

Ascorbic acid increases cell death in fibroblast and VERO cultures after oxidative and thermic stress

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Background: Ascorbic acid has been considered to act as a scavenger of free radicals generated in the cell after oxidative stress. Cultured rat fibroblasts and monkey kidney tumor cells (cell line VERO) were subjected to oxidative and thermic stress in the presence and absence of ascorbic acid in order to directly test this action.

Materials and Methods: The cell viability was analyzed by a cell proliferation assay (XTT), while cell death was studied by in situ DNA nick/end labeling (TUNEL), double vital Hoechst/Propidium iodide staining and nuclear morphology.

Results: The incubation of the cells with hydrogen peroxide for different times showed that after 2

hours the majority of the cells were dead and ascorbic acid did not protect fibroblast and VERO cells from this oxidative stress. The decrease of cell viability is dependent on hydrogen peroxide concentration and is not affected by the presence of increasing concentrations of ascorbic acid. Necrotic cell death occurs after a loss of cell membrane integrity without nuclear shrinking and lack of labeling by TUNEL. Similar results were obtained after inducing a thermic stress.

Conclusions: Under the present experimental conditions ascorbic acid did not protect the cells but rather increased the effects of both the oxidative and the thermic stress.

Introduction

A prolonged and massive intake of ascorbic acid, supposed to be beneficial to neoplastic patients¹, has been shown to induce tubulointerstitial nephropathy² and in general the problems of the action of ascorbic acid are still open since the condition in which the latter exerts an antioxidant or a pro-oxidant effect are not well established.

Fibroblasts possess specific transporters for ascorbic acid³ and in the cells the rates of radical generation and radical scavenging seem to be subjected to homeostatic regulation maintained by ascorbic acid and other endogenous antioxidants⁴. The antioxidant effect of ascorbic acid in blood is related to a mechanism of recycling by red cells ultimately dependent on the intracellular concentration of reduced glutathione⁵. The presence of ascorbate increases the intracellular concentration of hydrogen peroxide in erythrocytes and this has been related to its prooxidant effect⁶ ascribed to a Fe(III)-catalyzed extracellular oxidation of ascorbate. Recently, a pro-oxidant action has also been demonstrated in humans on diet supplemented with 500 mg/day of ascorbic acid⁷.

We investigated the effects of ascorbic acid on normal and neoplastic cells subjected to thermic (i.e. hot and cold shock) and oxidative stress induced by hy-

drogen peroxide in vitro. The aim and significance of this study was to assess the behavior of normal and neoplastic cells towards thermic and oxidative stress in the presence and absence of ascorbic acid by measuring cell death due to necrosis and/or apoptosis. We in fact used different types of stress to assess whether they may involve a common oxidative mechanism responsive to ascorbic acid.

Materials and Methods

Cell cultures: Primary fibroblasts were obtained from paws of newborn male Wistar rats by digestion with trypsin (2.25% in 15 mM phosphate buffer pH 7.2). Cells were cultured in a Dulbecco's Modified Eagle Medium (DMEM) supplied with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin (complete medium) in flasks and on gelatin-coated (gelatin dissolved in 2% phosphate buffer PBS) glass coverslips with a cell density of 30,000 cells/cm². A line of monkey kidney tumor (VERO) was cultured on the same medium under the same conditions.

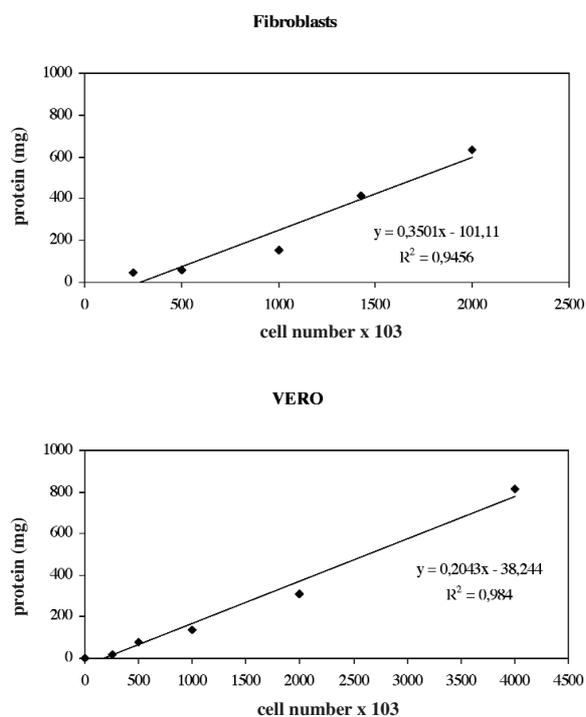
Thermic stress: After preliminary experiments to assess the optimal incubation times and temperatures the cells were incubated at 45°C for 10 min or, alternatively, at - 20°C for 20 min.

Oxidative stress: The oxidative stress was induced, 12 h after cell resuspension, by adding 1 mM hydrogen peroxide for 45 min in the presence and absence of 1 mM ascorbate, unless otherwise indicated.

Determination of glutathione: Total glutathione was spectrophotometrically determined at 412 nm with the recycling procedure by using Ellman's reagent.

Protein: Protein was estimated by the biuret method according to Gornall et al.⁸ in cell extracts prepared by one cycle of freezing-thawing followed by addition of 2.2 U/10⁶ cells aprotinin (Antagosan, Behring), mixing with Vortex and 1 min sonic irradiation with an Ultra-Turrax (Janke & Kunkle, GFR). The protein content was measured in different numbers of cells by a calibration experiment (Figure 1).

Figure 1. Protein content as a function of cell number in fibroblasts and VERO cells.



Cell viability: Cell viability was analyzed by the XTT assay (Cell Proliferation Kit II XTT, Roche) by growing 30,000 cells on 96-well plates in their growing medium. The cells, after thermic and oxidative stress, underwent treatment with the XTT reagents added to each well and incubated at 37° C for 4 h. After incubation, the plates were analyzed with a multiwell ELISA reader.

In situ DNA nick/end labeling (TUNEL): Cells were fixed for 30 min at room temperature in 4 % paraformaldehyde buffered at neutral pH. After washing with PBS, the cells were permeabilized by 0.1% Triton X-100 for 2 min at 4° C. The coverslips were then washed with PBS and in situ nick/end labeling of fragmented DNA was performed using terminal deoxynucleotidyl transferase (TdT) with fluorescein-conjugated nucleotides, as described by the

manufacturer. Negative control coverslips were prepared by substituting distilled water for the TdT enzyme and proceeding with the staining procedure. All coverslips were then washed three times with PBS, mounted in Elvanol and observed under an epifluorescence microscope (Zeiss). The labeled nuclei were easily identified from the counterstained nuclei and photographed.

Double vital Hoechst/propidium iodide staining: Double vital staining was performed according to Sandri et al.⁹. Thirty minutes before analyzing the cells 0.1 µg/ml Hoechst 33342 and 1 µg/ml Propidium Iodide was dissolved in the cell culture medium. Cells were mounted in Elvanol and red necrotic nuclei against blue counterstained normal nuclei were observed with an epifluorescence microscope, photographed and quantified.

Statistical analysis: The statistical significance of the data was determined using Student's t-test.

Results

Bioreductive defences in VERO cells and fibroblasts
Figure 1 allows us to evaluate the levels of protein concentration expressed per cell number.

Oxidative effects of hydrogen peroxide

The cells were subjected to different concentrations of hydrogen peroxide for various time intervals. When hydrogen peroxide and ascorbic acid were added together, a higher lethal effect was observed on the cells compared to what observed when hydrogen peroxide or ascorbic acid were added alone. After 120 minutes of exposure, most of the cells (>85%) were dead both in the presence or absence of ascorbic acid (Figure 2 A,B). VERO cells show similar results when exposed to hydrogen peroxide alone or combined with ascorbic acid (Figure 2 B). With decreasing concentrations of hydrogen peroxide there is an increase of cell survival but in any case ascorbic acid did not significantly protect the cells from the oxidative stress (Figure 2 C,D). Interestingly, at low concentration of hydrogen peroxide the presence of ascorbic acid seems to enhance its toxicity (Figure 3 C,D).

Thermic stress with hydrogen peroxide

In the presence of hydrogen peroxide VERO cells are more resistant than fibroblasts to the thermic stress but are more sensitive to oxidative stress when ascorbic acid was present (Figure 3 A,B).

Effect of ascorbic acid at different concentrations

Cell survival does not seem to be dependent on the concentration of ascorbic acid both for fibroblasts and VERO cells (Figure 4 A,B). In fact, increasing concentrations of ascorbic acid (from 0.1 mM to 50 mM) did not significantly protect the cells from oxidative and thermic stress.

Figure 2. Ascorbic acid (AA) was at 1 mM final concentration when incubated for different times with fibroblasts (a) and VERO cells (b) using hydrogen peroxide at a constant concentration of 1 mM. Dose-dependent effect of hydrogen peroxide (C,D) with ascorbic acid at a constant concentration of 1 mM at an incubation time of 30 min with fibroblasts (c) and VERO cells (d) The data are mean \pm -SE of 5 experiments. * Difference with control significant by Student's t test ($p < 0.001$).

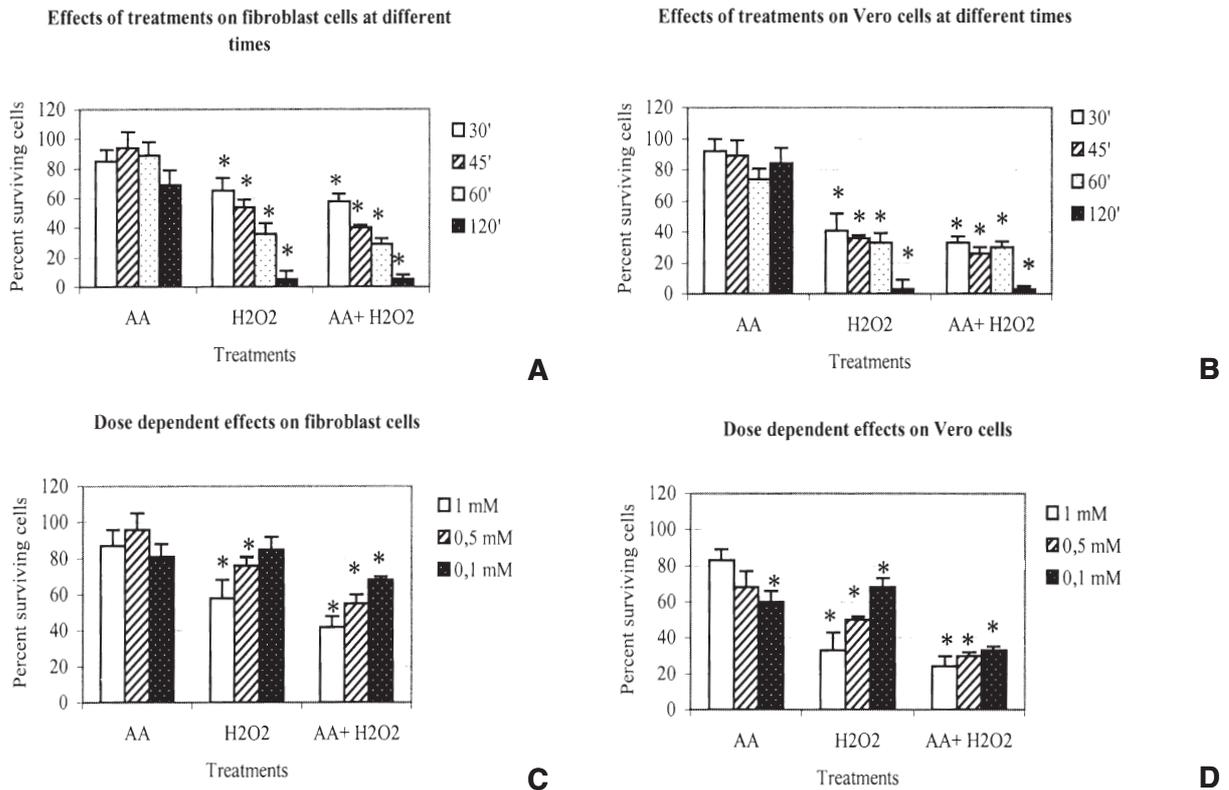
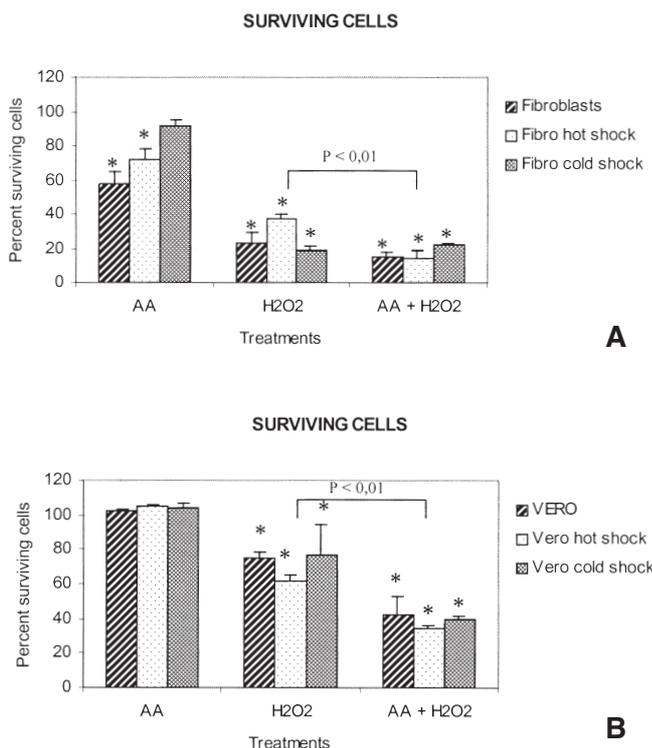


Figure 3. Cell survival after applying thermic shocks in the presence of 1 mM ascorbic acid and hydrogen peroxide. The data are mean \pm -SE of 4 experiments. * See Figure 2. The significance of the difference between treatment with hydrogen peroxide and ascorbate/hydrogen peroxide is indicated with the bar ($p < 0.001$).



Necrosis vs. apoptosis

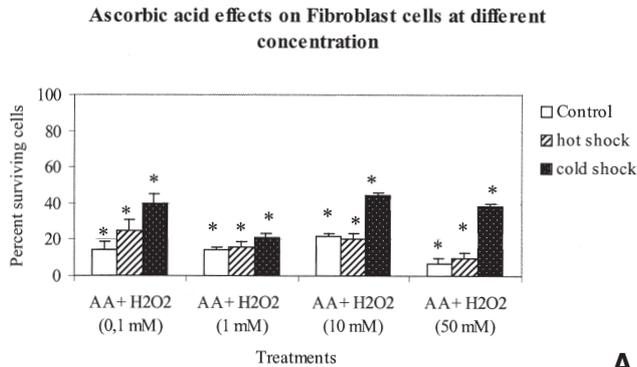
The mode of cell death of treated fibroblasts was analyzed to determine whether necrosis or apoptosis had occurred. Our data show a progressive increase of both necrotic and apoptotic cells which reach the highest value in the presence of a combination of ascorbic acid and hydrogen peroxide (Figure 5). The number of necrotic cells was always much higher than that of the apoptotic cells as indicated by the vital staining with propidium iodide which only stains nuclei of the cells with a damaged membrane, that is necrotic cells.

The effect of the treatments on VERO cells was demonstrated with Hoechst 33342 vital staining indicating both living and dead cells, and propidium iodide indicating dead cells. Morphology helps to distinguish the red necrotic cells from the equally red late apoptotic cells (i.e. cytoplasm shrinkage, chromatin condensation and fragmentation to form dense masses). Apoptosis was detected by two different methods giving similar results (Figure 6). Apoptotic cells reach the highest value after hydrogen peroxide treatment and ascorbic acid did not prevent apoptotic cell death. Fibroblasts behaved similarly (see Figure 5).

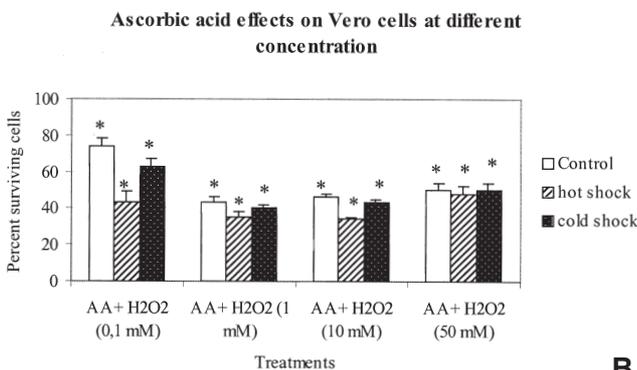
Discussion

B The present results document a different susceptibility of fibroblasts and VERO cells to thermic and ox-

Figure 4. Different ascorbic acid concentrations in the presence of 1 mM hydrogen peroxide with incubation time of 30 min with and without thermic stress. The data are mean \pm SE of 3 experiments. * See Figure 2.



A

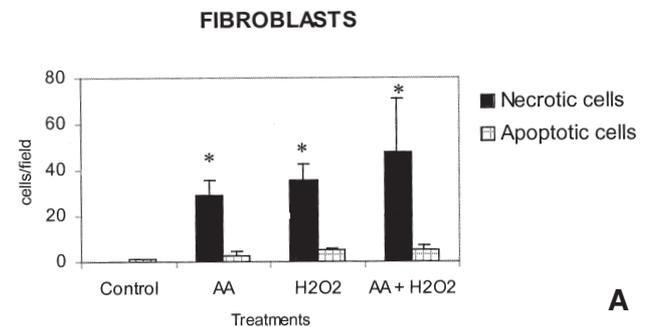


B

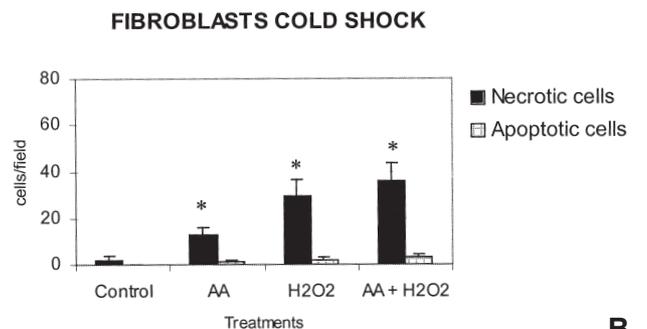
oxidative stress. The VERO cells appear to be less resistant at shorter times of exposure and higher concentration of ascorbic acid and hydrogen peroxide. The surviving fibroblasts on the contrary are less than the surviving VERO cells after the thermic stress in the presence of ascorbate and hydrogen peroxide. The combination of ascorbate and hydrogen peroxide is particularly toxic for fibroblasts subjected to thermic stress. The treatments induce a prevalent necrotic death while apoptosis appears to be rather limited with both types of cells. The pro-oxidant action of ascorbic acid generally observed under our experimental conditions is compatible with what has been observed by other authors¹⁰. The culture media in fact do not provide sufficient antioxidant protection from the cytotoxic effects¹¹. The relatively higher sensitivity of tumor cells to the pro-oxidant action of ascorbic acid may be related to their lower antioxidant defences and to the presence of transition metals¹². Ascorbic acid in fact has an antioxidant effect in the absence of metals, while it becomes a pro-oxidant when they are present^{6,13}. The cellular uptake of ascorbic acid is mediated by membrane transporters and regulated in a cell-specific manner^{3,4}. The antineoplastic effect of ascorbic acid has been related to its power of inducing a cytolytic and autophagic effect, cell membrane disruption, increased collagen synthesis, inhibition of cancer cell metabolism and proliferation¹⁵. Ascorbic

acid supplementation affects on the other hand iron status and increases bleomycin-induced DNA aberration in tumors¹⁶. Moreover, ascorbic acid has been observed to induce a time-dependent decrease in prostatic cancer cells viability *in vitro* through the production of hydrogen peroxide¹⁷ and the same has been observed with human urologic cancer cell lines¹⁸. Low levels of glutathione have been shown to favor a significant sensitization to oxygen and hydrogen peroxide toxicity in fibroblasts¹⁹ and the present results are in accord with those observations. Other recent observations may be relevant to the present results, such as those on a dehydroascorbate transporter²⁰ and on low doses (micromolar) of antioxidants which worsen the effects of the oxidative stress²¹. In addition, hydrogen peroxide effects are not prevented by short term (48 hours) exposure to ascorbic acid²², while the reduction of dehydroascorbate is a further cause of stress triggering apoptosis²³ and ascorbic acid autoxidation or prooxidation seem to depend on its environment²⁴.

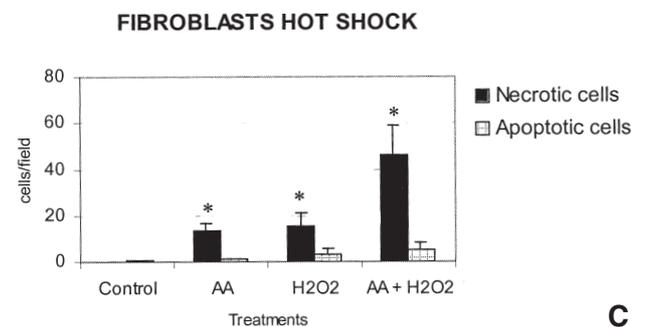
Figure 5. Necrotic and apoptotic fibroblasts found after applying thermic shock and hydrogen peroxide treatments under the conditions of Figure 2. The data are mean \pm SE of 3 experiments. * See Figure 2.



A

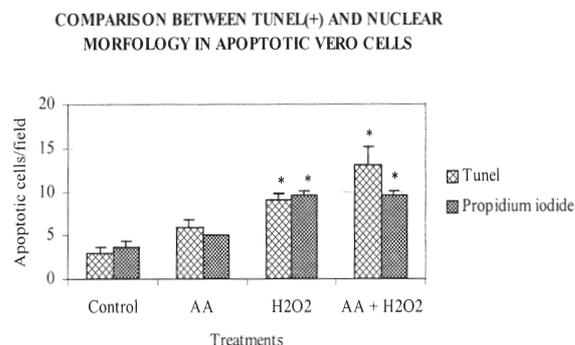


B



C

Figure 6. Apoptotic VERO cells measured by two methods after hydrogen peroxide treatments as in Figure 3. The data are mean \pm SE of 3 experiments. * See Figure 2.



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