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Pharmacological ascorbate triggers apoptotic pathway in liver cancer cells via up regulation of TNF-α

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Abstract

Ascorbic acid (Vitamin-C) is known to have tumor preventive and anti-tumor effects but the mechanism through which it regulates tumor progression needs to be elucidated. Probably, vitamin-C exerts its cytotoxic effects through TNFα mediated apoptotic pathway. TNFα induces two pathways simultaneously, that are, inflammatory and apoptotic pathways. In tumor cells the balance between apoptotic and inflammatory pathways is disturbed. TNFα induced NF-κB signaling is thought to be responsible for the inflammatory pathway and is implicated in many chronic diseases/disorders including cancer. Inhibition of inflammatory pathway at some point may allow only apoptotic pathway to proceed further, resulting in cancer cell death. This study was designed to evaluate the cytotoxic effect of ascorbic acid on Hepatocellular Carcinoma cells. Cell viability study was accessed after exposing the cells to increasing concentration of vitamin C. Expression of genes involved in signaling like TNFα and NF-κB was quantified. EC50 of Vitamin-C for Huh-7 was found to be 2.17mM. The expression profiling showed that TNFα was elevated, whereas the expression of NF-κB remained unchanged. In synthesis, the study showed that vitamin-C induced TNFα mediate apoptosis by shifting the balance from inflammatory to apoptotic pathway. These findings may be helpful in further understanding of the mechanism of cytotoxicity in cancer cells induced by high dose vitamin-C.

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Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of deaths worldwide. Major causes include Hepatitis B virus (HBV), Hepatitis C virus (HCV), alcohol consumption and dietary aflatoxins. Global number of cases diagnosed are approximately 560,000 and 550,000 deaths attributed to liver cancer. Due to the lack of effective and reliable treatment for this group of cancer, there is an urgent need for the investigation of new drugs, either stand alone or a combinatorial therapy that show reduced cytotoxicity and increased efficacy against cancerous cells. Currently, complementary and alternative medicine (CAM) is rapidly gaining attention around the globe. In CAM, many herbs and vitamins are used as therapeutic interventions. In particularly, higher dose of vitamin C when given through intravenous route showed anticancer effects, which can be an attractive and feasible option for the treatment of cancer. When administered intravenously, high-dose vitamin C acts as an anticancer drug, in contrast to oral vitamin C that keeps serum vitamin C level under 100μM, which functions as a nutrient (Levine *et al.*, 1996; Graumlich *et al.*; Campbell *et al.*, 1991; Cameron and Pauling, 1976).

Inflammatory cells are believed to enhance tumor genesis by virtue of non-specific pro-inflammatory cytokines including interferon gamma, tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6 (Aggarwal *et al.*, 2006; Robinson and Coussens, 2005). Among these, cytokine that is involved in a number of factors including apoptosis, cell survival, inflammation and immunity. It has been proposed to have a crucial role in the pathogenesis of cancers. Owing to its anticancer potential is currently used in the treatment of higher grade non-metastatic and metastatic malignancies (Eggermont *et al.*, 2003). Besides TNF-α, nuclear factor-kappa B (NF-κB) is homo- or heterodimer protein that constitutively expressed in fibro lamellar HCC (Li *et al.*, 2010). Studies have shown that most of the factors involved in tumor progression exert their effects through NF-κB (Carr *et al.*, 2014).

Upon activation, it is translocated to the nucleus and initiates transcription of crucial genes involved in tumor progression and inhibition of apoptosis. Furthermore, evidences suggest that NF-κB also has role in cancer stem-cell survival (Hewamana *et al.*, 2008).

NF-κB is essential component in TNF-α mediated inflammatory carcinogenic pathway. NF-κB, once activated by TNF-α signaling for cell survival, induces cell growth. Once down-regulated, it will result in cancer cell death via apoptosis, making NF-κB a promising potential target for cancer related therapeutic intervention (Pikarsky *et al.*, 2004).

The objective of this study is to delineate the plausible mechanism of anticancer action of vitamin C on inflammatory pathway of HCC cell line Huh-7, and to know the effect of increasing dose of vitamin C on the expression of inflammatory genes, TNF-α and NF-κB.

Materials and methods

Cell culturing

Huh-7 cell lines (ASAB cell culture bank) were revived according to standard protocol. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) media containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cultured cells were maintained at 37°C in incubator with 5% atmospheric CO_2.

MTT assay for cell viability

Cell cytotoxicity studies were conducted through MTT assay. Confluent cells were trypsinized and counted. Cells were seeded in 96 well plates so that each well contains $1x10^4$ cells. After cells were adhered, they were treated with different doses of Ascorbic acid ranging from 2mM to 10mM for 2 hours. Then cells were washed twice with PBS and they were allowed for additional 24 hours to grow. MTT was added to each well after 24 hours and incubated for 4 hours. DMSO was added to dissolve the colored crystals formed. Reading was taken at 570 nm through Elisa plate reader. The experiment was carried out in triplicates.

Gene expression studies

Huh-7 cells cultured in 6 well plates (cell count=1x10^6) were exposed to different concentrations of Ascorbic acid i-e.2,4,6 and 8mM respectively for 2 hours. After 2 hours media containing Vitamin C was removed from each well and washed with PBS. Fresh media was added and cells were allowed to grow for further 24 hours. After 24 hours RNA was isolated from cells using TRIZOL method. RNA was dissolved in diethyl pyro-carbonate (DEPC) treated water and incubated for 10-15 min at 55^{o}C.

Table 1. Primer sequences used during the PCR analysis

mRNA targets	Oligonucleotides (5' - 3')	
NF-κB	Forward	GCTTAGGAGGGAGAGCCCA
	Reverse	TATGGGCCATCTGTTGGCAG
TNFα	Forward	CCCAGGGACCTCTCTCTAATC
GAPDH	Reverse	ATGGGCTACAGGCTTGTCACT
	Forward	GCTCTCTGCTCCTCCTGTTC
	Reverse	TTCCCGTTCTCAGCCTTGAC

cDNA was synthesized by reverse transcription reaction following manufacturer protocol (Thermo Fisher Scientific). Gene expression profiling was done through Real Time PCR. Targeted gene primers and reaction profile were amplified on Real Time PCR (Model No. Applied Biosystem).

Maxima SYBR Green/ROX qPCR Master Mix (Catalog number: K0221 Thermo Scientific) was used. Reference housekeeping gene GAPDH was used to compare relative expression of target genes. Experiments were replicated in triplicates. The results were analyzed by Sequence Detection Software (ABI). The relative quantification was calculated as the difference between the mean Ct values of each target and reference gene GAPDH (Mean Ct = Ct (target gene) - mean Ct (GAPDH).

Statistical analysis

All statistical tests were carried out in Graph pad prism, version 6.0. The hypothesis was tested with Student's t-test. P < 0.05 as*, P <0.005 as**, P < 0.001 as*** were considered significant.

Results

Cytotoxic Effect of Vitamin C exposure on Huh 7 cell lines

Huh7 cells were observed under inverted phase contrast microscope at 40 X magnification before exposure to Vitamin C for 24 hours. After Vitamin C exposure, cells were observed again. The cells had undergone heavy apoptosis (Fig. 1).

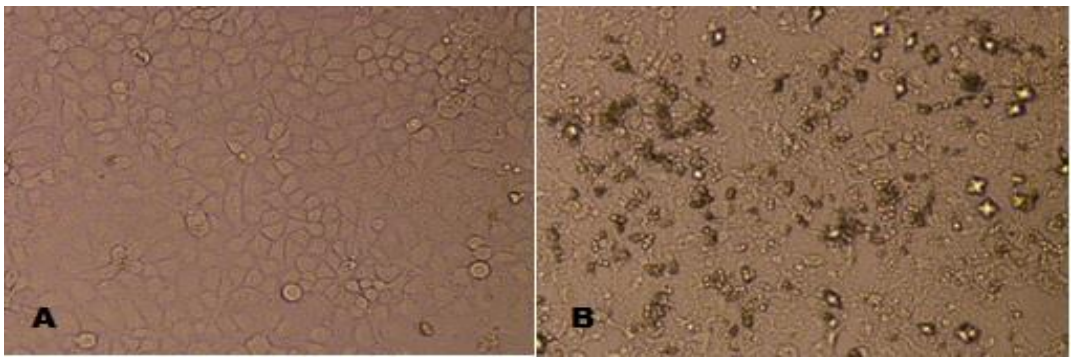

Fig. 1. Huh-7 cells before (A) and after (B) exposure to ascorbate.

Determination of cytotoxicity of Vitamin C through MTT Assay

Cells viability assay was done through MTT assay. MTT reagent contains a substrate [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] which is converted to colored formazan crystals by living cells. The amount of crystals can be measured by spectrophotometry at 570 nm wavelength.

The absorbance is thus directly proportional to number of viable cells. Vitamin C concentrations ranging from 2mM to 10mM were applied to cells. Cell viability of cells was decreased with increasing concentration of Vitamin C and EC50 value was found to be 2.17 mM. The MTT graph in (Fig. 2.) shows that Vitamin C decreases cell viability with increased concentration.

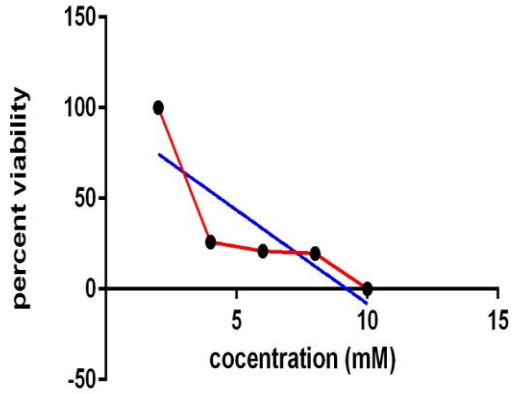

Fig. 2. Graph plotted between percent viability of Huh-7 cells and increasing concentrations of vitamin-C to determine EC $_{50}$ of Vitamin C for Huh-7 cell line.

Calculation of EC_{50} of Vitamin C for Huh-7 Cell Line

EC_{50} of Vitamin C for Huh-7 cell line was calculated by linear regression equation.

Linear regression equation:

Y = -21.22x +95.92

(Take Y=50)

50 = -21.22x +95.92

EC_{50} = X = 2.16

Expression analysis of TNFα

It was observed that Vitamin C significantly increases the expression of TNFα (p value=0.039) in a dose dependent manner with concentrations ranging from 2mM to 10mm as shown in (Fig. 3).

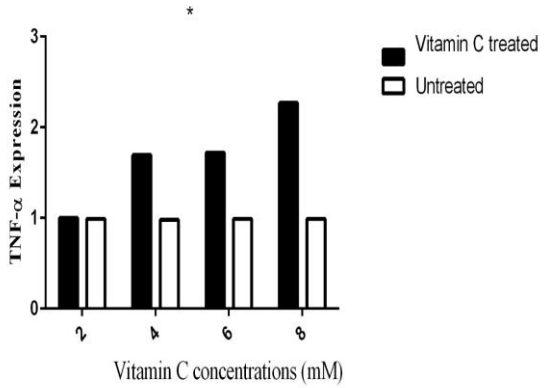

Fig. 3. Expression of TNFα in Huh-7 cell line exposed to increasing concentrations of Vitamin C (p value=0.039), compared to untreated Huh-7 cells. The expression of TNF α is significantly higher in ascorbate treated cells as compared to untreated cells.

Expression analysis of NF-κB

Effect of increasing concentrations of vitamin-C on expression level of NF-κ B was studied. There was no significant difference (p value=0.0554) of vitamin-C on expression level of NF-κ Bas shown in Fig 4.

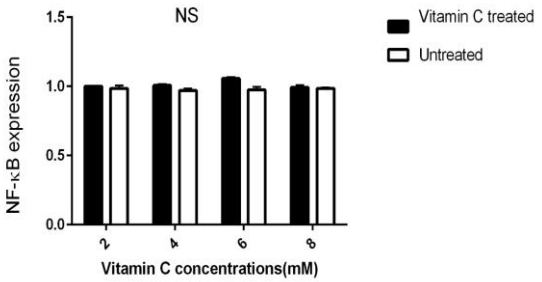

Fig. 4. Expression of NF-κ B in Huh-7 cell line treated with different concentrations of vitamin-C compared to untreated Huh-7 cells. The expression level of NF-κB in ascorbate treated cells is similar to that of untreated cells (p value=0.0554).

Discussion

High dose Vitamin C is toxic to many cancerous cell lines in vitro. Qi Chen *et al* reported that many of the cancerous cell lines are killed at the concentrations ranging from 1mm to 10mM while normal cells were found to be tolerant even at concentration of 20mM and greater (Chen *et al.*, 2005). Researchers postulated different mechanisms on the basis of their findings. Numerous studies have shown the production of Hydrogen peroxide being responsible for Vitamin C induced cytotoxicity of Cancer cells. However, in this study we aimed to study Vitamin C induced anti-tumor aspect of immune system.

In our study EC 50 of Vitamin C for liver cancer cell line Huh 7 was calculated to be 2.17mM. This concentration of Vitamin C is easily and safely achievable in vivo through Intravenous Vitamin C (Hoffer *et al.*, 2015). Relative expression of TNFα in Vitamin C treated Huh 7 cells was found to be increased in dose dependent manner, whereas relative expression of NF-κ B gene remained unchanged in Vitamin C treated Huh 7 cells.

TNFα is known to activate two independent pathways which diverge at the very early stage of TNFα signalling (Aggarwal *et al.*, 2005).

One pathway causes apoptosis whereas the other is involved in inflammation. The inflammation pathway is mediated by NF-κ B which is the key link of inflammation and cancer. Once activated, NF-κB signals TNFα to stop apoptotic pathway (Van Antwerp *et al.*, 1996; Lee *et al.*, 2004; Zhu *et al.*, 2011). In the absence of NF-κ B signalling, TNFα is considered as a potent anti-tumor gene and has been proposed to be used in therapy against many cancers (Nakamoto *et al.*, 1999).

In the present study, we observed that ascorbic acid up regulates expression of TNFα whilst keeping expression of NF-κ B un-affected. Bowie *et al.* in 2000 reported that Vitamin C inhibits activation of NFκ B by inhibiting blockade of Iκ B (Bowie and O'Neill, 2000). Iκ B activation is blocked by Iκ B Kinase (IKK) which in turn is mediated by p38. Vitamin C, affects expression of p38, causes its constitutive expression thus inhibiting blockade of Iκ B by IKK. Thus allowing Iκ B to inhibit activity of NF-κ B. This observation provides a supportive evidence in the favour of above conclusion that Vitamin C shifts TNFα from inflammatory and proliferative pathway towards apoptotic pathways. Our study provides another important aspect regarding the mechanism of cancer cell cytotoxicity through high dose Vitamin C.

Abbreviations

The following abbreviations are used in this manuscript:
HCC: Hepatocellular Carcinoma
TNF: Tumor necrosis factor
NF-κB: Nuclear Factor Kappa B
CAM: Complementary and Alternative medicines
MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
PBS: phosphate buffer saline
EC50: Effective Concentration 50

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