INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 11, No. 4, p. 359-364, 2017

RESEARCH PAPER OPEN ACCESS

Vitamin C retards the growth and motility of liver cancer cells huh 7 in wound healing assay

Muhammad Imran Raza, Sara Hassan, Wasifa Naushad, Hajra Sadia*

Department of Healthcare Biotechnology, Atta-Ur-Rahman, School of Applied Biosciences, National University of Sciences and Technology, Islamabad, Pakistan

Key words: Liver cancer cells, Vitamin C, Wound healing assay

http://dx.doi.org/10.12692/ijb/11.4.359-364 Article published on October 30, 2017

Abstract

Vitamin C has long been discussed as having anticancer potential but with little evidence on mechanism of action. Some clinical studies have been performed but with no conclusive results. Researchers have been calling for more studies. The aim of study was to investigate the anti-cancer role of high dose Vitamin C on growth and motility of Liver cancer cells Huh 7 in wound healing Assay. Huh-7 cells were cultured in complete medium (DMEM) at 37°C with 5% CO_2. For experimental study design, Huh 7 cells were divided in to two groups as: cells treated with Vitamin C and cell without Vitamin C treatment. To assess the viability of cancer cells upon vitamin C treatment, MTT assay was done. For growth kinetics, cells were plated in a 6 well plate and counted using haematocytometer at different time points. Cancer cell motility was assessed thorough two-dimensional migration wound healing assay. Statistical analysis was done using Graph-pad prism software. Vitamin C treatment inhibits the growth and motility of liver cancer cells. Percentage of wound closure in untreated is high as compared to Vitamin C treated cells which shows Vitamin C significantly retards the growth of Cancer cells ($P<0.05$). Vitamin C inhibits growth and motility of liver Cancer cells Huh-7 in wound healing assay.

* **Corresponding Author:** Hajra Sadia ✉ hajrasadia@gmail.com

Introduction

Hepatocellular carcinoma (HCC) is the most prevalent liver cancer and the third leading cause of deaths worldwide. Etiological agents of HCC include alcohol consumption, hepatitis B virus, Hepatitis C virus and dietary aflatoxins. Annually 560,000 cases of HCC are diagnosed and reported out of which the mortality rate is 550,000 (Abdel-Hamid *et al.,* 2011). With the mortality rate being so high and unavailability of effective treatment for HCC there is a dire need for the development a novel drug which can be a standalone drug or as combinatorial therapy that exhibits a promising anticancer activity with the added benefit of low cytotoxicity towards normal cells (McGlynn and London, 2005).

Presently the paradigm of medicine has shifted towards complementary and alternative medicine (CAM) and it is getting global attention. In CAM, many herbs and vitamins are used as therapeutic interventions (Levine *et al.,* 1996). Vitamin C is an essential micronutrient administered as a dietary supplement with a wide range of significant biological functions. Its prescribed RDA varies from 60mg/day in the US and 30mg/day in Britain. Vitamin C has the lowest toxicity in comparison to all the vitamins (Graumlich *et al.,* 1997). Moreover, when administered intravenously in high dose vitamin C has exhibited anticancer effects, hence it can be considered as a promising and feasible option for anticancer therapy and cancer prevention. It has been previously reported that oral consumption of Vitamin C makes it function as a nutrient and keeps the serum level of vitamin C low i.e. under 100µM whereas intravenous administration of high-dose vitamin C make it act as an anticancer drug (Campbell *et al.,* 1991; Cameron and Pauling, 1976).

Wound healing Assay is an easy to apply, cost effective, well developed assay designed to study cell migration, growth and proliferation in cell culture. The basic principle underlying this assay is to administer a scratch in the cell monolayer of the cells, seeded in multi-well plates, using a micropipette tip or syringe and subsequently monitoring the extent of cell growth and migration prior to exposure to the test drug- which in the case of this study is Vitamin C. The growth and migration is monitored in set intervals by taking pictures or by using time lapse microscopy (Liang *et al.,* 2007).

The objective of this study is to ascertain whether Vitamin C holds the potential to retard the migration and growth of hepatocellular carcinoma cells and therefore establishing its role as a potential candidate for anticancer therapy. The mechanism by which Vitamin C retards the growth and progression of cancer cells has not been elucidated yet and further work needs to be done in this regard.

Materials and methods

Study design

The study was designed to investigate experimentally the effect of Vitamin C (2mM) dose on the growth and motility of liver cancer cells in two separate groups. Untreated group was not treated with vitamin C and treated group was treated with 2mM dose of Vitamin C. For both groups, equal number of cells (250,000 cells/well) were used. Results were analysed as difference in the motility and growth of untreated and Vitamin C treated groups and statistical analysis was done using Graph pad prism.

Study Location

This study was conducted in cell culture Lab of Atta- ur-Rahman School of applied Biosciences, National University of Sciences and Technology (NUST) over the period of Six months from September 2016 to Feb 2017.

Cell line and media

Cryopreserved liver cancer cell line Huh 7 was taken from ASAB cell culture Bank. Cells were revived and cultured in DMEM under standard conditions and were kept in CO2 incubator. Huh 7 cells were maintained in their respective complete medium according to ATCC and NIH NIAID AIDS reagents guidelines. Complete medium (DMEM) was used for growing cells contains 10% fetal bovine serum (FBS).

Assessment of cancer cell viability

Equivalent numbers of Huh 7 cells with and without Vitamin C treatments were seeded in 96-well plates.

After 24 hours, cells were incubated with 20µl/well of 5mg/ml MTT reagent for 3.5 hours followed by removal of media, addition of 150µl/well of MTT solvent (0.1% NP-40 and 4 mM HCl in isopropanol) and rocking for 15 minutes. Absorbance was read at 590nm) to analyze viability.

Evaluation of cancer cell proliferation

Equivalent numbers (500,000) of Huh 7 cells with and without Vitamin C treatment were seeded in 6-well plates for 24, 48 and 96 hours. After each time point, cells were trypsinized and counted using hemocytometer. For each time point and treatment, cells were counted in triplicates. The formula used for counting was as follow:

$$\textbf{Total number of cell} = \left(\textit{total cells counted } x \frac{\textit{dilution factor}}{\textit{\# of squares counted}} x\ 10{,}000\right)$$

Migration (wound healing) assay

Confluent monolayers of Huh 7 cells (250,00 cells/well) were seeded in 6-well plates to form perfect confluent monolayer. The monolayers were scratched using a pipette tip (P200).

Fresh medium with and without 2Mm Vitamin C, was added to the wells and cells were allowed to migrate for the indicated time. Cells were imaged with Nikon Eclipse Ti microscope adjusted with a Nikon digital sight camera. Images were processed and cell migration was analyzed according to the relative wound closure (width of the wound) at different time points.

Statistics

Statistical analysis of significant differences was performed with unpaired t test assuming Gaussian distribution with Welch's correction (GraphPad Prism software). Error bars represent standard error of the mean (SEM). A probability (P) value of 0.05 or lower was considered significant.

Results

Cancer progress with unlimited proliferation and migration of cancer cells. Here, we confirm vitamin C treatment abrogates the proliferation (cell counting using hemocytometer) and motility of liver cancer cells in a two-dimensional migration assay (wound healing). Vitamin C treatment abrogates the growth of liver cancer cells by slowing down the growth of cell (Fig. 1a) at 24, 48, 72 and 96 hours but does not affect the viability of liver cancer cells (Fig. 1b). Untreated and treated cells (250,000 cells per treatment) at 0 hour were plated in 6 well plate and cells were allowed to grow up to 24, 48, 72 and 96 hours and counted at every time point and replated; MTT assay was done at each time point to check the viability of cancer cells. Untreated cells showed a significant increase (p-value 0.034) in the proliferation (number of cells/well) whereas cells treated with Vitamin C showed a non-significant increase (p-value 0.09328) in the number of cells at different time points. The results show that vitamin C abrogates the proliferation of cancer cells at a significant rate (p-value 0.0001) after 96 hours.

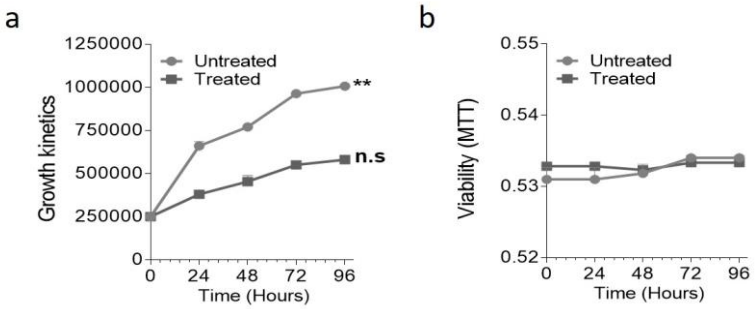

Fig. 1. Effect of Vitamin C treatment on the growth kinetics and viability of Huh 7 cells over a period of 0h to 96h. (a) Assessment of growth/proliferation of liver cancer cells with and without Vitamin C treatment at 0, 24, 48, 72 and 96 hours**.** (b) MTT metabolism assay for the viability of Vitamin C treated and untreated Huh 7 cells at 0, 24, 48, 72 and 96 hours. Error bars represent standard deviations. Significance was taken at P <0.01** and P <0.001***. n.s = not significant. Experiments were repeated more than three times with similar results.

We also confirmed that untreated liver cancer cells showed a progressive wound closure at different time points (0, 8 and 24h), but the less wound closure in vitamin C treated cells (Fig. 2). The width between the two black lines for each treatment was measured at 0 hr, 8 hr and 24 hr. Wound area for time 0 hr was taken as 100% and percentage of wound closure was calculated for 8 hr and for 24hr. Then percentage of wound closure between treated and untreated were compared. Our results showed that liver cells without Vitamin C treatment shows 100, 75 and 50% wound width at 0, 8 and 24 hours but very minimal wound closure in Vitamin C treated cancer cells as 100, 93 and 89% wound width. Our data suggests a significant wound closure at 8 hours (p-value 0.0010) and 24 hour (p-value 0.00023) for untreated and treated huh 7 cells.

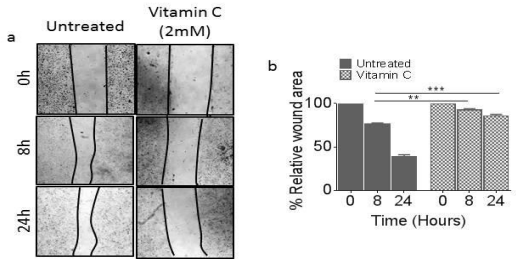

Fig. 2. Vitamin C broadly inhibits the motility of liver cancer cells Huh-7 cells via wound healing assay. a & b) Representative images and graphical representation of wound closure for Huh-7 untreated and Vitamin C treated Huh-7 cells (triplicates) at 0, 8 and 24 hours. Images were taken at 4X magnification. Black lines on images depict wound area. Error bars represent standard deviations. Significance was taken at $P<0.05^{*}$, $P<0.01^{**}$, $P<0.001^{***}$, and $P<0.0001^{****}$. n.s = not significant. Experiments were repeated more than three time with similar results.

Discussion

High dose Vitamin C in cancer treatment is being debated for a long time (Unlu *et al.,* 2016) but there is a little evidence how does Vitamin C acts against cancer cells. In recent days, there is an increased interest in studying Vitamin C as an adjunct treatment with chemotherapy (Hoffer *et al.,* 2015; Fritz *et al.,* 2014). Some studies have explored that Vitamin C Induced Ascorbate radical produces hydrogen peroxide which is toxic towards Cancer cells (Jacobs *et al.,* 2015; Stephenson *et al.,* 2013).

Chen *et al.* presented some data which was indicative of increased sensitivity of some cancer cell lines towards high dose concentration of Vitamin C. It was also postulated that cytotoxic effect might be due to production of hydrogen peroxide which is selectively toxic to cancer cells but not to normal cells (Chen *et al.,* 2008). Raza *et al* reported that cytotoxic effects of high dose Vitamin C is due to apoptosis caused by up regulation of TNFα in Liver cancer cells. High dose Vitamin C in Liver cancer cells shuts down the inflammatory pathway and shifts the pathway towards apoptotic pathway (Raza *et al.,* 2016).

Some studies have shown that Vitamin C inhibits the activation of NF-κB by interacting with a factor called NF-κB inducing kinase (NIK), NIK is a factor involved in the activation of NF-κB. Several strategies are being researched to block NIK to block activation of NF-κB (Carcamo *et al.,* 2002). Several clinical studies have been conducted in order to assess the safety of high dose Vitamin C in cancer patients and it was found that high dose Vitamin C is safe in majority of patients if the right protocol is used (Hoffer *et al.,* 2015). Proliferation and motility is considered as the important hallmarks of cancer. Liver cancer progress through the uncontrolled proliferation and migration of liver cells that leads to liver cancer development, progression of disease and metastasis. Our current findings from cell counting and MTT assay suggests that Vitamin C treatment significantly slows down the growth of liver cancer cells without having an effect on the viability of growing cells.

Wound healing assay is a very cost effective in vitro technique to assess the anti-cancer potential of a drug. In this study, we have implied wound healing assay to assess the anti- cancer and growth retarding effect of high dose Vitamin C on Huh 7 & liver cancer cells. As wound healing assay is based on the fact that any drug which has anti-cancer potential may slow down the motility of cancer cells. In our study the wound produced was observed after treatment with Vitamin C and the wound area was compared in Vitamin C treated cells with untreated controls. The percentage wound closure was significantly higher in untreated cells at 8 hours and 24 hours.

These results suggests that Vitamin C had slowed down the motility of Liver cancer cells. The growing evidences regarding the role of high dose Vitamin C in treatment of cancer is suggestive of further comprehensive clinical studies.

Conclusion

The nature, identity, and mechanism of action of the pro- and anti-motility factors of BST-2 are yet to be determined. However, experimental insights show that the pro-motility factors secreted by cells expressing wild type BST-2 possess proteolytic activity given the increased cell invasion in cells conditioned with the secretome.

Conflict of interest

The authors declared that no financial or any other conflict of interest exist.

Acknowledgements

We are thankful to higher education commission, Pakistan for research grant.

References

Abdel-Hamid NM, Nazmy MH, Mahmoud AW, Fawzy MA, Youssof M. 2011. A survey on herbal management of hepatocellular carcinoma. World Journal of Hepatology **3,** 175-83.

Cameron E, Pauling L. 1976. Supplemental ascorbate in the supportive treatment of cancer: Prolongation of survival times in terminal human cancer. PNAS **73,** 3685-9.

Campbell A, Jack T, Cameron E. 1991. Reticulum Cell Sarcoma: Two Complete 'Spontaneous' Regressions, in Response to High-Dose Ascorbic Acid Therapy. Oncology **48,** 495-7.

Carcamo JM, Pedraza A, Borquez-Ojeda O, Golde DW. 2002. Vitamin C Suppresses TNFα Induced NFκB Activation by Inhibiting Iκβα Phosphorylation. Biochemistry **41,** 12995-13002.

Chen Q, Espey MG, Sun AY, Pooput C, Kirk KL, Krishna MC, Khosh DB, Drisko J, Levine M. 2008. Pharmacologic doses of ascorbate act as a pro oxidant and decrease growth of aggressive tumor xenografts in mice. PNAS **105,** 11105-11109.

Fritz H, Flower G, Weeks L, Cooley K, Callachan M, McGowan J. 2014. Intravenous vitamin C and cancer: a systematic review. Integrative Cancer Therapies **13,** 280-300.

Graumlich JF, Ludden TM, Conry-Cantilena C, Cantilena LR, Wang Y, Levine M. 1997. Pharmacokinetic model of ascorbic acid in healthy male volunteers during depletion and repletion. Pharmaceutical Research **14,** 1133-9.

Hoffer LJ, Robitaille L, Zakarian R, Melnychuk D, Kavan P, Agulnik J. 2015. High-dose intravenous vitamin C combined with cytotoxic chemotherapy in patients with advanced cancer: a phase I-II clinical trial. PloS One **10,** e0120228.

Jacobs C, Hutton B, Ng T, Shorr R, Clemons M. 2015. Is there a role for oral or intravenous ascorbate (vitamin C) in treating patients with cancer? A systematic review. Oncologist **20,** 210-23.

Levine M, Conry-Cantilena C, Wang Y, Welch RW, Washko PW, Dhariwal KR. 1996. Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. PNAS **93,** 3704-9.

Liang CC, Park AY, Guan JL. 2007. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nature Protocols **2,** 329-33.

Mc Glynn KA, London WT. 2005. Epidemiology and natural history of hepatocellular carcinoma. Best Practice & Research Clinical Gastroenterology **19,** 3-23.

Raza MI, Sikandar A, Sadia A. 2016. Pharmacological Ascorbate triggers apoptotic pathway in Liver cancer cells via up regulation of TNF-α. International Journal of Biosciences **9,** 253-258.

Stephenson CM, Levin RD, Spector T, Lis CG. 2013. Phase I clinical trial to evaluate the safety, tolerability, and pharmacokinetics of high-dose intravenous ascorbic acid in patients with advanced cancer. Cancer Chemotherapy and Pharmacology **72,** 139-46.

Unlu A, Kirca O, Ozdogan M, Nayır E. 2016. High-dose vitamin C and cancer. Journal of Oncological Sciences **1,** 10-2.