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Cytotoxicity of Ascorbic Acid in a Human Colorectal Adenocarcinoma Cell Line (WiDr): In Vitro and In Vivo Studies

Ana Catarina Mamede [a b c], Ana Salomé Pires [a d], Ana Margarida Abrantes [a c], Sónia Dorilde Tavares [a c d], Ana Cristina Gonçalves [c e], João Eduardo Casalta-Lopes [a], Ana Bela Sarmento-Ribeiro [c e], Jorge Manuel Maia [f] & Maria Filomena Botelho [a c]

[a] Biophysics Unit, Institute of Biomedical Research on Light and Image (IBILI), Faculty of Medicine, University of Coimbra, Coimbra, Portugal

[b] CICS-UBI, Health Sciences Research Centre, University of Beira Interior, Covilhã, Portugal

[c] Centre of Investigation on Environment, Genetics and Oncobiology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal

[d] Faculty of Sciences and Technology, University of Coimbra, Coimbra, Portugal

[e] Applied Molecular Biology and Hematology Group, Faculty of Medicine, University of Coimbra, Coimbra, Portugal

[f] Faculty of Sciences, University of Beira Interior, Covilhã, Portugal

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Ana Catarina Mamede
Biophysics Unit, Institute of Biomedical Research on Light and Image (IBILI), Faculty of Medicine, University of Coimbra, Coimbra, Portugal; CICS-UBI, Health Sciences Research Centre, University of Beira Interior, Covilhã, Portugal, and Centre of Investigation on Environment, Genetics and Oncobiology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal

Ana Salomé Pires
Biophysics Unit, Institute of Biomedical Research on Light and Image (IBILI), Faculty of Medicine, University of Coimbra, Coimbra, Portugal, and
Faculty of Sciences and Technology, University of Coimbra, Coimbra, Portugal

Ana Margarida Abrantes
Biophysics Unit, Institute of Biomedical Research on Light and Image (IBILI), Faculty of Medicine, University of Coimbra, Coimbra, Portugal, and Centre of Investigation on Environment, Genetics, and Oncobiology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal

Sónia Dorilde Tavares
Biophysics Unit, Institute of Biomedical Research on Light and Image (IBILI), Faculty of Medicine, University of Coimbra, Coimbra, Portugal; Centre of Investigation on Environment, Genetics and Oncobiology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal; and Faculty of Sciences and Technology, University of Coimbra, Coimbra, Portugal

Ana Cristina Gonçalves
Centre of Investigation on Environment, Genetics and Oncobiology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal, and Applied Molecular Biology and Hematology Group, Faculty of Medicine, University of Coimbra, Coimbra, Portugal

João Eduardo Casalta-Lopes
Biophysics Unit, Institute of Biomedical Research on Light and Image (IBILI), Faculty of Medicine, University of Coimbra, Coimbra, Portugal

Ana Bela Sarmento-Ribeiro
Centre of Investigation on Environment, Genetics and Oncobiology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal, and Applied Molecular Biology and Hematology Group, Faculty of Medicine, University of Coimbra, Coimbra, Portugal

Jorge Manuel Maia
Faculty of Sciences, University of Beira Interior, Covilhã, Portugal

Maria Filomena Botelho
Biophysics Unit, Institute of Biomedical Research on Light and Image (IBILI), Faculty of Medicine, University of Coimbra, Coimbra, Portugal, and Centre of Investigation on Environment, Genetics and Oncobiology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal

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The first and second authors contributed equally to this work.
Address correspondence to Ana Catarina Mamede, Biophysics Unit, Institute of Biomedical Research on Light and Image (IBILI), Institute of Biomedical Research on Light and Image (IBILI)., Faculty of Medicine, University of Coimbra, Azinhaga de Santa Comba, Celas 3000-548, Coimbra, Portugal. Tel: +351239480240. Fax: +351239480258. Email: ana_mamede@hotmail.com

Vitamin C, available in its reduced form (ascorbic acid; AA) and in its oxidized form (dehydroascorbic acid; DHA), may act in physiological conditions as an antioxidant or pro-oxidant. The aim of this study is to evaluate the cytotoxic effects of pharmacological doses of AA in a human colorectal adenocarcinoma cell line (WiDr) in vitro, through spectrophotometry, clonogenic assays and flow cytometry, and in vivo with xenotransplanted Balb/c nu/nu mice. The results show that the reduced form of vitamin C induces an anti-proliferative and cytotoxic effect in adenocarcinoma colorectal cells under study. The results obtained *in vivo* after treatment with AA showed a large reduction in the rate of tumor growth. Such understanding can guide decisions about which colorectal cancer patients might potentially benefit from vitamin C pharmacologic therapy.

INTRODUCTION

In the past years, the number of patients affected by colorectal cancer has increased around the world and is now one of the leading causes of death in most developed countries. Risk factors for colorectal cancer are dependent on the region and even within countries because diet, lifestyle, and some hereditary factors may determine the incidence and prevalence of this pathology. In recent years, had been enormous research advances and huge improvements in the treatment of cancer; however, it is necessary to further develop additional and innovative approaches to treat this health problem (1–3).

Over the past few years, many studies have been carried out using cultured cells, animal models or patients to study vitamin C effect on tumor cells. Vitamin C, a lactone isolated in 1928 by Szent-Gyorgyi, is obtained by the human body from exogenous sources, notably in fresh fruits and vegetables, and is available in its reduced form (ascorbic acid; AA) and in its oxidized form (dehydroascorbic acid; DHA). The conversion reaction of AA to DHA, mediated by the reductase enzyme, is a process that generates free radicals and can promote the reduction or oxidation of a system. The molecular structure of AA and DHA, whose effects on cells are distinct, are similar to glucose because of multiple hydroxyl groups close to each other, as represented in Fig. 1 (4–7).

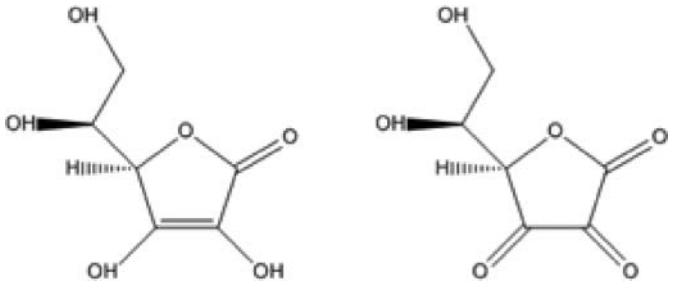

FIG. 1. Chemical structure of ascorbic acid (left) and dehydroascorbic acid (right).

The history of vitamin C in cancer is highly controversial (8). As a result of this controversy, the therapeutic effect of vitamin C has been discredited for years. Currently, new knowledge of pharmacokinetics and pharmacodynamics of vitamin C have stimulated interest in the revaluation of its application in the prevention and treatment of cancer (9–13). As an antioxidant, the main role of vitamin C is to neutralize free radicals because they need a pair of electrons to achieve its stability. As vitamin C is an excellent source of electrons, it can donate them to free radicals, reflecting its reduction capacity and decreasing oxidative stress. Vitamin C also acts as a prooxidant molecule, promoting the formation of reactive oxygen species (ROS) that impair cell viability. On the other hand, most tumor cells can't transport AA directly to its interior, the reason why the cells obtain vitamin C in its oxidized form, DHA. As mentioned above, tumor cells also show another peculiarity: the decrease of several antioxidant enzymes in relation to normal cells. Consequently, the increased production of hydrogen peroxide, coupled with the breakdown of the activity of antioxidant enzymes in cancer cells and the presence of transition metals, may result in the selective cytotoxicity of vitamin C and the subsequent revelation of its therapeutic potential (12).

Oxidative stress is a biochemical condition characterized by the imbalance between the presence of relatively high levels of toxic reactive species, especially ROS, and the antioxidative defence mechanisms. ROS are products of normal cellular metabolism and can be defined as organic/inorganic molecules containing one or more unpaired electrons in atomic or molecular orbitals that can damage cell at different levels. ROS, such as hydrogen peroxide, superoxide radical, hydroxyl radical, and singlet oxygen, are highly reactive and can be produced from both endogenous and exogenous substances. Potential endogenous sources include mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation. Exogenous factors include environmental agents and several modifiable factors such as diet, medications, and lifestyle. At high concentrations, ROS can be important mediators of damage to cell structures, like nucleic acids, lipids, and proteins, affecting enzyme activity, altering membrane function and modulating expression of genes that regulate cell differentiation and growth. On the other hand, at low/moderate concentrations, ROS are essential for life because of their role in many vital processes such as signal transduction, bactericidal activity of phagocytes, and induction of mitogenic responses (14–20).

Therefore, the aims of this work are to study the selective cytotoxicity of the reduced form of vitamin C and see how a human colorectal adenocarcinoma cell line is affected by this powerful antioxidant/prooxidant molecule through the evaluation of cell proliferation by spectrophotometry, clonogenic assays, and flow cytometry. In vivo studies with xenotransplanted *Balb/c nu/nu* mice, to verify the anticarcinogenic effect of AA, were performed.

MATERIAL AND METHODS

Cell Culture

Human colorectal adenocarcinoma cell line (WiDr), acquired from American Type Culture Collection (Rockville, MD), was cultured in Dulbecco's Modified Eagle's Medium (Sigma D-5648) supplemented with 100 μM sodium pyruvate (Gibco 11360), 10% heat-inactivated fetal bovine serum (Gibco 2010–09), and 1% antibiotic/antimycotic (100 U/mL penicillin and 10 μg/mL streptomycin, Gibco 15140–122). Cells were maintained at 37°C with 95% air and 5% CO_2.

Cell Proliferation

To evaluate the effect of AA (Sigma A5960) in cell proliferation, we used the colorimetric test MTT (3-(4,5-dimethylthiazolyl-2)2,5-diphenyltetrazolium bromide). The dehydrogenase enzymes, present in metabolically active cells, have the ability to cleave the tetrazolium ring of MTT and form dark blue formazan crystals that can subsequently be solubilized and quantified by spectrophotometry (21,22). For each experiment, cells were plated in 24 multiwells in a concentration of 50,000 cells/mL and kept in the incubator overnight to allow the cells attachment. After incubating cells with different AA concentrations during 1 and 4 h, the culture medium was replaced. After 24, 48, 72, and 96 h of rest, cell proliferation was evaluated: culture medium was removed, 900 μL of phosphate buffered saline (PBS) was added, and then a 150 μL of solution of MTT (5 mg/mL; Sigma M2128) in PBS. After 3 h, a 150μL of solution of isopropanol (Sigma 279544) in 0.04M hydrochloric acid (Sigma H1758) was added and the cells were resuspended. The contents of each well were transferred to a plate with 96 wells and the absorbance was quantified at 570 nm with a reference filter of 620 nm in an ELISA spectrophotometer (SLT-Spectra). The obtained results were analyzed and processed in software OriginPro 8.0, being the cytotoxicity expressed as the percentage of inhibition of cell proliferation correlated with control experiments. This allows the determination of the AA concentration that inhibits the culture cell proliferation in 50% (IC_{50}) through sigmoid fitting (Boltzman function). Each experiment was performed in duplicate and repeated in 3 different sets of tests.

Clonogenic Assay

Clonogenic assay aims to determine cell survival based on the ability of a single cell to grow and form a colony after the cells are subjected to the action of AA. For this study, 500 cells were seeded per well and after 24 h were incubated with AA at different concentrations. Two hours after exposure to vitamin C, cells were washed with PBS and new medium was added. After 5 days, the medium was changed and at tenth day the colonies were visualized. Culture medium was aspirated, cells were washed with PBS, and methanol (Sigma 34860) was added to fix the colonies, a procedure that was repeated twice. After the plates have dried, the crystal violet dye (Sigma M2128; 0.5% diluted in methanol) was added. Subsequently, the plates were washed with warm water and allowed to dry, after which the number of colonies were counted and the plate efficiency and survival factor determined.

$$Plate\ efficiency\% = \frac{Number\ of\ counted\ colonies}{Number\ of\ seeded\ colonies} \times 100$$

$$Survival\ factor\% = \frac{Plate\ efficiency\ of\ treated\ samples}{Plate\ efficiency\ of\ control\ samples} \times 100$$

Flow Cytometry

To study/characterize the redox intracellular environment after incubation of AA at different concentrations, we determined by flow cytometry the cell viability, the ROS production, the expression of reduced glutathione (GSH) and the changes of mitochondrial membrane potential. The analysis was performed using a 6 parameter, 4-color FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 15 nW argon laser. For each assay, at least 10^4 events was collected using Cell Quest software (Becton Dickinson, San Jose, CA)) and analyzed using Paint-a-Gate software (Becton Dickinson, San Jose, CA)). For flow cytometry analysis, 10^6 cells were seeded per well and after 24 h were incubated with AA at different concentrations. One hour after exposure to vitamin C, cells were washed with PBS and new medium was added. After 24 h, cells were examined by flow cytometry.

Cell Viability

To evaluate cell viability, annexin-V/propidium iodide (AV/PI) incorporation assay was used. One of the main features of cell death by apoptosis is the redistribution of plasma membrane phosphatidylserine, a phospholipid that, in apoptotic cells, is translocated from the inner to the outer leaflet of the plasmatic membrane and binds to AV. Complementarily, PI, which does not permeate viable cells, binds to deoxyribonucleic acid (DNA) intercalating between the bases on late apoptotic and necrotic cells (23). In this assay, 10^6 cells were incubated during 15 min in binding buffer with 1 μL of AV (Kit Immunotech, Marseille, France) and 5 μL of PI (Kit Immunotech, Marseille, France). Subsequently, cells were excited at a wavelength of 525 nm for AV and 640 nm for PI, collecting 10^4 events to assess the percentage of viable, early apoptotic, late apoptotic/necrotic, and necrotic cells (24).

Detection of Intracellular Peroxides

2,7-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), a lipid permeable and non-fluorescent compound, is cleaved by intracellular esterases by entering cells and leads to 2,7-dichlorodihydrofluorescein ($DCFH_2$). In the presence of peroxides, $DCFH_2$ is oxidated with formation of dichlorofluorescein (DCF), a highly green fluorescent compound. The emitted fluorescence is proportional to the concentration of intracellular peroxides (25). A cell suspension of 10^6 cells was incubated with 5 μM of $DCFH_2$-DA (Sigma D6883) for 1 h at 37°C in

the dark. After washing the cells with PBS, the analysis was performed with an excitation wavelength of 504 nm, being the emission wavelength 529 nm. The results are presented as mean fluorescence intensity (MFI) values.

Detection of Superoxide Radical

Dihydroethidium (DHE) easily crosses cell membranes and is converted by superoxide radical to ethidium, a red fluorescent compound that merges the DNA remaining inside the cell (26). A cell suspension of 10^6 cells was resuspended in PBS and incubated with 5 μM of DHE (Sigma 37291) dissolved in DMSO (Sigma D8418) for 10 min at 37°C in the dark. After washing the cells with PBS, the analysis was performed with an excitation wavelength of 620 nm. Results are presented as MFI values.

GSH Expression

The expression of GSH, an antioxidant defence, was performed by flow cytometry using the fluorescent compound mercury orange. This compound binds stoichiometrically to mercurial sulfhydryl groups with the formation of fluorescent ducts. However, this compound faster reacts with GSH than with the sulfhydryl groups of proteins and the reaction product emits an intense red fluorescence when excited with an argon laser at a wavelength of 488 nm (27). A cell suspension of 10^6 cells was incubated with 4 μL of mercury orange (Sigma 83377) in acetone (Sigma 650501) for 15 min at room temperature in the dark. After washing the cells with PBS, the analysis was performed with an excitation wavelength of 620 nm. Results are presented as MFI values.

Mitochondrial Membrane Potential Measurement

The lipophilic cationic 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) is a molecule able to selectively enter the cell and which exists in 2 forms, monomers (M) and aggregates (A), depending on the state of polarization/depolarization of the mitochondrial membrane. When the membrane potential is high, the JC-1 forms aggregates that emit red fluorescence (590 nm). In turn, as the mitochondrial membrane potential decreases or in cases where the membrane is depolarized, JC-1 is excluded from mitochondria and remains in the cytoplasm in the form of monomers that emits green fluorescence (529 nm). Thus, the ratio between the intensities of green and red fluorescence (M/A), determined by flow cytometry, provides an estimate of mitochondrial membrane potential (28). To perform JC-1 (Invitrogen, T-3168) incorporation, the cells were incubated at a final concentration of 5 mg/mL in DMSO during 15 min at 37°C in the dark. Subsequently, the cells were washed with PBS and analyzed by flow cytometry. The results are presented as aggregate/monomer fluorescence intensities ratio.

In Vivo Studies

In order to verify the reduction of tumor growth rate after the daily injection of AA in mice, it was developed a methodology to evaluate in vivo the role of vitamin C reduced form in cancer. Thus, 8×10^6 WiDr cells were inoculated on the back of *Balb/c nu/nu* mice and, during several days, the body weight of mice and tumor size were monitored, as well as the behavior and general health of the mice. When tumor volume reached 300–500 mm^3, an aqueous solution of AA with a concentration of 150 mg/kg was daily injected intraperitoneally during 12 days. Tumor volume was estimated according to Dagrosa et al. (29). After therapy, the mice were killed by cervical dislocation. In this experience, AA was not administered to control animals. During experiments, animals were kept in filtertop cages under constant temperature and humidity and maintained on sterilized diet and water ad libitum. The animals were kept under sterile conditions in cycles of 12 h of dark/light.

Statistical Analysis

Results were analyzed using the software PASW (Predictive Analysis SoftWare) version 18. In comparison of all the conditions we used the Kruskal-Wallis test, whereas comparisons 2-by-2 were made with the Mann-Whitney test. A significance level of 5% ($P < 0.05$) was considered for all comparisons. The in vivo results were analyzed through a prospective study, in which the estimation of Kaplan-Meier to construct survival curves was made. We considered that an event occurred if the tumor volume was 3 times the initial volume. The curves were compared using the log-rank test and a significance level of 2% ($P < 0.02$) was considered.

RESULTS

To evaluate the effects of vitamin C in WiDr cells, we examined the effect of AA at different concentrations and for different periods of incubation and rest in the proliferation of cells using MTT assay. Table 1 represents the biological response of WiDr cells after 1 and 4 h of exposure to AA with 24, 48, 72, and 96 h of cellular rest. As can be seen in Table 1, exposure of WiDr cells to AA for 1 h results in an IC_{50} equal to 20.4 mM after 24 h of rest, being registered a slight increase in IC_{50} value at 48 h of rest (25.1 mM). After 1 h of incubation with AA and 72 and 96 h of rest, there was an IC_{50} value equal to 18.5 mM and 4.54 mM, respectively. On the other hand, when cells are exposed to AA for 4 h, there is a decrease in the IC_{50} value registered for all times of rest: 4.36 mM (24 h), 5.8 mm (48 h), 3.85 mM (72 h), and 1.73 mM (96 h).

Clonogenic assays are an asset to test the cytotoxicity of vitamin C as they allow one to analyze the effects of AA in cells after a long period of time and determine the outcome of the vitamin C on cells. Through this technique, it was observed that as the concentration of AA increases, the survival factor of cells significantly decrease ($P < 0.05$), as can be seen in Fig. 2. When cells are treated with a concentration of 0.5 mM, there

TABLE 1
Half maximal inhibitory concentration of human colorectal adenocarcinoma cell line (WiDr) cells after exposure to ascorbic acid

Exposure time (h)	Rest time (h)	IC_{50} (mM)	R^2
1	24	20.40	0.96
	48	25.10	0.82
	72	18.50	0.98
	96	4.54	0.93
4	24	4.36	0.93
	48	5.80	0.91
	72	3.85	0.93
	96	1.73	0.99

WiDr cells were exposed to AA for 1 or 4 h and after 24, 48, 72, and 96 h of cellular rest, cellular proliferation was calculated. IC_{50} (half maximal inhibitory concentration) were obtained using the equation of the sigmoid fitted curves (Boltzman function). R^2 is the coefficient of determination of the fitted curves. Each experiment was performed in duplicate and repeated in three independent sets of tests.

is a reduction factor of survival of 44.88% relatively to control. At concentrations of 2, 3, and 5mM, the reduction of survival factor is more marked, being always above 80%.

The assessment of cell viability was performed by flow cytometry using the AV/PI incorporation assay. This technique allows one to distinguish different cell populations: viable cells (VC), cells in early apoptosis (EAC), cells in late apoptosis/necrosis (LAC/NC) and necrotic cells (NC). Cytometry studies show that as the concentration of AA increases, cell death also increases, as shown in Fig. 3. Thus, AA induces a statistically significant decrease ($P < 0.05$) in cell viability in a dose-dependent way. Note that, at a concentration of 45 mM,

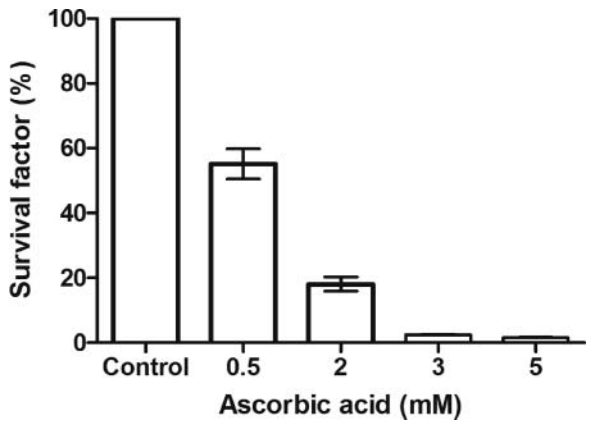

FIG. 2. Survival factor of human colorectal adenocarcinoma cell line (WiDr) cells after treatment with ascorbic acid. The cells were exposed to different concentrations of AA (0.5, 2, 3, and 5 mM) and were subsequently observed the formation of colonies by the crystal violet dye. The results represent the average of 3 independent studies ± standard deviation.

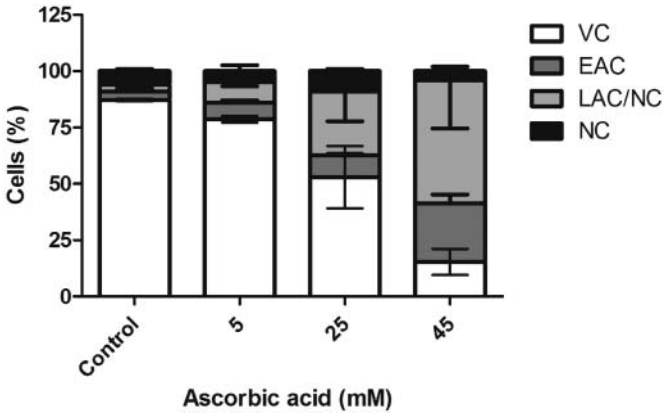

FIG. 3. Cell viability by flow cytometry using dual staining with AV and PI. Figure represents the percentage of viable cells (VC), in early apoptosis (EAC), in late apoptosis/necrosis (LAC/NC), and necrosis (NC) after treatment with ascorbic acid. The results express the average of 3 independent experiments ± standard deviation.

cell death reaches 86%, being 26% attributed to the early apoptosis, 56% to late apoptosis/necrosis and 4% to necrosis.

Given the importance that the ROS may have in the mechanisms of cytotoxicity of vitamin C, it was determined the expression of peroxides and superoxide radical. As can be seen in Fig. 4, there is an increase in the intracellular production of peroxides when cells are treated with 5 and 25 mM of AA. When these cells are exposed to 45 mM, the production of peroxide decreases to about half of the value recorded for control. In the comparison of all the conditions, it was found that there are significant differences ($P < 0.05$).

To evaluate the production of superoxide radical, we resort to DHE and the respective analysis of the fluorescence intensities

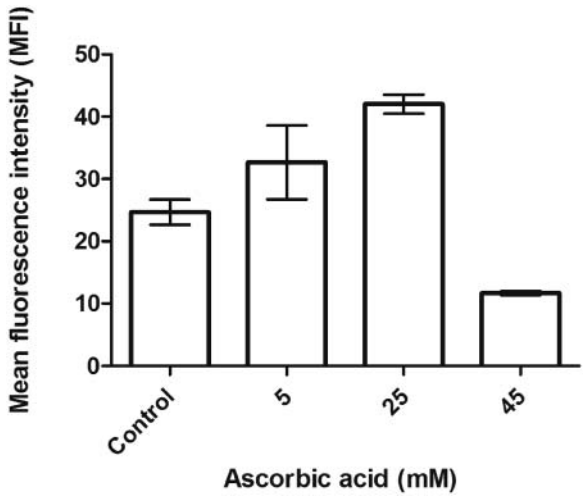

FIG. 4. Production of peroxides by flow cytometry using $DCFH_2$-DA. The cells were treated with ascorbic acid and subsequently the production of peroxides was detected. The results are expressed as mean fluorescence intensity and express the average of 3 independent experiments ± standard deviation.

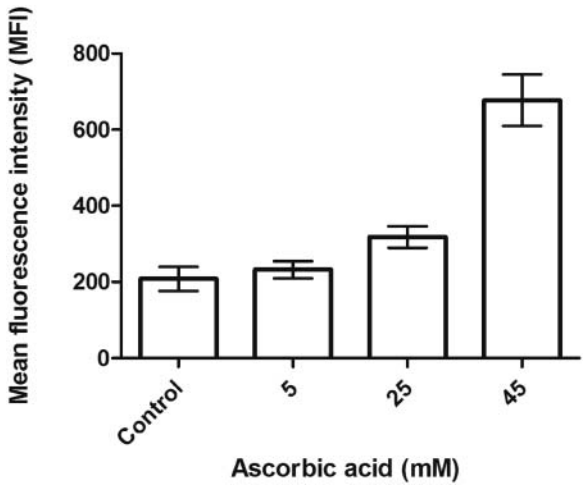

FIG. 5. Production of superoxide radical by flow cytometry using DHE. The cells were treated with ascorbic acid and subsequently the production of superoxide radical was detected. The results are expressed as MFI and express the average of 3 independent experiments ± standard deviation.

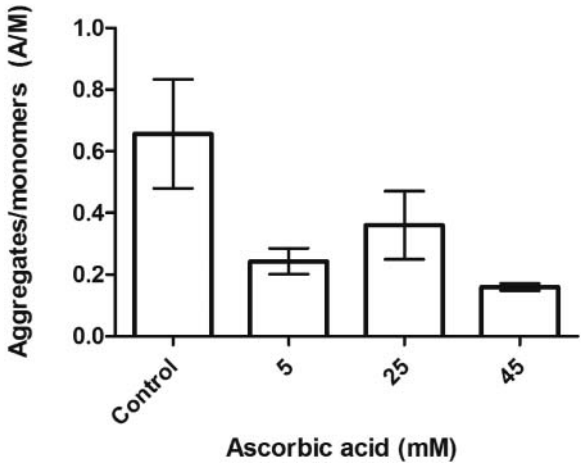

FIG. 7. Analysis of mitochondrial membrane potential by flow cytometry using the fluorescent probe JC-1. The cells were treated with ascorbic acid and subsequently the mitochondrial membrane potential was detected. The results are expressed as the ratio between aggregates and monomers and express the average of 3 independent experiments ± standard deviation.

by flow cytometry. From the analysis of Fig. 5, it is possible to observe that, as the concentration of AA increases, the production of superoxide also increases. It should be noted that this statistically significant increase ($P < 0.05$) is very pronounced when the cells are incubated with 45 mM (superoxide production is almost 4 times higher than the control).

To evaluate the expression of intracellular GSH, we used the orange mercury probe and the analysis of fluorescence intensities were performed by flow cytometry. Through the analysis of the Fig. 6, we can see that when cells are incubated with AA, the expression of GSH increases in a statistically significant way ($P < 0.05$) relatively to control. It should be noted that there is a slight decrease in expression of GSH (about 8% less) at a concentration of 45 mM when compared to concentration of 25 mM.

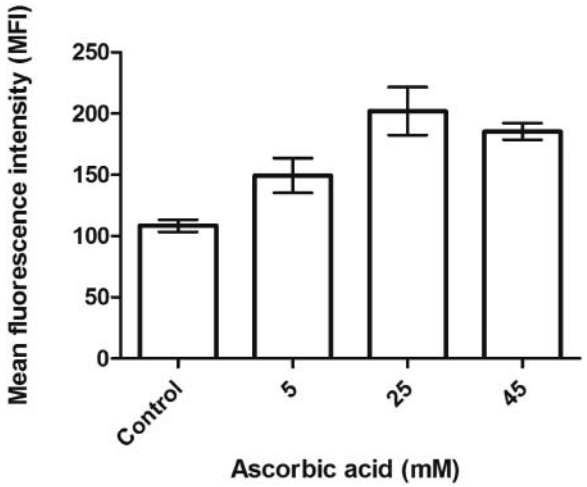

FIG. 6. Expression of GSH by flow cytometry using orange mercury. The cells were treated with ascorbic acid and subsequently the expression of GSH was detected. The results are expressed as MFI and express the average of 3 independent experiments ± standard deviation.

Fig. 7 shows the ratio between aggregates and monomers (A/M) in WiDr cells after treatment with AA. As we can see, there is a marked decrease in mitochondrial membrane potential in WiDr incubated with AA compared to control, which is more relevant at concentration of 45 mM. Statistical analysis of these data showed no statistically significant differences for 5, 25, and 45 mM.

With regard to *in vivo* studies, and as represented in Fig. 8, there are significant differences between the 2 groups (control and under treatment) with respect to achieve a relative volume of 3 (log-rank test, $P = 0.002$). Half of the sample reached this volume controls on the 4th day, whereas half of the controls reached this value at 12 days. Not all mice in the latter group reached the relative volume considered. Note that no changes were recorded in animal weight over time.

DISCUSSION

Vitamin C is a nutrient whose benefits are long been known and widely disseminated, being the majority because of its antioxidant action. However, some studies suggest that this nutrient may have a preventive and therapeutic role in cancer disease because of its potential prooxidant activity, promoting the formation of ROS that induce selectively cancer cell death. Because of the discussion that remains in the scientific community about the benefits/harms of large doses of vitamin C in the prevention and treatment of cancer, it is imperative to carry out more studies to infer the true function of this antioxidant/prooxidant in human health and disease. This study aimed to evaluate the mechanisms of action of vitamin C

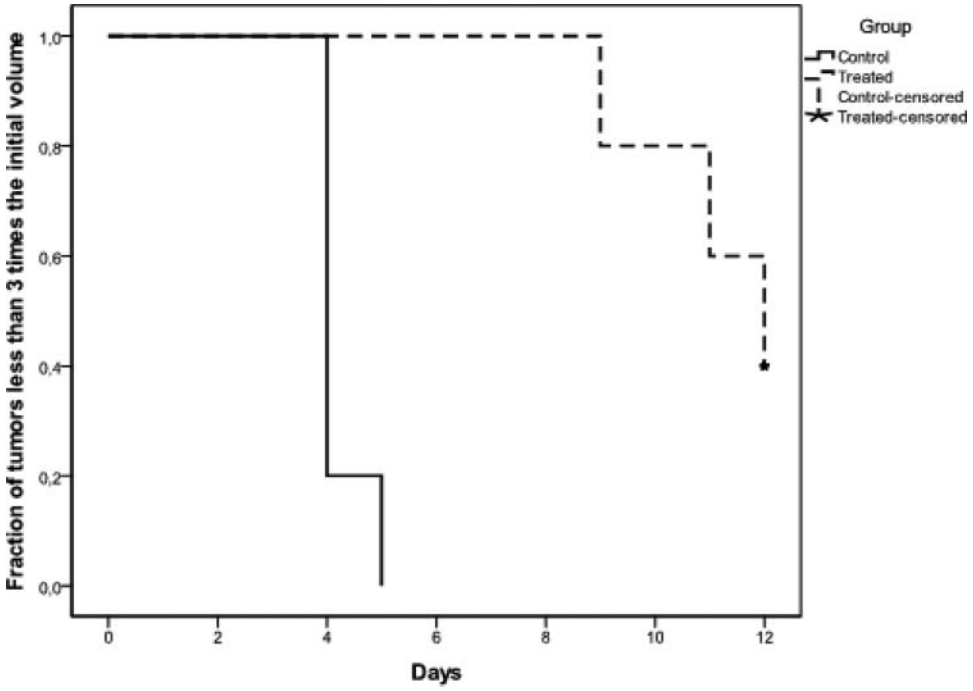

FIG. 8. Evolution of tumor growth over 12 days of therapy with ascorbic acid (AA). Over 12 days, Balb/c nu/nu xenografts were subjected to intraperitoneal therapy with AA. Xenotransplanted Balb/c nu/nu not treated with AA was used as a control. The results represent the average of 5 control mice and 5 mice that undergo therapy.

through various techniques, seeking results that represent an asset for the assessment of the applicability of vitamin C in cancer disease.

According to the results obtained by the MTT assay, we can see that when WiDr cells are exposed to AA for 1 h and allowed to rest for 24, 48, 72, or 96 h, the recorded IC_{50} decreases progressively as the rest time increases. The same behavior was observed when the cells are left in contact with AA for 4 h. However, there are notable differences in the value of IC_{50} obtained for all times of rest, being always much smaller for incubation times of 4 h when compared with incubation times of 1 h. When cells are incubated for 1 and 4 h and allowed to stand 48 h, there is a slight increase in value when compared with the IC_{50} value obtained for the 24 h. This behavior can be a sign that the amount of drug that penetrates in the cell was not enough to reduce cell proliferation but rather to trigger some mechanism of resistance to AA at 48 h.

To confirm and reinforce the results obtained by the method of MTT, several assays were performed to analyze the reproductivity of the cell death results. That is, it was determined if the cell had long-term capacity to proliferate after treatment with different concentrations of AA in the range 0.5–5 mM. These studies, called by clonogenic assays, allow verifying the marked reduction of survival factor of WiDr cells after treatment with AA. These studies let assessing the longer-cell responses after treatment with AA, unlike the MTT technique, which only allow to evaluate the response of cells to certain drugs over short periods of time. By the results obtained by the clonogenic assays, it appears that the cells lose their reproductive capacity as the concentration of AA increases.

After confirming the reduction of proliferation and cell survival in the cell line under study, it was necessary to understand the cellular mechanisms through which these effects appear. For that, we used the flow cytometry technique, which allows us to analyze cell viability by double staining with AV/IP, the presence of ROS by staining with $DCFH_2$-DA and DHE, the expression of intracellular GSH through the orange mercury staining, and the alteration of mitochondrial membrane potential through the fluorochrome JC-1. AV/PI assay allow the assessment of cell viability and distinguishes different types of cell death. Thus, this double staining showed that as the concentration of AA increases the cell death in WiDr also increased, with the majority of cell death occurring by late apoptosis or necrosis.

One mechanism that has been suggested for the selective cytotoxic effect of vitamin C in tumor cells involves oxidative stress (30,31). Thus, an increase of oxidative stress coupled with a decrease of the antioxidant enzyme by a factor of 10 to 100, as well as an increase of transition metals and the oxidation of AA to DHA in tumor cells, can lead to selective cytotoxicity in this type of cells. Because ROS play an important role in the cytotoxic action of vitamin C, we resorted to the use of 2 specific probes for the detection of peroxides and superoxide radical, the $DCFH_2$ and DHE, respectively. Analyzing the results obtained, it was found that when WiDr cells are exposed to 45 mM of AA the production of hydrogen peroxide is lower, whereas the production of superoxide radical is higher relatively to the

control. The concentration of 25 mM of AA in WiDr induces an increase of this 2 reactive oxygen species, whereas for the concentration of 5 mM there is only a slight increase compared to control. Besides the cytotoxicity, with high concentrations of vitamin C it was also observed a marked decrease in the levels of peroxides, suggesting a conversion into more cell damaging species, as the hydroxyl radical or the nitrite peroxide.

Although these reactive species were not measured, this can be a reasonable justification because in the presence of free iron, the hydrogen peroxide is converted by the Fenton-Habber Weiss reaction into hydroxyl radical, and the superoxide anion in the presence of nitric oxide can be converted into nitrite peroxide. These species are highly reactive and toxic to the cell, leading to cell death. Because the detection of superoxide anion by the probe DHE is a relatively specific reaction, this probe can also detect nitrite peroxide. The increase of fluorescence intensity detected after incubation of WiDr to 45 mM of AA, could in fact be due to the presence of reactive nitrogen species. Note that, as higher the production of reactive species, higher the cell death, as confirmed by linking the results obtained by flow cytometry.

To ascertain the level of the antioxidant defences present in tumor cells, it was analyzed by flow cytometry the expression of intracellular GSH using as fluorescent probe, the orange mercury. Knowing that GSH is a reducing agent of peroxides, it is expected that as greater the production of hydrogen peroxide, the greater the expression of intracellular GSH. Thus, after exposure of WiDr to 45 mM of AA, the expression of intracellular reduced glutathione is higher relatively to the control despite the decrease of hydrogen peroxide, as stated above. The explanation for this could be the rapid conversion of hydrogen peroxide to other damaging reactive species.

To confirm the involvement of mitochondria in the biological effects of vitamin C, we evaluate the mitochondrial membrane potential with JC-1 probe. Our studies are consistent with the foregoing and confirm the results obtained in the studies of proliferation, observing a decrease in the ratio A/M under conditions in which vitamin C induced cell death in WiDr cells. However, statistical analysis of these data showed no statistically significant differences for 5, 25, and 45 mM.

To finalize this study, the authors developed a methodology that allowed to study in vivo the role of vitamin C in cancer disease by reducing the rate of tumor growth after daily intraperitoneal injection of AA. Several pharmacokinetic studies have clearly demonstrated that vitamin C concentrations in plasma and tissues are tightly controlled in terms of ingesta, because the oral route of administration is limited by the elimination of vitamin excess by the urinary system. As a consequence, oral administration of vitamin C cannot achieve plasma concentrations greater than 50–100 μM and for this reason, oral supplementation of vitamin C appears to have no influence on the treatment of cancer patients. Thus, by studying the best way to administrate AA to *Balb/c nu/nu*, we did not consider the oral route. Of the 3 possible routes, the intravenous was ideal due to much higher plasma concentrations of vitamin C obtained (6,32). However, given the frailty of the tail vein of the mice used and the need of a daily injection of AA, we preferred the administration through the intraperitoneal route. From the results, it can be seen that tumor growth in mice undergoing therapy has stagnated, while tumor growth at the control mice continued to increase, a fact which shows that AA may have an effective role in the approach to treatment as is widely uptaken by tumor cells. Verrax and Calderon (33) also conducted a study involving the daily administration of AA to xenotransplanted animals with hepatocellular carcinoma cells and also found that the rate of tumor growth decreased over thirty days. Yeom et. al. (34) also reported that administration of a high concentration of AA inhibits tumor growth in *Balb/c* mice implanted with sarcoma 180 cancer cells through the restriction of angiogenesis.

CONCLUSION

In this work, which main objective was to contribute to the clarification of the mechanisms of action of vitamin C in cancer, we can draw several conclusions. For *in vitro* studies conducted in colorectal adenocarcinoma cells, its main objective was to evaluate the anti-proliferative and cytotoxic effects of vitamin C. From our results, we can conclude that the reduced form of vitamin C induces an inhibitory effect on the cell line studied. This anti-proliferative effect is associated with a cytotoxic effect due to the decreased cell viability and consequent increase in cell death by apoptosis/necrosis. This cytotoxic effect of AA may be related to the production of ROS, including the conversion of peroxides in more reactive and toxic species, such as the hydroxyl radical and nitrite peroxide, which is consistent with cell death by late apoptosis/necrosis observed. Mitochondrial dysfunction is another mechanism that is related to oxidative stress and can contribute to the cytotoxic effect of high concentration of vitamin C in colorectal adenocarcinoma cells. Finally, it was proven *in vivo* that the reduced form of vitamin C stabilizes tumor growth and may contribute for cancer therapy.

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