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## Effect of ascorbic acid and hydrogen peroxide on mouse neuroblastoma cells

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### Abstract

Ascorbic acid is one of the antioxidant compounds widely used against free radical stress. The present study was undertaken to examine whether ascorbic acid and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), alone or in combination, could influence cell viability. The murine neuroblastoma cell line, N2a, was used to perform a dose response curve for ascorbic acid. It was observed that ascorbic acid alone at physiological concentrations (0.1–0.4 mM) did not cause any cell death. However, at pharmacological concentrations (1–6 mM), ascorbic acid caused dose-dependent cell death. The lethal concentration at which 50% cells were killed (LC<sub>50</sub>) was determined to be approximately 3.141 mM ascorbic acid at 24 h. H<sub>2</sub>O<sub>2</sub> up to 300 μM alone did not cause significant cell death. In the combined treatment, when the cells were treated with ascorbic acid at physiological concentrations (0.4 mM) and H<sub>2</sub>O<sub>2</sub> at 400 μM, higher rates of cell death were observed compared to the cell death rates caused by either compound alone. Subsequent experiments revealed that cell death was partly mediated through the loss of total glutathione levels in the cells. These data suggest that the combination of ascorbic acid and H<sub>2</sub>O<sub>2</sub> is disadvantageous for cancer cell survival. Further studies are required to ascertain the physiological significance of these observations.

### Keywords

ascorbic acid; hydrogen peroxide; glutathione; neuroblastoma cells

### Introduction

Cancer is a major health problem that accounts for more than seven million human deaths worldwide annually (1). In the US alone, 555,500 Americans succumbed to the disease in 2002, which translates into more than 1,500 deaths daily (2). Recent reports indicate that cancer has become the number one cause of mortality in America, followed by coronary heart disease. Traditionally, the main therapeutic strategy in the fight against cancer is surgery and radiation. This approach is beneficial when the tumor is localized in a particular area in the body. However, in the case of disseminated cancers, the preferred approach to curb cancer cells is chemotherapy, where synthetic chemicals are used. Unfortunately, most of these chemotherapeutic drugs do not specifically target the tumors in the body and thus result in severe undesirable side-effects on the normal cells in cancer patients (3). Although natural products have recently been viewed as an alternative source of effective anticancer agents (4–11), they have yet to yield much success. Therefore, due to the differences in the

internal and external tumor environments, it has become evident that a single chemotherapeutic agent does not suffice to kill tumor cells completely. A possible approach to this problem may be combination chemotherapy, where two or more compounds at non-toxic doses are employed to kill cancer cells selectively. Ascorbic acid is one of the naturally and abundantly occurring compounds ranging from 0.1 to 0.4 mM in the cells (12). While its antioxidant properties are well known worldwide, its use as an effective anticancer substance is highly controversial (13). For instance, ascorbic acid has been reported to exhibit anti-carcinogenic and antimutagenic activities (14,15); however, its adverse effects have also been well documented (16,17).

Studies have shown that under certain conditions, ascorbic acid functions both as an anti- and pro-oxidant in cells (18). Considering its safety at pharmaceutical doses (19,20), ascorbic acid could be an ideal candidate for the combined treatment of malignant tumors. While the antioxidant activity of ascorbic acid is of little assistance in controlling tumor cell proliferation, its pro-oxidant action in the presence of certain compounds could help damage cancer cells (21). Initially, to explore the feasibility of this approach, we tested ascorbic acid in the presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) on murine neuroblastoma cells. Subsequently, total glutathione levels in these cells were evaluated.

## Materials and methods

### Cell culture

All routine chemicals used in this study were of analytical grade. The central nervous system derived murine neuroblastoma cell line, N2a (CCL-131) was purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained as a monolayer culture in RPMI-1640 (modified) medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml) in a 5% CO<sub>2</sub> incubator at 37°C. Cell counts and cell viability were assessed using 0.4% trypan blue dye exclusion assay on a hemocytometer under a light microscope. Dye stained cells (blue) were counted as dead cells, while cells without dye were counted as viable cells. The actual cell numbers were determined by multiplying the diluted number of cells with the initial cell numbers. Cell viability exceeded >90%. Cells were diluted in complete RPMI medium and seeded in culture plates for the experiments.

### Treatment of cells

Cells were seeded in flat bottom 96-well microtiter plates at a starting density of  $2 \times 10^4$  cells per well in a total volume of 190 µl growth medium supplemented with 10% regular FBS. The cells were allowed to adhere to wells in the incubator overnight. They were then treated (typically 60–70% confluent) with increasing concentrations of ascorbic acid (0.1–6 mM) or H<sub>2</sub>O<sub>2</sub> (100–400 µM) in a final volume of 10 µl. Stock and working stocks of ascorbic acid or H<sub>2</sub>O<sub>2</sub> were always prepared fresh in phosphate-buffered saline (PBS). In all the experiments, cells in the medium alone or cells in medium equal volumes of PBS served as the controls. These plates were incubated for 24 h continuously without further renewal of the growth medium in a 5% CO<sub>2</sub> in air at 37°C with the plates capped in the standard manner.

### Evaluation of cytotoxicity

Cytotoxicity was evaluated by dye uptake assay using crystal violet, as previously described (5). As per this procedure, the cells were fixed with 0.25% glutaraldehyde, followed by gentle washing and stained with crystal violet dye. The dye was recovered and the optical density measurements were obtained at 540 nm in a plate reader. The average absorbance values of the controls were taken as 100% cell viability. From the treated and control

absorbance values, the percentage of cells killed was determined by the following equation:  $[1-(T/C)] \times 100$ , where T is the average absorbance values of treated cells and C is the average absorbance values of control cells.

### Estimation of cellular glutathione levels

N2a cells were treated with ascorbic acid (0.4 mM) or H<sub>2</sub>O<sub>2</sub> (100 μM) or with a combination of both, for 24 h. Cellular glutathione was assayed according to the method described by Smith *et al* (22). In brief, following incubation, the cells were deproteinized with 2% 5-sulfosalicylic acid (10 μl/well) for 30 min at 37°C, followed by the addition of 90 μl of reaction mixture containing 0.416 mM sodium ethylene diamine tetraacetic acid (EDTA), 0.416 mM nicotinamide adenosine dinucleotide phosphate (NADPH), 0.835 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and 0.083 mM sodium phosphate buffer, pH 7.5. The mixture was incubated at 37°C for 30 min. The absorbance was measured at 412 nm on a plate reader.

### Statistical analysis

The experimental results were presented as the means ± standard error mean (SEM). The data were analyzed for significance by one-way ANOVA and then compared by Dunnett's multiple comparison tests using GraphPad Prism Software, Version 3.00 (San Diego, CA, USA). A P-value of <0.05 indicated a statistically significant difference. The lethal concentration at which 50% cells were killed (LC<sub>50</sub>) values were determined from the graphs where both curves crossed (23).

## Results

### Biphasic activity of ascorbic acid on cells

In our preliminary studies, ascorbic acid at a concentration of 0.1–0.7 mM did not cause any cell death even after 24 h of exposure (Fig. 1). In the subsequent experiments, the concentrations of ascorbic acid were adjusted between 1 and 6 mM. Ascorbic acid was found to cause a significant ( $P < 0.05$ ,  $n = 12$ ) dose-dependent decrease in the cell viability in comparison to the control. The percentage of cell viability ( $\pm$ SEM) was  $78 \pm 1.6$ ,  $69 \pm 5.9$ ,  $51 \pm 4.8$ ,  $30 \pm 1.4$ ,  $20 \pm 1.0$  and  $12 \pm 1.2$  at 1, 2, 3, 4, 5 and 6 mM ascorbic acid, respectively (Fig. 1). The LC<sub>50</sub> was found to be approximately 3.141 mM after 24 h (Fig. 2). The non-toxicity of ascorbic acid at physiological concentrations and the dose-dependent cytotoxicity at pharmacological doses clearly demonstrate the biphasic behavior of ascorbic acid.

### Effect of H<sub>2</sub>O<sub>2</sub> on cells

The role of H<sub>2</sub>O<sub>2</sub> on cell viability was evaluated at different concentrations for 24 h. It was found that in comparison to the untreated control, H<sub>2</sub>O<sub>2</sub> did not cause a drastic decrease in cell viability at a dose of up to 300 μM ( $P > 0.05$ ,  $n = 12$ ); however at 400 μM, there was significant ( $P < 0.05$ ,  $n = 12$ ) cell death (Fig. 3). The percentage of cell viability ( $\pm$ SEM) was  $100 \pm 0.0$ ,  $98 \pm 1.3$ ,  $92 \pm 4.3$  and  $73 \pm 4.3$  at 100, 200, 300 and 400 μM H<sub>2</sub>O<sub>2</sub>, respectively. The estimated LC<sub>50</sub> was found to be >400 μM H<sub>2</sub>O<sub>2</sub>.

### Combined effect of ascorbic acid and H<sub>2</sub>O<sub>2</sub> on cell viability

The role of ascorbic acid was tested on cell viability in the presence of H<sub>2</sub>O<sub>2</sub>. The concentration of ascorbic acid was fixed at 0.4 mM, which represents the physiological concentration (12), and the cell viability was tested in the presence of 400 μM H<sub>2</sub>O<sub>2</sub> for 24 h. Ascorbic acid alone at a dose of 0.4 mM did not induce cell death in comparison to the control. On the other hand, H<sub>2</sub>O<sub>2</sub> alone at a dose of 400 μM caused approximately 27% cell death. However, when combined with 0.4 mM ascorbic acid, H<sub>2</sub>O<sub>2</sub> at 400 μM caused a

sharp increase ( $P < 0.05$ ,  $n = 12$ ) in cell death (Fig. 4). For instance, when both  $H_2O_2$  and ascorbic acid were present together, cell death increased to 71%. Decreasing the concentration of  $H_2O_2 < 300 \mu M$  in the presence of 0.4 mM ascorbic acid did not cause significant cell death (data not shown).

### Effect of ascorbic acid and $H_2O_2$ on glutathione levels

Our results showed that ascorbic acid (0.4 mM) and  $H_2O_2$  (100  $\mu M$ ) alone at non-toxic doses caused a significant depletion in the total glutathione levels in the cells (Fig. 5) after 24 h of treatment. However, these levels decreased further when ascorbic acid and  $H_2O_2$  were present together. Since we used glutathione reductase in the assay, the total glutathione measurements in our study represent both the oxidized (GSSG) and reduced form (GSH) of glutathione.

## Discussion

Although chemotherapy is the best option for the treatment of disseminated tumors, the severe side-effects are the primary drawbacks of this approach. One of the novel strategies to circumvent the problem is to follow combination chemotherapy, where two or more drugs are tested simultaneously. Since this method is expected to achieve higher potency due to the synergistic interaction of chemical compounds, the major advantage is that it employs lower concentrations of individual components in the combined drugs. Thus, this procedure not only significantly decreases the undesirable side-effects, but it also prevents drug resistance by the tumor cells. In the present study, we investigated combining two compounds for an effective response on cells under *in vitro* conditions, using ascorbic acid and  $H_2O_2$ .

We observed that ascorbic acid exhibited biphasic behavior, where it was found to be inactive at physiological concentrations (Fig. 1) and cytotoxic at pharmacological doses (Fig. 2). The biphasic observations were consistent with earlier reports (20). At higher doses, ascorbic acid significantly killed cancer cells with an  $LC_{50}$  of 3.141 mM. The cytotoxicity results are in agreement with ones from previous studies (20). If the potency of ascorbic acid is increased further (i.e., if the  $LC_{50}$  is decreased) by mixing with another compound, this approach of the combination of drugs may become a more effective strategy to kill cancer cells selectively. In our experiments, we combined ascorbic acid at non-toxic doses with  $H_2O_2$  and evaluated the cytotoxicity on the cells. While  $H_2O_2$  itself did not cause severe toxicity to the cells (Fig. 3), the combination of ascorbic acid and  $H_2O_2$  resulted in significantly higher rates of cell death (Fig. 4). Since the main function of ascorbic acid in the body is to act as an antioxidant against free radicals, the higher death rate due to the combination of ascorbic acid with  $H_2O_2$  clearly suggests that ascorbic acid could also act as a pro-oxidizing agent, an observation consistent with previous studies (21).

Another very common antioxidant substance in the cells is glutathione. In our study, treatment with ascorbic acid alone was found to decrease the total glutathione levels of the cells. Although ascorbic acid is an effective antioxidant substance against oxidative stress in the cells, it was striking to observe that ascorbic acid at non-toxic doses depleted significant levels of glutathione (Fig. 5). This observation may suggest that ascorbic acid released certain oxidative species in the aqueous media. This speculation is consistent with earlier reports, in which the pro-oxidant action of ascorbic acid was shown to be the consequence of circumstantial conditions (21). On the other hand,  $H_2O_2$  alone at non-toxic doses also caused a significant decrease in the total glutathione levels in the cells. These levels decreased further when ascorbic acid and  $H_2O_2$  were added together to the cells (Fig. 5). The combination assay results were notable from the therapeutic point of view. For instance, it was shown that tumor cells have higher levels of glutathione than normal cells (24). The

presence of elevated glutathione levels in malignant tumors often limits the effectiveness of chemotherapy (25). However, from our study, it is evident that by combining both ascorbic acid and H<sub>2</sub>O<sub>2</sub> at non-toxic doses, a significant decrease in the glutathione levels in the cancer cells could be achieved, which may lead to effective death of cancer cells. In conclusion, considering the safety and the benefits of ascorbic acid, it can be an ideal candidate in combinational studies in clinical trials against malignant tumors.

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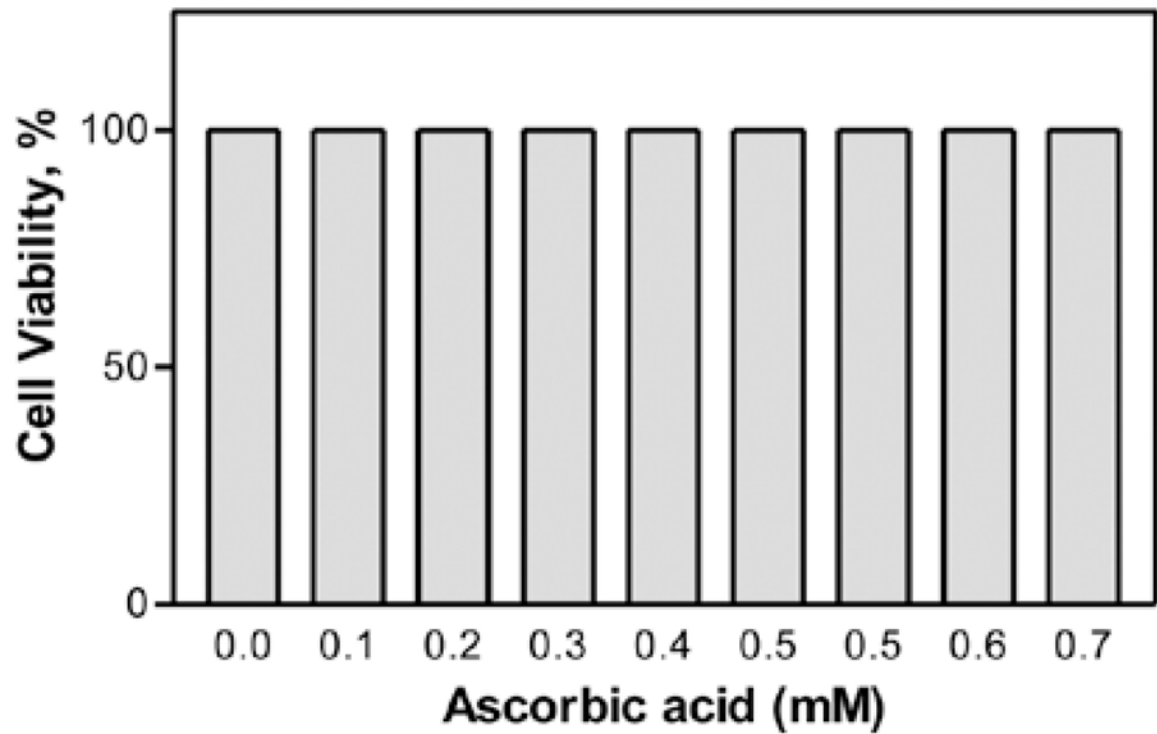
## Abbreviations

<b>DTNB</b>	5,5-dithiobis-2-nitrobenzoic acid
<b>EDTA</b>	ethylene diamine tetraacetic acid
<b>FBS</b>	fetal bovine serum
<b>LC<sub>50</sub></b>	lethal concentration at which 50% cells were killed
<b>NADPH</b>	nicotinamide adenosine dinucleotide phosphate
<b>PBS</b>	phosphate-buffered saline

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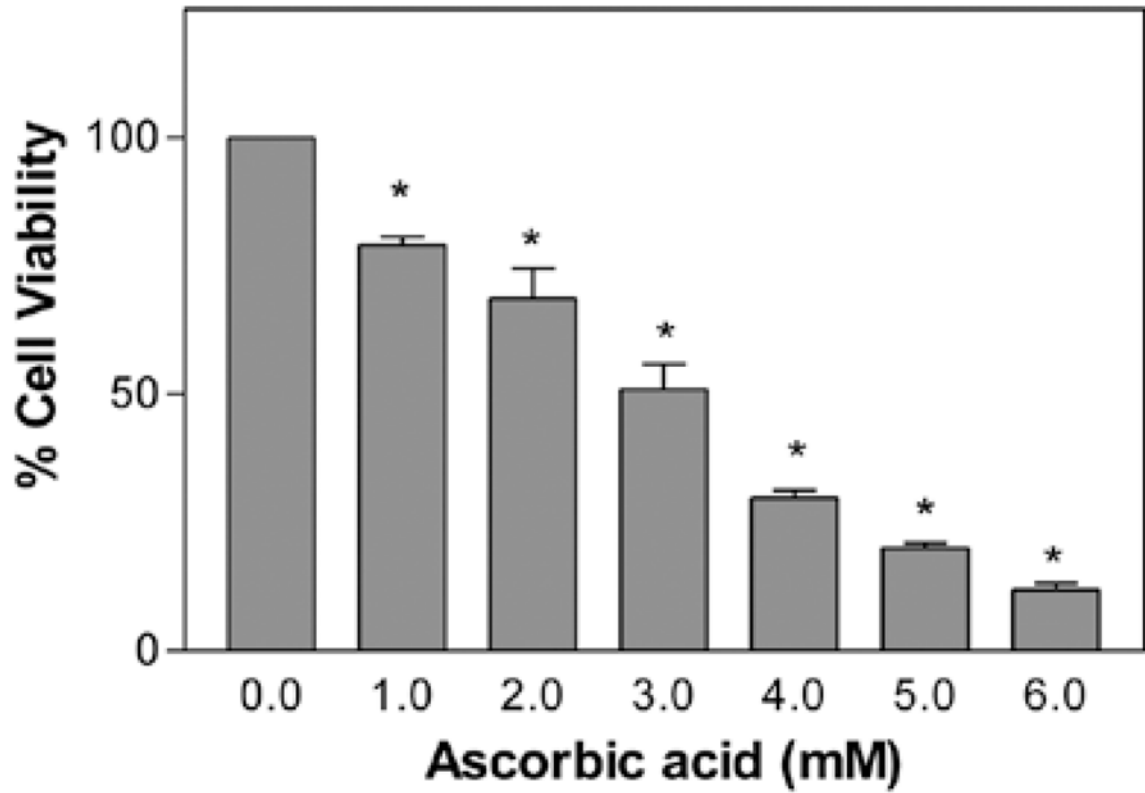
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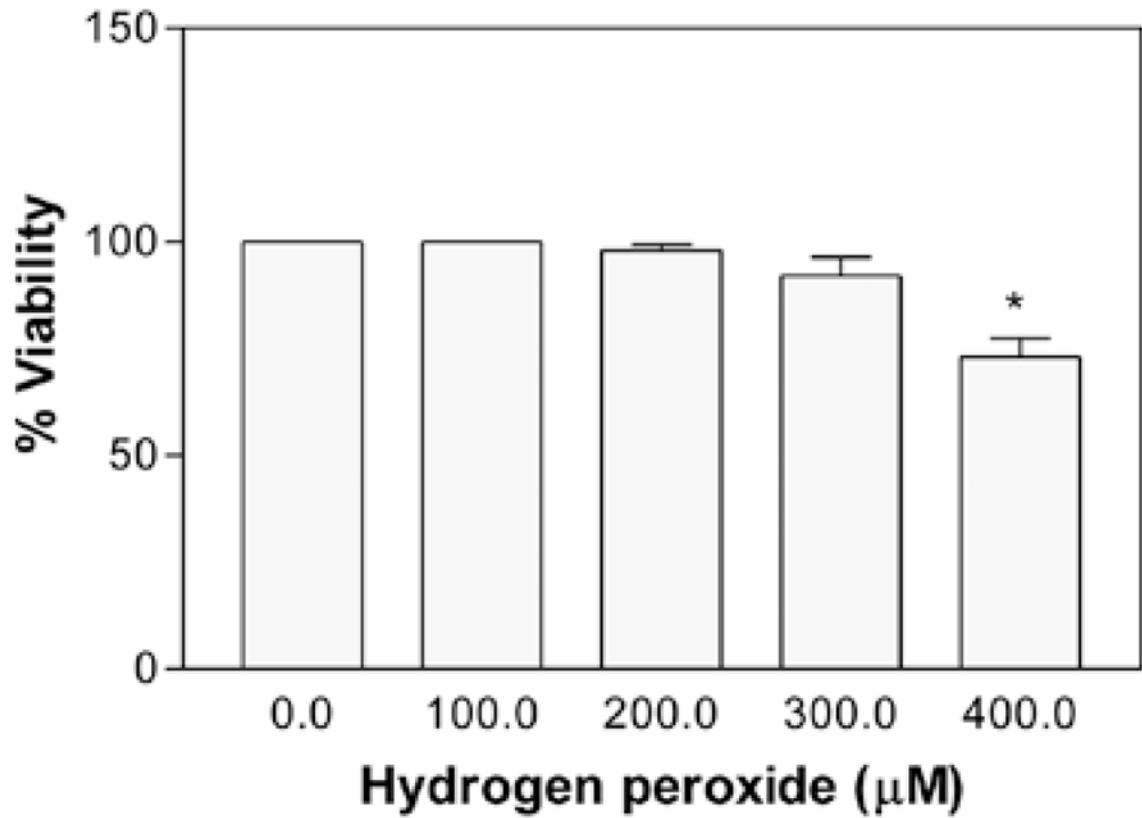


**Figure 1.** Effect of low ascorbic acid concentrations (physiological) on murine neuroblastoma cell viability. The cells with a starting density of  $2 \times 10^4$  were treated with ascorbic acid for 24 h. Data are represented as the means  $\pm$  SD (n=12, \*P>0.05 denotes statistically significant differences vs. control).



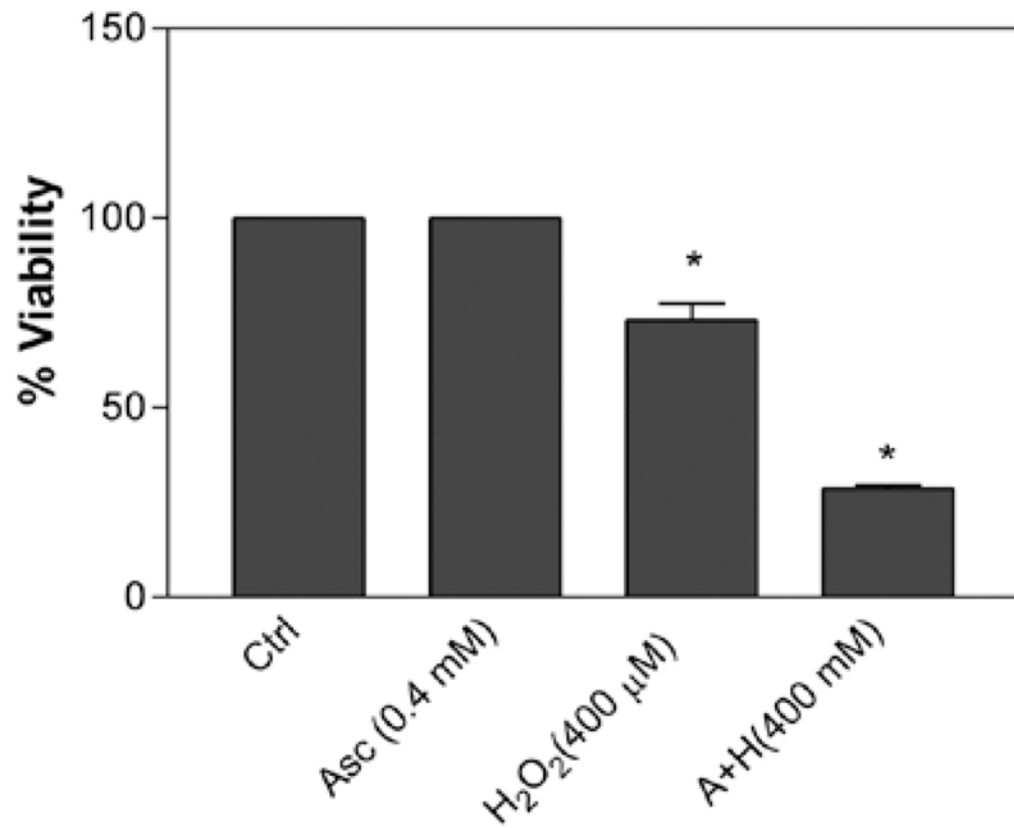


**Figure 2.** Effect of high ascorbic acid concentrations (pharmacological) on murine neuroblastoma cell viability. The cells with starting a density of  $2 \times 10^4$  were treated with ascorbic acid for 24 h. Data are represented as the means  $\pm$  SD (n=12, \*P<0.01 denotes statistically significant differences vs. control).

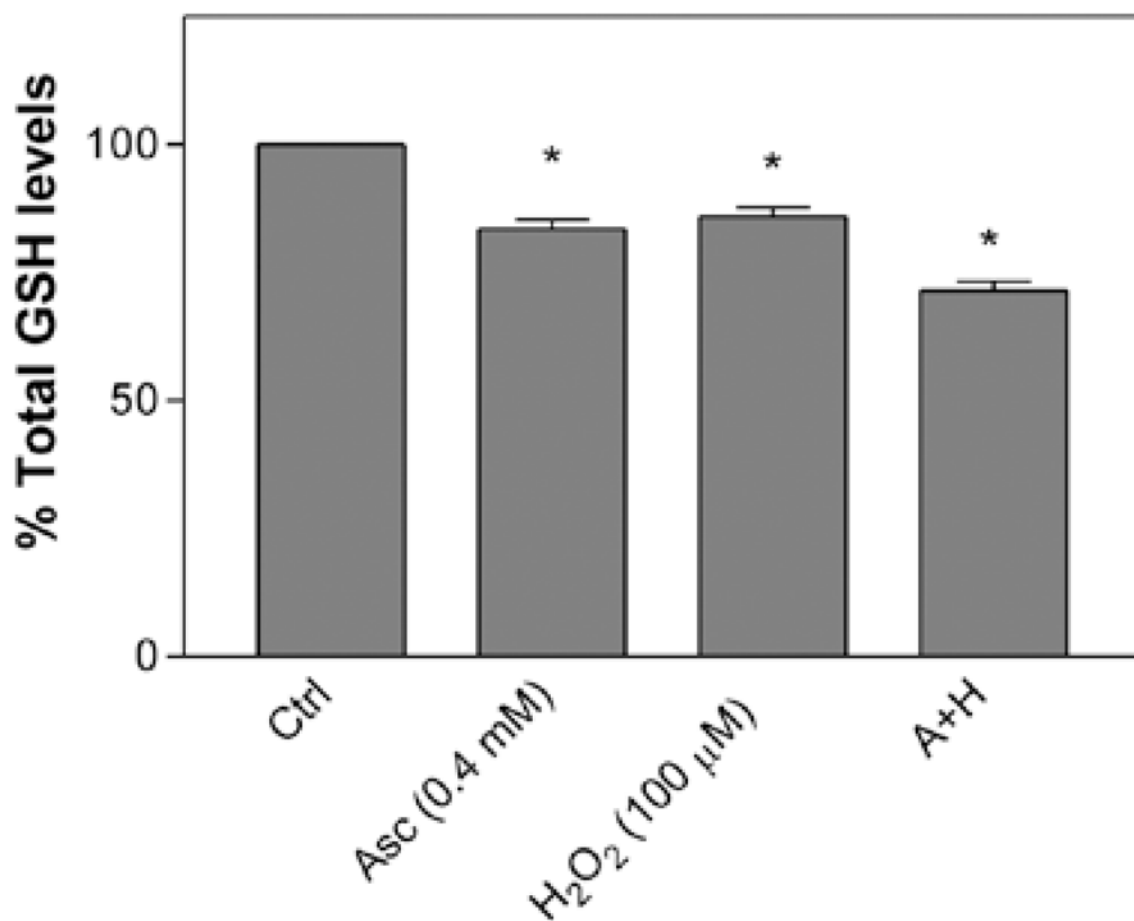


**Figure 3.**

Effect of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) on murine neuroblastoma cell viability. The cells with a starting density of  $2 \times 10^4$  were treated with  $\text{H}_2\text{O}_2$  for 24 h. Data are represented as the means  $\pm$  SD (n=12, \*P<0.05 denotes statistically significant differences vs. control).



**Figure 4.** Effect of the combination of ascorbic acid (0.4 mM) and H<sub>2</sub>O<sub>2</sub> (400 μM) on murine neuroblastoma cell viability. Cells were exposed for 24 h. Data are represented as the means ± SD (n=12, \*P<0.01 denotes statistically significant differences vs. control).



**Figure 5.** Effect of the combination of ascorbic acid (0.4 mM) and H<sub>2</sub>O<sub>2</sub> (100 μM) on total glutathione levels in murine neuroblastoma cells. Cells were treated for 24 h and assayed for GSH levels. Data are represented as the means ± SD (n=12, \*P<0.05 denotes statistically significant differences vs. control).