Tropical Journal of Pharmaceutical Research June 2018; 17 (6): 1043-1048
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v17i6.9

Original Research Article

Antiproliferative and apoptotic effects of high-dose vitamin C in cholangiocarcinoma cell line

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Sent for review: 12 January 2018 *Revised accepted: 16 May 2018*

Abstract

Purpose: *To evaluate the antiproliferative and apoptotic effects of high-dose vitamin C in cholangiocarcinoma cell line (CCA).*
Methods: *Sulforhodamine B colorimetric assay was used to determine cell proliferation in human K100-CCA cell lines. The expression of anti-apoptotic Bcl-2 protein/ pro-apoptotic Bax protein was evaluated by Western blot analysis.*
Results: *Ascorbic acid inhibited the growth of CCA in a dose-dependent manner. The half-maximal inhibitory concentration (IC_{50}) of ascorbic acid on K100 at 24 h was 55 ± 9.4 mM. It was observed that treatment with ascorbic acid resulted in the reduction of glutathione and increased hydrogen peroxide contents in the cells. Moreover, disturbance of mitochondrial membrane potential occurred in ascorbic acid-treated cells in a dose-dependence manner. The ratio of Bcl2/Bax decreased in CCA treated with ascorbic acid.*
Conclusion: *The results show that the anti-proliferation effect of ascorbic acid in CCA may be attributable to the modulation of expressions of Bcl-2 and Bax proteins and dissipation of the mitochondrial electrochemical potential gradient, which is an early event leading to apoptosis.*

Keywords: *Vitamin C, Cholangiocarcinoma, Apoptosis, Glutathione, Mitochondrial membrane potential*

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INTRODUCTION

Cholangiocarcinoma (CCA) is a rare form of cancerous tumour that originates from the biliary epithelial cells. It is prevalent in the Southeast Asian region and is associated with symptoms like liver fluke infection (caused due to *Opisthorchis viverrini* and *Chlonorchis sinensis*). This condition leads to other secondary symptoms like cell injury, prolonged inflammation, and reparative biliary epithelial cell proliferation. Due to prolonged inflammation, a local environment is created with the dominance of cytokines and other growth [1] Currently, radical surgery is considered to be the most preferred therapy for CCA as it has high curative potential. However, in most cases, the patients are referred for surgical intervention at an advanced stage of the disease, due to which the surgery may not give the predicted outcome [2]. In addition to surgery, chemotherapy is considered to be another approach for treating

CCA in inoperable patients. However, the currently-used chemotherapeutic drugs have not shown satisfied results in terms of survival benefit [3]. Hence, there is a need to develop new and advanced chemotherapeutic drugs aiming at high rates of survival with low side effects.

The use of ascorbic acid as a potent chemotherapy agent in treating cancer and its effect in minimising the disease symptoms has a controversial history [4]. Several studies have reported using a high dose of ascorbic acid for treating cancer [5-9]. Furthermore, studies have shown that ascorbic acid treatment led to enhanced prolongation and quality of life. However, later studies showed its disadvantages in treating cancer patients [10,11]. In a previous study, a high dose of pharmacological ascorbic acid concentration was found to induce *in vitro* cell death and *in vivo* tumour growth inhibition [12].

Hydrogen peroxide (H_2O_2) is an oxidative molecule involved in many immune reactions of the body. Hence, high-dose ascorbic acid treatment leads to increased production of H_2O_2 in human connective tissues. In case of healthy cells, intracellular quenching of H_2O_2 takes place through natural anti-oxidants present in the cells. However, the cancer cells lack sufficient levels of anti-oxidants to quench H_2O_2, leading to its accumulation. This causes a condition of increased oxidative stress, and the cancer cells eventually undergo apoptosis [13-15].

Only a few research works have focused on the effect of ascorbic acid on CCA cell line. Thus, the present study aimed at evaluating the antiproliferative and apoptotic effects of high-dose vitamin C or ascorbic acid treatment on K100 CCA cell line.

EXPERIMENTAL

Cell culture

The study included the human CCA cell line K100 cells, which were provided by Dr. Banchob Sripa of the Department of Pathology, Faculty of Medicine, Khon Kaen University. K100 cells were routinely cultured in Ham's F12 media supplemented with 12.5 mM N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid, pH maintained at 7.3, with 100 U/mL penicillin, 100 unit/mL streptomycin sulphate, and 10 % foetal calf serum. The culture was maintained under an atmosphere of 5 % CO_2 at 37 °C [16]. The cells were subcultured every 2-3 days before the cells reached 80% confluency using 0.25 % trypsin–EDTA, and the medium was renewed after an overnight incubation.

Sulforhodamine B assay

A 96-well culture plate was used to seed the cultured CCA cells at a density of 5×10^3 cells/well. After overnight culture, the cells were pre-treated with ascorbic acid (0.5 - 100 mM) for 24 h. Cell proliferation was determined by *sulforhodamine B (*SRB) colorimetric assay. Briefly, the cells were washed with phosphate buffer saline (PBS) and fixed with 10 % (w/v) trichloroacetic acid and *stained* with 0.4 % SRB in 1 % acetic acid for 30 min. The cells were washed with 1 % (v/v) acetic acid to remove excess dye. The protein-bound dye was dissolved in 10 mM Tris base solution (pH 10.5) and incubated for 20 min. A microreader plate was used to determine the absorbance. Cell growth inhibition was expressed in terms of percentage of untreated control absorbance following subtraction of mean background absorbance. The IC_{50} concentration (50% inhibition of cell growth) was calculated from the dose-response curves. The experiment was performed in triplicates.

Measurement of glutathione (GSH) and hydrogen peroxide (H_2O_2) contents

The cells were treated with 50 mM ascorbic acid for 24 h. Glutathione (GSH) and H_2O_2 activities were assessed with GSH assay kit (Item No. 703002, Cayman Chemical Company) and H_2O_2 assay kit (Item No. ab102500, *Abcam*, Cambridge, UK).

Measurement of mitochondrial transmembrane potential

The dissipation of mitochondrial transmembrane potential leads to an early apoptotic event. To measure the change in $\Delta\Psi_m$, 96 black well plate cell culture, at a density of 10,000 cells/well, was maintained overnight. Then the culture was treated with 50 mM ascorbic acid for 6 h. The assay followed the previously described procedure [17], using the cationic, lipophilic dye, 5,5′,6,′-tetrachloro-1,1′,3,3′ tetraethyl-benzimidazolyl carbocyanine iodide (JC-1) (Clayman Chemical) staining with few modifications. The cultured plate was centrifuged at 1,000 rpm for 5 min at room temperature. The cultured medium was then washed off and loaded with JC-1 dye and left for 20 min for staining. The excess stain was washed by centrifugation, and the culture was again incubated in the assay buffer. Fluorescent microscope was used to analyse $\Delta\Psi_m$ with an emission wavelength of 535 nm and

an excitation wavelength of 485 nm. Red fluorescence was observed, which signified J-aggregates formed by JC-1 present in a healthy mitochondrial matrix. In the case of depolarised mitochondria, green fluorescence was observed due to efflux of JC-1 into the cytoplasm, where it exists in the monomeric form. If the fluorescence shifted from red to green, this indicated depolarisation of $\Delta\Psi_m$.

Western blot analysis of BAX and Bcl_2

Cells were treated with 50 mM ascorbic acid for 24 h. Then the cells were washed with PBS, lysed with RIPA buffer [150 mMNaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mMTris–HCl (pH 7.4), 50 mM glycerophosphate, 20 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4] with the addition of protease inhibitor cocktail (M221: Amresco, OH, USA). Charged separation of the protein samples was done by SDS-PAGE gel electrophoresis (with 8 – 10 % SDS polyacrylamide gel). This electrophoresis process was followed by western blotting. The obtained bands were transferred onto a PVDF membrane. The membranes were blocked with 5 % (w/v) skimmed milk powder in Tris-buffered saline (TBS) containing 0.1 % Tween-20 at room temperature for 1 h. The PVDF membranes were incubated with primary antibody in PBS at 4 °C overnight. After washing with PBS, the blots were incubated with the HRP-conjugated secondary antibodies at room temperature for 1 h. After washing away the unbound antibodies, the blots were incubated in ECL substrate solution (Super Signal West Pico Chemiluminescent Substrate: ThermoScientific, IL, USA). The densities of the specific protein bands were visualised and captured by Image Quant™ 400 (GE HealthCare).

Statistical analysis

Data are presented as mean ± SD. Analysis of variance (ANOVA) was used to determine significant differences between each experimental group. An ANOVA on-rank test was also performed for the non-parametric test. The level of significance was set at $p < 0.05$ using SigmaStat software version 4 (Systat Software, Inc. California, USA).

RESULTS

Effect of ascorbic acid on cancer cell proliferation

The effect of ascorbic acid on cell proliferation was evaluated by SRB assay in K100 CCA cell line (Figure 1). Based on these measurements, the IC_{50} value of ascorbic acid on K100 at 24 h was determined to be 55 ± 9.4 mM. The antiproliferation effect of ascorbic acid depended on dose and time. These results confirmed the anti-proliferative effects of ascorbic acid on CCA cell line.

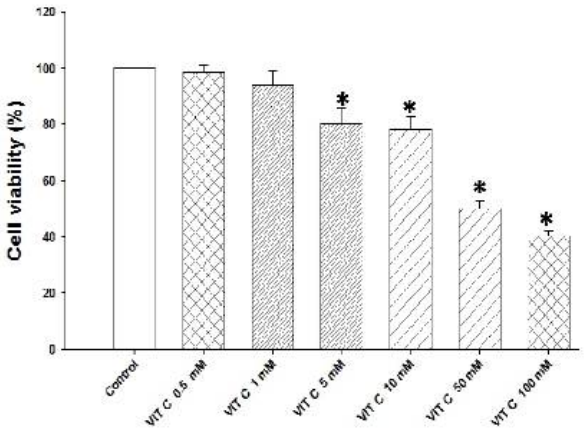

Figure 1: Effect of ascorbic acid on cancer cell proliferation. K100 cells were treated with varying concentrations of ascorbic acid, ranging from 0.5 to 100 mM, for 24 h. The analysis was done by sulforhodamine B assay. Each bar represents the mean ± SD (n = 3)

Effect of ascorbic acid on GSH and H_2O_2 contents

The basal GSH content was decreased (Figure 2), whereas the H_2O_2 content in cell was increased in cells treated with ascorbic acid (VIT C) compared with control ($p < 0.05$) (Figure 3).

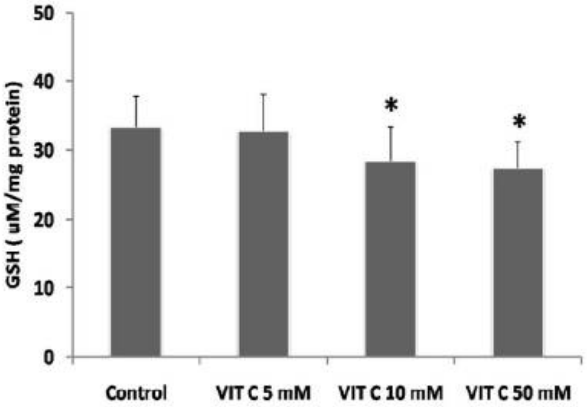

Figure 2: Effect of ascorbic acid on **GSH** content in CCA. K100 cells were treated with varying concentrations of ascorbic acid, ranging from 5 to 50 mM, for 24 h. Values are mean ± SD (n = 3). *Significantly different compared with control ($p < 0.05$)

Effect of ascorbic acid on dissipation of mitochondrial membrane potential

Due to the accumulation of J-aggregates in the

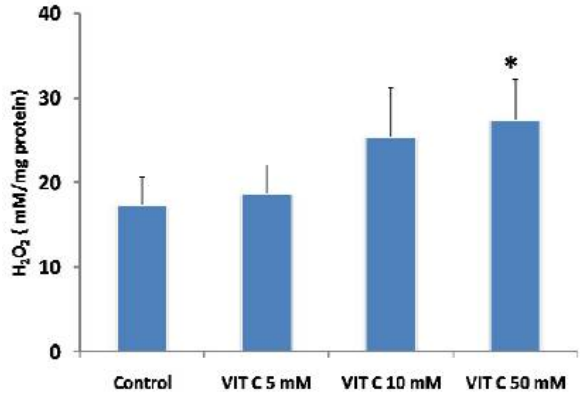

Figure 3: Effect of ascorbic acid on H_2O_2 content in CCA. K100 cells were treated with varying concentrations of ascorbic acid (range, 5-50 mM) for 24 h. Values are mean ± SD (n = 3). *Significantly different compared with control ($p < 0.05$)

untreated control cells, mitochondria exhibited red fluorescence, representing intact $\Delta\Psi_m$. Treatment with ascorbic acid led to the rapid depolarisation of $\Delta\Psi_m$, which was marked by green fluorescence of JC-1 monomeric forms presented in the cytosol (Figure 4). On using 5-50 mM concentrations of ascorbic acid, a disturbance in the mitochondrial membrane potential (□Ψm) was detected in ascorbic acid-treated cells, which was a dose-dependent phenomenon.

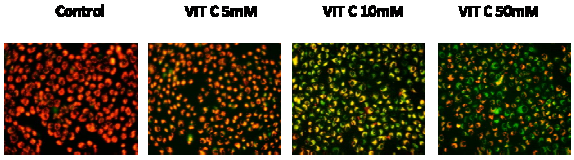

Figure 4: Effect of ascorbic acid on mitochondrial membrane potential. K100 was treated with THC for 6 h. $\Delta\Psi_m$ change was examined using JC-1 staining method. Fluorescence microscopy was performed with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The experiments were performed in duplicate

Expression of Bcl2 and BAX

The pro-apoptotic activity of ascorbic acid is attributed to its ability to modulate the expression of Bcl2 and Bax proteins. In the present study, there was a dramatic increase in the expression of Bax protein, whereas a significant decrease in synthesis of Bcl2 protein was marked when the cells were treated with ascorbic acid. The ratio of Bcl2/Bax is shown in Figure 5.

DISCUSSION

We observed that the treatment with ascorbic acid resulted in a reduction of GSH and increased H_2O_2 contents in cells. Although the mechanism and physiological relevance remain to be fully understood, the presence of ascorbic acid leads to the production of H_2O_2 [13,15]. It is well known that H_2O_2 is involved in the redox control of several physiological processes including cell proliferation and apoptosis [13,18].

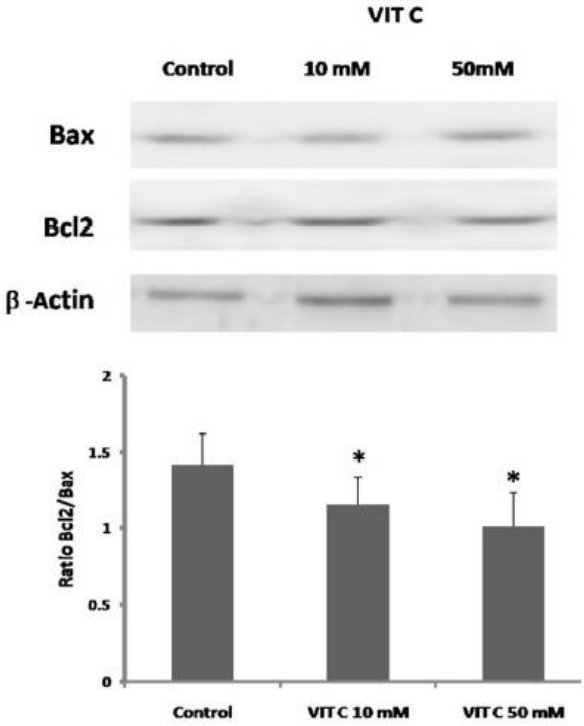

Figure 5: Expression *of BAX and Bcl2 in CCA.* The images shown are representative of experiments with similar results. Each bar represents mean ± SD ($n = 3$; *$p < 0.05$ compared with control)

Since ascorbic acid produced H_2O_2 and causes the reduction of GSH, this imbalance of redox state may disrupt the membrane potential of mitochondria present inside the cells. In our study, we found that disturbance of the mitochondrial membrane potential (ΔΨm) was detectable in ascorbic acid-treated cells. The ΔΨm is maintained by the respiratory chain complexes to generate the high energy ATP. Disturbance of the ΔΨm results in cellular energy crisis subsequently led to cell death [19]. A small change in mitochondrial permeability transition (MPT) could depolarise the mitochondria, whereas the increasing number of MPT due to immense ROS, for instance, can lead to necrosis and apoptosis [19]. Ascorbic acid has been shown to disturb the integrity of ΔΨm by a mechanism associated with reduced GSH and increased H_2O_2 content in cells.

Apoptosis is regulated and executed by different interplays of many genes responsive to various stimuli. The major apoptotic regulatory genes are Bcl-2 and p53 [20]. The p53 gene induces

apoptosis by direct or indirect modulation of the expressions of Bcl-2 family proteins (Bax and Bcl-2) [21]. The Bcl-2 proteins play a crucial role in controlling apoptosis through the regulation of permeability of the mitochondrial membrane. Bax is a pro-apoptotic protein found in the cytoplasm. Along with other pro-apoptotic proteins, Bax protein promotes apoptosis through the formation of homodimeric and heterodimeric complexes. This results in the formation of channels in the mitochondrial membrane, facilitating the release of cytochrome c and apoptosis-inducing factors (AIFs) into the cytoplasm. In contrast, the anti-apoptotic Bcl-2 protein localises the outer mitochondrial membrane and mediates its anti-apoptotic effects by stabilising the integrity of the mitochondrial membrane [20-23]. In this study, we also found that the pro-apoptotic effect of ascorbic acid is attributed to its ability to modulate the expression of Bcl2 and Bax proteins.

CONCLUSION

In conclusion, our results showed that the mechanism of anti-proliferation effect of ascorbic acid in CCA cells may be attributable to the modulatory expression of Bcl-2 and Bax proteins and dissipation of the mitochondrial electrochemical potential gradient, which is an early stage of cell death.

DECLARATIONS

Acknowledgement

This work was supported by a grant (no. TP 2/41/2017) from the Thammasat University, Thailand.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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