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Apoptosis in Human Papillomavirus-Induced Cervical Cancer Cells By Higher Doses of Ascorbic Acid

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ABSTRACT

Objective: To assess the anti-cancer role of ascorbic acid using high doses against the cervical cancer cell line and to establish a cell culture facility in the laboratory.

Study Design: In vitro study.

Place and Duration of Study: Department of Biochemistry and Molecular Biology, Army Medical College, Rawalpindi Pakistan, from Aug 2014 to Jul 2015.

Methodology: HeLa cells were grown in Dulbecco's modified eagle medium, and upon reaching confluency, cells were challenged with different doses of ascorbic acid. The MTT assay was employed to study the cytotoxic effects of increased doses of ascorbic acid on HeLa cells.

Results: An increasing dose of ascorbic acid was found to be cytotoxic toward HeLa cells, and its EC50 was found to be between 5-6μM. A high dose of ascorbic acid is selectively cytotoxic to HeLa cell lines of cervical cancer, which may imply the treatment of cervical cancer patients. NF- kB inducing kinase is a salient protein in TNFα-induced NF-kB activation. Ascorbate has been reported to inhibit that activation which has anti-apoptotic roles in tumour progression. CB-Dock was employed to propose the binding sites for NIK-Ascorbate interaction, which might be a therapeutic target for cancer cells.

Conclusion: The study concludes that high dose ascorbic acid is cytotoxic to cervical cancer cells HeLa and may have important implications in treating cervical cancer patients. Therapeutic intervention of this vitamin may contribute to suppressing cancer development.

Keywords: Ascorbic acid, Cervical cancer, HeLa cells, MTT assay.

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INTRODUCTION

Cancer refers to the uncontrolled division of the body's cells. The abnormal regulation of tumour suppressor genes might even have a role in cancer development. Due to their altered expression, certain oncogenes are not suppressed by their tumour suppressor genes. The altered expression triggers abnormal cell proliferation.[1] More than 19 million cases of cancers were reported (breast cancer being the most prevalent) worldwide, along with 10 million deaths from cancer in 2020. Cervical cancer was observed to be ninth in number in cancer types for the year 2020.[2] Human papillomavirus (HPV) induced cervical cancer occurrence was higher in a meta-analysis. It was observed that women with human immunodeficiency virus (HIV) had a higher risk of having cervical cancer than those with no HIV infection.[3] HPV has been regarded as the most common type of virus, spreading as a sexually transmitted disease. There are various strains of the virus, among them; HPV16 and HPV18 have been classified to cause cancer, contributing to a higher risk of cervical cancer.[4]

Various therapeutic approaches have been employed to combat cancers, including anti-inflammatory agents and antioxidants. Ascorbic acid has been proved to be an important vitamin after its discovery.[5] A study published by Chen *et al,* documented that a higher plasma concentration of ascorbate results in the accumulation of hydrogen peroxide that is preferably toxic to cancer cells. Catalases are present in the blood; normal cells have an extensive blood supply. As a result, catalase in blood converts toxic hydrogen peroxide into non-toxic water and oxygen, whereas cancer cells lack extensive blood supply and do not have enough catalase to decompose hydrogen peroxide. As a result, hydrogen peroxide damages cancer cells (can cause breaks in DNA and mitochondria).[6] Ascorbic acid therapeutic implications have been extensively reported in various other types of cancers. The role of ascorbic acid in the oral squamous cell line was studied. It was found to have induced cell apoptosis by DNA damage and energy depletion and the cell cycle arrest at G0 and G1 phases.[7]

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Nuclear factor light chain enhancer of activated B cells (NF-κB) has regulatory roles in cell cycle progression and inflammation. Various inflammatory cytokines trigger the activation of NF-κB, tumour necrosis factor-alpha (TNFα) being the most common. An NF-κB inducing kinase (NIK) is another crucial protein in TNFα-induced NF-κB activation.[8] NIK has been reported to phosphorylate inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ), which thus phosphorylates inhibitory kappa B-alpha (IkBα) to activate NF-κB activation.[9] NF-κB has been observed to induce the expression of antiapoptotic genes. In this way, it facilitates the tumour cells to escape apoptosis.[10] Ascorbic acid has been found to suppress the TNFα-induced activation of NF-κB in cancer cells. In an experimental study, higher luciferase activity was observed in cells incubated with TNFα.

We aimed at studying the effect of ascorbic acid at higher concentrations, inducing cytotoxicity to cervical cancer cells (HeLa cell line). This study assessed the cytotoxic effects of ascorbic acid in human cervical cancer cell lines by evaluating the cell viability. In this study, we report the anti-cancer role of ascorbic acid on the human cervical cancer cells, which marks the therapeutic potential of ascorbic acid in combating cancer in higher doses.

METHODOLOGY

This in vitro study was carried out at the Department of Biochemistry and Molecular Biology, Army Medical College, Rawalpindi, from August 2014 to July 2015. The ethical approval was obtained from the Ethical Review Committee (No. ERC/ID/154) and was conducted in compliance with the Declaration of Helsinki.

Phosphate buffer saline, fetal bovine serum (FBS), L-ascorbic acid, and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St Louis, Missouri, USA), while HeLa vial and MTT reagent were from Sigma Aldrich (Taufkirchen, Germany). DMEM media, 1% penicillin-streptomycin and trypsin were from Bio West (France). RPMI media and trypan blue dye were from Invitrogen (MA, US).

HeLa cervical carcinoma cells were revived using standard protocols. The HeLa vial (RRID: CVCL-0030) was washed twice with phosphate-buffered saline (5x-PBS) by centrifuging (1 tablet PBS per mL of distilled water and autoclaved afterwards). The pellet was washed again with PBS and re-suspended in 500µL RPMI media. Cells were then cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS (Sigma-Aldrich, Cat#F9665) and 1% penicillin/streptomycin. The cells were cultured in a 37°C incubator with 5% atmospheric CO2.HeLa cells were subcultured in 96 well plates after harvesting them using trypsin (Bio West France, Cat#L0932). Cells were counted using a trypan blue dye exclusion assay. The following formula was used for cell counting with trypan blue and hemocytometer (Abcam, USA). Cells per mL= (number of cells/number of squares) $x10^4$

0.1 molar L-ascorbic acid solution was freshly prepared, and pH was adjusted with NaOH. Ascorbic acid in concentrations ranging from 2µM to 10µM was applied to plates, and cells were incubated for 2 hours. Then cells were washed with PBS. Fresh DMEM media (Bio West France, Cat# P0103) was added to cells after washing them, and further were grown for 24 hours. A 10% DMSO was used as a positive control. Same concentrations of ascorbic acid (with no cells) were used as blank.

After the exposure to ascorbic acid (24 hrs), an MTT reagent was added to measure the viable cells (10 µL). The plate was covered in aluminium foil to protect the reagent from light and incubated at 37°C for 4 hours before adding 10µL DMSO. The readings were taken at 490nm. The experiment was carried out in triplicates. MTT reagent contains a substrate (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) converted to coloured formazan crystals by living cells. The absorbance was measured at 570nm in the ELISA reader. EC50 was then measured by plotting a graph against each ascorbic acid concentration and absorbance.

A docking simulation was performed to propose the possible binding of ascorbic acid in the living cells. NIK as kinase protein was chosen for binding interaction to determine the inhibitory action of ascorbic acid. The crystal structure was retrieved from Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB, PDB), NF-kB inducing kinase (ID, 4G3D). In addition, the 3D structural file for L-Ascorbic acid was retrieved from PubChem (ID 54670067). These retrieved structures were then subjected to cavity blind docking through a web server, CB Dock.

All the tests were carried out in Graph pad prism, version 6.0. EC50 of vitamin C for the HeLa cell line was calculated by the linear regression equation.

RESULTS

HeLa cells were observed under an inverted phase-contrast microscope (Olympus, Tokyo, Japan) at

40X magnification before exposure to ascorbic acid for 24 hours. After the exposure, cells were observed again. The cells had undergone heavy apoptosis (Figure-1).

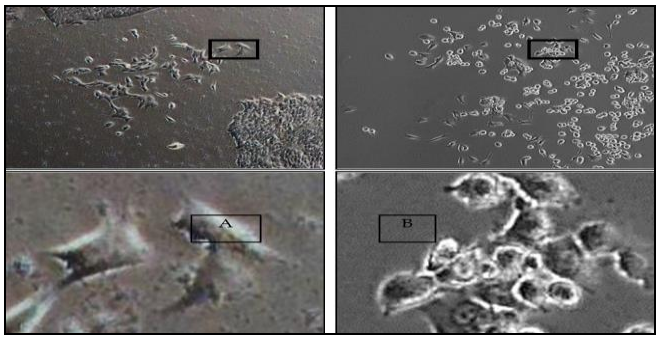

Figure-1: HeLa cells (a) before and (b) after exposure to ascorbate

The concentration of ascorbic acid ranging from 2µM to 10µM was applied to cells. Cell viability decreased with increasing concentration, and the EC50 value was observed to be 6.75µM (Figure-2).

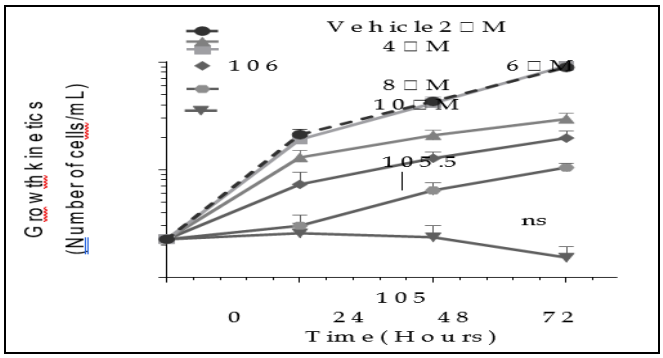

Figure-2: Growth kinetic curve plotted between number of cells against time elapsed

Graphs were plotted between absorbance, different concentrations of ascorbic acid, and percentage viability (Figure-3). Y=-10.66x+122.0, (taking Y as 50) 50=-10.66x+122.0 EC50=X=6.75.

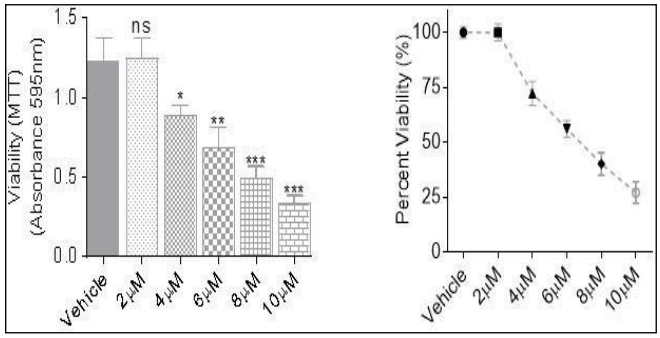

Figure-3: A is graph between absorbance and different concentrations of vitamin C; B is graph between percent viability and different concentrations vitamin C.

Ascorbate has been docked to NIK as an inhibitory target. This ligand establishes interactions, including hydrogen bonding and hydrophobic and polar interactions (Figure-4).

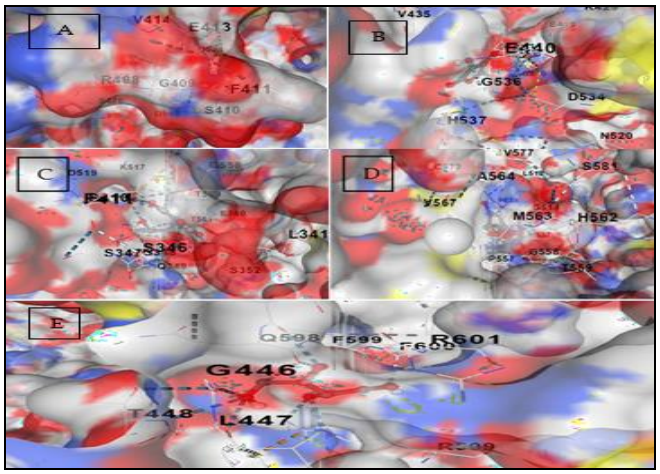

Figure-4: An illustration of 3D docked poses of NIK-Ascorbate interaction at different position

Figure Legends

Fig-1 The human papillomavirus originated HeLa cell line was treated with different ranges of ascorbate to screen cell apoptosis, the cells were grown under controlled conditions and represent the growth with no exposure to ascorbate (a), while the cells were given different concentrations of ascorbate and positive apoptosis was observed (b) under a microscope, triggered by ascorbate.

Fig-2 A plot representing the growth kinetics of cells when treated with different ranges of ascorbate (2-10µM), against the time.

Fig-3 Graphs illustrate cell viability and the absorbance with different concentrations of ascorbate, the cellular activity is indicated to be reduced with increasing concentration of ascorbate (a), the cell number is shown to be declined when employed with a high range of ascorbate (b).

Fig-4 A computational illustration of 3D docked poses of the NIK protein interacted with ascorbate, representing the possible binding sites for proposing protein inhibition.

DISCUSSION

Ascorbic acid-loaded cells revealed little activity induced by TNFα. Over time, the cells accumulated with ascorbic acid exhibited a 50% inhibition in the reporter protein activity induced by TNFα.[11] Due to the lack of effective and reliable treatment for some

advanced cancers, there is an urgent need for investigation of new drugs that show increased efficacy against the cancerous cells. Among many other herbs and vitamins, high-dose intravenous ascorbic acid is one option for the treatment of cancer.[12] A study of nine cancer patients undergoing IVC (intravenous vitamin C) therapy revealed positive outputs.[13] The anti-cancer role of ascorbate in animal models has been well established, however, the clinical efficacy is somewhat contentious regarding the use of high doses of ascorbic acid against cancer in humans.[14] Besides playing an important role in immune stimulation, antibody response, immunoglobulin level, and many other biochemical effects, ascorbate has the lowest toxicity of all vitamins.[15] Experimental evidence suggests that ascorbic acid supplement plays an eminent role in cancer development. An increase in the pharmacological concentration shows a specific role in suppressing the growth and development of cancerous cells in vivo and in vitro. Its experiments in this aspect are encouraging and need more light to end this biological rigor.[16]

This vitamin has been the main focus in chemotherapy as its treatment is non-toxic and shows expected results in most cases. Various studies had been conducted previously to find out the possible therapeutic role of ascorbic acid. We tested the hypothesis of ascorbic acid's role in anti-cancer in the cervical cancer cell lines. Our study found the intense apoptosis of HeLa cancer cells after treating them with higher concentrations of L-Ascorbate. Different concentrations of the ascorbate were used to observe the possible effects of ascorbate on cancer cells when treated with a different range of concentrations. A range of 2µM to 10µM was used against cancer cells. The cell viability was reduced effectively at 6.75µM for EC50 value. Transcription of the vitamin C transporter protein (SVCT2) is altered in the human breast cells. A US-based study comprising 113 cases revealed the reduced vitamin C transporter protein expression in human breast cancer tissues. The use of ascorbate (100µM) remarkably changed the expression of transporter protein, suggesting its role in breast cancer treatment.[17]

Su *et al*, reported inhibition of thyroid cancer cells and cell apoptosis by varying doses of ascorbate after cultured human thyroid cancer cells were injected into an animal model of mice.[18]

In support of our study findings, entailing the potential role of high doses of ascorbate, findings from a study carried out on Brazilian women who tested positive for HPV marked a significant association between dietary intake and persistent infection. The study observed a minimum risk for persistent HPV infection with high intakes of ascorbic acid. Management of viral infections using antioxidants has been previously reported to be effective. Oxidative stress has been shown to increase HIV replication induced by increased activity of NF-kB.[19] A research based on ascorbic acid therapy and two drugs revealed a significant reduction in the cell. Cycle-related proteins, reduced levels of anti-oxidative transcription factors and mitochondrial dysfunction.[20]

The findings from our study reveal a significant role of ascorbic acid in higher concentrations in cancer cells. A study revealed apoptosis in the human colon cancer cell line, induced by vitamin C. The vitamin triggered apoptosis by way of modulating calcium influx.[21] Findings from the study exhibited cancer cell death (in human breast carcinoma and human colon cancer cell lines) by ascorbic acid through blocking energy metabolism.[22]

Using inexpensive and non-toxic ascorbic acid as therapy for reducing malignancies and viability of cancer cells was promoted initially by the clinical study of Cameron and Pauling. Unfortunately, we do not have effective treatment for advanced cancer patients. The pharmaceutical role of ascorbic acid provides us with the light of hope in the medical field, so there is a need to devise an effective treatment to fight back against this fatal disease.

CONCLUSION

Certain cancer cells have been observed to present high sensitivity toward high doses of ascorbic acid in various invitro studies. This vitamin also enhances the chemosensitivity of certain other cancer cell types. The study concludes that a high dose of ascorbic acid is selectively cytotoxic to cervical cancer cells and may have potential implications in treating cervical cancer patients. Therapeutic intervention of this vitamin may contribute to suppressing cancer development.

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Conflict of Interest: None.

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Author's Contribution

ZAB: did manuscript writing and drafting, AM, AR & IM: Carried out experimental work and analyzed the data, AG: Revised the manuscript, AM: Added to the intellectual contents of the manuscript.

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