the density of 20,000 cells per well. After 24 h, ASC was added for 30 min before the administration of 5 µM CellROX Orange Reagent (Invitrogen) for the next 30 min. Then, the cells were washed with DPBS and medium was replaced with FluoroBrite DMEM (supplemented as described above) [22]. Fluorimetric analyses of ROS levels were performed with Leica DMI6000B system (excitation: 546 nm). At least 40 cells from at least 3 independent experiments (N > 3) were taken for the quantification of relative ROS levels with ImageJ software. Each image was subjected to thresholding and background substraction followed by fluorescence intensity measurements. Short exposure time (~150 ms) was secured to avoid unspecific photoredox reactions.

2.6. Implantation of gliomablastoma cells

For transplantation, U87 cells were trypsinized, counted with Coulter Z2 Counter and resuspended at the final density of 5×10^5/5 µl in DMEM without supplements. Cells were administered in aseptic conditions to male Wistar rats, 200–230 g body weight (N ≥ 6 for each condition, Department of Neuroanatomy). Before the procedure, the animals were anesthetized with 2–3%, inhaled isoflurane Aerrane (Baxter) in oxygen (total gas input 2 l/min). The animals were placed in a stereotaxic apparatus, then the incision was made on the head skin. The injection site was determined stereotaxically (coordinates: antero-posterior: 0.30 mm; medio-lateral: 3.0 mm; dorso-ventral: 5.0 mm, [23]). After drilling a hole in the cranium (without affecting the meninges), a 27 gauge needle on a Hamilton syringe was introduced into the skull using the stereotactic frame. To avoid uncontrolled displacement of the implanted cells following the local pressure changes, the needle was kept in place for 1 min, then 5×10^5 cells in 5 µl volume was delivered at a constant rate of 1 µl/min. After the injection, the needle was left in the place of injection for 3 min before it was gently pulled out. Then, the incision on the head was stitched and the animals were placed in home cages. During the entire procedure, the animals



(caption on next page)

Fig. 1. The effect of ASC on the motility of U87, AT-2 and 3T3 cells. (A) U87 cells were cultivated for 24 h and treated with ASC for 1 h. Their motility was estimated afterwards with time-lapse videomicroscopy using integrated modulation contrast (IMC) optics. Circular diagrams and dot plots depict cell trajectories and the movement parameters (Distance and Cell displacement) at the single cell level, whereas column plots show their averaged values at the population level (registered for 8 h; N = 50). Statistical significance was analyzed with non-parametric Mann-Whitney test (**$p < 0.01$; *$p < 0.05$). Error bars represent standard error of the mean (SEM). Scale bar = 100 μm. Inserts show cell morphology 24 h after the administration of ASC. The motility of AT-2 (B) and 3T3 cells (C) was analyzed as in A. In addition, the effect of ASC on cell viability was estimated by trypan blue test (insert). Statistical significance of the differences was estimated with non-parametric Mann-Whitney test (*$p < 0.05$). Error bars represent standard deviation (SD) calculated from at least 3 independent experiments (N > 3). Note that ASC induces a dose-dependent inhibition of tumor cell motility and less prominent effects on the motility of 3T3 cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were kept in a stereotaxic frame under isoflurane anaesthesia. After 4 and 7 days post implantation the animals were anesthetized with Aerrane and injected into the tail vein with ASC solution (1 g/kgBM or 2 g/kgBM, which referred to ca. 5–10 mM ASC in the body fluids). The solution was administered with Medipan 610 infusion pomp with 80 μl/min flow rate. The day before and at the day of implantation animals were injected with 10 mg/kgBM CsA (Novartis), and vaccinated each day 5 mg/kgBM CsA to minimize the immune response. After 10 days, the animals were anesthetized by intraperitoneal administration of Euthanasol vet (Fatro) at a dose of 50 mg/kg and subjected to perfusion-fixation with 3.7% paraformaldehyde (at room temperature), their brains were removed and stored for histological procedures in 3.7% paraformaldehyde in 0.01 M PBS, pH 7.4 at 4 °C. All in vivo experiments were approved by local ethical commission (Decision No. 219/2018).

2.7. Analysis of tumor growth within brain tissue

For immunofluorescence analyses, the brains were sectioned on a vibratome (Leica VT1000 S) into 30 μm thick coronal slices stored in 0.01 M PBS, pH 7.4, at 4 °C. The slices were assessed by immunocytochemical staining to estimate the extent of tumor invasion/the distribution of tumor cells and to identify the areas of reactive changes within the host brain. For this purpose, free-floating slices were blocked for 1 h with 5% fetal bovine serum in 0.01 M PBS/0.1% Triton X-100. Primary antibodies against glial fibrillary acid protein (rabbit anti-GFAP, DAKO Z0334, 1:2000) and against human nuclear antigen (mouse anti-HuN, Merck MAB1281, 1:1000) were applied in blocking solution and the slices were incubated overnight at 4 °C with shaking. Slices were then rinsed three times with blocking solution (5 min. each) and incubated with the appropriate fluorescence-labeled secondary antibodies (goat anti-mouse AlexaFluor546 and goat anti-rabbit AlexaFluor488, Thermo Fisher Scientific, 1:1000) in blocking solution for 2 h. Then the specimens were washed with PBS. Slices were mounted on glass slides using Mowiol (Sigma-Aldrich) and analyzed with the wide-field Leica DMI6000B fluorescence microscope using tile-scan module and L5 and N2.1 filter cubes to visualize AlexaFluor488 and AlexaFluor546 respectively. Images were subjected to the fluorescence intensity thresholding in ImageJ software (1.8.0_112 version) to identify glial scar (GFAP-positive) and tumor infiltration (HuN-positive) areas. Quantitative analyses of at least 10 slices per sample (N = 10) were followed by the calculation of Region of Interest (ROI)/whole brain slice area ratio.

2.8. Blood morphology and biochemistry

For the analyses of blood morphology (erythrocyte count, thrombocyte count, total white blood cell count, hemoglobin, and erythrocyte indices), 200 μl of rat blood was collected into Microvette 100K3E EDTA tubes (Sarstedt) and measured with SCIL Vet ABC Analyzer according to manufacturer protocol. For biochemical analyses, 200 μl of rat blood was collected into S-Monovette 4.5 ml lithium heparin tubes (Sarstedt) and centrifuged (400*g*, 10 min). Supernatant (serum) was analyzed using Spotchem EZ SP-4430 and the test strips (Arkray) according to manufacturer protocol.

2.9. Hemolysis assay

To estimate hemolytic potential of ASC and ascorbic acid, hemolytic tests were conducted on the samples of human and rat venous blood diluted in CPDA (citrate-phosphate-dextrose-adenine) solution. 20 μl of each blood sample was added to 1 ml of Hanks' Balanced Saline Solution (HBSS; Corning) and incubated for 1 h in the presence of 1 to 14 mM ASC or ascorbic acid (Sigma) in Eppendorf tubes. Then, the samples were centrifuged (400*g*, 5 min) and 100 μl of each supernatant was placed into 96-wells microplate (Sarstedt). Hemolysis was assessed by measurements of supernatant absorbance at 405 nm with the plate reader (Thermo Scientific) with the blood incubated in distilled water as a positive (100%) control. Experiments were conducted in triplicate.

2.10. Statistical analyses

All data were presented as mean ± SEM from at least three independent experiments (N > 3). The statistical significance of the differences was tested with the non-parametric Mann-Whitney *U* test. Statistical significance was shown at *$p < 0.05$; **$p < 0.01$. Data processing was performed in Origin Pro 2016 software.

3. Results

3.1. ASC impairs the viability and invasiveness of U87 and AT-2 cells in dose dependent manner

To investigate the impact of sodium ascorbate (ASC) on the viability and invasive properties of GBM cells, we performed time-lapse videomicroscopy analyses of the movement of human (ASC-auxotrophic) GBM U87 and T98G cells immediately after the administration of high ASC doses (1–5 mM). As reference lines, we used (ASC autotrophic) rat prostate cancer AT-2 cells and mouse 3T3 fibroblasts [2]. When administered at the concentration of 1 mM, ASC reduced the motile activity of U87 and T98G cells to ca. 30% of control values (Figs. 1A and S1). An even more prominent inhibition of GBM cells movements was observed in the presence of 5 mM ASC. This effect was accompanied by morphological changes that suggested cell necrosis, especially in the presence of 5 mM ASC (Fig. 1A). It may explain the lack of U87 and T98G motility in the presence of 10 mM ASC (not shown). Interestingly, prostate AT-2 cells showed similar reactivity to ASC (despite the autotrophic rat origin), whereas the morphology of rodent 3T3 fibroblasts was remained unaffected even in the presence of 5 mM ASC (Fig. 1C).

ImageStream analyzes of AnnexinV/PI-stained cells confirmed a relatively strong necrotic response of U87 cells within 2 h after the administration of 5 and 10 mM ASC (up to 60% for U87 cells; Fig. 2A). No apoptotic bodies and nuclear distortions were observed at the initial ASC treatment phases. ImageStream analyses demonstrated numerous AnnexinV$^+$ cells, accompanied by the signs of nuclear excision from intracellular compartments. However, ASC did not induce the necrosis of U87 cells when administered at the concentration of 1 mM, which indicates that the effect of 1 mM ASC on U87 motility (cf. Fig. 1A) does not result from its interference with cell viability. Similar dose-dependence of pro-necrotic action of ASC was observed in AT-2 populations (Fig. 2B), whereas no necrotic effect of ASC was seen in 3T3

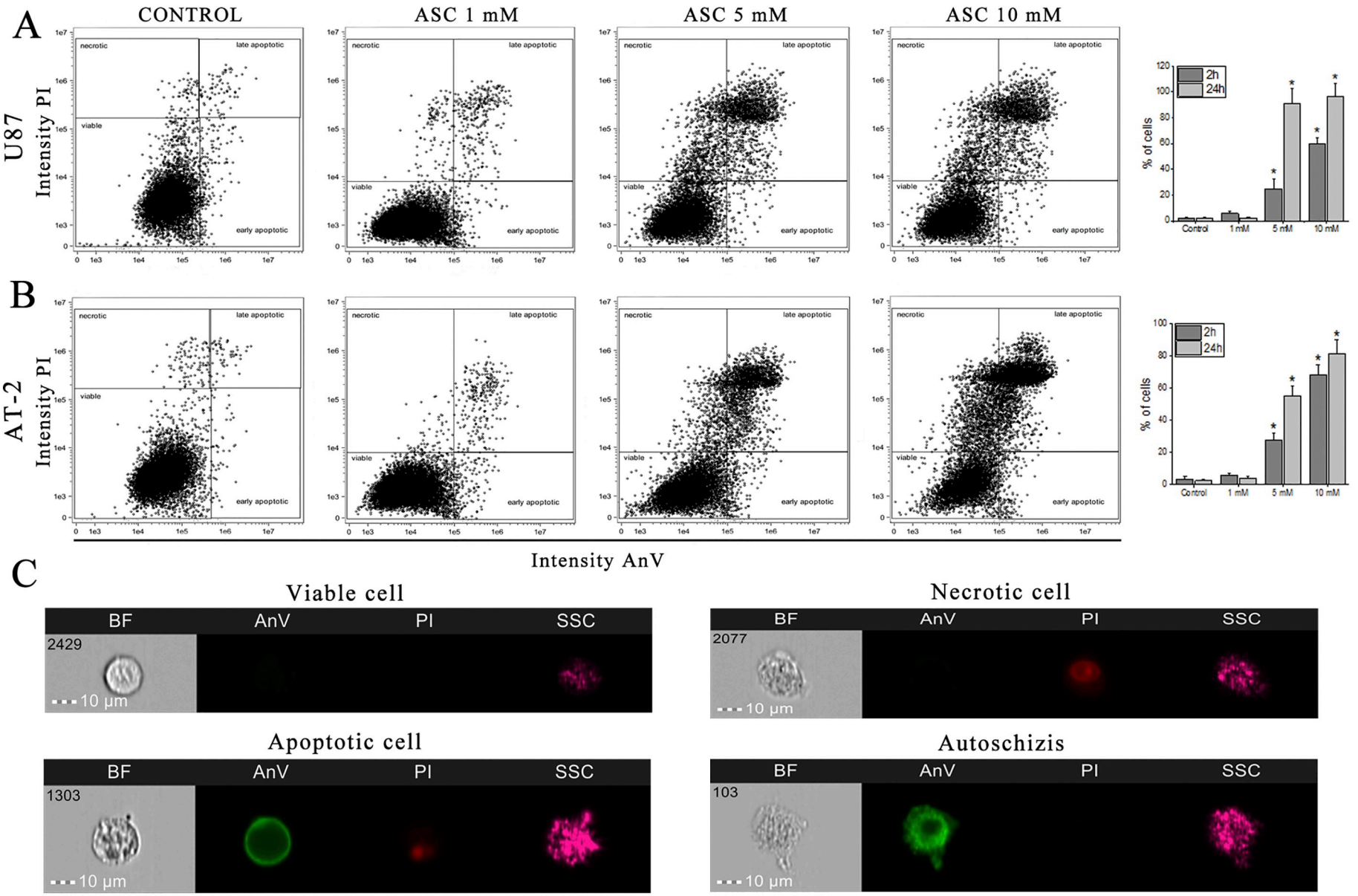


Fig. 2. Pro-apoptotic/cytotoxic activity of ASC in U87 populations. (A) U87 cells were treated with ASC for 1 h, followed by their incubation in control medium for 2 and 24 h, before their harvesting and staining with propidium iodide/AnnexinV. Column plots represent the percentage of PI and/or AV-positive cells. For each condition, 1500 cells were analyzed with ImageStream® flow cytometer, followed by IDEAS 6.2-assisted data processing. Statistical significance was estimated with non-parametric Mann-Whitney test (*$p < 0.05$). Error bars represent standard deviation (SD) calculated from at least 3 independent experiments ($N > 3$). (B) AT-2 cells were treated with ASC and analyzed as in A. (C) Cells were treated as in A. Disruption of cell membrane and excision of intracellular/perinuclear compartments was estimated by IMC microscopy. Abbreviations: BF: bright field, AnV: Annexin V, PI: propidium iodide, SSC: side scatter. Note the prominent and dose-dependent necrotic-response of U87 and AT-2 cells to 5 mM and 10 mM ASC.

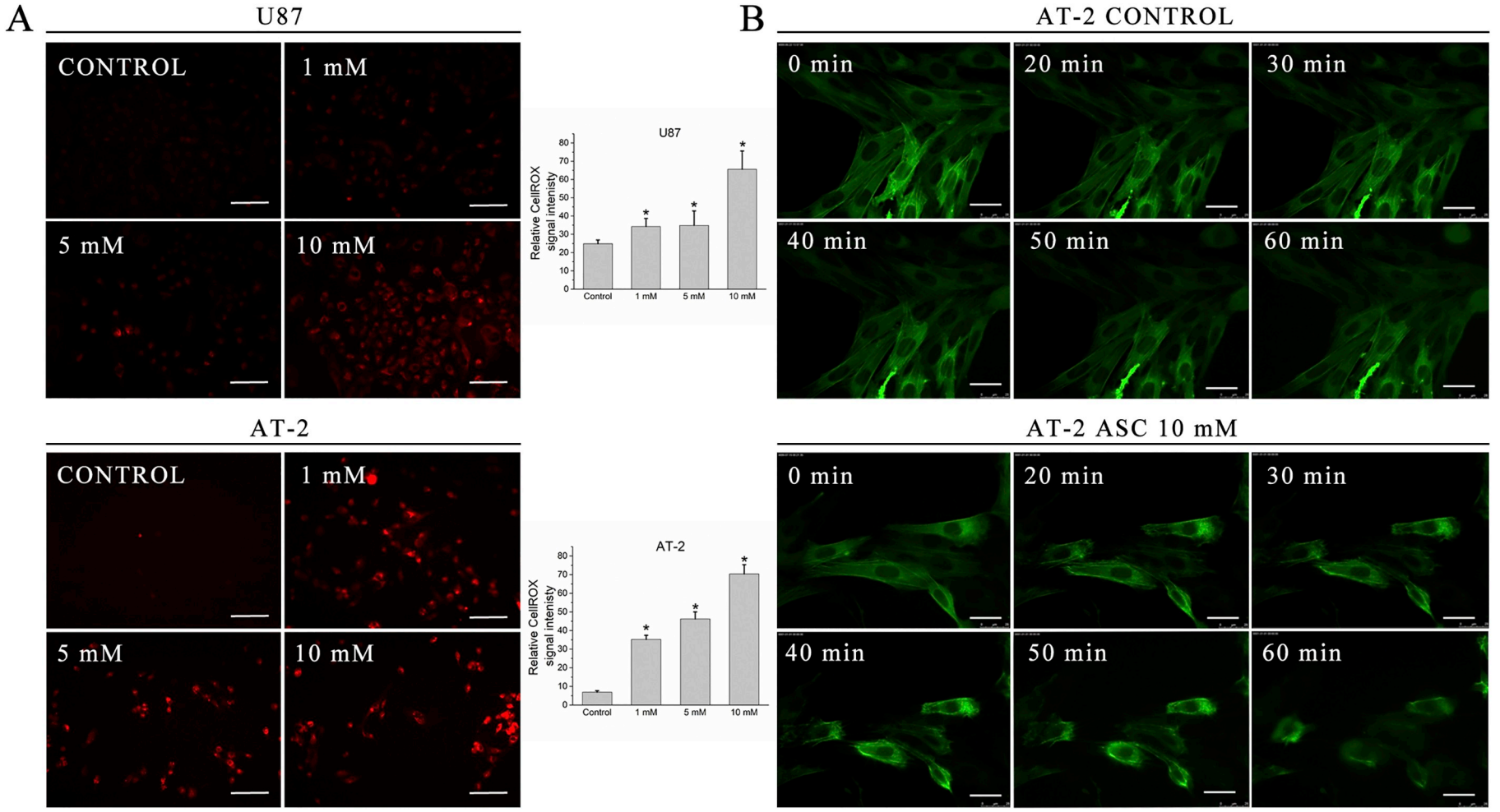


Fig. 3. The effect of ASC on ROS levels and actin cytoskeleton architecture in U87 and AT-2 cells. (A) Cells were treated with ASC for 1 h, incubated with CellROX Orange Reagent and intracellular ROS levels were visualized with fluorescence microscopy. Column plots represent the ROS levels/cell that were calculated on the basis of intracellular CellROX-specific fluorescence intensity in at least 40 cells/sample and normalized against the control. Statistical significance was calculated with non-parametric Mann-Whitney test (**$p < 0.01$; *$p < 0.05$) on the basis of data from 3 independent experiments (N > 120). Scale bar = 100 μm. (B) AT-2 cells were transduced with Cell Light™ Actin-GFP, Bac Mam 2.0, treated with 10 mM ASC and F-actin dynamics were visualized immediately afterwards (in 10 min intervals for 1 h) with time-lapse fluorescence videomicroscopy. Data are representative for 3 independent experiments (N = 3). Scale bar = 25 μm. Note that ASC induces ROS production, followed by the cell shrinkage, membrane damage and excision of the cytoplasm.

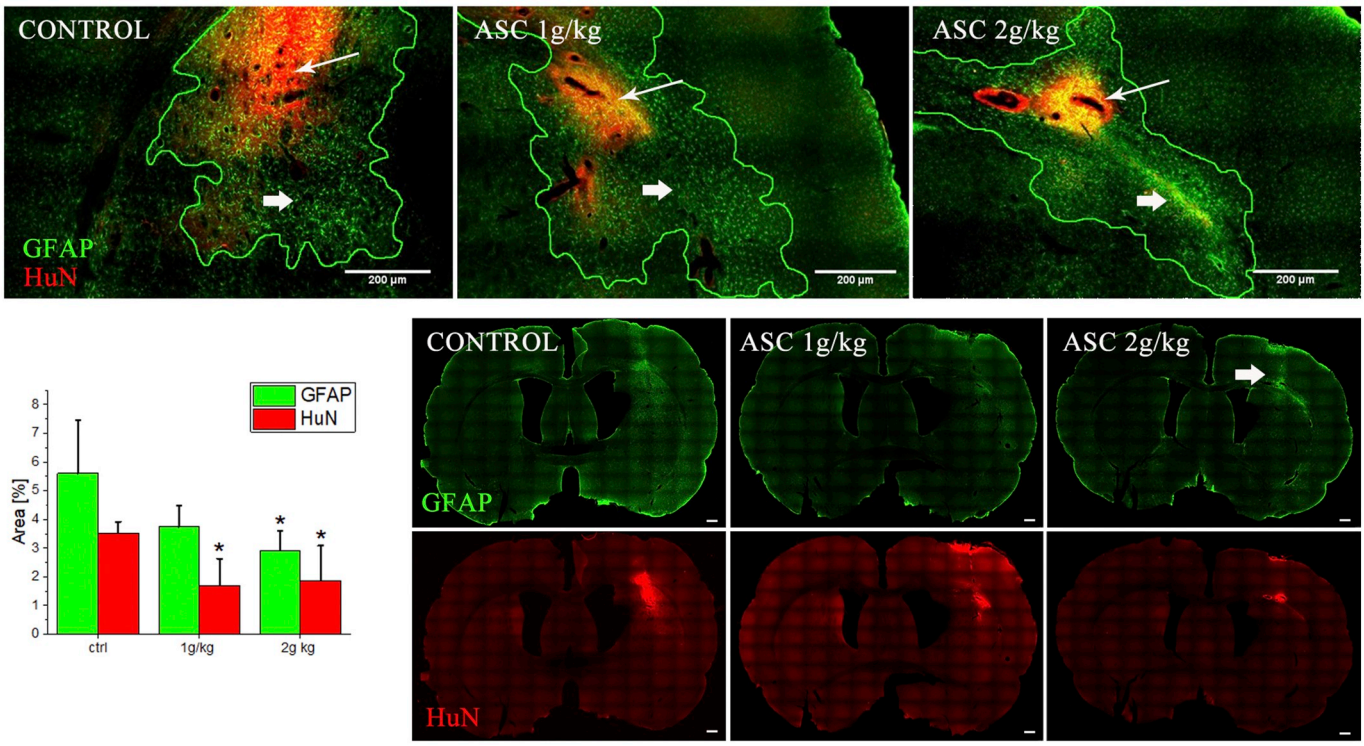


Fig. 4. Effect of intravenous administration of ASC on U87 tumor development in rodent brains. U87 tumor-bearing rats were subjected to intravenous injection of ASC at 3rd and 7th day after cell implantation. Brains were isolated, perfused and fixed after 10 days, followed by their sectioning and staining against GFAP (astrocytes) and HuN (tumor cells). (A) Intravenous administration of ASC inhibits tumor invasion in vivo and seems to reduce the size of neoplasia. Concomitantly, considerable recruitment of glial cells (green; thick arrows) into compartments occupied by glioblastoma cells (red; slim arrows) were observed. Green contours show glial scars formed within locus of tumor implantation and development. (B) Quantitative analysis of the signals from glial scars (GFAP) and implanted tumor cells (HuN) in relation to whole area of analyzed specimens. Statistical significance was calculated with non-parametric Mann-Whitney test (*$p < 0.05$; N = 10 for each condition. Scale bar = 200 µm) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

populations. Interestingly, abundant apoptotic cells (up to 90%) were noticed upon the long-term (24 h) incubation of U87 and AT-2 cells in the presence of 5 and 10 mM ASC. These data show the heterogeneity of U87 and AT-2 cells responsiveness to ASC. More sensitive cell sub-populations react to ASC with the induction of "autoschizis" [20], which leads to the fast plasma membrane break and nuclear excision. Concomitantly, apoptosis is induced in less susceptible cells. However, in both cases ASC impairs the invasive potential of tumor cells.

3.2. ASC-induced impairment of cell viability and invasiveness depends on ROS overproduction

Reactive oxygen species (ROS) have previously been described to participate in cellular responses to ASC [13]. To identify the mechanism (s) of ASC effect on U87 and AT-2 viability and motility, we further estimated ROS levels in ASC-treated cells with a CellROX assay. A dose-dependent increase of ROS was observed in U87 and AT-2 cells 2 h. after ASC administration (Fig. 3A). Concomitantly, dramatic re-arrangements of the cytoskeleton organization were revealed in 10 mM ASC-treated cells transfected with BacMam baculovirus bearing actin-GFP sequence. F-actin disorganization proceeded in time leading to the disappearance of F-actin bundles after 60 min (Fig. 3B). These data confirm that ASC interferes with the viability of tumor cells in a ROS-dependent manner. This observation explains relatively fast motility arrest observed in 10 mM ASC-treated 87 and AT-2 populations.

3.3. ASC inhibits glioma growth in vivo

To further estimate the systemic consequences of the pro-necrotic activity of ASC on GBM cells, we estimated its effect on the progression of U87 tumors in Wistar rat brains. Statistically significant decrease of tumor areas and glial scars was observed in animals intravenously injected with ASC (twice at 3rd and 7th day post implantation; Fig. 4). Concomitantly, blood analyses did not show any signs of hemolysis or biochemical disorders after ASC administration (Figs. 5 and 6). These data indicate that tissues and organs of ASC-treated animals are not affected by ASC or by the remnants of necrotic cells. Finally, we compared pro-hemolytic activity of high doses (14 mM) ASC and ascorbic acid (AA) to further assess the safety of intravenous ASC administration (Fig. 6). Spectrophotometric analysis of the supernatants isolated from blood samples incubated with ASC or AA for 1 h demonstrated slight hemolysis at low AA concentrations and almost complete hemolysis in the presence of 14 mM AA. Morphological analyses of erythrocytes clearly showed the swelling of AA-treated erythrocytes and disruption of their membranes. In turn, ASC exerted no hemolytic effect even at 14 mM concentration. These data indicate that high doses of ASC can inhibit GBM progression via the induction of cells necrosis, which has minute systemic effects.

4. Discussion

Systemic side-effects of tumor chemotherapy enforce premature treatment cessations. Together with the microevolution of drug-resistant tumor cell populations, these side-effects result in accelerated deaths of oncologic patients. Recently, the application of ascorbic acid and its derivatives was suggested for supportive chemotherapy to interfere with tumor recurrence [18]. Numerous reports speculate on the potential of vitamin C in tumor therapy, however this matter remains controversial in the light of other reports on its inefficiency and potential adverse effects. Our current data demonstrate the significant impairment of glioblastoma multiforme (GBM) cell viability and invasiveness in the presence of millimolar sodium ascorbate (ASC) doses in

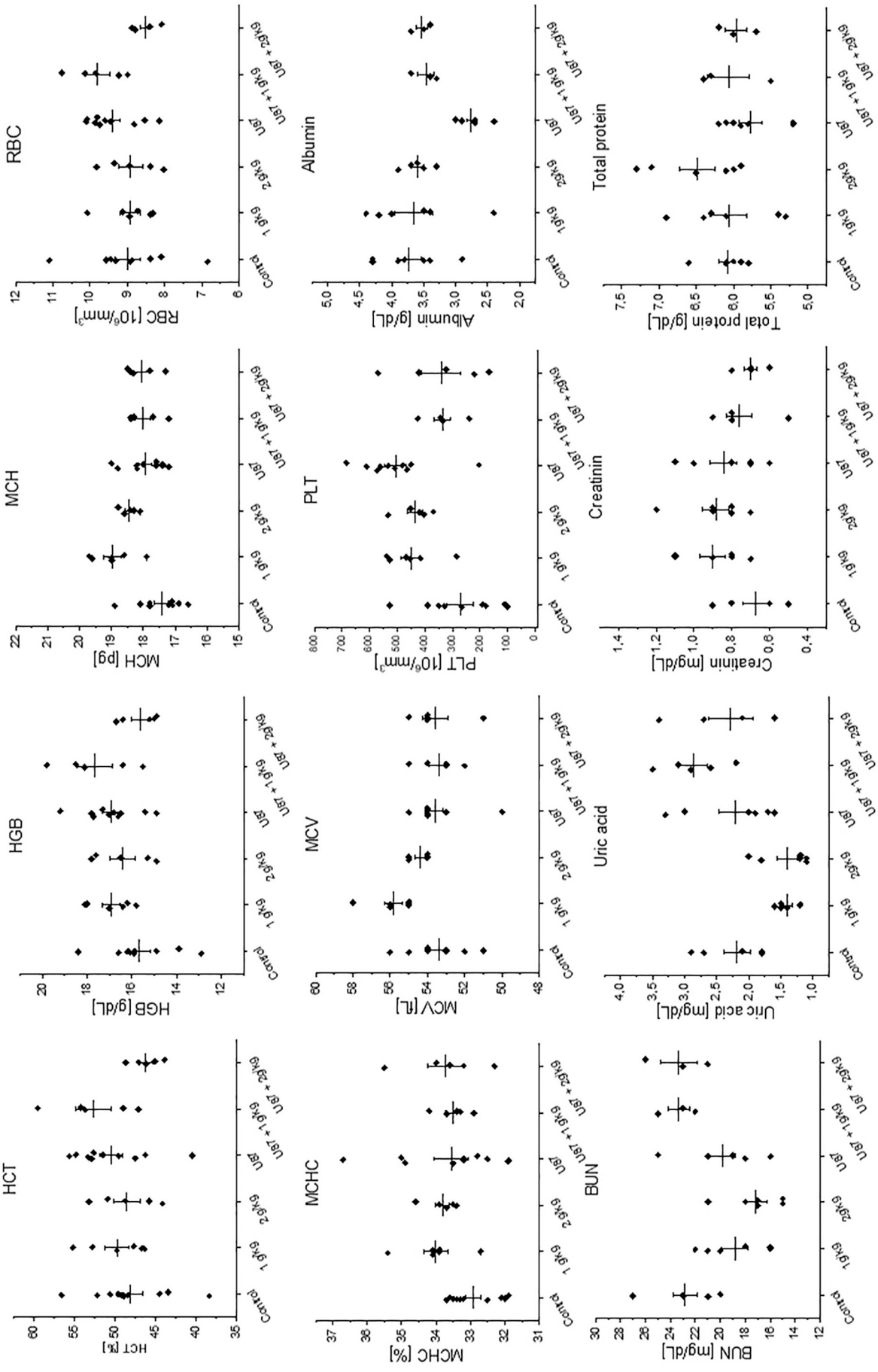


Fig. 5. Effect of intravenous administration of ASC on blood morphology and biochemical parameters. Blood of examined rats was analyzed for morphological and biochemical parameters, which illustrate the safety of intravenous administration of ASC. Bars represent standard error of mean (SEM), N ≥ 5. Note the lack of hemolytic effects and nephrotoxic aftermath after the intravenous administration of ASC.

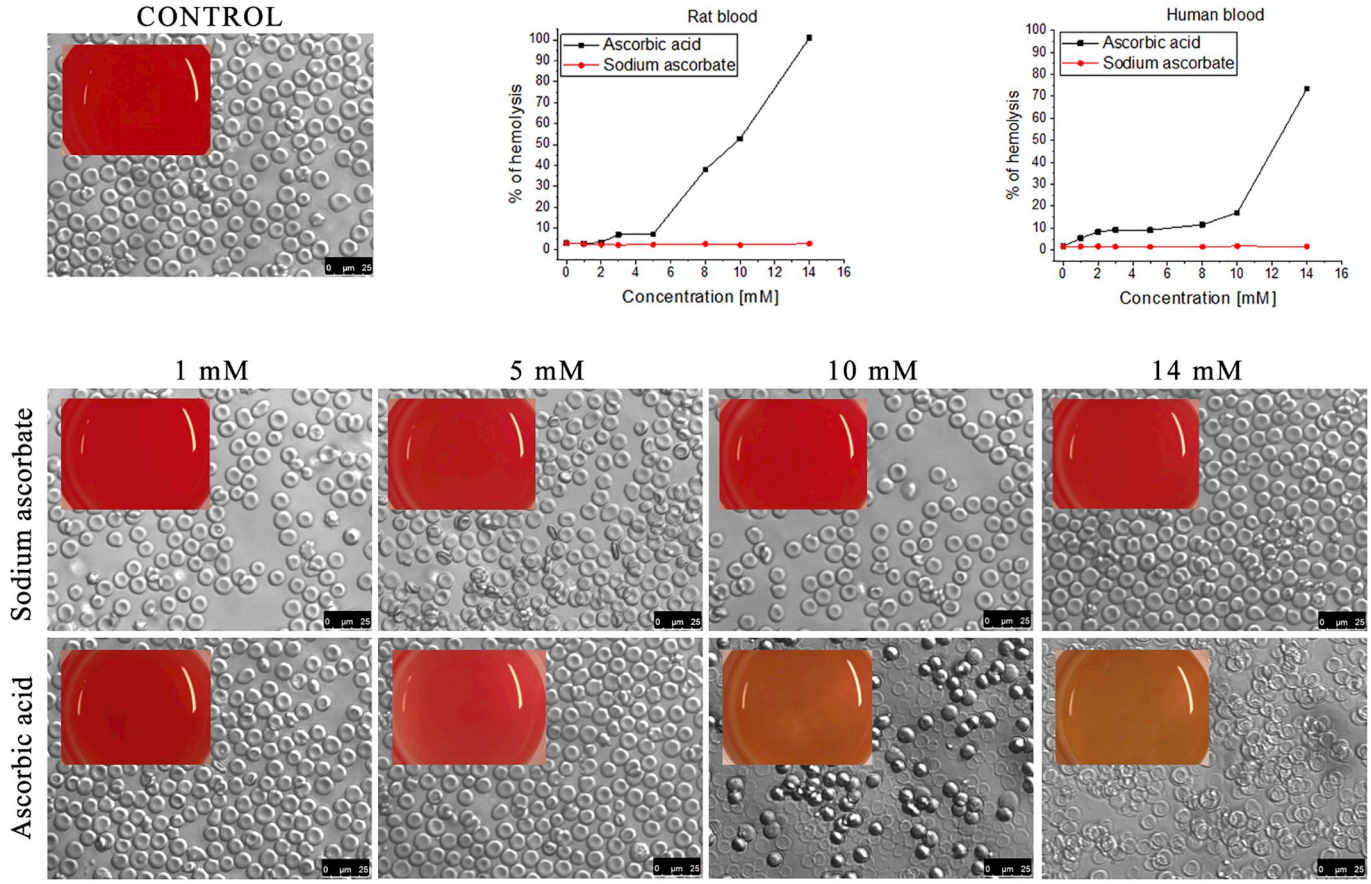


Fig. 6. ASC does not show pro-hemolytic potential in silico. Blood samples were diluted in Hanks' Balanced Saline Solution, followed by ASC or AA treatment for 1 h. After centrifugation, supernatant absorbance (λ = 405 nm) was measured to estimate the intensity of hemolysis in comparison to blood incubated in distillated water. Morphology of erythrocytes was visualized with IMC optics. Inserts visualize blood samples re-suspended in HBSS solution after 2 h of incubation in the presence of ASC or AA. Note the lack of hemolytic effect of 14 mM ASC and a pronounced AA-induced lysis of rat and human erythrocytes.

vitro and in the in vivo rat model of GBM. Together with the corresponding data on prostate cancer (PC) cells, these observations may confirm the suitability of ASC for adjunctive GBM and PC treatment. On the other hand, the ASC-induced attenuation of human GBM tumor progression in rats was accompanied by the necrotic death of substantial portion of GBM cells, which may add to the potential risks of this strategy.

ROS play a significant role in the mechanisms that regulate cell migration [24]. We have previously shown that ascorbic acid administered at low (micromolar) concentrations inhibits the migration in WC256 cells via its interference with physiologic production of ROS [25]. Here, we show the impairment of GBM and prostate cancer cell viability and migration in the presence of millimolar ASC concentrations, which is apparently related to ROS overproduction in intracellular compartments. Uncontrolled oxidative stress usually leads to remodeling or destruction of cytoskeleton in cancer cells [26,27]. Presumably, this mechanism is responsible for actin cytoskeleton rearrangements and motility arrest in ASC-treated populations. In our hands, the inhibitory effect of ASC on GBM cell motility was also accompanied by heterogeneous viability-related cell reactions to this agent. A rapid necrotic-like death was observed in a fraction of ASC-treated cells, which was manifested by cell swelling, membrane break and the release of cytoplasm. Apparently, “autoschizis”-related violent cell reactions to high ASC doses replace the apoptosis in “hyper-sensitive” GBM cells. This cell death mechanism has been described as a self-excision of cytoplasm and was till now observed only in the presence of vitamin C and menadione [19,20]. In our hands, this phenomenon was followed by the induction of apoptotic program in the cells that initially survived ASC treatment. It confirms the heterogeneity of the ASC-sensitivity of GBM cells. Biological relevance of both processes is illustrated by the corresponding effects of ASC in prostate cancer cell populations and by the inhibition of GBM tumor growth and spreading after ASC application in vivo, estimated by measurements of neoplasia and glial scars areas in rat brains. Thus, our data support previous notions on anti-tumor activity of ASC. However, they also show the violence of GBM cell reactions to ASC, which can potentially cause local inflammation and the disturbance of systemic homeostasis.

Pro-necrotic activity of millimolar ASC is an important issue that should be thoroughly addressed before the implementation of vitamin C and its derivatives into chemotherapeutic programs. Previously, the risk of nephrolithiasis (kidney stones; [3,28]) and renal failure was reported in patients with renal disorders after long-term oral ASC supplementation [29]. On the other hand, high serum concentrations of vitamin C, which show pro-oxidative and pro-necrotic activity (1–14 mM), can only be achieved by its intravenous application, which does not contribute to renal disorders [30,31]. Perhaps therefore we did not observe any distinct systemic side-effects of ASC application in experimental animals. In particular, intravenous a double (in 3-days interval) ASC administration at 1 g/kgBM and 2 g/kgBM doses did not evoke any detectable systemic effects in vivo. Neither did the biochemical and morphological analyses of rat blood and sera reveal any significant changes in rat blood morphology (HGB, HCT, MCH RBC, MCHC and MCV). Accordingly, we did not see any signs of organ damage, especially these reminiscent of renal disorders. ASC effects remain in contrast to previous results on hemolytic potential of ascorbic acid. They indicate that “autoschizis” does not evoke systemic adverse

effects in the rat model. Thus, they suggest the perspectives of ASC application in adjunctive GBM chemotherapy, even though the consequences of "autoschizis" in humans require further study.

On the other hand, cellular reactions evoked by ascorbate anions strongly depend on their local concentrations. Therefore, the lack of systemic adverse ASC effects in the rat model could also be explained by the discrepancies between ASC concentrations in vitro and in vivo. Theoretically, ASC administration at 1 g/kg BM and 2 g/kg BM doses should give ca. 5–10 mM of ASC in body fluids. However, the organisms developed efficient uptake systems to prevent local ASC deficiency, which may result in differences in ASC content between organs/tissues. The uptake and transport of ascorbate in the brain tissue is determined by SVCT2 (ASC transporter; [32]) and by GLUT1/3 (DHA transporters; [32,33]). They can lead to ASC accumulation at relatively high (millimolar) concentrations both in neurons and cerebrospinal fluid. ASC concentrations can reach millimolar values in central nervous system of experimental rats due to the possible up-regulation of SVCT2 expression in neurons and astrocytes that results from the induction of oxidative stress within brain tissue [33]. Recently, these transporters were proposed in increase the efficiency of ascorbate-based cancer therapy [34]. Moreover, tumor development usually results in the disruption of blood-brain-barrier continuum, which can increase the local bioavailability of ASC. On the other hand, tumor hypoxia may attenuate toxicity of ascorbate [35]. Notably, the rats are ASC-autotrophic, which implies that their systemic tolerability to ASC may be much higher than of humans. In this context, relatively strong reactions of rat AT-2 cells to ASC indicate that the sensitivity of tumor cells to this compound is not related to their brain origin and to systemic auxotrophy. Collectively, these data support the notion on the anti-cancerogenic potential of millimolar ASC. However, they also leave several issues unaddressed. They concern in particular the potential incompatibility of rat and human research models, which make questionable the simple extrapolation of the data from animal models to human therapy.

In general, our data confirm ASC potential for adjunctive tumor therapy. They stay in concordance with the results of recent case report on a significant improvement in glioblastoma multiforme patient after intravenous ASC administration [36]. Accordingly, the efficiency of the combined vitamin C/temozolomide/radiotherapy treatment is currently under investigation [ClinicalTrials.gov Identifier: NCT02344355]. Despite our promising results and the participation of ASC in the brain protection against redox imbalance, the assertions on the application of vitamin C in tumor treatment are still premature as not sufficiently supported by clinical studies on humans [37–40]. Human trials of high-dose vitamin C supplementation in conventional cancer therapies appear to have several limitations due to lack of rigor in trial design. Next, the consideration of high-dose vitamin C/ASC application in clinical trials must include its ability to induce hemolytic anemia (and even mortality), mainly in subjects with glucose-6-phosphate dehydrogenase deficiency [18,41]. Hemolytic responses are absent in ASC-treated rats; however, rats are non-auxotrophic to ASC, therefore this observation can hardly be extrapolated to humans. Furthermore, the systemic consequences of ASC-induced "autoschizis" should be evaluated in humans. Due to significant discrepancies and controversies in the topics related to application of high doses of vitamin C in oncological trials, present oncological guidelines do not recommend the use of vitamin C in a cancer therapy. Should they be introduced, they must be tailored to individual patients and should not imply chemotherapy replacement. Even though the present state of the art on ASC activity does not justify the introduction of vitamin C/ASC in a tumor therapy, further research may expand our knowledge on its therapeutic potential. It can also help to develop methods that optimize its delivery to the interior of tumor loci.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116657.

Funding

Faculty of Biochemistry, Biophysics and Biotechnology of Jagiellonian University was a partner of the Leading National Research Center (KNOW) supported by the Ministry of Science and Higher Education. This work was financially supported by the Polish National Science Centre (UMO-2015/19/D/NZ3/00273 to D.R. and, in part, UMO-2015/17/B/NZ3/01040 to J.C.).

Acknowledgements

We wish to thank Dr. Elżbieta Karnas and Dr. Ewa Zuba-Surma for technical support with ImageStream analyses.

Authors' contributions

Damian Ryszawy — study conception and design, data analysis and final manuscript preparation.

Maciej Pudełek — study conception and design, data collection, and manuscript preparation.

Jessica Catapano — data collection.

Małgorzata Ciarach — data collection.

Zuzanna Setkowicz — data collection, data analysis, and manuscript preparation.

Ewa Konduracka — data analysis and conceptual work.

Zbigniew Madeja — data analysis and conceptual work.

Jarosław Czyż — study design, data analysis and manuscript preparation.

Declarations of Competing Interest

The authors declare no conflict of interest.

References

[1] G. Waris, H. Ahsan, Reactive oxygen species: role in the development of cancer and various chronic conditions, J. Carcinog. 5 (2006) 14.
[2] G. Drouin, J.R. Godin, B. Pagé, The genetics of vitamin C loss in vertebrates, Curr. Genomics 5 (2011) 371–378.
[3] J. Lykkesfeldt, A.J. Michels, B. Frei, Vitamin C, Adv. Nutr. 5 (2014) 16–18.
[4] H.M. Oudemans-van Straaten, A.M. Spoelstra-de Man, M.C. de Waard, Vitamin C revisited, Crit. Care 18 (2014) 460.
[5] S. Park, The effects of high concentrations of vitamin C on cancer cells, Nutrients. 5 (2013) 3496–3505.
[6] Q. Chen, M.G. Espey, M.C. Krishna, J.B. Mitchell, C.P. Corpe, G.R. Buettner, E. Shacter, M. Levine, Pharmacologic ascorbic acid concentrations selectively kill cancer cells: action as a pro-drug to deliver hydrogen peroxide to tissues, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 13604–13609.
[7] S.J. Padayatty, H.D. Riordan, S.M. Hewitt, A. Katz, L.J. Hoffer, M. Levine, Intravenously administered vitamin C as cancer therapy: three cases, CMAJ 174 (2006) 937–942.
[8] A. Salazar-Ramiro, D. Ramírez-Ortega, V. Pérez de la Cruz, N.Y. Hérnandez-Pedro, D.F. González-Esquivel, J. otelo, B. Pineda, Role of redox status in development of glioblastoma, Front. Immunol. 26 (7) (2016) 156.
[9] T.C. Jorgenson, W. Zhong, T.D. Oberley, Redox imbalance and biochemical changes in cancer, Cancer Res. 73 (2013) 6118–6123.
[10] C.H. Yeom, G.C. Jung, K.J. Song, Changes of terminal cancer patients' health-related quality of life after high dose vitamin C administration, J. Korean Med. Sci. 22 (2007) 7–11.
[11] J.E. Kim, J.S. Kang, W.J. Lee, Vitamin C induces apoptosis in human colon cancer cell line, HCT-8 via the modulation of calcium influx in endoplasmic reticulum and the dissociation of bad from 14-3-3β, Immune Netw. 12 (2012) 189–195.
[12] E. Fischer-Fodor, N. Miklasova, I. Berindan-Neagoe, B. Saha, Iron, inflammation and invasion of cancer cells, Clujul Med. 88 (2015) 272–277.
[13] S. Ohno, Ohno Yumiko, N. Suzuki, G. Soma, M. Inoue, High-dose vitamin C (ascorbic acid) therapy in the treatment of patient with advanced cancer, Anticancer Res. 29 (2009) 809–816.
[14] J. Duconge, J.R. Miranda-Massari, M.J. Gonzalez, J.A. Jackson, W. Warnock, N.H. Riordan, Pharmacokinetics of vitamin C: insights into the oral and intravenous administration of ascorbate, P. R. Health Sci. J. 27 (2008) 7–19.
[15] Harakeh S, Diab-Assaf M, Khalife JC, Abu-el-Ardat KA, Baydoun E, Niedzwiecki A, El-Sabban ME, Rath M. Ascorbic acid induces apoptosis in adult T-cell leukemia, Anticancer Res. 27 (2007) 289–98.
[16] G. Wang, T. Yin, Y. Wang, *In vitro* and *in vivo* assessment of high-dose vitamin C against murine tumors, Exp. Ther. Med. 12 (2016) 3058–3062.

[17] C.N. Zhao, Y. Li, X. Meng, S. Li, Q. Liu, G.Y. Tang, R.Y. Gan, H.B. Li, Insight into the roles of vitamins C and D against cancer: myth or truth? Cancer Lett. 431 (2018) 161–170.
[18] E. Klimant, H. Wright, D. Rubin, D. Seely, M. Markman, Intravenous vitamin C in the supportive care of cancer patients: a review and rational approach, Curr. Oncol. 25 (2018) 139–148.
[19] J. Gilloteaux, J.M. Jamison, D. Arnold, E. Ervin, L. Eckroat, J.J. Docherty, D. Neal, J.L. Summers, Cancer cell necrosis by autoschizis: synergism of antitumor activity of vitamin C: vitamin K3 on human bladder carcinoma T24 cells, Scanning 20 (1998) 564–575.
[20] J. Gilloteaux, J.M. Jamison, D.R. Neal, M. Loukas, T. Doberzstyn, J.L. Summers, Cell damage and death by autoschizis in human bladder (RT4) carcinoma cell resulting from treatment with ascorbate and menadione, Ultrastruct. Pathol. 34 (2010) 140–160.
[21] D. Ryszawy, M. Sarna, M. Rak, K. Szpak, S. Kędracka-Krok, M. Michalik, M. Siedlar, E. Zuba-Surma, K. Burda, W. Korohoda, Z. Madeja, J. Czyż, Functional links between Snail-1 and Cx43 account for the recruitment of Cx43-positive cells into the invasive front of prostate cancer, Carcinogenesis 35 (2014) 1920–1930.
[22] M. Pudełek, J. Catapano, P. Kochanowski, K. Mrowiec, N. Janik-Olchawa, J. Czyż, D. Ryszawy, Therapeutic potential of monoterpene α-thujone, the main compound of Thuja occidentalis L. essential oil, against malignant glioblastoma multiforme cells in vitro, Fitoterapia 134 (2019) 172–181.
[23] G. Paxinos, C. Watson, The Rat Brain in Stereotaxic Coordinates, Academic Press, New York, 1986.
[24] J. Sroka, Z. Madeja, Reactive oxygen species in regulation of cell migration. The role of thioredoxin reductase, Postepy Biochem. 55 (2009) 145–152.
[25] E. Wybieralska, M. Koza, J. Sroka, J. Czyz, Z. Madeja, Ascorbic acid inhibits the migration of Walker 256 carcinosarcoma cells, Cell. Mol. Biol. Lett. 13 (2008) 103–111.
[26] D. Zhu, K.S. Tan, X. Zhang, A.Y. Sun, G.Y. Sun, J.C. Lee, Hydrogen peroxide alters membrane and cytoskeleton properties and increases intercellular connections in astrocytes, J. Cell Sci. 118 (2005) 3695–3703.
[27] W.G. Hu, Q.P. Lu, Impact of oxidative stress on the cytoskeleton of pancreatic epithelial cells. Exp Ther Med. 2014 Nov;8(5):1438-1442. Epub 2014 Sep 18. Intravenously administered vitamin C as cancer therapy: three cases, CMAJ 174 (2006) 937–942.
[28] J. van der Reest, E. Gottlieb, Anti-cancer effects of vitamin C revisited, Cell Res. 26 (2016) 269–270.
[29] S.J. Padayatty, M. Levine, Vitamin C: the known and the unknown and Goldilocks, Oral Dis. 22 (2016) 463–493.
[30] B.L. Auer, D. Auer, A.L. Rodgers, The effect of ascorbic acid ingestion on the biochemical and physicochemical risk factors associated with calcium oxalate kidney stone formation, Clin. Chem. Lab. Med. 36 (1998) 143–147.
[31] M. Prier, A.C. Carr, N. Baillie, No reported renal stones with intravenous vitamin C administration: a prospective case series study, Antioxidants (Basel) 7 (2018).
[32] C. Wohlrab, E. Phillips, G.U. Dachs, Vitamin C transporters in cancer: current understanding and gaps in knowledge, Front. Oncol. 7 (2017) 74.
[33] J.M. May, Vitamin C transport and its role in the central nervous system, Subcell. Biochem. 56 (2012) 85–103.
[34] M.F. McCarty, Expression and/or activity of the SVCT2 ascorbate transporter may be decreased in many aggressive cancers, suggesting potential utility for sodium bicarbonate and dehydroascorbic acid in cancer therapy, Med. Hypotheses 81 (2013) 66470.
[35] T. Sinnberg, S. Noor, S. Venturelli, A. Berger, P. Schuler, C. Garbe, C. Busch, The ROS-induced cytotoxicity of ascorbate is attenuated by hypoxia and HIF-1alpha in the NCI60 cancer cell lines, J. Cell. Mol. Med. 18 (2014) 530–541.
[36] N. Baillie, A.C. Carr, S. Peng, The use of intravenous vitamin C as a supportive therapy for a patient with glioblastoma multiforme, Antioxidants (Basel) 7 (2018).
[37] R.M. Abou-Jawde, J. Reed, M. Kelly, et al., Efficacy and safety results with the combination therapy of arsenic trioxide, dexamethasone, and ascorbic acid in multiple myeloma patients: a phase 2 trial, Med. Oncol. 23 (2006) 263–272.
[38] J.R. Berenson, J. Matous, R.A. Swift, R. Mapes, B. Morrison, H.S. Yeh, A phase I/II study of arsenic trioxide/bortezomib/ascorbic acid combination therapy for the treatment of relapsed or refractory multiple myeloma, Clin. Cancer Res. 13 (2007) 1762–1768.
[39] J.S. Welch, J.M. Klco, F. Gao, et al., Combination decitabine, arsenic trioxide, and ascorbic acid for the treatment of myelodysplastic syndrome and acute myeloid leukemia: a phase I study, Am. J. Hematol. 86 (2011) 796–800.
[40] P.R. Subbarayan, M. Lima, B. Ardalan, Arsenic trioxide/ascorbic acid therapy in patients with refractory metastatic colorectal carcinoma: a clinical experience, Acta Oncol. 46 (2007) 557–561.
[41] J. Quinn, B. Gerber, R. Fouche, K. Kenyon, Z. Blom, P. Muthukanagaraj, Effect of high-dose vitamin C infusion in a glucose-6-phosphate dehydrogenase-deficient patient, Case Rep. Med. (2017).