Combined arginine and ascorbic acid treatment induces apoptosis in the hepatoma cell line HA22T/VGH and changes in redox status involving the pentose phosphate pathway and reactive oxygen and nitrogen species

Bau-Shan Hsieh\textsuperscript{a}, Li-Wen Huang\textsuperscript{b}, Shu-Jem Su\textsuperscript{c}, Hsiao-Ling Cheng\textsuperscript{a}, Yu-Chen Hu\textsuperscript{a}, Thu-Ching Hung\textsuperscript{a}, Kee-Lung Chang\textsuperscript{a,d,*}

\textsuperscript{a}Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
\textsuperscript{b}Department of Medical Laboratory Science and Biotechnology, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
\textsuperscript{c}Bachelor Degree Program of Health Beauty, Department of Medical Technology, School of Medicine and Health Sciences, FooYin University, Kaohsiung 83101, Taiwan
\textsuperscript{d}Department of Biochemistry, Faculty of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan

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Abstract

Arginine is a physiological substrate for nitric oxide synthase to generate nitric oxide (NO), which can influence tumor cell survival, while ascorbic acid is selectively toxic for cancer cells. This study explored the effect of an arginine/ascorbic acid combination on human cancer cell lines. The hepatoma cell line HA22T/VGH was the most sensitive of the tested cells to combination treatment. A combination of 5.74 mM of arginine and 0.57 mM of ascorbic acid induced HA22T/VGH cell death through apoptosis and an increase in levels of reactive oxygen species and NO, as well as its stable products NO\textsubscript{2} and NO\textsubscript{3}. The combination also reduced the activity of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and transaldolase in the pentose phosphate pathway, a major mechanism for producing NADPH, resulting in a marked decrease in intracellular NADPH levels. A dramatic decrease in intracellular glutathione (GSH) levels, a decrease in the mitochondrial membrane potential, ATP depletion and release of cytochrome c were also seen. Caspase-9 and caspase-3 were activated, apoptotic protein Bax expression increased and the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL decreased. These results suggest that this combination induced HA22T/VGH cell death by interfering with redox state regulation by a reduction in pentose phosphate pathway activity and increasing oxidative and nitrosative stress.

Keywords: Arginine; Ascorbic acid; Pentose phosphate pathway; Redox state; HA22T/VGH; Nutrient mixture therapy

1. Introduction

Arginine is a dibasic, cationic amino acid which is essential for humans and is involved in a number of biosynthetic pathways. It is also a physiological substrate for nitric oxide synthase to generate nitric oxide (NO). NO, one of the smallest biological products of mammalian cells, is pleiotropic and mediates diverse functions. It protects cells from death at a low concentration, but becomes cytotoxic at high concentrations [1]. NO interacts with superoxide anion to produce peroxynitrite (ONOO\textsuperscript{−}), which can induce p53-dependent apoptosis [2]. There is increasing evidence that NO regulates tumor cell survival and influences cancer metastasis. The ultimate effect of NO on tumor cell survival is dictated by multiple factors, including levels of NO production and the genetic and epigenetic makeup of the tumor cells [3].

Ascorbic acid (vitamin C) plays important physiological roles as a reducing agent, antioxidant, free radical scavenger and enzyme cofactor. Although it is not generally considered to be cytotoxic, it acts as a pro-oxidant and causes oxidative stress in cells [4], inhibits cell growth [5–7] and induces apoptosis at a high concentration (≥1 mM) [8,9]. It has a controversial history in cancer treatment [10], but, recently, it was reported that pharmacological ascorbate concentrations (0.3–20 mM) are selectively toxic for cancer cells due to the formation of ascorbate radicals and H\textsubscript{2}O\textsubscript{2} [11,12].

Some nutrient mixtures have been found to have an antitumor effect on many kinds of cancers in humans [13–15]. Importantly, in contrast to the toxic side effects of current mono and double chemotherapy, nutrient mixtures have been shown to be safe therapeutic agents [16,17]. Recently, Waheed Roomi et al. [13,14] reported that a nutrient mixture containing lysine, proline, arginine, ascorbic acid and green tea extract can suppress the growth of human...
The human hepatoma cell line HA22T/VGH was purchased from the Food Industry Research and Development Institute in Taiwan (BCRC number: 60168) and was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 0.1 mM nonessential amino acids (Gibco BRL Life Technologies, Grand Island, NY, USA). The human skin keratinocyte cell line HaCaT, human hepatoma cell line HepG2 and human malignant melanoma cell line A375 were purchased from ATCC and cultured in DMEM. Human peripheral blood mononuclear cells isolated from healthy persons and the human basal cell carcinoma cell line BCC-1/KMC (from Kaohsiung Medical University) were cultured in RPMI-1640 (Gibco). All the above culture media also contained 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% l-glutamine (Gibco). Human primary skin keratinocytes were cultured in commercialized keratinocyte serum-free medium (Gibco). Human peripheral blood mononuclear cells isolated from healthy persons were harvested by trypsinization and resuspended in Hanks’ balanced salt solution (Sigma), nonessential amino acids (Gibco BRL Life Technologies, Grand Island, NY, USA). The human skin keratinocyte cell line HaCaT, human hepatoma cell line HepG2 and human malignant melanoma cell line A375 were purchased from ATCC and cultured in DMEM. Human peripheral blood mononuclear cells isolated from healthy persons and the human basal cell carcinoma cell line BCC-1/KMC (from Kaohsiung Medical University) were cultured in RPMI-1640 (Gibco). All the above culture media also contained 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% l-glutamine (Gibco). Human primary skin keratinocytes were cultured in commercialized keratinocyte serum-free medium (Gibco). Human skin primary skin melanocytes were cultured in Medium 254 containing growth supplement (Gibco). All cells were incubated at 37°C in an atmosphere of 5% CO2/95% air. l-Arginine, l-ascorbic acid, bis-dehydro-l-ascorbic acid and other chemicals were obtained from Sigma (St. Louis, MO, USA).

After treatment, the cells were detached by trypsin treatment, the suspension centrifuged at 300×g and the supernatant discarded. The cell pellets were resuspended in serum-free medium and an equal volume of 0.4% Trypan blue solution (Gibco). After incubation at room temperature for 3 min, viable cells were counted in a hemocytometer. All counts were performed in triplicate.

2.1. DNA Fragmentation

After treatment, the cell pellet was incubated overnight at 50°C in lysis buffer (100 mM Tris, 20 mM EDTA, 0.2% SDS, 5 mg/ml of proteinase K), then 5 mg/ml of RNase A was added and the mixture incubated for 2 h at 50°C, followed by DNA extraction with phenol/chloroform/isoamyl alcohol (25:24:1). Approximately 50–100 μg of DNA was loaded on a 2% agarose gel containing 0.5 μg/ml of ethidium bromide, and electrophoresis was carried out for 1 h at 50 V, after which the gel was photographed under ultraviolet light [20].

2.1.1. Western blotting

Western blotting was performed as described in our previous study [21]. Cytoplasmic extracts were prepared using ice-cold lysis buffer and incubation on ice for 15 min. After centrifugation, protein in the supernatant was quantified using a Bio-Rad Laboratories kit (Bio-Rad Laboratories, Hercules, CA, USA) and 60 μg of protein per lane was electrophoresed on 10% or 12% SDS-polyacrylamide gels. After transfer of the protein to nitrocellulose membranes, the membranes were blocked at room temperature for 1 h in phosphate-buffered saline (PBS) plus 0.5% Tween 20 containing 5% fat-free powdered milk, then were incubated for 2 h at 25°C with antibodies against human procaspase-9, procaspase-3, Bcl-2, Bcl-xL, Bax, cytochrome c or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, the membranes were incubated for 90 min at 25°C with the appropriate horseradish peroxidase-labeled secondary antibody (PharMingen, San Diego, CA, USA) and the proteins visualized by chemiluminescence detection (PerkinElmer Life Sciences, Inc., Boston, WA, USA). β-Actin was used as the internal control.

2.5. Measurement of ROS and NO levels

2′,7′-Dichlorodihydrofluorescein diacetate (H2DCF-DA) and 4′-amino-5-methylamino-2′,7′-dihydrofluorescein diacetate (DAF-FM diacetate) (Molecular Probes, Eugene, OR, USA) were used to measure intracellular ROS and NO production, respectively. In brief, 10 μM H2DCF-DA or DAF-FM diacetate was added to the culture medium 30 min before the end of treatment. At the end of treatment, the cells were harvested by trypsinization and resuspended in Hanks’ balanced salt solution (Sigma), then the fluorescence of dichlorofluorescein formed from either the oxidation of H2DCF-DA by cellular oxidants or the reaction of DAF-FM diacetate with NO was measured using a FACScan flow cytometry (Beckman Coulter-Epics XL; Beckman Coulter, Inc., Fullerton, CA, USA) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Data were analyzed using WinMDI 2.8 software.

2.6. Fluorometric assay of nitrite (NO2−) and nitrate (NO3−)

After treatment, HA22T/VGH cells were washed twice with PBS and scraped harvested in PBS at 4°C. The cell pellet was resuspended in 100 μl of PBS and sonicated at 20-s intervals for 2 min at 4°C and the cell lysate was centrifuged at 12,000×g for 10 min at 4°C, then the supernatant was collected and analyzed immediately. NO2− plus NO3− levels were determined on a spectrophotometer (Model LS-55, Perkin Elmer, Ltd., Beaconsfield, UK) using a commercial kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer’s instructions with a standard curve of sodium nitrite. The results were normalized to 1 mg of cellular protein. Cellular proteins were quantified using a Bio-Rad Laboratories kit.

2.7. Measurement of mitochondrial membrane potential

The mitochondrial membrane potential change (∆Ψm) is defined as a change in the electrochemical gradient. The mitochondrial ∆Ψm was measured using a fluorescent cationic dye, DiOC6(3) [3] (Molecular Probes). HA22T/VGH cells were seeded in six-well plates (Corning, Inc., Corning, NY, USA) and incubated overnight, then underwent treatment for 6 h before being incubated with 40 nM DiOC6(3) for 15 min at 37°C. The cells were then trypsinized and resuspended for analysis by FACScan flow cytometry (Beckman Coulter-Epics XL) [22]. Data were analyzed using WinMDI 2.8 software.

2.8. Measurement of ATP

HA22T/VGH cells (2×10⁵/well) were seeded in six-well plates (Corning, Inc.), incubated with drugs for 6 h, scraped off and centrifuged. The cell pellet was dissolved in deionized distilled water and ATP levels measured using a commercial luciferin/luciferase kit (Promega, Madison, WI, USA) and a luminometer (Hidex, Turku, Finland).

2.9. Determination of glutathione (GSH) and NADPH

Cell lysate supernatant was prepared as in the nitrite and nitrate assay above, and GSH levels and NADPH levels were determined using commercial kits purchased from Cayman Chemical or Biovision (Mountain View, CA, USA), respectively. Results were obtained by comparison with a standard curve and normalized to 1 mg of cellular protein. Proteins were quantified using a Bio-Rad Laboratories kit.

2.10. Analysis of pentose phosphate pathway enzyme activities

The activities of glucose-6-phosphate dehydrogenase (6PGD) and 6-phosphogluconate dehydrogenase (6PGD) and transaldolase (TAL), the rate limiting enzymes of the pentose phosphate pathway, were assayed. G6PD activity was measured in 6PGD assay solution (120 mM Tris, 10 mM MgCl2, pH 7.7, 2 mM glucose 6-phosphate, 0.9 mM NADP) and 0.1 U/ml of 6PGD, while 6PGD activity was measured in 6PGD assay solution (120 mM Tris, 10 mM MgCl2, pH 7.7, 2 mM 6-phosphogluconcate and 0.5 mM NADP). TAL activity was measured in the presence of 10 μg of 0.6-glycerophosphate dehydrogenase/triosephosphate isomerase (1:6 ratio) in 40 mM triethanolamine, 5 mM EDTA, pH 7.6, 3.2 mM d-fructose 6-phosphate, 0.2 mM erythrose 4-phosphate and 0.1 mM NADP. Assays were performed at room temperature and monitored by continuous reading of the absorbance at 340 nm for 10 min on a spectrophotometer (Model DU-640, Beckman), then the activity was calculated and expressed as units per minute per milligram of protein [23]. Proteins were quantified using a Bio-Rad Laboratories kit.

2.11. Statistical analysis

All data are expressed as the mean±S.D. The significance of differences was determined by ANOVA followed by Fisher’s Exact Test. Statistical analyses were performed using SAS (version 6.011, SAS Institute, Inc., Cary, NC, USA). A P value <0.05 was considered statistically significant.

3. Results

3.1. Effects of arginine and/or ascorbic acid on cell viability

To gain an initial insight into the effect of arginine or ascorbic acid on the viability of HA22T/VGH cells, the cells were treated with different concentrations of arginine or ascorbic acid for 24 h. As shown in Fig. 1A, arginine had no effect even at the high concentration of 1.0 mM, whereas ascorbic acid significantly reduced cell viability at concentrations higher than 0.57 mM. At 0.85 mM of ascorbic acid, cell viability was reduced by 28% compared to untreated control cells.
The effects of a combination of 5.74 mM of arginine and different concentrations of ascorbic acid were then tested. As shown in Fig. 1B, 5.74 mM of arginine plus 0.57 mM of ascorbic acid caused a significant reduction in cell viability to 50% of that in controls, while each alone had no, or little, effect (Fig. 1A).

Different concentrations of ascorbic acid combined with 5.74 mM of arginine were used to treat different kinds of cells and the IC50 was evaluated. As shown in Table 1, the IC50 values for different cell types covered a wide range, with a trend for the more malignant cells to be more sensitive. HA22T/VGH cells were the most sensitive of the tested cells and were chosen for further study. When combinations of 5.74 mM of arginine with different concentrations of ascorbic acid were tested on HA22T/VGH cells, the IC50 was seen at 0.57 mM of ascorbic acid. If the ascorbic acid was replaced by its oxidized form, dehydroascorbic acid, no cytotoxic effect was seen (Fig. 1C).

3.2. Apoptosis induction

DNA fragmentation electrophoresis showed that apoptosis occurred in HA22T/VGH cells treated for 24 h with the combination (Fig. 2). To determine whether this combination-induced apoptosis was associated with altered expression of apoptosis-regulating proteins, HA22T/VGH cells were treated for 12 h with arginine and/or ascorbic acid and protein expression was measured by Western blotting. During apoptosis, procaspase is activated by cleavage at specific residues to generate active caspase. As shown in the immunoblots in Fig. 3, 12-h treatment of HA22T/VGH cells with combined arginine/ascorbic acid, but neither agent separately, resulted in a significant decrease in procaspase-9 levels and a marked decrease in procaspase-3 levels, indicating caspases were activated. A significant increase in Bcl-2 expression and a decrease in Bcl-xL expression were seen with either arginine or ascorbic acid treatment, whereas both were markedly decreased with the combination treatment. In addition, arginine alone caused a significant increase, and ascorbic acid alone caused a significant decrease, in expression of the apoptotic protein Bax, while the combination caused a marked decrease. Neither treatment alone had any effect on cytochrome c release, while the combination caused a marked increase.

3.3. Cell death in addition to apoptosis

We then examined whether pretreatment with a caspase inhibitor abolished the apoptotic cell death induced by combined arginine/...
Fig. 3. Effects of the arginine/ascorbic acid combination on the expression of apoptosis-related proteins. After treatment for 12 h with arginine (Arg, 5.74 mM) and/or ascorbic acid (AA, 0.57 mM), levels of apoptotic proteins (procaspase-9, procaspase-3, Bax and cytochrome c) and of anti-apoptotic proteins (Bcl-2 and Bcl-xL) in the cytosol fraction were examined by Western blotting, with β-actin as the internal control. (A) shows a typical example and (B) shows the relative density of the band compared to that in untreated cells (control), taken as 100%. The results are expressed as the mean±S.D. for three separate experiments. *P<.05 compared to the untreated controls; #P<.05 compared to the Arg+AA combination.
ascorbic acid treatment. As shown in Fig. 4A, no DNA fragmentation was seen in cells treated for 24 h with the arginine/ascorbic acid combination when the cells were incubated with Z-LEHD-FMK (a caspase-9 inhibitor), Z-DEVD-FMK (a caspase-3 inhibitor) or Z-VAD-FMK (a caspase family inhibitor) for 30 min before, and during, the combined treatment. However, the caspase inhibitor-pretreated groups still showed lower cell viability than the corresponding control group (Fig. 4B), showing that cell death was not completely prevented by the caspase inhibitor and that another kind of cell death was induced by the combination.

3.4. Increase in oxidative and nitrosative stress

To investigate the effect of arginine and/or ascorbic acid on oxidative and nitrosative stresses, HA22T/VGH cells were treated with 5.74 mM of arginine and/or 0.57 mM of ascorbic acid for 6 h, then ROS and NO levels, as well as those of its final products NO$_2^-$ plus NO$_3^-$, were determined. As shown in Fig. 5A, neither agent alone had any major effect on ROS production, but a dramatic increase in ROS levels was seen with the combined arginine/ascorbic acid treatment. As shown in Fig. 5B, as expected, addition of arginine, a precursor of NO, enhanced NO production, but, interestingly, ascorbic acid also enhanced NO production to the same degree as arginine and there was a synergistic effect with the combination. As shown in Fig. 5C, only the combination had any effect on the levels of NO$_2^-$ plus NO$_3^-$. These data indicate that the arginine/ascorbic acid combination causes noticeably increased oxidative and nitrosative stress in HA22T/VGH cells.

3.5. Decrease in the mitochondrial membrane potential and ATP levels

Decreased mitochondrial permeability is an early indication of cellular apoptosis. The mitochondrial $\Delta \Psi_m$ is defined as a change in the electrochemical gradient and was measured by flow cytometry using a fluorescent cationic dye, DiOC$_6$(3). In nonapoptotic cells, the positively charged dye enters the negatively charged mitochondria, whereas this does not occur in apoptotic cells, in which the mitochondria have lost their charge. Fig. 6A shows that, after 6 h of treatment, arginine had no effect on $\Delta \Psi_m$, but ascorbic acid caused a significant decrease and the combination caused a marked decrease. As shown in Fig. 6B, neither arginine nor ascorbic acid alone caused a significant change in intracellular ATP levels, whereas, in accordance with the marked change in $\Delta \Psi_m$, the combination resulted in significant decreased ATP levels.

3.6. Decrease in GSH and NADPH

By reacting directly with free radicals in a nonenzymatic reaction, GSH is important for cellular defense against reactive oxygen damage.
We therefore examined the GSH content of HA22T/VGH cells after arginine and/or ascorbic acid treatment for 6 h. Neither agent alone had any effect on the intracellular GSH content, whereas the combination caused a marked decrease (Fig. 7A). NADPH acts as the electron donor cofactor in the glutathione reductase reaction, so NADPH is critical in maintaining appropriate levels of GSH. As GSH redox homeostasis is dependent on NADPH [24], the changes in the NADPH content of the arginine- and/or ascorbic acid-treated groups were dramatically decreased, showing the same trend as the change in GSH levels (Fig. 7B).

3.7. Reduction in pentose phosphate pathway enzyme activities

The pentose phosphate pathway is a major mechanism for the production of NADPH. Since NADPH levels were decreased by the arginine/ascorbic acid combination, we examined whether the combination had any effect on the pentose phosphate pathway. The pentose phosphate pathway is composed of oxidative and nonoxidative phases; G6PD and 6PGD are key enzymes of the oxidative phase, while TAL is the rate-limiting enzyme of the nonoxidative phase [23,25]. HA22T/VGH cells were treated with arginine and/or ascorbic acid for 6 h, then G6PD, 6PGD and TAL activity was measured. As shown in Fig. 8, neither arginine nor ascorbic acid alone had any effect on the activities of these three enzymes, but the combination cause a marked reduction in all three activities.

4. Discussion

In this study, we found that the combination of arginine and ascorbic acid had a selective effect on human cancer cell lines and almost no effect on normal human cells. The combination of 5.74 mM arginine and 0.57 mM ascorbic acid induced apoptosis and changed the redox status of HA22T/VGH cells, in which levels of ROS and NO and its stable products NO$_3^-$ and NO$_2^-$ were increased, while intracellular NADPH and GSH levels were dramatically decreased. In addition, it reduced the activities of G6PD, 6PGD and TAL, the key enzymes of the pentose phosphate pathway, a major mechanism for producing NADPH. These findings suggest that this combination induced HA22T/VGH cell death by interfering with the regulation of the redox state.

NO is generated in the conversion of L-arginine to L-citrulline by the NADPH-dependent NO synthase, which requires tetrahydrobiopterin and FAD as cofactors [26,27]. Our results showed that addition of arginine increased NO production (Fig. 5B), indicating that NADPH was consumed. A previous report demonstrated that ascorbic acid
promotes NO synthesis by enhancing the availability of tetrahydrobiopterin and increasing the affinity of NO synthase [28]. In accordance with this report, we observed that NO production was increased by ascorbic acid and that the combination had a synergistic effect and resulted in a marked increase in NO production, suggesting that more NADPH would be needed for the marked increase in NO production induced by combination treatment. However, the activities of the three key enzymes of the pentose phosphate pathway, a major mechanism for producing NADPH, were reduced (Fig. 8), which would worsen the reductive state. In addition to an increase in NO production, an increase in NO2- and NO3- (Fig. 5C) and ROS (Fig. 5A) was found with the combination treatment, suggesting the oxidative state was augmented. Reactive oxygen and nitrogen species are central to the cell death pathway [29], and regulation of the redox state is important in protecting cells against oxidative and nitrosative stress.

The most abundant endogenously produced antioxidant in eukaryotic cells is the tripeptide GSH, which is mainly responsible for the maintenance of the cellular redox state. GSH synthesis from glutamate, cysteine and glycine is catalyzed sequentially by γ-glutamylcysteine ligase and GSH synthase [30]. More recent studies have shown that GSH levels play a critical role in determining sensitivity and resistance to apoptosis in many cells [31]. Maintenance of GSH homeostasis is dependent on NADPH produced by the pentose phosphate pathway [30]. In fact, the role of the pentose phosphate pathway is to keep glutathione in the reduced state (GSH) and protect sulfhydryl groups and cellular integrity from oxygen radicals. Thus, an increase in G6PD, 6PGD and TAL activities, which allows the pentose phosphate pathway to generate more NADPH, prevents GSH oxidation and cell death [32,33]. In contrast, the combination treatment used in the present study reduced the activities of these enzymes, leading to a decrease in GSH levels (Fig. 7A). The worsened reductive state and GSH depletion could trigger mitochondria collapse, which finally leads to cell death [34,35]. Consistent with this, our data showed a decrease in ΔΨm (Fig. 6A), depletion of ATP (Fig. 6B) and increased cytchrome c release (Fig. 3) after combination treatment.

It has been reported that cancer cells have higher glucose metabolism and pentose phosphate cycle activity than normal cells [36,37]. In addition, different cancer cells show different levels of ROS generation and different mitochondrial sensitivities to ROS [38–40]. Recently, Chen et al. [12] reported that the efficacy of ascorbic acid treatment in cancers is dependent on their mitochondrial sensitivity to H2O2. Xie and Huang [3] reported that whether NO or its final products NO2- and NO3- caused necrotic or apoptotic death was dependent on the baseline NO levels and cell type. These results may explain why the combination of arginine and ascorbic acid had a selective effect on cancer cells and not on normal cells.

Nutrition supplementation is beneficial for cancer patients by accelerating immune responses, compensating for malnutrition and providing anti-oxidative defense [19,41]. In accordance with other reports [13–15,18], the present study showed that a nutrient mixture could act directly on tumor cells, suggesting that nutrition supplementation in cancer treatment might be multifunctional. The relatively high dose of the arginine/ascorbic acid combination used in this study may raise concerns about safety in clinical application. The doses of arginine and ascorbic acid used were chosen for two reasons: firstly, because each alone was not cytotoxic in our system, and, secondly, both arginine and ascorbic acid have been used at high doses in humans with no side-effects [42–44]. However, it may be possible to decrease the dosages of both components in the combination.

In conclusion, this study shows that a nutrient mixture of arginine and ascorbic acid with low toxicity for normal cells induces the death of some cancer cells and offers promise for use in clinical treatment. However, more basic and clinical studies are needed to test this possibility.


