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Effect of Ascorbic Acid on SH-SY5Y Cells at Different pH Levels

Okan SANCER[1,2], Pınar ASLAN KOŞAR[1]

[1] Innovative Technologies Application and Research Center, Genetic Research Unit, Suleyman Demirel University, Isparta, Türkiye

[2] Faculty of Medicine, Department of Medical Biology, Suleyman Demirel University, Isparta, Türkiye

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Abstract

Objective

Cancer is a complex disease, and many different methods are used to treat it. Ascorbic acid is one of the nutritional supplements used to prevent the proliferation of cancer cells. Therefore, the effect of ascorbic acid at different pH levels on SH-SY5Y cells was investigated.

Material and Method

The cytotoxic effect of ascorbic acid at different pH levels and concentrations, and its effect on cell proliferation and gene expression were studied on SH-SY5Y cells.

Results

The pH and ascorbic acid levels that decreased the viability of the SH-SY5Y cell line and inhibited migration in wound healing were pH:8 and IC_{50}:4.15. In addition, it was determined that p53 expression level increased ($p<0.05$) and MDM2 and AKT1 expression levels decreased ($p<0.05$) at the pH and IC50 values mentioned above.

Conclusion

The findings of this study show that AA applied at high pH affects the viability of SH-SY5Y cells, inhibits their migration capacity, and alters the expression levels of p53, MDM2, and AKT1 genes.

Keywords: Ascorbic acid, Migration, pH, Proliferation, Viability

Introduction

Cancer is characterized as a complex disease that can be altered by genomic and epigenomic factors that occur with the change of gene expression in cells, causing cells to live longer and proliferate (1). There is substantial evidence that ascorbic acid (AA) has anticancer properties and has been proposed as a potential anticancer agent (2-4). The pharmacological use dose of AA has been redesigned for the treatment of treatment-resistant cancer cells in combination with radiotherapy, monotherapy, and chemotherapeutic

Correspondence: O.S. / okansancer@hotmail.com
Received: 06.01.2025 • Accepted: 23.03.2025
ORCID IDs of the Authors: O.S: 0000-0001-7935-5004; P.A.K: 0000-0003-2602-5145

drugs in various cancers, including breast cancer (5), colorectal cancer (6) melanoma (7), gastric cancer (8) and pancreatic cancer (9). A dose of 10 mM AA has been observed to cause apoptosis in neuroblastoma and melanoma cells and has been shown to act as an important modulator of the growth of murine myeloma cells in an in vitro colony assay (10). Some studies with vitamin C have shown that it causes cytotoxicity through the depletion of adenosine triphosphate in cancer cells (9, 11). Therefore, it can be proposed that vitamin C exerts an effect on the intracellular metabolism of malignant cells by disrupting the redox balance of H_2O_2; this represents a promising area for further investigation.

The process of metabolic reprogramming that occurs in cancerous cells is frequently accompanied by an increase in the acidity of the extracellular matrix. Measurement of pH in tumour tissue using microelectrode, magnetic resonance or fluorescence results in an extracellular pH ranging from 6.5 to 6.9. A notable characteristic of many tumours is the variation in pH levels across their structure These levels are usually higher near the surface of the tumour and lower in the middle. Surfaces formed by highly metastatic cells have a pH ranging from 6.1 to 6.4, whereas, for not metastatic tumours, the pH ranges from 6.7 to 6.9 (12).

The level of p53 in cells is positively correlated with the level of DNA damage and can vary. The presence of low levels of p53 leads to the arrest of the cell cycle, while high levels of this protein lead to apoptosis. Activation of PI3K/AKT leads to inhibition of p53 through activation of MDM2, another tumour suppressor. MDM2 is an oncoprotein that regulates tumour formation. The p53-induced expression of PTEN results in the formation of a p53/PTEN interaction, which in turn suppresses cell survival through the inhibition of the PI3K/AKT signaling pathway. PTEN forms a complex with p53 and affects the transcriptional activity of p53 by controlling its DNA binding. MDM2 is phosphorylated for nuclear translocation by the AKT kinase (13). Furthermore, it is known that p53 and PTEN interact and regulate each other at the transcriptional and protein levels. This could be an important control mechanism for switching between survival and death. PTEN stabilizes the p53 protein in two ways: by triggering the AKT-MDM2 complex and by increasing the acetylation of the p53 protein. It is therefore likely that the decreased p53 activity observed in PTEN-lacking tumor cells is due to this mechanism. As noted above, the PTEN and p53 proteins interact with each other and represent among the most significant control mechanisms for cells to switch between survival and death (14).

In addition to all this information, although the use of ascorbic acid as a natural antioxidant is widespread, it is unclear how it will affect SH-SY5Y cells at different pH levels. In the present study, we aimed to evaluate the effect of dose-dependent ascorbic acid on cytotoxicity, migration, and p53, MDM2, PTEN, and AKT1 expression in SH-SY5Y cells at different pH levels.

Material and Method

Cell Culture

SH-SY5Y (ATCC, USA) cells were incubated with Dulbecco's Modified Eagle Medium (Capricorn, Germany) supplemented with 10% fetal bovine serum (Sigma-Aldrich, USA) and 100 IU/mL penicillin, 10 µg/mL streptomycin (Sigma-Aldrich, USA).

MTT Assay

In our study, an MTT assay was performed according to the study of Riss et al. (15). The cells were seeded in 96-well plates at a seeding density of 10,000 cells/well, and then left to grow for 24 hours before treatment with ascorbic acid (Sigma) and different pH. Cells were then treated with 0, 2, 4, 6, 8, 16 mM AA and 6, 7, 8 pH for 24 hours. The pH of the cell culture medium was adjusted four times a day with HCl and NaOH to maintain a constant pH value (12). A multiscan plate reader (Synergy HTX BioTek, USA) was used to record optical densities at 570 nm. The percentage of viability of the cells was calculated according to the method of Yeap et al. (16).

Cell Migration Assay in Vitro

The cell migration assay was employed to ascertain fundamental cell migration characteristics, including speed, persistence, and polarity. A total of $1x10^4$cells were seeded into each well of a 6 well plate. The cells were incubated at 37°C in 5% CO_2 for 24 hours to allow them to adhere to the surface and form confluent monolayers. These confluent monolayers were then scratched with a sterile pipette tip. This left a ~ 0.4 mm wide scratch. To remove detached cells, the wells were washed with a culture medium (17). The culture medium was refreshed four times for 24 h with media containing IC_{50}= 5.46 mM, 3.66 mM, 4.15 mM AA, and 6,7,8 pH, respectively. The pH of the cell culture medium was adjusted with HCl and NaOH to maintain a constant pH value (12). Each wound size was visualized using an inverted microscope. The wound closure rate (%) was calculated using ImageJ software (18).

Real Time PCR Expression Analysis

The IC_{50} concentrations determined for pH=6, pH=7,

and pH=8 were applied to SH-SY5Y cells for 24 h, respectively. The real-time PCR expression analysis was conducted in accordance with the manufacturer's methodology. The primer sequences used were as follows; p53 F: TCTACAAGCAGTCACAGCACAT, p53 R: CAACCTCAGGCGGCTCATAG MDM2 F: TGGCGTGCCAAGCTTCTCTGT, MDM2 R: ACCTGAGTCCGATGATTCCTGCT, PTEN F: CGACGGGAAGACAAGTTCAT, R: AGGTTTCCTCTGGTCCTGGT, AKT1 F: TCTATGGCGCTGAGATTGTG, R: CTTAATGTGCCCGTCCTTGT, ACTB F: CATGTACGTTGCTATCCAGGC, R: CTCCTTAATGTCACGCACGAT. The results were normalized using the ACTB gene expression data. The CT values of the target genes were determined, and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ (Livak method).

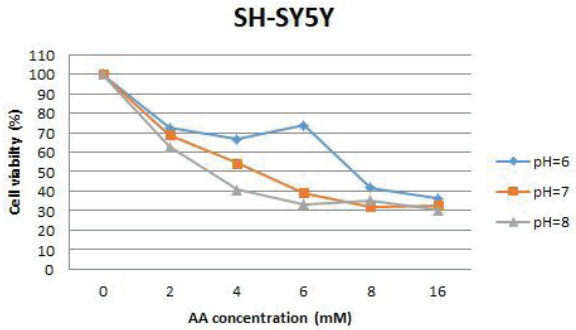
Statistical Analysis of Data

The statistical analysis was conducted using GraphPad Prism v.8 (San Diego, CA, USA). To evaluate the differences between the groups, the Student's t-test was employed. The level of significance was set at $p < 0.05$.

Results

MTT Assay

The effect of AA and different pH values on the viability of SH-SY5Y cells was evaluated by MTT assay. Six different concentrations of AA (0, 2, 4, 6, 8, 16 mM) and three different pH (6, 7, 8) media were used in this study. Results showed that the combination of pH 8 and AA (IC50) provided the most effective reduction in proliferation. (Fig. 1). IC50 values determined depending on pH change were 5.46 mM for pH=6, 3.66 mM for pH=7, and 4.15 mM for pH=8.



Cell	IC50 (AA, mM)		
	pH=6	pH=7	pH=8
SH-SY5Y	5.46	3.66	4.15

Figure 1
Cell viability and inhibition of SH-SY5Y cells due to different AA and pH levels. Values are mean ± SD of triplicate value. *p<0.05 vs. control group.

Migration Assay

Cell migration assay was performed to determine the effect of different AA and pH levels on the migration of SH-SY5Y cells. The migration of SH-SY5Y cells was inhibited by 31.57% for pH=6, 77.27% for pH=7, and 89.51% for pH=8, respectively, compared to the control group (Fig. 2).

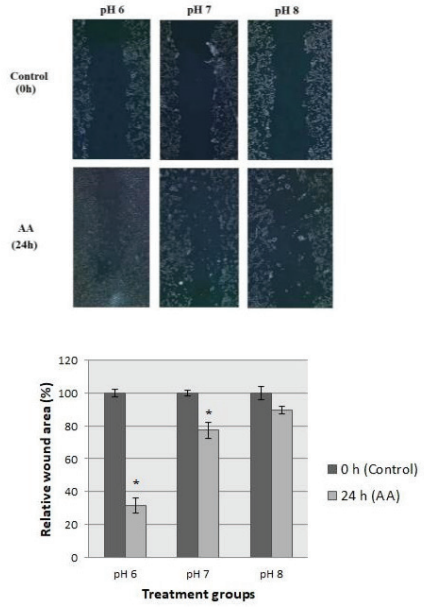


Figure 2
The SH-SY5Y cells were cultivated until they reached confluence, after which a scratch was made to create a wound. The combination treatment has been observed to have a more pronounced effect on cell migration. The initial scratch and 24-hour observation results are presented as the gap area. Each bar represents the mean ± SD. (*p<0.05, compared to control group).

Expression Analysis

In the context of p53, MDM2, and AKT1 expression results, a statistically significant outcome was observed in the comparison between pH 8 and pH 8 + AA ($p < 0,05$) Conversely, no statistically significant outcome was observed in the remaining experimental comparisons ($p > 0.05$) (Fig. 3).

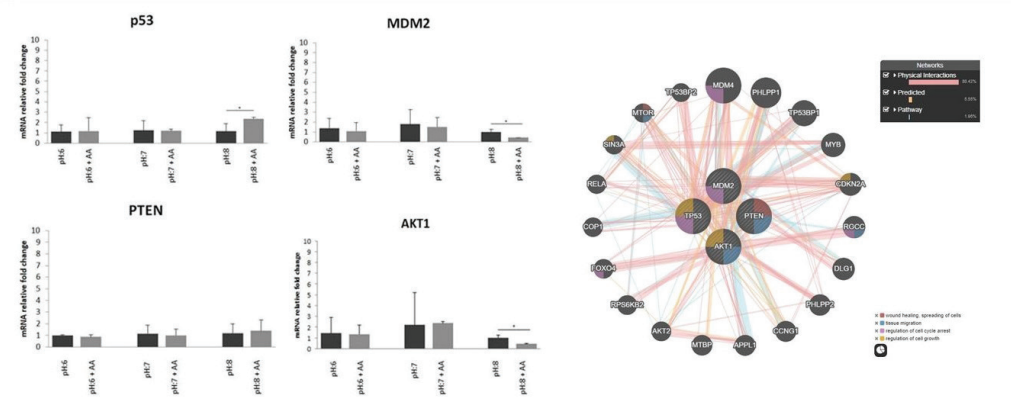


Figure 3

Relative mRNA expression results and statistical comparison between groups. Values are expressed as mean ± SD. * p<0.05. Gene network and function info from the Genmania database (https://genemania.org/).

Discussion

High-dose vitamin C is a common treatment option among complementary and alternative medicine practitioners for a range of various diseases. Except for the known complications of vitamin C in individuals with renal failure or glucose-6-phosphate dehydrogenase deficiency, high-dose intravenous vitamin C appears to be a safe intervention. However, it is emphasized that complementary and alternative medicine practitioners should be informed about the use of vitamin C in patients with cancer, and chronic and incurable diseases and should be careful about unexpected harm, drug interaction, or benefit (19).

Recent study information on the physiological properties of Vit-C, its pharmacokinetics, and results from preclinical reports indicate that high-dose Vit-C can be used effectively in the treatment of various tumor types (20). Tumour cells have been observed to rapidly consume glucose for glycolysis, resulting in the rapid production of lactate. This process enables tumour cells to obtain the energy they require to sustain their proliferation, regardless of the oxygen content present. Therefore, the higher metabolic rate of tumor cells has been considered as the main cause of the acidic tumor microenvironment (21). Tumours have a distinctive microenvironment, characterized by elevated temperatures, high expression of specific enzymes, a tendency towards a reduction in redox potential, and an acidic pH of approximately 6.5 (22).

Cancer cells can evade acid stress by activating and expressing proton and lactate transporters and exchangers. Therefore, they have an extracellular acidic and intracellular alkaline pH gradient. The alteration in the acid-base balance of tumour cells has been linked to an increase in several key characteristics, including proliferation, evasion of apoptosis, metastatic potential, aggressiveness, invasiveness, treatment resistance, and immune evasion. Reversing the pH gradient may be one of the most promising anticancer strategies paving the way for the development of new and innovative therapies. These include tumor-targeted pH-sensitive antibodies and pH-sensitive nanoparticle conjugates with anticancer drugs. An alternative approach is the oral or parenteral use of buffer systems, such as sodium bicarbonate, to neutralize tumour acidity. While buffering therapy does not pose any problems against standard treatment methods, it makes it possible to use different combinations to increase the effectiveness of the treatment (23).

The anticancer potential of pharmacological AA has been established in several cancer cells. It is known that pH may be a critical effect factor for multiple anticancer therapies. As a result of the investigation of the therapeutic effect of AA on cell lines PC3 and DU145 cultured at different pHs, it was shown that acidic pH inhibited AA uptake in PCa cells and weakened the cytotoxic activity of pharmacological ascorbic acid (12). In other studies, high doses of L-ascorbic acid reduced the viability of the HT29 cell line in vitro (24), and the combination of AA and selenium had an additional chemopreventive effect on the HCT116 and SH-SY5Y cell lines (1). It was observed that a 99.6% pure titanium plate was coated with 596.29 nmol ascorbic acid after application of 5 M NaOH and applied to hBCCs, MDA-MB-231 cells.

The results obtained after this application showed that it synergistically inhibited the proliferation, spreading, and migration of the cells (25).

AA contributes to its anticancer effects by regulating several key processes, including cell growth and differentiation, DNA methylation, the activity of the Ten-eleven translocation family protein (TET), hypoxia-inducible factor-1α (HIF-1α) signaling, and the regulation of gene expression, including transcription factors such as p53, NF-κB, and AP-1, as well as vascular endothelial growth factor (VEGF). These processes can modulate the expression of tumour suppressor genes and oncogenes (26). p53, one of the tumour suppressor proteins, has an important role in the regulation of factors such as cell cycle, apoptosis, aging, cell proliferation, and differentiation. In addition, it has been shown that its expression level changes in various cancer types and it is known to have an important effect (27).

MDM2 functions as an inhibitor of the p53 protein, thereby modulating the p53 signaling pathway (28). MDM2 inhibition has been shown to induce p53-mediated reversal of the Bcl2/Bax ratio and lead to cell death in PTEN-deficient colorectal cancer cells. PTEN is an important negative effector of the PI3K signaling pathway that can inhibit the activation of Akt and other downstream kinases (29). PTEN is a tumour suppressor gene that can halt cell proliferation and encourage cellular apoptosis (28). AKT, the cell survival oncoprotein (also referred to as protein kinase B), has a pivotal function in several processes that promote cell survival, proliferation, growth, and migration. This gene is over-activated in human cancers and is known to be closely associated with poor prognosis and treatment resistance (30).

In this study, the combination of pH=8 and 4.15 mM applied to SH-SY5Y cells was found to be the optimum treatment affecting the mechanism of these cells. Furthermore, p53 expression increased, while MDM2 and AKT expression decreased in this combination ($p < 0.05$). PTEN expression increased, but there was no statistical change.

The maintenance of acid-base balance (pH) is a fundamental requirement for the survival of cells. Due to the rapid proliferation of cancer cells, CO_2 and lactic acid production as a result of intensive respiratory requirements disrupt the acid-base balance of the cells and lead to a change in pH. A more profound comprehension of metabolism and pH regulation in cancer cells is imperative for the advancement of diagnostic instruments and novel therapeutic interventions, thereby enhancing the well-being of cancer patients. This study shows that AA applied at low pH has little effect on the proliferation of SH-SY5Y cells, but at high pH, the acid-base balance of the cells is disturbed and AA decreases proliferation in these cells. However, although it was observed that AA has anticancer effects in SH-SY5Y cells at different pHs in vitro, it should be noted that this effect cannot be compared with in vivo and should be supported by in vivo studies.

Conflict of Interest Statement
There is no conflict of interest.

Ethical Approval
Not applicable.

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This research has not received any financial support from organizations in any sector.

Availability of Data and Materials
Data is available on request from the authors.

Author Contributions
OS: Conceptualization, methodology, investigation, administration, visualization, data curation, formal analysis writing-original draft.

PAK: Conceptualization, methodology, investigation, supervision, administration, writing—review a editing.

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