Abstract

Background. Chromosomal instability (CIN), defined as abnormality in chromosome structure or number, is the hallmark of malignancies. The role of vitamin C in cancer treatment is controversial and its effect on CIN is still an open field of research. In this work, we tried to study the effect of high-dose L-ascorbic acid (L-AA) on CIN-induced (CIN<sup>hi</sup>) and non-CIN-induced (CIN<sup>lo</sup>) cervical cancer cells.

Objectives. The aim of this study was to explore the effect of high-dose L-AA on CIN in the cervical cancer cell line (HeLa) cells.

Material and methods. The HeLa cells (CIN<sup>hi</sup> and CIN<sup>lo</sup>) were treated with 2 doses (5 mM and 8 mM) of L-AA for 24 h and 48 h. They were then analyzed by micronucleus (MN) scoring, cell ploidy and flow cytometry, the latter regarding γH2AX expression. Cell viability was assessed by the methylthiazol tetrazolium (MTT) and Annexin V assays.

Results. Treatment of CIN<sup>hi</sup> cells with L-AA led to a decrease in MN score (colchicine – 71.5 ±4.95, 67.5 ±0.71; L-AA (5 mM) – 49 ±7.07, 46.5 ±4.95; L-AA (8 mM) – 42 ±9.89, 41 ±1.41, at 24 h and 48 h, respectively; p < 0.05). Treatment of CIN<sup>lo</sup> cells with L-AA resulted in increased MN score (5 mM – 45 ±7.07, 36 ±4.24; 8 mM – 34.5 ±4.95, 31 ±1.41, at 24 h and 48 h, respectively; control – 15.5 ±0.71, 12.5 ±0.71; p < 0.05) and reduction in cancer cell viability (control – 100%; L-AA (5 mM) – 76.32% ±28.73, 72.74% ±20.30; L-AA (8 mM) – 66.14% ±19.13, 66.99% ±19.99, at 24 h and 48 h, respectively; p < 0.05). The expression of γH2AX was high in both groups at 48 h (mean CIN<sup>hi</sup> = 19.42%, CIN<sup>lo</sup> = 21.14%; control = 1.19% and 1.58%, respectively) with the 8 mM dose of L-AA.

Conclusions. L-Ascorbic acid was found to have a differential effect on CIN<sup>hi</sup> and CIN<sup>lo</sup> HeLa cells, which may be due to differences in oxidation status of these 2 categories.

Key words: cancer, vitamin C, chromosomal instability
Introduction

Chromosomal instability (CIN) is the defining feature of most malignancies. Many environmental factors, including dietary influences, may contribute to CIN. L-ascorbic acid (L-AA), commonly known as vitamin C, is a water-soluble vitamin with pro- and antioxidant roles. The role of L-AA in cancer is controversial, with studies variably reporting its anti- and pro-cancer effects. Although various mechanisms have been proposed for its anti-cancer effects, the exact mode of action of L-AA on cancer cells is not well understood. Yun et al. in their work on the effect of a high dose of vitamin C in colorectal cancer demonstrated its cytotoxicity to KRAS and BRAF mutated cancer cells, owing to the uptake and reduction of its oxidized form, dehydroascorbate. More recently, vitamin C has been found to be effective in targeting cancer stem cells. Although it has been suggested that it may selectively inflict DNA damage in cancer cells, there is no conclusive data on the effect of L-AA on DNA damage and CIN in cancer. We carried out this study with the aim of evaluating the effect of high-dose L-AA on CIN in the cervical cancer cell line (HeLa) cells.

Material and methods

The HeLa was used in the study to evaluate the effect of high-dose L-AA on cancer cells. The HeLa cells were obtained from a commercial source (National Centre for Cell Science, Pune, India) and maintained in the RPMI (Roswell Park Memorial Institute) 1640 formulation with 10% fetal calf serum (FCS) at 37°C in an atmosphere of 5% carbon dioxide (CO₂). The cells were plated in 6-well culture plates for incubation with drugs and all the experiments were carried out thrice. For the induction of CIN, the cells were treated with metaphase-arresting drug colchicine (0.2–200 µg/mL) for 24 h and 48 h, respectively. Chromosomal instability in the cells was assessed by staining them with Giemsa stain and counting the number of micronuclei.

![Graphs](image)

Fig. 1. A – micronucleus (MN) score at different concentrations of colchicine. Note that the highest score was found with 200 µg/mL at 48 h (encircled). This concentration was therefore chosen to induce chromosomal instability (CIN) in our study. B – percentage of viability of the HeLa cells in different treatment groups at 24 h and 48 h (double bars). Note the decreased viability in non-CIN-induced (CIN_lo) cells upon treatment with L-ascorbic acid (L-AA). However, in CIN-induced (CIN_hi) cells, no decrease was observed. C, D – MN score in CIN_lo and CIN_hi cells, respectively. Note that there was a higher score in the former group as compared to the control, whereas in the latter group, the score was found to be lower when compared to colchicine treatment alone.
(MN) present per 1,000 cells under a light microscope. The dose of colchicine causing maximum CIN was chosen for the induction of CIN in further experiments. Both CIN-induced (CIN\textsubscript{hi} = CIN high) and non-CIN-induced (CIN\textsubscript{lo} = CIN low) cells were then treated with 2 doses of L-AA (5 mM and 8 mM) for 2 time periods of 24 h and 48 h, respectively. At the end of the incubation period, the effect on parameters, like cell viability (methylthiazol tetrazolium (MTT) assay), apoptosis (Annexin V assay) and CIN MN counting, and the expression of DNA double-strand breaks (DSB) marker (γH2AX), was assessed.

**Results**

**Micronucleus score after treatment with colchicine**

Upon treatment with colchicine, the HeLa cells showed a variable number of MN (9–93/1,000 cells). However, the maximum number of MN (93/1,000 cells) was noted with 200 µg/mL at 48 h. Therefore, the latter dose and duration was used for the induction of CIN in HeLa cells (Fig. 1A).

**Effect of L-ascorbic acid on proliferation and viability of CIN\textsubscript{hi} and CIN\textsubscript{lo} HeLa cells**

A reduction in proliferation and viability of CIN\textsubscript{lo} cells was noted upon treatment with L-AA at both doses (5 mM – 76.32% ±28.73%, 72.74% ±20.30%; 8 mM – 66.14% ±19.13%, 66.99% ±19.99%, at 24 h and 48 h, respectively; p < 0.05, paired t-test). However, the reduction in viability was totally absent or only minimal in CIN\textsubscript{hi} cells (Fig. 1B).

**Effect of different doses of L-ascorbic acid on micronucleus scoring in CIN\textsubscript{hi} and CIN\textsubscript{lo} HeLa cells**

Treatment of CIN\textsubscript{hi} HeLa cells with L-AA led to a significant decrease in MN score at both durations (colchicine alone – 71.5 ±4.95, 67.5 ±0.71; CIN\textsubscript{hi} (5 mM) – 49.00 ±7.07, 46.5 ±9.95; CIN\textsubscript{hi} (8 mM) – 42 ±9.89, 41 ±1.41, at 24 h and 48 h, respectively; p < 0.055, paired t-test) (Fig. 1C). However, in CIN\textsubscript{lo} cells, the MN score upon treatment with vitamin C (5 mM = 45 ±7.07, 36 ±4.24; 8 mM = 34.5 ±4.95, 31 ±1.41 at 24 h and 48 h, respectively) was found to be significantly higher than in the untreated control (15.5 ±0.71, 12.5 ±0.71, p-value <0.05, paired t-test) at both durations of treatment (Fig. 1D).

![Fig. 2. A – percentage of apoptosis in HeLa cells upon treatment with various drugs. No significant increase was observed; B, C – distribution of cells in different phases of the cell cycle in different treatment groups: i) control; ii) L-ascorbic acid (L-AA) 5 mM; iii) L-AA 8 mM; iv) colchicine; v) colchicine + L-AA 5 mM; vi) colchicine + L-AA 8 mM, at 24 h and 48 h, respectively. Note the G2/M arrest caused by colchicine at both durations, and the presence of the majority of cells in the S and G2/M phases in L-AA-treated groups with or without colchicine.]

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Effects of vitamin C on HeLa cells

A. Sindhwani, S. Muthusamy, A. Bhatia. Effects of vitamin C on HeLa cells

Effect of L-ascorbic acid on apoptogenicity of CIN\textsubscript{hi} and CIN\textsubscript{lo} cells

Annexin V assay showed no significant increase in the percentage of apoptotic cells with any of the drugs used in our experiments (Fig. 2A).

Effect of L-ascorbic acid on the cell cycle of CIN\textsubscript{hi} and CIN\textsubscript{lo} HeLa cells

Treatment with colchicine resulted in cell cycle arrest at the G2/M phase (87.50% and 65.47% at 24 h and 48 h, respectively). When the cells CIN\textsubscript{hi} were treated with both doses of L-AA, the majority of cells were found to be distributed in the S and G2/M phases of the cell cycle at both treatment durations. The cells CIN\textsubscript{lo} were found to be mainly distributed in the S phase at 24 and the G2/M phase at 48 h, respectively (Fig. 2B,C).

Effect of L-ascorbic acid on the expression of DNA damage response marker γH2AX in HeLa cells

An increase in the percentage of γH2AX-positive cells was noted in both CIN\textsubscript{lo} and CIN\textsubscript{hi} cells, particularly after treatment with 8 mM L-AA (mean γH2AX expression – 19.42%, 21.14%; control – 1.19%, 1.58%, respectively) after 48 h of treatment (Fig. 3). However, there was no significant change in the expression of γH2AX in colchicine-treated cells.

Discussion

Cancers are heterogeneous in nature. Some degree of numerical and structural defect in chromosomes, termed as CIN, is present in most tumors. The current literature has emphasized the presence of populations of more differentiated and more aggressive cells in cancers, the latter harboring more genetic defects and being less susceptible to therapy. Therefore, the agents being tested for targeting cancer should be investigated in the light of their effects on different populations of cells present in cancer.

Many past studies have stressed the beneficial effect of high-dose vitamin C intake in cancer. Many papers have described the inhibitory effect of millimolar concentrations of L-AA in vitro. However, many other studies have reported contrary results.\textsuperscript{2–4,7} In view of the controversial literature on the effect of L-AA on cancer cells, we undertook the present work to investigate its effect on HeLa cells harboring CIN.

To induce a higher degree of CIN, we treated the HeLa cells with a high dose of colchicine.\textsuperscript{8} The 2 types of cells, when treated with vitamin C, showed paradoxical results.

![Graph showing the expression of γH2AX in HeLa cells](image-url)
Vitamin C was found to enhance CIN in CIN\textsubscript{lo} cells, at the same time decreasing their viability and proliferation. However, in CIN\textsubscript{hi} cells, vitamin C was observed to reduce CIN, at the same time increasing their viability and proliferation. We hypothesize that this may be due to cell death being triggered by L- AA via the induction of DNA damage beyond the repair capacity of the cancer cells in the former case. In the latter case (CIN\textsubscript{hi} cells), treatment with L- AA was probably decreasing CIN to sublethal levels by unknown mechanisms, allowing the more aggressive population of cells to proliferate (Fig. 4). Some of the previous studies carried out on lymphocytes have also demonstrated the genotoxic potential of L-AA, especially in the presence of oxidizing agents like H\textsubscript{2}O\textsubscript{2} or heavy metals like iron. The induction of single-strand breaks in the presence of oxidative stress was suggested as the mechanism behind the toxicity described above. Other studies have shown the protective effect of vitamin C on cisplatin-induced chromosome aberrations in lymphocytes in cancer patients.\textsuperscript{9,10} Interestingly, a study carried out on the HeLa cells by Azqueta et al. did not show any effect of L- AA on DNA strand break or base excision repair; however, the concentration used in their study was much lower (up to 200 µM) than that used by us.\textsuperscript{11} High-dose vitamin C has also been proposed to cause increased DNA damage via the generation of reactive oxygen species (ROS) in gene-mutated cancer cells.\textsuperscript{5}

Chromosomal instability is a double-edged sword. Up to a certain threshold, it is believed to increase cell proliferation; however, beyond that threshold, it is known to cause cell death.\textsuperscript{12} The results of our study, together with an increase in the expression of DNA damage response marker γH2AX in both categories of cells, demonstrate the clastogenic potential of L- AA on the HeLa cells, especially during longer exposure (48 h). However, whereas the clastogenicity of L- AA was able to cause killing in CIN\textsubscript{lo} cells, it was not found to reduce the viability of CIN\textsubscript{hi} cells. The differential effect on 2 categories of cells may help to explain the contradictory results of the studies trying to investigate the effect of vitamin C on cancer cells.

A complex effect was observed on the cell cycle upon treatment with L- AA. Colchicine, being a mitotic spindle inhibitor, resulted in cell cycle arrest in the G2/M phase. Treatment of these cells with L- AA resulted in the distribution of the cells to the S and G2/M phases. Also, in these CIN\textsubscript{hi} cells, the S-phase fraction (SPF) was seen to increase. Treatment with L- AA alone showed complex effects with a mild increase in SPF. Previous studies have also highlighted that the irregular cell cycle kinetics observed with L- AA, with cell cycle arrest in the S, G2M and G0/G1 phases, is dependent on the cell line being studied, and that this inhibition of cell division promoted necrosis of the malignant cells. In those studies, L- AA was found to act by regulating the activity of various molecules related to cell cycles, such as Cdc25C, Chk2-p53-p21\textsubscript{waf1/cip1}, etc.\textsuperscript{13–16}

The apoptosis assay carried out in our study did not demonstrate much increase in the population of the apoptotic cells, thereby indicating that L- AA may be causing cell death by mechanisms other than apoptosis. Previous studies have also reported different mechanisms of cell death caused by vitamin C like autoschizis (cell splitting), apoptosis progressing to pyknosis/necrosis on prolonged treatment, etc.\textsuperscript{15–18} The exact modes of cell death involved in vitamin C induced cytotoxicity, therefore, remain to be investigated.

Thus, our study suggests that treatment with L- AA may have different effects on CIN\textsubscript{hi} and CIN\textsubscript{lo} cancer cells. The genotoxic and cytotoxic effect of high-dose L- AA on CIN\textsubscript{lo}, but not on CIN\textsubscript{hi} HeLa cells, may be due to a difference in the oxidative status of these cell types. However, it needs to be confirmed in future studies. Moreover, our results also point toward the clastogenic potential of L- AA, as indicated by the increased MN score and increased expression of DNA damage response marker γH2AX.
However, the preliminary data obtained from the present study needs to be confirmed, using more robust studies, employing small interfering RNA (siRNA)-based methods for the induction of CIN and molecular techniques, like fluorescent in situ hybridization, for the measurement of CIN.

References