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Iron decreases cytotoxicity of vitamin C in Molt- 4 cells

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ABSTRACT

Vitamin C has been suggested to play a beneficial role in the prevention and treatment of cancer. However, its *in vivo* efficacy against cancer is inconsistent even at very high doses. We hypothesized that a high concentration of iron may reduce or eliminate the effectiveness of vitamin C cytotoxicity towards cancer cells. Molt-4 (human leukemia) cells and normal human leukocytes were incubated with holotransferrin (0, 1.25, 2.5, or 5 mg/mL) for an hour. Cells were then treated with sodium ascorbate (0, 64, 128, or 256 µM) and live cells were counted after various times of incubation. At concentrations of 256 µM and 128 µM, ascorbate alone killed all Molt-4 cells in 48 hr and 72 hr, respectively ($p<0.001$, compared to untreated control), without significantly affecting leukocytes ($p>0.05$). Preincubation with holotransferrin significantly inhibited the cytotoxicity of ascorbate toward Molt-4 cells. High concentrations of iron may be responsible for the reduced efficacy of vitamin C in killing leukemia cells and may explain the contradictory results obtained from clinical studies.

KEYWORDS: vitamin C, Molt-4 cells, leukocytes, cytotoxicity, iron

INTRODUCTION

Ascorbic acid or vitamin C is a nutrient essential for human survival and plays a key role in human health and diseases. Unlike most other mammals, humans are incapable of synthesizing vitamin C from glucose. Previous research has shown that supplementation of vitamin C, in addition to normal dietary intake, may be necessary to maintain optimal health. Most prominently, Pauling [1] concluded that the average adult required an ideal daily intake of 2.3 g or more of vitamin C. It is considered to be very safe even at high doses [2]. Vitamin C is present in all human tissues [3] at varying concentrations [4] with some of the highest concentrations found in adrenal, pituitary, brain, and eye tissues [5]. Physiologically, in addition to its primary role as an anti-oxidant, it is critical in the synthesis of collagen, carnitine and a variety of neurotransmitters [6]. Humans have evolved highly efficient mechanisms to transport and maintain sufficient intracellular levels of vitamin C. Due to structural similarities between glucose and vitamin C, glucose transporters (GLUTs) can facilitate the uptake of dehydroascorbic acid (oxidized vitamin C) into the cell where it is converted into ascorbic acid. Also, sodium-coupled vitamin C transporters (SCVTs) facilitate the uptake of ascorbate (reduced vitamin C).

Cancer cells show even more efficient mechanisms of vitamin C transport; because tumor cells have extremely high demand for glucose they show an increased expression of GLUTs [7]. In melanoma cells, for example, the rate of dehydroascorbic acid uptake is ten-fold higher than in melanocytes [7]. This leads to a hundred fold higher intracellular ascorbate concentration in some cancer cells, even though rates of ascorbate transport via SCVTs are approximately equal in both normal and cancer cells [7].

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Cameron *et al*. [8] proposed a significant role for vitamin C in the occurrence, progression and outcome of cancers. Ascorbate has been shown to kill various types of cancer cells *in vitro* [9-12] and *in vivo* [13, 14] by apoptosis [11, 15-19].

Iron is crucial for cell division and proliferation. It is transported from plasma into cells by the iron-carrying protein transferrin. Due to rapid growth and perturbed iron metabolism cancer cells have a high iron demand. Thus, cancer cells have a higher concentration of cell surface transferrin receptors [20, 21] for iron uptake, compared to normal cells. Even at extremely low temperatures (4°C), cancer cells can take up iron via transferrin receptors using holotransferrin as the carrier protein [21]. At higher temperatures (37°C), cancer cells rapidly take up iron via two main mechanisms: The holotransferrin-transferrin receptor pathway mentioned above and also direct internalization of iron [21].

Previous researchers have shown that iron is a promoter of carcinogenesis in animal studies [22, 23] and have also implicated it in the growth of a variety of human cancers [24-26]. High levels of dietary iron increase the risk of breast, colon, ovarian and other cancers in humans [27-29]. Iron increases oxidative stress-induced DNA damage in normal cells via the generation of free radicals [30]. Cells with damaged DNA may undergo repair, apoptosis or transformation [31, 32]. One recent study has shown that vitamin C may decrease iron uptake in human melanoma cells [33] and this may induce cell apoptosis.

Two studies by Pauling and Cameron [34, 35] showed vitamin C increased mean survival rates in cancer patients. However, subsequent studies [36, 37] have failed to find any significant beneficial effect of high doses of vitamin C supplementation on cancer survival rate. These controversial reports have been reviewed by Gonzalez *et al*. [38].

Vitamin C has both pro-oxidant and anti-oxidant properties, thus researching the role of minerals such as iron in enhancing or interfering with its anti-cancer activities may be critical to clinical applications. Because ascorbic acid has been shown to react with metals and generate free radicals [39], we hypothesized that the contradictory results in the above mentioned studies may be due to variations in intracellular iron levels in humans. One of us has previously shown that ascorbate is toxic to the human Molt-4 leukemia cells [10]. The current research was designed to investigate the role of iron (in the form of holotransferrin, an iron-bound protein that we [40] and other researchers [21] have previously used to raise iron content in cells) in vitamin C cytotoxicity in Molt-4 leukemia cells.

METHODS

Testing with Molt-4 cells

Molt-4 cells (American Type Culture Collection, Manassas, VA) were grown in 100 % humidity at 37°C in 5 % CO_2 in air, in RPMI-1640 medium (Life Technologies, Gaithersberg, MD) with 10 % fetal bovine serum (Hyclone, Logan, UT). Twenty-four hours after adding culture medium to the cells (1:1), aliquots of cell samples were treated with 0, 1.25, 2.5, or 5 mg/ml human holotransferrin (Sigma Chemical Co, St. Louis, MO). After 1 hr of incubation, various concentrations of sodium ascorbate (64, 128 and 256 µM) were added to the cell samples. Cell viability was not determined because it was not correlated with cell loss, as rapid dead cell disintegration was observed. Using a Reichert light microscope, dead cells were excluded based on morphological criteria, (chromatin condensation, formation of apoptotic bodies, shrinkage of cytoplasm and blebbing plasma membrane with irregular outline), as previously described [41] and live cells were counted immediately before (0 hr) and at 24, 48, and 72 hr after addition of ascorbate.

Testing with healthy leukocytes

Human whole blood obtained from a finger prick was diluted 1:100 with complete RPMI-1640 and divided into aliquots of 1 ml. These samples were subjected to the same procedures of holotransferrin and sodium ascorbate treatment as described above for Molt-4 cells. After thoroughly suspending cells, 10 µl of blood samples were mixed well with 10 µl of 10 µg/ml of acridine orange in PBS and 10 µl of this cell suspension was loaded

in a hemocytometer chamber. Based on morphological criteria for exclusion of dead leukocytes (as mentioned above for Molt-4), live cells were counted using a Reichert fluorescent microscope with an excitation filter of 490 nm, a 500 nm dichroic filter, and an emission filter of 515 nm (FITC filter combination). Acridine orange is a DNA intercalating dye and stains DNA and RNA only. For easier counting of leukocytes in the presence of red blood cells, acridine orange fluorescent staining was essential. Fluorescent dye was not used in counting Molt-4 cells because there is no interference of red blood cells in light microscopy.

Data analysis

Experiments were conducted 4 times using Molt-4 cells and 3 times using leukocytes and means and standard deviations of data points were calculated and plotted. Data (total live cell count) were analyzed by one-way ANOVA and difference between two treatment points by the Newman-Keuls test. A p value less than or equal to 0.05 was considered statistically significant.

DNA diffusion assay

To confirm our optical findings and to visualize the mode of cell death in Molt-4 cells, we also performed the DNA diffusion assay, according to a previously published protocol [40]. Briefly, a small aliquot of Molt-4 cells (50 µl) was centrifuged at 500g for 5 min and the pellet was resuspended in 10 µl PBS and mixed with 100 µl of 0.7% high-resolution 3:1 agarose (Amresco, Solon, OH).

Microgels were made using 100 µl of the above mixture on clear window frosted slides (Mac & Sons Specialty Glass, Titusville, FL) coated with one layer of dried agarose. A final layer of microgel was made with 200 µl of 2% SFR agarose (Amresco, Solon, OH). Slides were processed for alkaline lysis and neutralization and ethanol and spermine were used for precipitating DNA as described previously [40]. Dry slides were stained with 50 µl of YOYO-1 dye for visualization of cells. This technique allows differentiation of healthy cells, and also between apoptotic and necrotic cells. An apoptotic cell shows a circular gradient of granular DNA with a dense central zone and lighter hazy outer zones, giving the overall appearance of a halo, whereas necrotic cells show an unusually large homogeneous nucleus with a clearly defined boundary.

RESULTS

In untreated control cultures and in cultures treated with holotransferrin alone, Molt-4 cell counts significantly ($p<0.001$) increased as a function of time. There was no significant difference between the control and holotransferrin-treated cultures (**Figure 1A**). Also, in cultures treated with the lowest concentration of ascorbate (64 µM) and in cultures treated with various concentrations of holotransferrin alone, and a combination of the two, cell counts significantly ($p<0.001$) increased as a function of time (**Figure 1B**). Ascorbate alone, at 128 µM and 256 µM, significantly inhibited the growth of Molt-4 cells after 24 hours ($p<0.001$). At concentrations of 128 and 256 µM, all cells were killed at 72 hrs and 48 hrs, respectively (**Figures 1C** and **D**). Holotransferrin at all concentrations protected Molt-4 cells from ascorbate cytotoxicity at all time points (**Figures 1B, C,** and **D**).

Figure 2A, B, C, and **D** show number of human leukocytes (Mean $\pm$ SD, $n = 3$) at 0, 24, 48, and 72 hr time points respectively with different treatment combinations of sodium ascorbate and holotransferrin. In general, there was a significant reduction in leukocyte count over time ($p<0.05$), but there was no significant effect of any concentration of ascorbate, holotransferrin, or combination of the two.

Data of Molt-4 cells and leukocytes at the 24-hr time point are presented in **Figures 3** and **4**, respectively. Ascorbate at 128 and 256 µM significantly ($p<0.001$) reduced the number of Molt-4 cells whether they were pre-treated or not with the highest concentration of holotransferrin (5.0 mg/ml) (**Figure 3**). At 24 hr, there was no significant difference in Molt-4 cell count, treated with the highest concentration of holotransferrin alone when compared to control ($p>0.05$). However, the highest concentration of holotransferrin in combination with ascorbate at 128 or 256 µM

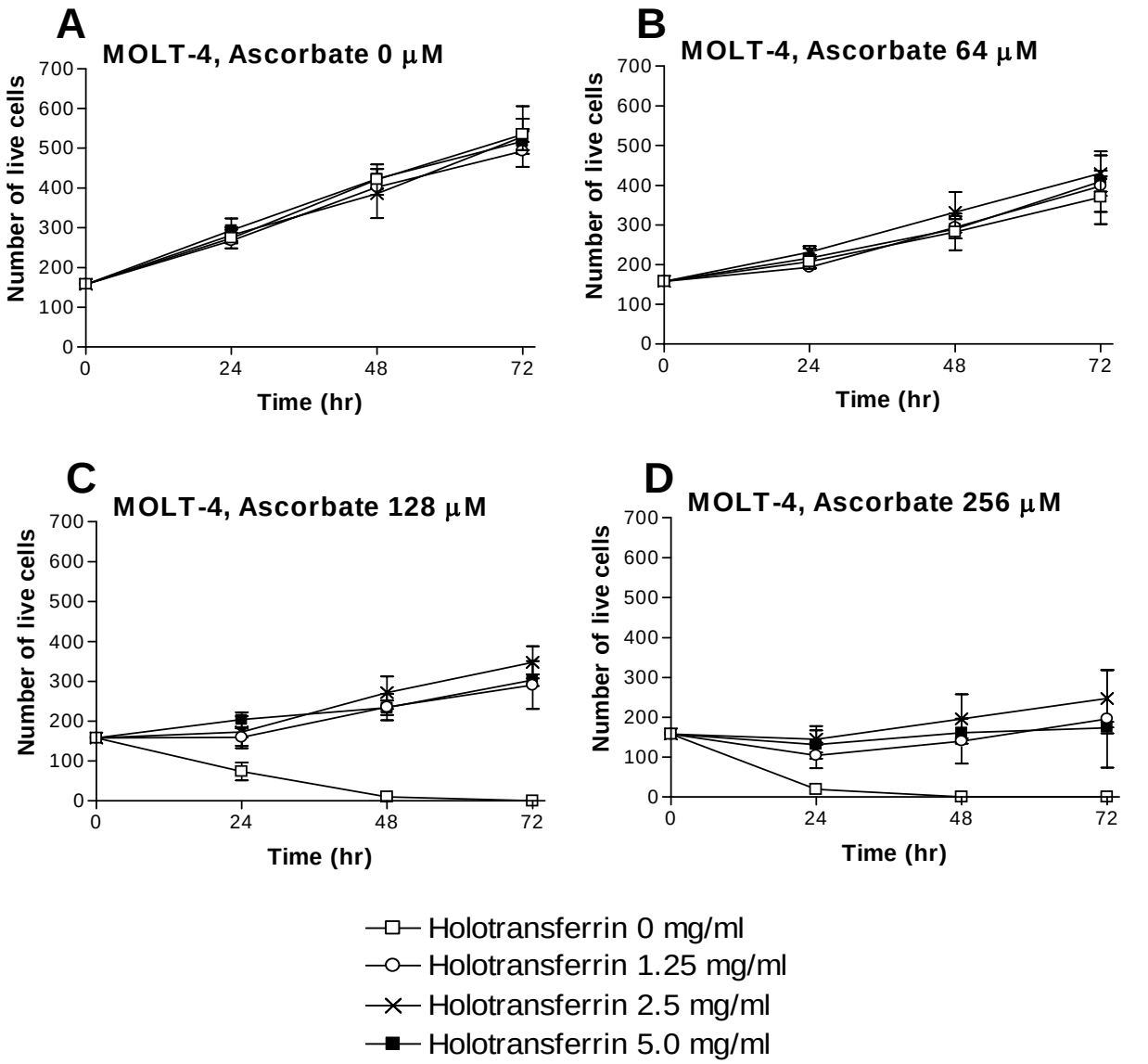

Figure 1 (A-D). Graphs showing number of Molt-4 cells (Mean ± SD, N = 4) at 0-, 24-, 48- and 72 hr time points with different treatment combinations of sodium ascorbate and holotransferrin.

significantly increased the number of surviving Molt-4 cells when compared to ascorbate (128 or 256 µM) alone ($p<0.001$). This indicates a protective effect of holotransferrin on the cytotoxicity of ascorbate on Molt-4 cells.

As shown in **Figure 4**, cell counts in leukocytes treated with ascorbate alone (128 or 256 µM) or in combination with holotransferrin at the highest concentration (5.0 mg/ml) did not show a significant difference ($p>0.05$) from control or from each other.

In Molt-4 samples processed for the DNA diffusion assay, all dead cells were observed to be undergoing apoptosis. No necrotic cells were seen. Some characteristics of typical live Molt-4 cells and typical apoptotic Molt-4 cells observed with a fluorescent microscope are shown in **Figure 5**. Results of apoptotic incidence in Molt-4 cells processed for the DNA diffusion assay for apoptosis (data not shown) were similar to those obtained using morphological criteria (see above and **Figures 1-4)**.

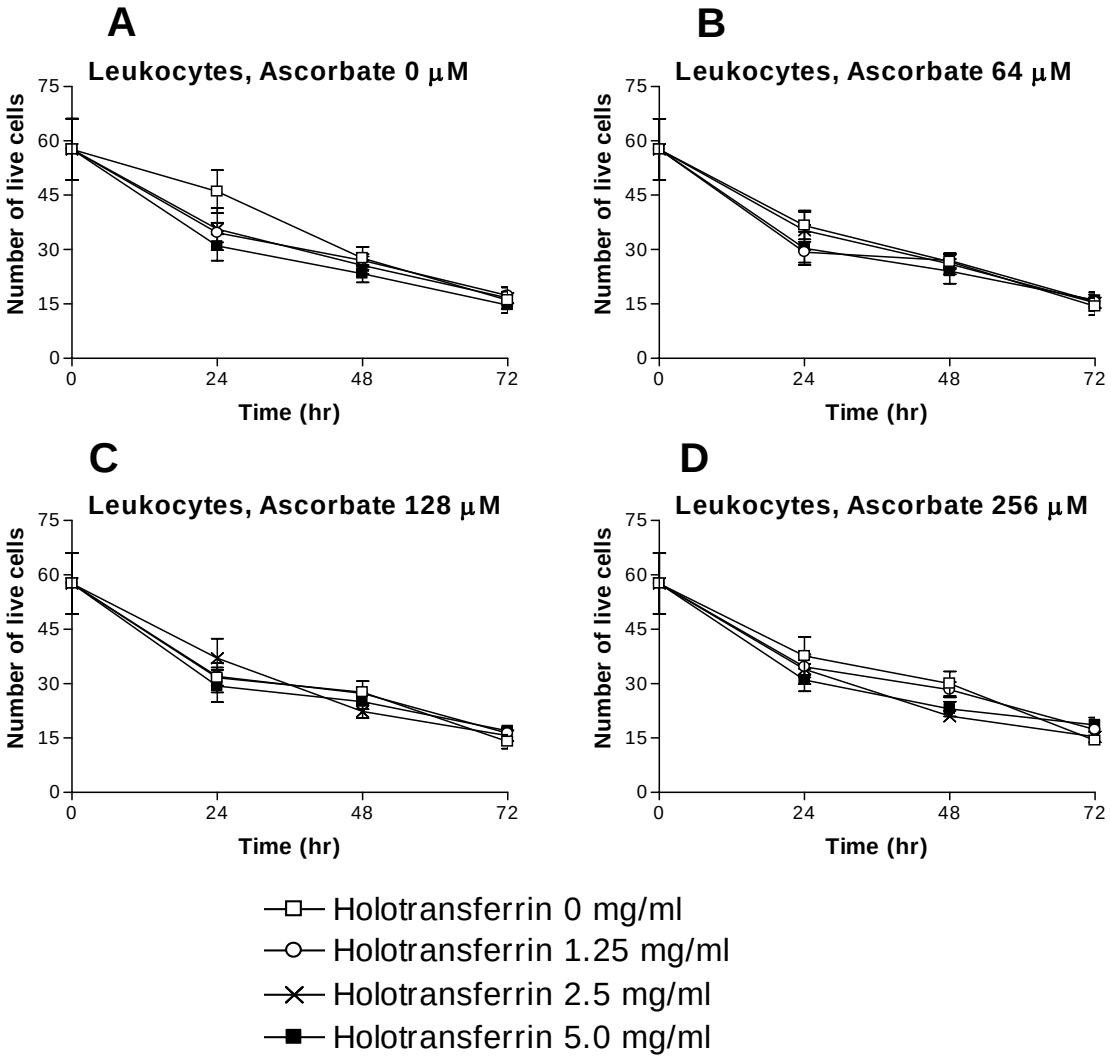

Figure 2 (A-D). Graphs showing number of human leukocytes (Mean ± SD, N = 3) at 0-, 24-, 48- and 72 hr time points with different treatment combinations of sodium ascorbate and holotransferrin.

DISCUSSION

We did not observe any significant cell loss of leukocytes treated with any concentration of ascorbate at any time point. However, there was a time dependent loss of leukocytes in all cultures, treated as well as controls. Resistance of leukocytes to ascorbate has previously been reported [10] and may be due to higher cellular defenses, such as antioxidants and DNA repair enzymes, efficient metabolism of ascorbate, utilization of ascorbate, and expulsion or sequestering of metal ions, particularly iron and copper. Furthermore, the lack of ascorbate cytotoxicity to leukocytes may be due to cellular macromolecules (such as proteins, RNAs, and lipids) in leukocytes that are not easily destroyed by ascorbate compared to those in Molt-4 cells.

Our study shows that sodium ascorbate (vitamin C) induced cytotoxicity in Molt-4 cells, in time- and dose- dependent manners. Cell death occurred mostly via apoptosis (data not shown). Apoptosis has been shown to be a primary mode of cell death in cancer cells treated with chemotherapeutic agents [42]. In our study, the cytotoxic effect of vitamin C was reduced when Molt-4 cells were incubated in complete RPMI 1640 having higher concentrations of holotransferrin (to increase intracellular iron levels) for an hour prior to

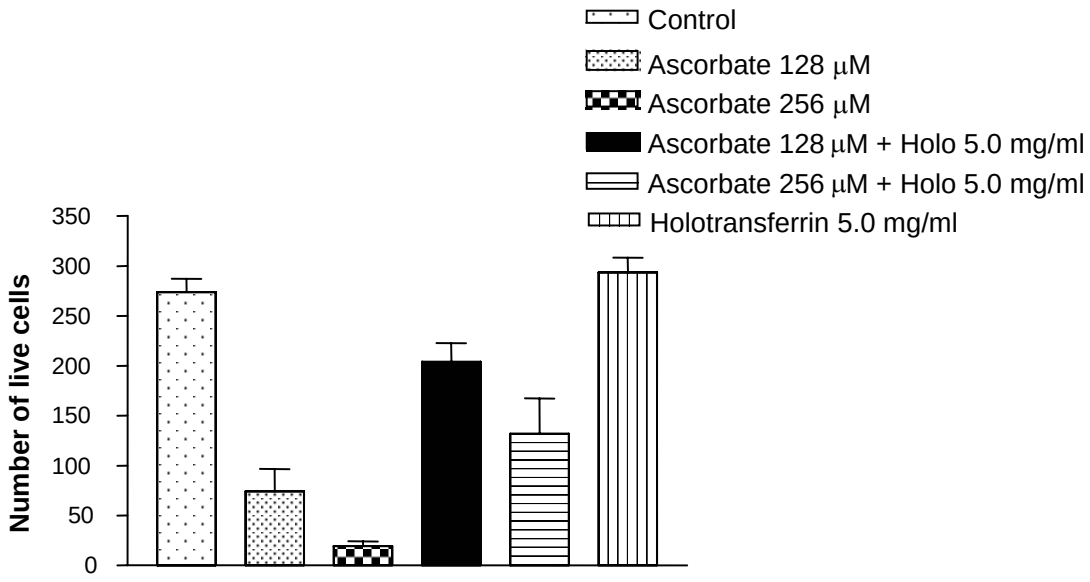

Figure 3. Bar graphs showing number of Molt-4 cells at 24 hrs after treatment with sodium ascorbate, with and without addition of holotransferrin. Each bar represents mean ± SD (n = 4). Ascorbate alone, at doses of 128 and 256 μM is very effective in eliminating Molt-4, while ascorbate in combination with the highest concentration of holotransferrin (5.0 mg/mL) is ineffective.

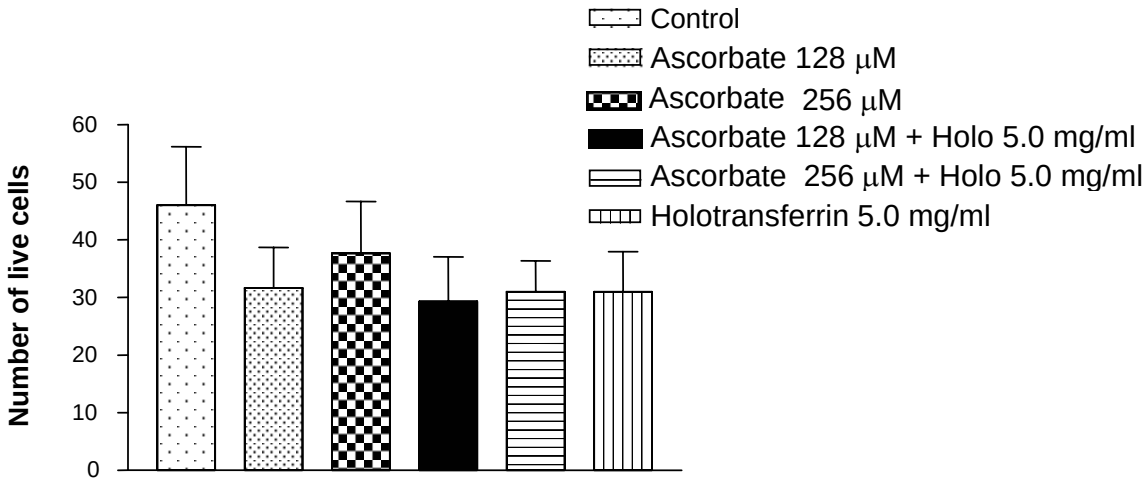

Figure 4. Bar graphs showing number of human leukocytes at 24 hrs after treatment with sodium ascorbate with and without addition of holotransferrin showing no significant effect in any of the treatment groups as compared to control. Each bar represents mean ± SD (n = 3).

ascorbate treatment. Previous studies have shown that sodium ascorbate has selective toxicity towards leukemia cell lines, HL-60 [43], Molt-4 [10], and Molt-3 [44] by induction of apoptosis. The main mechanisms proposed for this cytotoxicity is iron-related. Vitamin C has been shown to decrease cellular iron uptake by down-regulating transferrin receptors, in neuroblastoma PC-12 cells and melanoma cells [19, 33]. This down-regulation of transferrin receptors may lower intracellular iron. Iron is critical to a host of cellular metabolic processes and DNA synthesis. Iron is required for the formation and action of the enzyme ribonucleotide reductase,

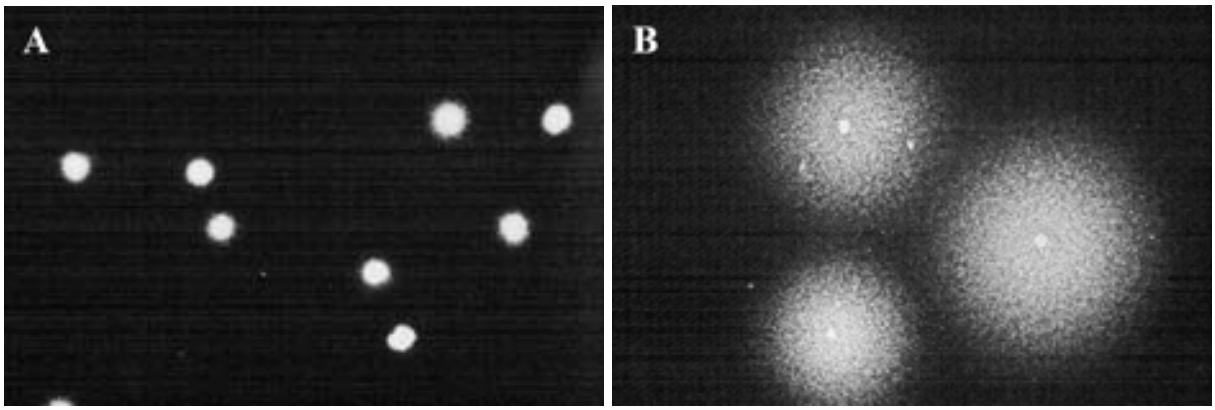

Figure 5. Photomicrographs of MOLT-4 cells processed using the DNA diffusion assay for apoptosis detection, **(A)** untreated, showing 8 normal cells and **(B)** treated with 256 µM sodium ascorbate for 48 hrs, showing three apoptotic cells (Magnification: 200×, dye: YOYO-1).

which catalyzes the conversion of ribonucleotides to deoxyribonucleotides, an event essential for DNA synthesis. Thus, without iron, the cell may accumulate DNA damage, leading to cell death [33]. Root-Bernstein *et al*. [45] hypothesized that toxicity of ascorbate may also be due to a variety of other mechanisms, including increased oxidation products from the conversion of dehydroascorbate to ascorbate within the cells, disruption of glucose utilization and inhibition of facilitative glucose uptake. Increased uptake of glucose and glycolysis occur in most cancer cells [46], which are associated with increased levels of Glut-1 transporters [47, 48]. Another mechanism of action was proposed by Lee [11], who observed an ascorbate-induced rapid and sustained rise of intracellular calcium [24] leading to cell death in a hepatoblastoma cell line.

Lowering cellular iron contents using desferroxamine has been shown to reduce apoptosis by artemisinin dimers in several cancer cell lines [49], likewise increasing intracellular iron using holotransferrin has been shown to increase apoptosis in Molt-4 cells [40]. These findings indicate that high intracellular iron contents are required for the cytotoxic action of some anticancer agents, such as artemisinin dimers and dihydroartemisinin, while low levels of iron are essential for the anticancer effect of others such as vitamin C.

Human leukocytes treated with ascorbate showed no significant effects in this study. Contrary to normal serum concentrations of 52 to 112 µM [50, 51], intracellular concentration of ascorbate in normal cells is in the millimolar range, as it is actively taken up by most cells, such as brain cells (1-2 mM) [16, 52], neutrophils (10-20 mM) [53], and lymphocytes (1.5-3.5 mM) [54-56]. Vitamin C may be considered safe in doses of several grams and serum concentrations of up to 200 µM are achievable by oral administration [57, 58]. The effective concentrations in our study were 128 µM (which is twice the average normal serum concentration) and 256 µM. These concentrations (128-256 µM) are readily achievable through oral administration and should be effective against a variety of cancer cells, provided intracellular iron concentrations are low.

CONCLUSION

Ascorbate alone, without iron supplementation, in low concentrations killed Molt-4 leukemia cells but did not affect human leukocytes. High concentrations of iron may reduce the efficacy of ascorbate in killing leukemia cells *in vitro*. This effect may be a factor in the contradictory results obtained from previous clinical studies on vitamin C. Our study suggests that the anti-cancer effect of vitamin C treatment depends upon intracellular iron concentration.

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