Determination of Anticancer Mechanism of Ascorbic Acid in Huh7 Cell Line

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To...

My Mentor,

Prof. Dr. Abid Nisar (Late)

Thanks for always believing in me...

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Abeera Sikandar

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Hepatocellular carcinoma is the fifth most common cancer and third major cause of death worldwide. Vitamin C is known to have tumor preventive and anti-tumor effect but its anti-cancer mechanism still needs to be determined. In the present study anti-tumor effect of Vitamin C through inflammatory pathway has been evaluated. Expression of inflammatory genes was determined through qPCR after 24 hour exposure to Vitamin C. EC_{50} of Vitamin C for Huh-7 was also determined using MTT cell viability assay, which was 2.17 mM. Expression profiling showed that expression of TNFα, a cytokine involved in apoptotic as well as proliferative and inflammatory pathway, was increased whereas the expression of NF-κB, a transcription factor involved in shifting TNF-α from apoptotic to inflammatory pathway, and Cox-2, an inflammatory and cancer biomarker, was decreased. The study showed that Vitamin C induced TNFα mediated apoptosis in Huh7 cells by shifting TNFα from inflammatory to apoptotic pathway. This study can prove to be helpful in determining new highly effective anti-cancer therapy with lesser side effects.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and third leading cause of deaths worldwide. HCC accounts for major mortalities worldwide however its worldwide distribution is quite variable. 80 % of disease burden of HCC is limited to countries in Asia and sub-Saharan Africa. Mostly, in high risk countries, major causes of the disease are HBV and dietary aflatoxins, whereas in low risk countries, major causes include HCV and alcohol consumption. Worldwide approximately 560,000 cases are diagnosed each year and 550,000 deaths due to liver cancer occur. In almost every geographic region, incidence of HCC is 2 to 4 times higher in men than that in women (McGlynn *et al.*, 2005). In Pakistan, HCC is a leading cause of death and accounts for 60%-90% of all primary liver malignancies. The symptoms of HCC include weight loss, blood in the stool, jaundice, fatigue and fluid in abdomen (Ogunbiyi *et al.*, 2001).

Development of HCC is a slow and gradual process during which several genetic and epigenetic changes accumulate in hepatocytes leading to increased expression of mitogens which ultimately cause increased proliferation. HCC can be traced back to hepatotropic viruses including Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) induced inflammation, aflatoxins, alcohol consumption and toxic chemicals. The inflammation leads to liver damage and cirrhosis which ultimately develops into HCC. During the prolonged pre-neoplastic stage, liver is suffering with hepatitis or cirrhosis or both. The upregulated hepatocyte cycling leads to formation of a monoclonal population of aberrant and dysplastic cells which express telomeric erosion, increased telomerase expression, microsatellite instability and genetic and chromosomal abnormalities. The production of pre-neoplastic cells may be the result of disruption in structure and function

of a number of genes involved in cell cycle regulatory pathways. These changes significantly alter microenvironment and matrix of liver. Genetic alterations are manifested in phenotypic alterations of the cells. Foci of phenotypically altered hepatocytes appear which subsequently replaced by foci and nodules of dysplastic cells. (Thorgeirsson and Grisham, 2002; Farazi *et al.,* 2006)

Hepatitis B Virus affects about 2 billion people around the world and cause estimated 320,000 deaths per annum. Approximately 30-50% of HBV-associated deaths are because of HCC (Lavanchy *et al*., 2004). The correlation between increasing incidence of HCC in patients with high levels of HBV DNA in serum shows the strength of relationship between HCC and HBV (Chen *et al*., 2006). Hepatitis C Virus infects about 170 million people globally (Chisari *et al*., 2005). About 20% of chronic HCV infections lead to cirrhosis, and 2.5% to HCC (Boven *et al*., 2005). The virus related mechanism of HCC development is complex and involves both viral and host factors (Farazi *et al*., 2006).

Alcohol consumption is one of the major causes of HCC.Chronic alcohol consumption is associated with increased production of pro-inflammatory cytokines through monocyte activation (McClain *et al*., 2002) and inducing increased concentration of circulating endotoxin which in turn activate Kupffer cells to produce many chemokines and cytokines (including TNFα, interleukin-1β (IL1β), IL6 and prostaglandin E2, thus causing adverse effects on hepatocytes. Chronic ethanol exposure make hepatocytes more sensitive to TNF-α induced cytotoxicity (Hoek *et al*., 2002) thus setting the grounds for chronic hepatocyte damage, regeneration, cirrhosis and thus ultimately HCC. There is a possibility that HCC during HCV and HBV may result because of high cell replication rate in an attempt to replace the cells infected by virus (Block *et al*., 2003).

Other causes of HCC include iron overload, non-alcoholic fatty liver disease, schistosomal co-infection, potentially hepatotoxic medications and environmental contaminants may also have important effects (Farazi *et al.*, 2006). Several types of inflammations can cause initiation and promotion of cancer. Infections like hepatitis B or C and liver damage due to alcoholism cause prolonged activation of immune cells which lead to chronic inflammation. Chronic inflammation provide suitable microenvironment for the development of HCC (Karin *et al.*, 2006; Wu *et al.*, 2009; Mantovani *et al.*, 2008). In 90 percent of cancers, environmental factors play a crucial role of which inflammatory microenvironment is one of the important factor. 25% of all the cancer cases are caused due to inflammation (Aggrawal *et al*., 2009). Inflammation play a crucial part in initiation, promotion and metastatic steps of the tumorigenic process (Grivennikov *et al.*, 2010). Fig. 1.1.shows the schematic diagram explaining causative agents and their mechanism of development of HCC.

The abnormal expression of any factor involved in control of inflammation leads to altered expression of cancer related genes and genes involved in apoptosis and cell proliferation. There is also evidence that inflammatory cells and immune modulators promote tumor development and metastasis (Eiro *et al*., 2012). Inflammatory cells are believed to enhance tumorigenesis by virtue of non-specific pro-inflammatory cytokines including interferon gamma, tumor necrosis factor (TNF), interleukin(IL)-1, alpha, beta, and IL-6 (Aggarwal *et al*., 2006; Robinson *et al*., 2005).

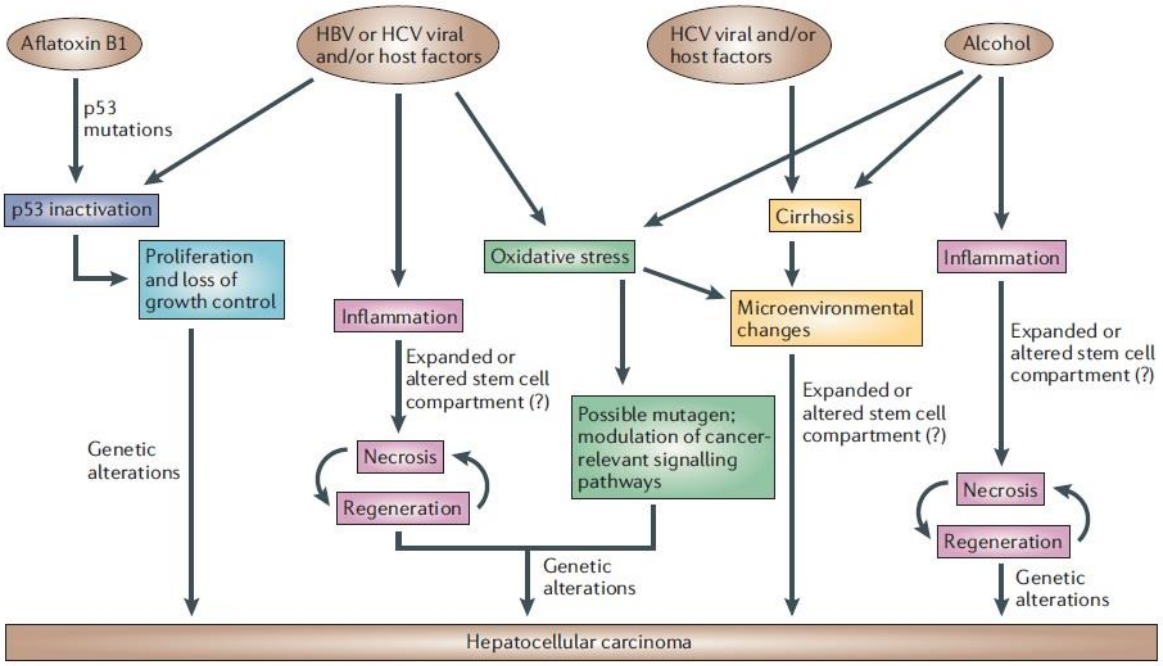


Figure 1.1. Pathogenesis of HCC. The figure shows the factors that lead to development of HCC and their respective mechanisms. Most of the causative agents of HCC cause inflammation, liver damage and cirrhosis which ultimately lead to HCC (Adapted from Farazi *et al.*, 2006).

Activated nuclear factor-kappa B (NF-kB) is a transcription factor that initiates transcription of cell proliferative and anti-apoptotic genes, and is believed to be one of the main factor involved in inflammation related tumorigenesis. All of these factors collectively act as initiators of proliferation and inhibitors of apoptosis and thus promote tumorigenesis.

1.1 Tumor Necrosis Factor- alpha (TNF-α) and its Dual Role

Tumor necrosis factor is a cytokine which is involved in a number of factors including apoptosis, cell survival, inflammation and immunity. TNF- has been postulated to have a crucial role in the pathogenesis of cancers. It is one of the most important pro-inflammatory cytokines involved in the growth, differentiation, cellular function and survival of many cells (Yang *et al.,* 2011). It is currently used in the treatment of local advanced and metastatic malignancies. TNF-α binds with TNF receptor 1 and 2 (TNFR-1, TNFR-2) and activates many signal transduction pathways which play role in a number of cellular functions. Several laboratory studies have been carried out in the past decade to understand the antitumor mechanism of TNF-α. It has been observed that TNF-α attacks tumor associated vasculature (TAV) and induce hyper-permeability and destroys vascular lining. Thus results in selective accumulation of antitumor drugs in tumor sites, and ultimately its destruction (Horssen *et al*., 2006).

Tumor necrosis factor-alpha is mainly produced by activated macrophages, T-lymphocytes and natural killer cells (NK cells). Low level of expression is due to fibroblasts, smooth muscle and tumor cells. TNFα acts by binding to TNF receptor 1 (Vandenabeele *et al*., 1995). TNFR1 is an important member of death receptor family that plays part in inducing apoptotic cell death (Ashkenazi *et al*., 1998). TNFR1 is also widely studied due to its dual role. In addition to the induction of apoptosis, it also have an ability to introduce cell survival signals. Binding with TNFα causes TNFR1 to trimerize and release silencer of the death domain protein (SODD) (Takada *et al*., 2003). TNFR-associated death domain (TRADD) is then recruited to DD of TNFR-1 and binds the adaptor proteins receptor interacting protein (RIP), TNFR-associated factor-2 (TRAF-2)

and Fas-associated death domain (FADD) (Rath *et al*., 1999). These adaptor molecules then activate key molecules that carry out intracellular signaling. During signaling of apoptosis, TNFR1 activates FADD which in turn recruits and activates pro-caspase-8 thus initiating a cascade of events leading to apoptosis regulated by caspases, also including mitochondria in the process. Ultimately the caspase signaling cascade activates endonucleases including EndoG, thus causing DNA fragmentation (Degterev *et al*., 2003).

When TNFα signals survival, TRAF-2 is recruited which initiates a pathway of phosphorylation thus ultimately activating cFos/cJun transcription factors through mitogen-activated protein kinase (MAPK) and cJun N-terminal kinase (JNK) (Natoli *et al*., 1997).

NF-kB is activated when TNFα is signaling survival. NF-kB once activated signals TNFα to stop apoptosis pathway (Chu *et al*., 1997). Both NF-kB and cFos/cJun transcription factors activate transcription of anti-apoptotic, proliferative, immunomodulatory and inflammatory genes. NF-kB is major survival factor which inhibits TNFα induced apoptosis. Inhibition of NF-kB is expected to improve efficiency of apoptotic inducing treatments against cancer (Beg *et al*., 1996). NF-kB expression is abnormal or constituent in many malignancies and is known to have a role in tumorigenesis (Dolcet *et al*., 2005; Lin *et al*., 2003).

1.2 Role of NF-κB in HCC

Studies have shown that NF-kB is constitutively expressed in fibrolamellar HCC (Li *et al*., 2010). The studies have shown that most of the factors involved in tumor progression are controlled by NF-kB, a cytoplasmic transcription factor which is controlled

by many cytokines (Carr *et al*, 2014). It is transferred to nucleus when activated and initiates transcription of crucial genes involved in tumor progression and inhibition of apoptosis. Evidence suggest that NF-kB also have role in cancer stem-cell survival (Guzman *et al*., 2007). It up-regulates expression of anti-apoptotic genes including bcl2 like 1 (bcl-x1), B-cell lymphoma 2 (bcl-2), x-linked inhibitor of apoptosis protein, cellular FLICE-inhibitory protein, inhibitor of apoptosis-1 (IZP)-1 and IAP-2, and also proliferative genes including cyclin D1, cmyc and Cox-2. Recent studies have shown NF-kB to be helpful in diagnosis, prognosis of cancer in patients. It provides a very suitable microenvironment for new tumors for proliferation, angiogenesis and metastasis (Dobrovolskaia *et al*., 2005; Karin *et al*., 2002; Luo *et al*., 2005). It is also been targeted in anticancer treatment (Aggarwal *et al*., 2009). NF-kB is activated in majority of tumors as a result of signaling of the neighboring pro-malignant cells (Grivennikov and Karin, 2010; Yu *et al*, 2009). Li *et al*., in 2010 reported its constitutive expression in fibrolamellar HCC (Li *et al*., 2010).

1.3 Role of Cyclo-oxygenase 2 in HCC

Cyclo-oxygenase 2 (COX-2) has also been proposed as a target for anticancer treatment (Xu *et al*., 2002). COX-2 is inducible enzyme which produces prostaglandins in inflammatory and tumor conditions (Kujubu *et al*., 1991; Hla *et al*., 1992). It has been found to be associated with tumorigenesis of many tumors including colon carcinoma, colorectal carcinoma, gastric carcinoma and squamous cell carcinoma (Eberhar *et al*., 1994; Sano *et al*., 1995). In these cancers, an upregulated concentration of COX-2 mRNA have been observed. It used to be considered a universal marker of cancer, (Han *et al*., 1990; Subbaramaiah *et al*., 1996) but some cancers do not show overexpressed COX-2,

including breast carcinoma, basal cell carcinoma and mucinous ovarian carcinoma (Ristimaki *et al*., 1997; Kargmann *et al*., 1995; Leong *et al*., 1996). Thus it was suggested that overexpression of COX-2 is not a universal phenomenon associated with cancer but it is cancer specific.

In case of HCC, expression COX-2 have been shown to be associated with tumor grade and differentiation. COX-2 mRNA have been found to be in high concentration relative to surrounding tissues in well differentiated HCC tissues (Hepatology, 1999).

A study has shown that inhibition of COX-2 inhibition in HCC tissues lead to growth inhibition and death of tumor cells via apoptosis. Another study revealed that COX-2 overexpression in HCC patients lead to enhanced tumor growth by increased angiogenesis. Kern *et al*., implanted huh-7 xenografts in nude mice and observed that selective COX-2 inhibition caused reduction in tumor size (Kern *et al*., 2004).

It is involved in many hallmarks of cancer, including downregulation of apoptosis (Tsujii *et al*., 1995; Shiff *et al*., 1995; Han *et al*., 1990; Elder *et al*., 1996) it also aids tumor growth under stress condition e.g. hypoxia. Its bioproduct prostaglandin E2 (PGE2) increases cell survival by stimulation of Ras-MAPK signaling (Kaidi *et al.,* 2006). It is also involved in suppression of antigrowth signals. It has been shown to down-regulate the TGFβ type II receptor, which is involved in cell growth suppression signaling (Tsujii *et al*., 1995).

Upregulation of COX-2 has also been shown to promote angiogenesis (production of new blood vessels) which is another hallmark of cancer. Overexpressed COX-2 promotes angiogenesis by upregulating VEGF (vascular endothelial growth factor) and

other basic fibroblast growth factors which are involved in developing new blood vessels (Hanahan *et al*., 1996; Tsuji *et al*., 1998; Jones *et al*., 1999). PGE2, bioproduct of COX-2 has been shown to reduce activation of antitumor cytotoxic CD8+ T cells, thus allowing tumor cells to evade immune system (Harris *et al*., 2002; Harizi *et al*., 2005; Ahmadi *et al*., 2008).

1.4 Relationship between COX-2, TNFα and NF-κB

Nuclear factor- kappa B is essential component in TNF alpha mediated inflammatory carcinogenic pathway. NF-kB, once activated by TNFα signaling for cell survival, induces cell growth. If NF-kB is silenced, it will result in cancer cell death via apoptosis. that NF-kB is promising potential target for anticancer treatment (Pikarsky *et al*., 2004). TNFα cannot initiate apoptosis without the activation of RNA and protein inhibitors which can only be achieved through inhibition of NF-κB (Makris *et al*., 2000). Although there are many inflammatory modulators play part in development of inflammation, it is well established that major promotor of inflammation is NF-κB which does so by initiating transcription of many inflammatory mediators (Li *et al*., 2002). NF-κB is also involved in activation of transcription of proliferative and anti-apoptotic genes (Liu *et al*., 1996; Beg *et al*., 1996; Van *et al*., 1996; Wang *et al*., 1996) thus it also has a well-accepted role in tumor development (Li *et al*., 2002). The important anti-apoptotic genes that have been activated by NF-κB include specific inhibitor of caspase 8 activation c-FLIP (Micheau *et al*., 2001), caspase inhibitors cIAP1 and cIAP2 and the anti-apoptotic B-cell leukemia/lymphoma 2 (Bcl2) and family Bcl-XL (Karin *et al*., 2002). It also initiates transcription of COX-2 (Ke *et al*., 2006). It is an established fact that expression of COX-2 and NF-κB is upregulated in case of HCC. Thus causing the disruption in apoptotic

pathway and shifting TNF-α towards proliferative pathway (Lei *et al*., 2010). Fig. 1.2. Shows the relationship between TNF-α, COX-2 and NF-κB in a simplified way.

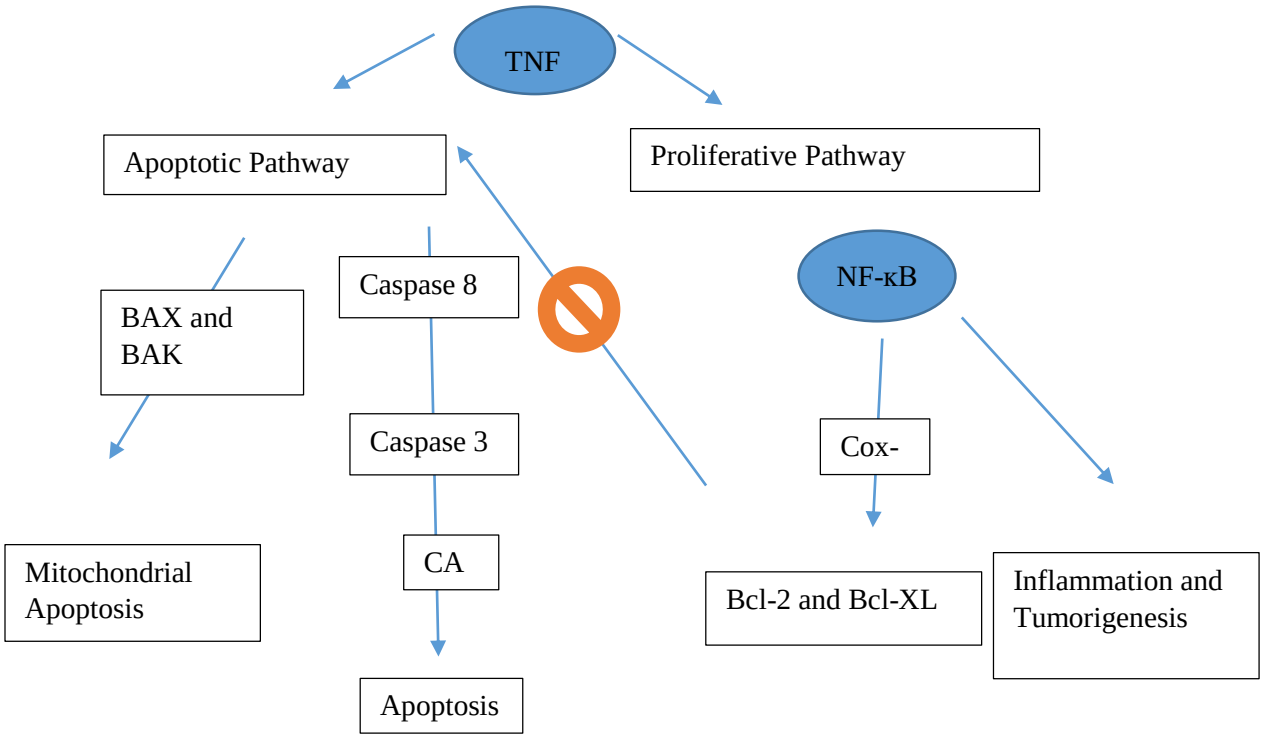


Figure.2.2. Relationship between TNFα, COX-2 and NF-κB. In HCC, upregulation of NF-κB and COX-2 cause disruption of apoptotic pathway and shift TNF-α towards proliferative pathway. Adapted from Pikarsky *et al*., 2004; Makris *et al*., 2000; Li *et al*., 2002; Liu *et al*., 1996.

1.5 Current Treatment Options for HCC

A number of treatment options have been proposed but the prognosis has been very poor. Resection is only reasonable option left but the percentage of HCC patients undergoing resection remains very low, because of the invasion of the cancer to portal or hepatic vein, the spread of cancer in whole liver and metastasis. Some other treatment

options including cryosurgery, radiation therapy and chemotherapy are also present but their efficacy is very limited (Venook, 1994).

Chemotherapy in HCC has remained largely controversial because of the low response rate. Very few single chemotherapeutic agents show response rate of >10%. Combination therapy is not very promising either. As it result in increased cytotoxicity without any considerable increase in efficacy compared to single chemotherapeutic agent. (Whang-Peng and Chao, 1998).

Other Treatment options include percutaneous treatments (radiofrequency tumor ablation or percutaneous ethanol injections). Many palliative treatments are also available. The average response rate of these treatments is quite low i.e. 20-60% with severe side effects including deterioration of liver function, Liver abscesses, and some cases massive liver necrosis and portal vein thrombosis (Luc *et al*., 2007).

Due to the lack of effective and reliable treatment for this group of cancer, there is an urgent need for investigation of new drugs and/or combination of drugs that show reduced general cytotoxicity and increased efficacy against cancerous cells. Now a days, complementary and alternative medicine (CAM) is becoming popular all over the world. Among many other herbs and vitamins, high dose intravenous Vitamin C is one option for treatment of cancer. Besides having minimum side effects, it has also been shown to be considerably effective in preventing tumors. Vitamin C has an established role in prevention of various types of cancers (Block *et al*., 1991). Vitamin C increases survival in cancer patients by 20 times (Cameron and Pauling, 1976; Cameron and Pauling, 1978) and increases life quality of cancer patients (Murata *et al*., 1982; Campbell and Cameron, 1991). It has also proved helpful in the treatment of cancer if right protocols are used. It

has been shown that intravenous administration of Vitamin C increase the plasma concentration of Vitamin C by many folds as compared to that achieved by oral administration. High dose Vitamin C, when administered intravenously act as drug, in contrast to oral vitamin C which keeps serum Vitamin C level under 100 µM and acts as a nutrient (Graumlich *et al*., 1997; Levine *et al*., 1996).

Vitamin C is known to have the lowest toxicity of all vitamins. Its higher dosages may cause diarrhea, intestinal distention and gas. Other problems that may originate in high doses are urinary extraction of calcium, iron and manganese; increased absorption of iron; increased urinary oxalate or uric acid levels (only in a small subgroup of population) (Scott *et al.,* 2006).

The above discussion leads to the hypothesis that being an anticancer agent, Vitamin C should have an inhibitory effect on inflammation which is one of the causes and of cancer, and it should downregulate the expression of inflammatory biomarkers in cancer cell line. We hypothesize that ascorbate induce TNFα mediated apoptosis in Huh7 cells by downregulating NF-κB and COX-2, thus shifting TNFα from inflammatory and proliferative pathway to apoptotic pathway.

Aim of the study

To evaluate anti-cancer mechanism of Vitamin C in Huh-7 cell line through inflammatory pathway.

Objectives

- To evaluate EC_{50} of ascorbate for HuH7 cell line through MTT cell viability assay
- To study the effect of ascorbate on expression level of TNFα, NF-κB and COX-2 through quantitative PCR

REVIEW OF LITERATURE

Hepatocellular Carcinoma is the fifth most common cancer and third leading cause of cancer related deaths worldwide, with above 500,000 mortalities per annum (Altecruze *et al.,* 2009). Major cause of HCC is HBV infection, followed by HCV. Other factors include alcohol abuse and liver damaging chemicals (aflatoxins etc). Liver cancer cause 1 in 40 deaths throughout the world (WHO, 2002).

The process of development of HCC is slow and gradual during which several genetic and epigenetic changes accumulate in hepatocytes leading to increased expression of mitogens which ultimately cause increased proliferation. There is a possibility that HCC during HCV and HBV may result because of high cell replication rate in an attempt to replace the cells infected by the virus (Block *et al.,* 2003). Before HCC, there is a prolonged phase of persistent hepatitis or cirrhosis or both, during which cell cycle upregulated by production of growth factors and due to epigenetic mechanisms is that lead to HCC. That results into production of monoclonal populations of dysplastic and abnormal cells which express telomeric erosion, increased telomerase expression, microsatellite instability and genetic and chromosomal abnormalities. The production of pre-neoplastic cells may be t

The result of disruption in structure and function of a number of genes involved in cell cycle regulatory pathway (Thorgeirsson *et al.,* 2002). These changes significantly alter microenvironment and matrix of liver. Genetic alterations are manifested in phenotypic alterations of the cells. Foci of phenotypically altered hepatocytes appear which subsequently are replaced by foci and nodules of dysplastic hepatocytes (Thorgeirsson *et al.,* 2002).

Inflammation plays a key role in development of HCC. 25% of all the cancer cases are caused due to inflammation (Aggrawal *et al.,* 2009). Inflammation play a critical part in initiation, promotion and metastatic steps of the tumorigenic process. It is observed that persistent chronic inflammation caused by HCV or HBV infection, makes the ground for development of cancer. Cirrhosis due to alcohol consumption may also lead to prolonged chronic inflammation and thus HCC (Karin *et al.,* 2006; Wu *et al.,* 2009).

There are many modulators and activators that control the process of inflammation. Which include TNFα, NF-κB and COX-2. TNFα is an important modulator that controls many biological processes simultaneously, including inflammation, cell proliferation and apoptosis (Rath *et al.,* 1999). It signals down to two independent pathways via TNFR1, one is cell proliferative pathway and the other is apoptotic pathway (Degterev *et al.,* 2003; Natoli *et al.,* 1997). The cell proliferative pathway activates NF-κB which is a key factor involved in transcription of anti-apoptotic, pro-inflammatory and proliferative genes. NF-κB is the main factor that signals TNFα to stop apoptotic signaling and shifts it to proliferative and inflammatory pathway (Beg *et al.,* 1996). The genes it transcribes include Bcl2 and Bcl-XL which are important anti-apoptotic factors (Lin and Karin, 2007), and COX-2 which is a key inflammation activator (Eberhart *et al.,* 1994; Sano *et al.,* 1995).

Currently many anticancer therapies target these inflammatory modulators for tumor reduction. TNFα is induced in many cancer therapies to initiate apoptosis in cancer cells (Horssen *et al.,* 2006). Whereas NF-κB and COX-2 are inhibited in many anticancer treatments to downregulate inflammation and cancer cell proliferation (Aggarwal *et al.,* 2009; Xu *et al.,* 2002).

2.1. Treatment Options

A number of treatment options have been proposed for HCC but the prognosis has been very poor. Resection is only reasonable option left but the percentage of HCC patients undergoing resection remains very low, because of the invasion of the cancer to portal or hepatic vein, the spread of cancer in whole liver and metastasis. Some other treatment options including cryosurgery, radiation therapy and chemotherapy are also present but their efficacy is very limited (Venook, 1994).

Chemotherapy in HCC has remained largely controversial because of the low response rate. Very few single chemotherapeutic agents show response rate of >10%. Combination therapy is not very promising either. As it result in increased cytotoxicity without any considerable increase in efficacy compared to single chemotherapeutic agent. (Whang-Peng and Chao, 1998).

Other Treatment options include percutaneous treatments (radiofrequency tumor ablation or percutaneous ethanol injections). Many palliative treatments are also available. the average response rate of these treatments is quite low i.e. 20-60% with severe side effects including deterioration of liver function, liver abscesses, and some cases massive liver necrosis and portal vein thrombosis, (Luc *et al.,* 2007).

Now a days, complementary and alternative medicine (CAM) is becoming popular all over the world. Among many other herbs and vitamins, high dose intravenous Vitamin C is one option for treatment of cancer. Besides having minimum side effects, it has also been shown to be considerably effective in preventing tumors. There have been proposed a number of hypothesis explaining the antitumor effect of Vitamin C but the exact

mechanism of ascorbate's action against cancer cells is still unknown (Bonilla Poras *et al.*, 2011). There are a number of possibilities including ascorbic acid simultaneously affecting apoptotic pathways, pro-oxidant damage and increased oxidation of ascorbate at high concentrations in plasma thus to unstable metabolite de-hydro ascorbic acid which can be damaging (Jingjing *et al.*, 2006; Bonilla-Poras *et al.*, 2011).

It has been studied by Jing-jing *et al.*, in 2006 that combined effect of arsenic trioxide (A_2O_3) and Vitamin C on HCC cells and observed increased apoptosis in cells exposed to both $A_2 O_3$ and Vitamin C than cells exposed to $A_2 O_3$ only. They established that Vitamin C stimulates A_2O_3 to induce apoptosis in HCC cells through oxidative pathway (Jing-jing *et al.*, 2006). Several other studies have proven Vitamin C to be apoptosis inducing agent in cancer cells in-vitro (Bonilla-Porras *et al.*, 2011).

A study by Mikinova *et al.*, in 2012 showed that higher dose of intravenous (IV) vitamin C than recommended dietary dose show reduction in inflammation in cancer patients which in turn is associated with tumor size reduction (Mikinova *et al.*, 2012). Padayatty *et al.*, reported that the mean peak values of plasma Vitamin C concentrations achieved by IV Vitamin C administration were 6.6 folds higher than those achieved by oral administration, for 1.25 g Vitamin C (Padayatty *et al.*, 2004).

The clinical studies have shown that if Vitamin C is administered orally, the plasma concentration is maintained below 100 µM, by decreasing absorption and increasing urine excretion as soon as oral dose exceeds 200 mg (Graumlich *et al.*, 1997; Levine *et al.*, 1996). Whereas intravenous injection increases the plasma concentrations of Vitamin C to high levels by bypassing the intestinal absorption step. Thus intravenous Vitamin C when given in high dose act as drug against cancer. According to pharmacokinetic modeling, plasma

vitamin C concentration can reach 220 µM for maximum tolerated oral dose, i.e. 3 g at 4 hours interval. Whereas intravenous administration of 100 g Vitammin C can increase plasma Vitamin C concentration to as much as 15,380 µM (Cameron and Campbell, 1974). This makes it clear that intravenous administration of Vitamin C can increase plasma concentrations of Vitamin C beyond the limits of oral dose.

Another favorable phenomenon in anticancer activity of Vitamin C is that, Vitamin C tends to accumulate near solid tumors in higher concentrations as compared to the surrounding normal tissues (Honegar *et al.,* 1988; Langemann *et al.,* 1989; Agus *et al.,* 1999). Piaza *et al.,* in 1997 gave as much as 4 g of vitamin C per day for short term to radiotherapy patients and reported improvement in recovery (Piaza *et al.,* 1997). Huber, Schneider and Kahr used 0.5 to 2 g Vitamin C in combination with other vitamins daily on terminal cancer patients and achieved a standstill period in which tumor growth stopped for a considerable period of time (Tsujii *et al.,* 1998).

Some researchers have shown that H_2O_2 plays a central role as a toxic agent in high-dose vitamin C therapy. H_2O_2 formed due to high extracellular ascorbate concentrations, diffuse across the cell membrane (Antunes *et al.,* 2000) and cause breaks in DNA and mitochondria. Mitochondria in some cancer cells may have increased sensitivity towards H_2O_2 (Hyslop *et al.,* 1988, Comelli *et al.,* 2003; Ahmad *et al.,* 2005). Tumor cells die when exposed to H_2O_2 in less than 30 minutes (Schraufstatter *et al.,* 1985; Lee *et al.,* 1999).

Seminal studies carried out by Chen *et al.,* showed that ascorbate concentrations higher than 1 mM cause a buildup of hydrogen peroxide (H_2O_2) which in turn kills cancer cell preferably. The study showed that the concentration of ascorbate at which half of the cells die (EC_{50} value) is less than 5 mM, but for normal cells, it is more than 20mM. This

phenomenon makes ascorbate selectively toxic towards cancer cells. Cell death is caused by extracellular concentration of ascorbate and H_2O_2. Detectable levels of H_2O_2 are formed by ascorbate only in presence of 0.5-10% serum. Human whole blood inhibits H_2O_2 formation. Its formation is also dependent on time and ascorbate concentration. Most probably plasma catalase and red blood cell glutathione peroxidase (GP) destroy H_2O_2. Thus, Chen *et al.,* concluded that ascorbate at certain concentration (pharmacological dose) acts as prodrug and forms H_2O_2 which in turn is selectively toxic towards cancer cells (Chen *et al.,* 2005; Chen *et al.,* 2007).

Ascorbate has been used in combination with other drugs and have shown positive effects. There have been proposed a number of hypothesis explaining the antitumor effect of Vitamin C. Cameron and Pauling's hypothesis suggests that it combats cancer by increasing collagen synthesis and thus maintains the integrity of ground substance and keeps the cells in place thus preventing metastasis (Cameron and Pauling, 1974). Cameron and Pauling also suggested that cancer patient's show reduced immunocompetence due to lymphocyte ascorbic acid depletion. General deficiency of Vitamin C in state of cancer may also lead to hormonal imbalance which affects host's resistance towards cancer invasive growth (Cameron and Pauling, 1974).

Cancer patients in general suffer from Vitamin C deficiency. Moreover, Vitamin C appears to be required in higher concentration than normal in state of cancer (Reddy *et al.,* 1996; Jones *et al.,* 1993; Qiau *et al.,* 1998). The induction of powerful carcinogen methylacholanthrene have been shown to give a boost to Vitamin C production in experimental animals (Crew *et al.,* 2000). Since human has left unable to synthesize his own vitamin C over the course of evolution, and depend upon the extrinsic sources to meet

his dietary requirements, a higher intake of Vitamin C is needed in state of cancer to keep intrinsic resistance mechanisms working in order to fight off cancer (Cameron and Pauling, 1974).

According to laboratory data, ascorbate is effective against a number of cell lines (Benade *et al.,* 1969; Bram *et al.,* 1980). In most of the cases, extracellular concentration as low as 100-200 µM is effective, but in some, concentrations in mM are required (Reodran *et al.,* 1995). Many studies have shown benefits of combined administration of Vitamin C along with chemotherapeutic agents (Block *et al.,* 2007). The fact that ascorbate selectively accumulates at the tumor site, is a pharmacological advantage, in addition to the tumoricidal effect of Vitamin C. Thus high dose intravenous administration of Vitamin C will make ascorbate accumulate at the cancer site and provide sufficient concentration of ascorbate, required for antitumor activity. A study have shown that Vitamin C induces apoptosis in leukemia cells by oxidative stress mechanism (Bonilla-Porras *et al.,* 2011).

Reports by Padayatty *et al.,* showed that dose intravenous Vitamin C treatment not only reduced tumor progression but also improved health status of the patients (Padayatty *et al.,* 2006). Carr *et al.,* in 2014 gave high dose intravenous Vitamin C to a large number of patients undergoing chemotherapy and reported decrease in chemotherapy related side effects and increase in quality of life (Carr *et al.,* 2014).

Despite the number of studies being carried out to determine anticancer activity of Vitamin C, the exact mechanism of action of Vitamin C against cancerous cells is still not known. Due to lack of effective treatments against HCC, there is a need to evaluate the anti-cancer mechanism of Vitamin C against HCC and determine its mode of action. The

present study is a humble effort to meet this need. In this study, we have tried to determine the anti-cancerous mechanism of Vitamin C through its anti-inflammatory action.

MATERIALS AND METHODS

3.1 Cell culture

Huh-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) media containing 5 % fetal bovine serum (FBS) and 1% penicillin/streptomycin. Huh-7 vial was thawed at 37^{o}C after retrieving from -80^{o}C freezer and washed twice with 5x PBS (1 tablet PBS per ml of distilled water, autoclaved afterwards). The cells were cultured in 37^{o}C incubator with 5% atmospheric CO_2.

3.2 Exposure of Huh7 cells to Vitamin C

Huh-7 cells were treated with different doses of ascorbic acid and the cytotoxic effect was determined through MTT assay.

3.2.1. Day1: Cell Seeding

Huh7 cells were cultured in 96 well plates after harvesting them using trypsin. Cells were counted using trypan blue exclusion assay and seeded in 96 well plate ($1x10^4$ cells per plate). Following formulae was used for cell counting with trypan blue and haemocytometer.

Cells per mL = (number of cells / Number of squares) x 10^4

Cells were cultured overnight.

3.2.2. Day 2: Drug Exposure

Fresh media with 2, 4, 6, 8 and 10 mM vitamin C was added to cultured cells. 10% DMSO was used as positive control. Same concentrations of Vitamin C (with no cells) were used as blank.

3.2.3. Day 3: Determination of cytotoxicity of Vitamin C through MTT assay

10 µL of MTT reagent was added to each well. The plate was covered up in an aluminum foil to protect the reagent from light and incubated at 37 ^{o} C for 3 hours before adding 10 µL DMSO. The readings were taken at 490 nM. The experiment was carried out in triplicates.

3.3 Expression Profiling of Huh-7 cells

Huh7 cells were cultured in 6 well plates (cell count=$1x10^6$) and exposed to 5 dilutions of Vitamin C viz. 2, 4,6,8 and 10 mM for 24 hours following RNA extraction by TRIZOL method. The major steps of RNA extraction were separation, precipitation, washing and solubilization. The cells grown in culture flask were directly lysed with in the flask. 1.5 mL of TRIZOL reagent was added to cell culture flask. 40 µL of 5 N glacial acetic acid (1 mL of glacial acetic acid with 2.48 mL of water) was then added and mixed vigorously by repeated pipetting, to lyse the cells. Lysed samples were kept at room temperature for 5minutes to allow complete dissociation of nucleoprotein complexes. Lysates were supplemented with 200 µL chloroform and mixed vigorously for 15 seconds. The resulting mixture was kept at room temperature for 5 minutes and centrifuged at 14000 rpm for 20 min at 4^{o}C. Following centrifugation, the mixture was separated into a brownish

phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase whereas DNA and proteins were in the interphase and organic phase, respectively. Aqueous phase was transferred to a fresh micro-centrifuge tube and RNA was precipitated by mixing with 500 µL iso-propanol. Mixture was kept at room temperature for 5-10 min and RNA was pelleted by centrifugation at 14,000 rpm for 15 min at 4ºC. RNA precipitate formed a gel-like or white pellet at the bottom of the tube. The supernatant was removed and RNA pellet was re-suspended in 1 mL of 75% ethanol by vortexing. RNA suspension was spun at 10,000 rpm for 5 min at 4ºC. Ethanol was removed and RNA pellet was briefly air-dried for 5 min. RNA was dissolved in diethyl pyro-carbonate (DEPC) treated water and incubated for 10-15 min at 55ºC - 60ºC.

3.3.1. Expression Analysis of COX-2, NF-κB and TNFα genes

3.3.1.1. cDNA Synthesis

cDNA was synthesized by reverse transcription reaction using reverse transcriptase (All the chemicals were purchased from Fermentas). The reverse transcription reaction contains 1 x RT buffer, 100 ng/µL of RNA, 5 µM oligos, 1.5 mM dNTPs (each) and 10 U of reverse transcriptase in DEPC water. The reverse transcription reaction was carried out in 20 µL of total volume. 2 µg of RNA and oligos were diluted with DEPC water and incubated at 70ºC for 10 min. The reaction tube was kept on ice and 10 µL of 5 x RT buffer and 2 µL of dNTPs mix (from 10 mM each), 40 U of reverse transcriptase along with its buffer and 20 U of Ribolock RNase inhibitor were added. The reverse transcription was carried out at 42ºC for 60 min followed by inactivation of reverse transcriptase at 70ºC for 10 min. The cDNA was used directly for PCR or stored at –20ºC until further use.

3.3.1.2. Expression analysis

Targeted gene primers and reaction profile were amplified on Real Time PCR (Model No. 7300 Applied Biosystem). Maxima SYBR Green/ROX qPCR Master Mix (Catalog number: K0221 Thermo Scientific) was used. Each 25 µL reaction mixture contained SYBR green 12.5 µL, 1:3 diluted cDNA 3 µL, NF water 7.5 µL, 1 µL forward primer and 1 µL reverse primer.

3.3.1.2.1. q-PCR Profile

The initial denaturation step was carried out at 95 ºC for 10 minutes, followed by denaturation, at 95 ºC for 30 sec. annealing step was 60 seconds long and temperature was 60 ºC. The extension step after that was performed at 72 ºC for 60 sec.

RNA levels of the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined as reference for calculating the relative expression levels of the target genes. All samples were run at least in duplicates. The results were analyzed by Sequence Detection Software (ABI). The relative expression level was calculated as the difference between the mean Ct values (calculated from the duplicates) of each target and reference gene GAPDH (Mean Ct = Ct (target gene) - mean Ct (GAPDH). The relative expression level was calculated as the difference between the mean Ct of genes and housekeeping gene, GAPDH.

3.3.1.3. q-PCR Result Calculation

Ct values of the target genes were compared to the reference housekeeping gene GAPDH and fold change in the expression of targeted markers was calculated as follows:

Ct Value of GAPDH in control = X1

Ct Value of GAPDH in Experimental = X2

Ct Value of Target Gene in Control = Y1

Ct Value of Target Gene in Experimental = Y2

ΔCt Control = (Ct of Target in Control) - (Ct of GAPDH in Control) = Y1- X1= ΔCt1

ΔCt Exp = (Ct of Target in Exp.) - (Ct of GAPDH in Exp.) = Y2- X2= ΔCt2

ΔΔCt = (ΔCt Exp.) – (ΔCt Control) = ΔCt2- ΔCt1

Fold Change = 2 (-ΔΔCt)

Copy Number /100 copies of GAPDH= 2 (-ΔCt)*100

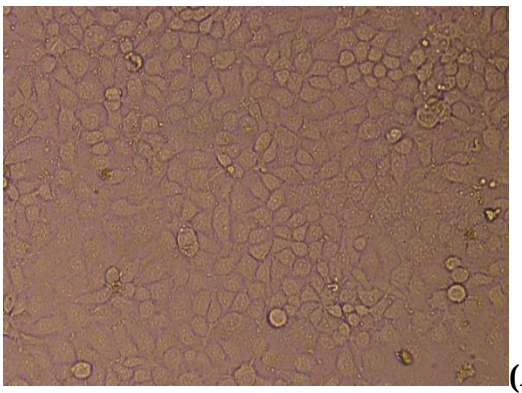
3.5.2. Statistical analysis

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

RESULTS

4.1 Cytotoxic Effect of Vitamin C exposure on Huh7 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. 4.1).


(A)

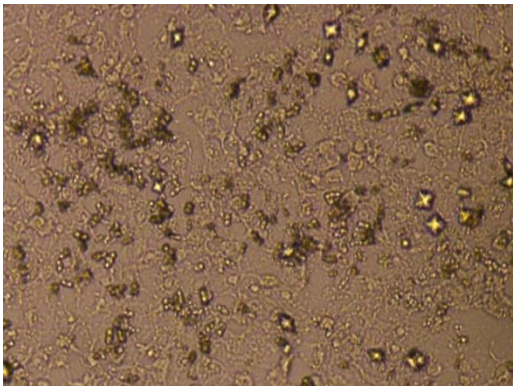
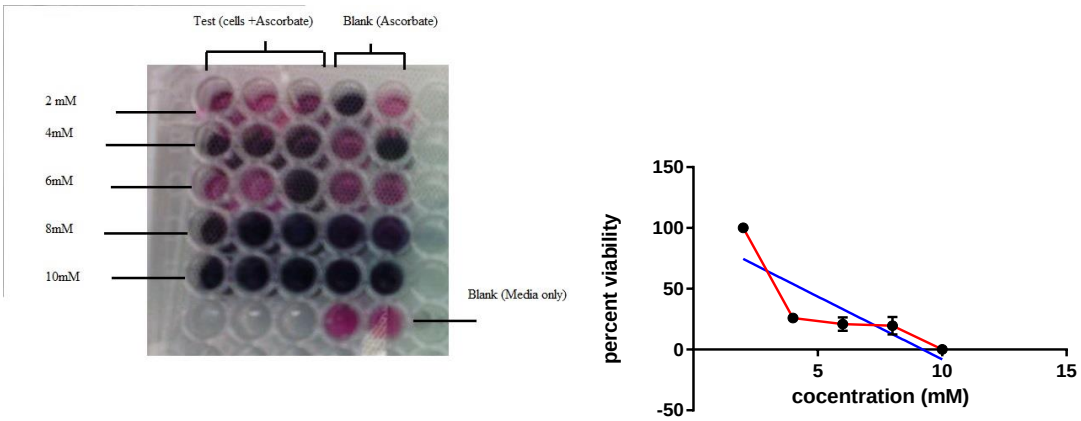

(B)

Figure 4.1. Huh7 cells before (A) and after (B) exposure to ascorbate for 24 hours.

4.1.1. Determination of cytotoxicity of Vitamin C through MTT Assay

Cells were cultured (cell count=$1x10^4$) in 96 well plates for 24 hours before exposing to increasing concentrations of Vitamin C (Fig. 4.14.). After 24 hours exposure to Vitamin C, MTT reagent was added to measure the viable cells. 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. The MTT graph in figure 14.15 shows that Vitamin C decreases cell viability with increased concentration.



(A) **(B)**

Figure 4.2. Determination of cytotoxicity of Vitamin C through MTT Assay. A: MTT reagent added to cells after exposure to increasing concentrations of Vitamin C. increasing concentration of Vitamin C shows false positive results because of reducing potential of Vitamin C. To eliminate the false results due to Ascorbate concentrations, absorbance of blank ascorbate of respective concentrations is subtracted from absorbance of the test wells containing the cells. B: 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.

4.1.1.1. Calculation of Percent Viability

Absorbance of test = X

Blank = B

Absorbance of Control = Y

Percent Viability = (X – B/ Y – B) x 100

4.1.1.2. 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)

$50 = -21.22x + 95.92$

$EC_{50} = X = 2.16$

4.1.2. Expression Analysis

To measure the expression levels of three pro-inflammatory genes including TNFα, NF-κB and COX-2 in Vitamin C treated Huh7 cells and compare them to those of untreated cells. Huh-7 cells were cultures in 24 well plates (cell count= 10^6 cells per well). Cells were treated with increasing concentrations of Vitamin C for 24 hours. RNA of the cells was isolated after the treatment and used to perform cDNA synthesis and then qPCR. All the experiments were carried out in duplicates.

4.1.2.1. Primer Optimization

Primers for all genes (TNF-α, COX-2, GAPDH and NF-κB) were optimized to amplify their respective genes specifically. Dissociation curves are shown in Figures 4.2, 4.3, 4.4 and 4.5 respectively. Single peak shows primer is optimized and no secondary structures are formed.

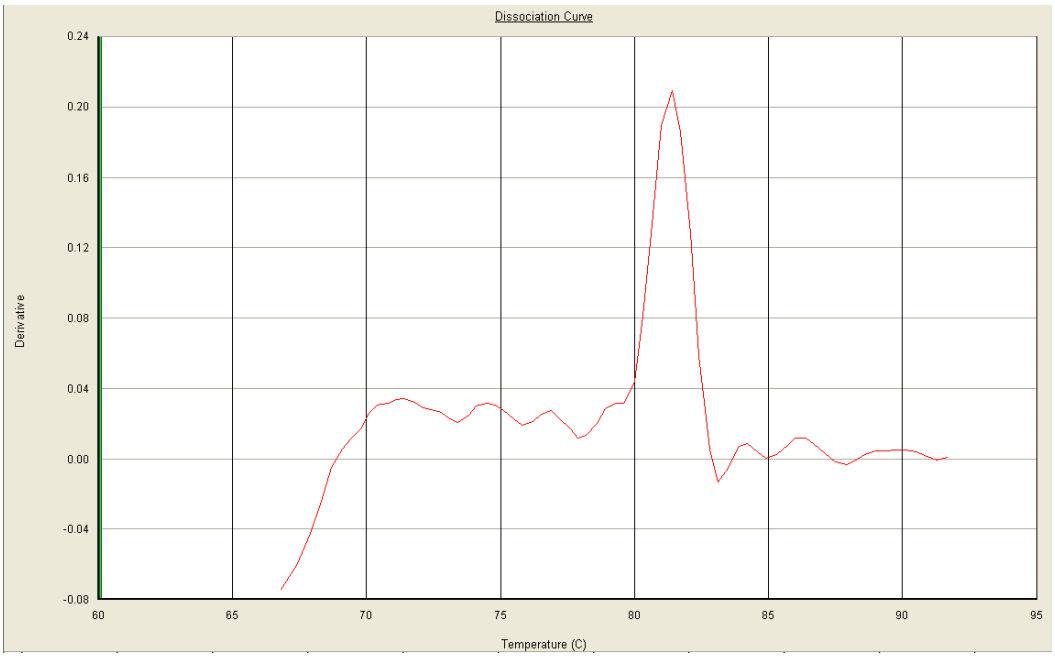


Figure. 4.4. Optimization of TNF*α* Primers: Single peak shows that primers are optimized and no secondary structures are formed.

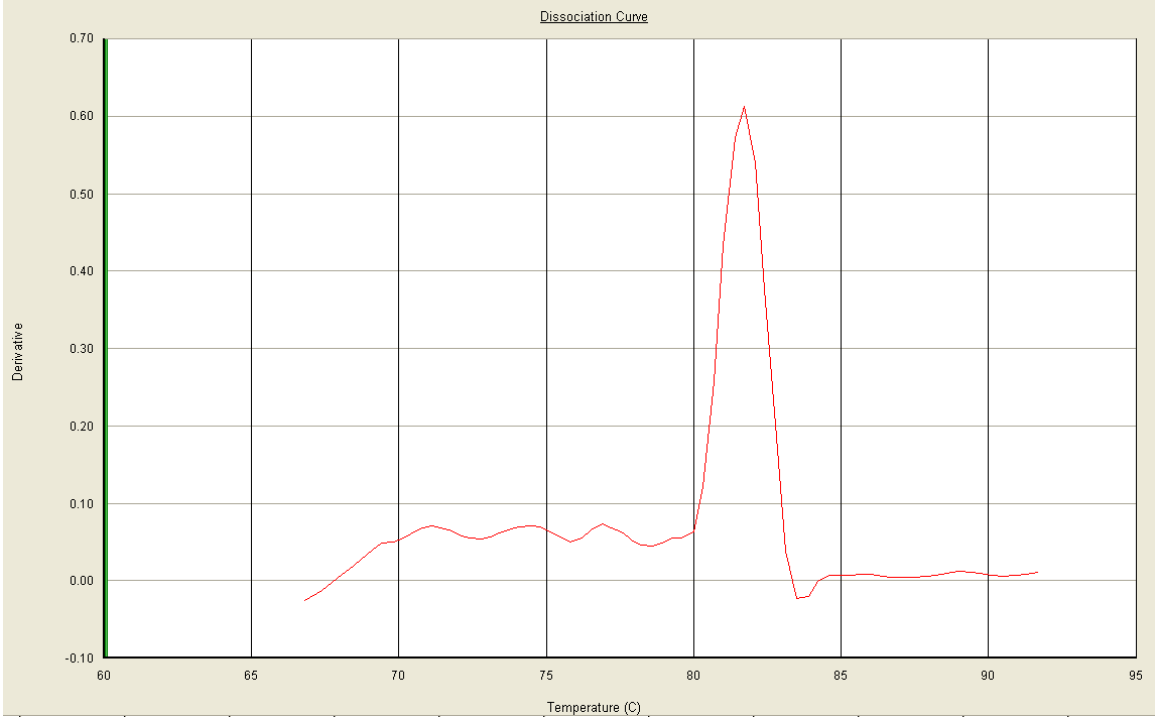


Figure. 4.5. Optimization of COX-2 Primers: Single peak shows that primers are optimized and no secondary structures are formed.

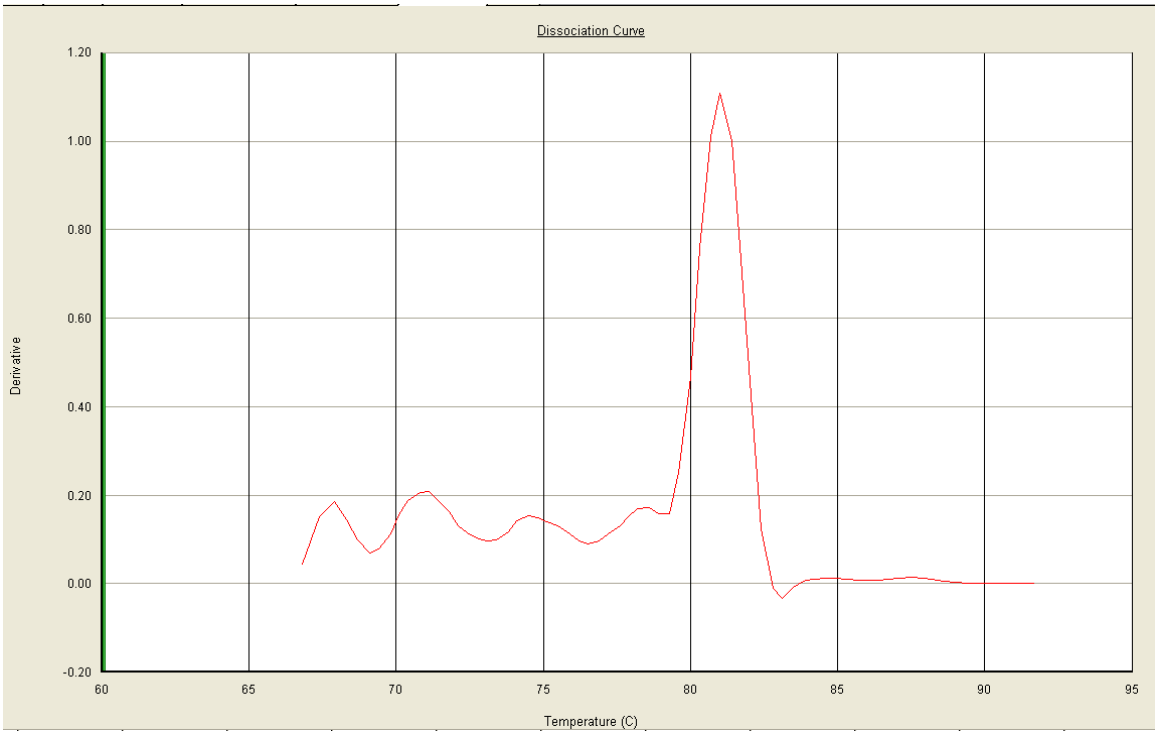


Figure. 4.6. Optimization of GAPDH Primers: Single peak show that primers are optimized and no secondary structures are formed.

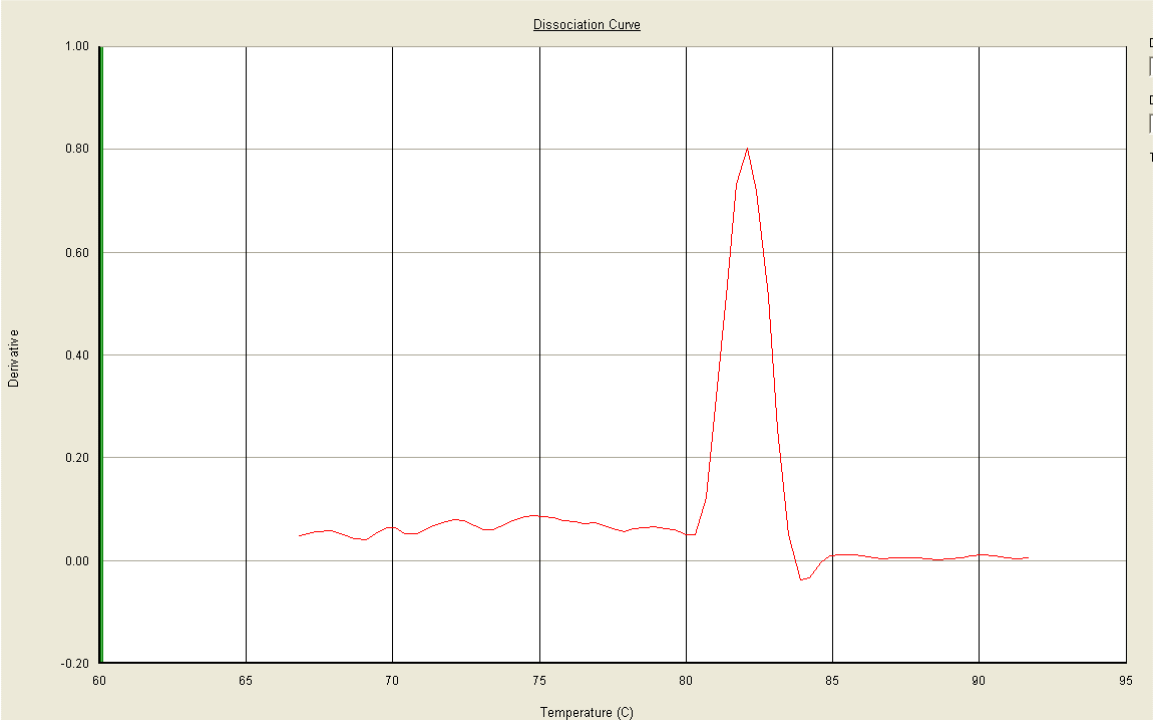


Figure. 4.7. Primer Optimization of NF-κB: Single peak show that primers are optimized and no secondary structures are formed.

4.1.2. Expression analysis of COX-2, TNFα and NF-κB

It was observed that Vitamin C exposure at all concentrations caused significant decrease in expression level of COX-2 with p value being 0.0002(Fig.12, Fig. 4.13). A significant decrease (p value=0.0009) in expression level of NF-κB was also observed at 10 mM concentration of Vitamin C (Fig. 4.10). At the other concentrations, i.e. 2,4,6 and 8 mM, the expression of NF-κB remained constant (Fig. 4.8, Fig 4.9). On the other hand, ascorbic acid caused a significant increase (p value=0.015) in the expression level of TNFα (Fig.4.6 -4.7).

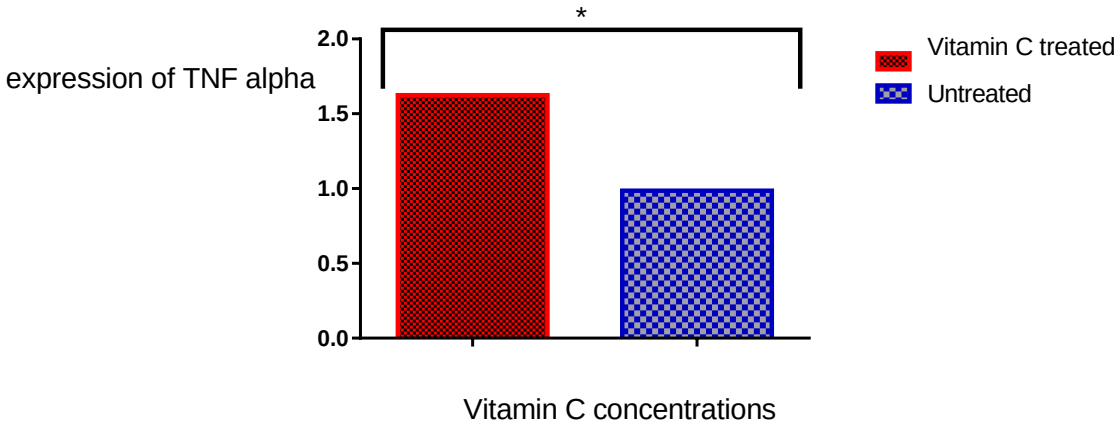


Figure.4.8. Expression analysis of TNFα. Expression of TNFα in Huh-7 cell line exposed to increasing concentration of Vitamin C, compared to untreated Huh-7 cells. The expression of TNF α is significantly higher in ascorbate treated cells as compared to untreated cells.

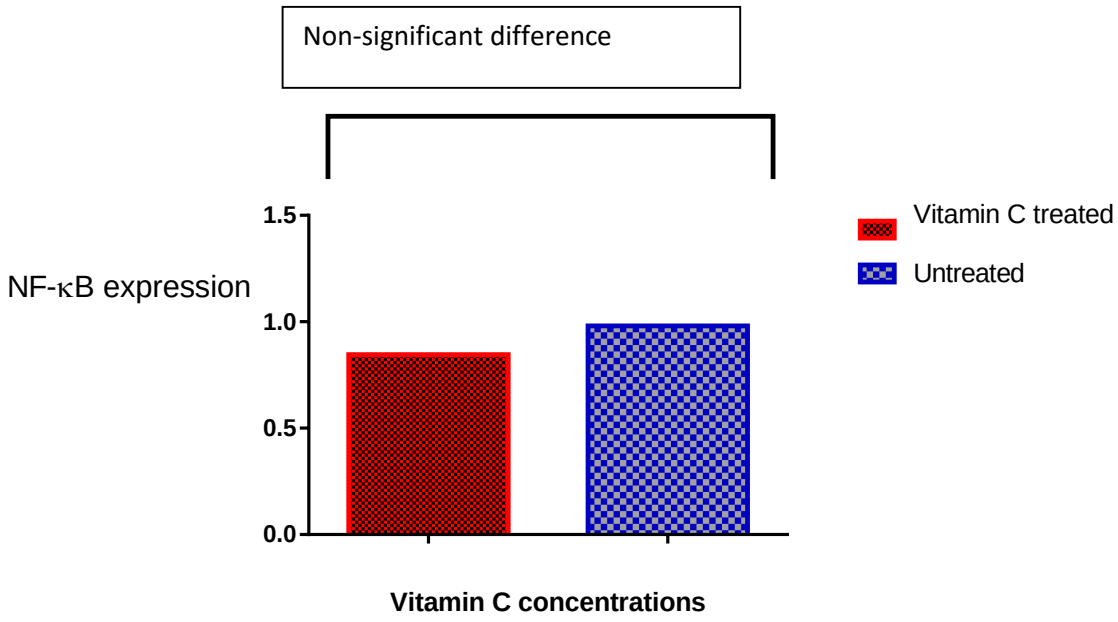


Figure.4.9. Expression Analysis of NF-κB. Expression of NF-κB in Huh-7 cell line treated with different concentrations of Vitamin C compared to untreated Huh-7 cells. The expression level of NF-κB in ascorbate treated cells is similar to that of untreated cells.

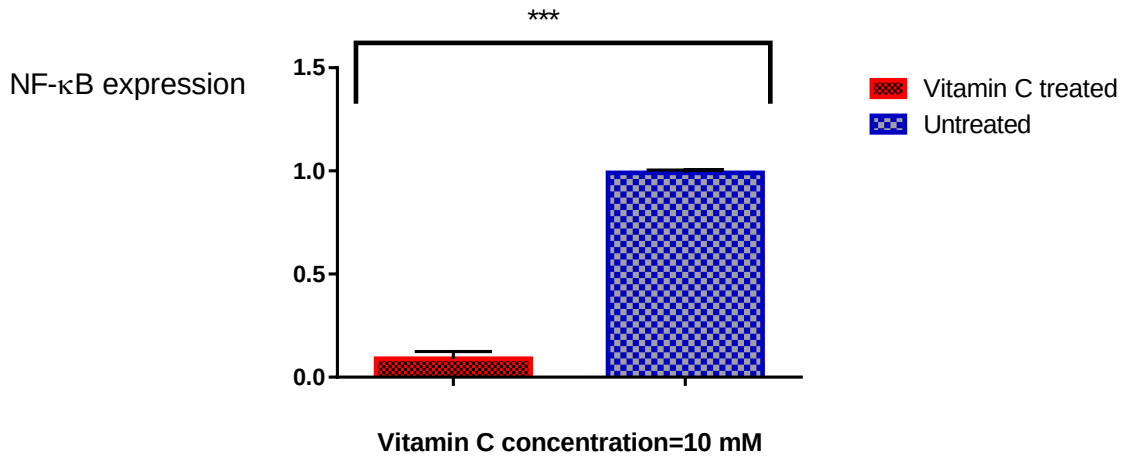


Figure.4.10. **Expression analysis of NF-κB**. Expression of NF-κB in Huh-7 cell line treated with 10 mM Vitamin C compared to untreated Huh-7 cells. Expression of NF-κB in cells treated with 10 mM ascorbate is significantly lower as compared to untreated cells.

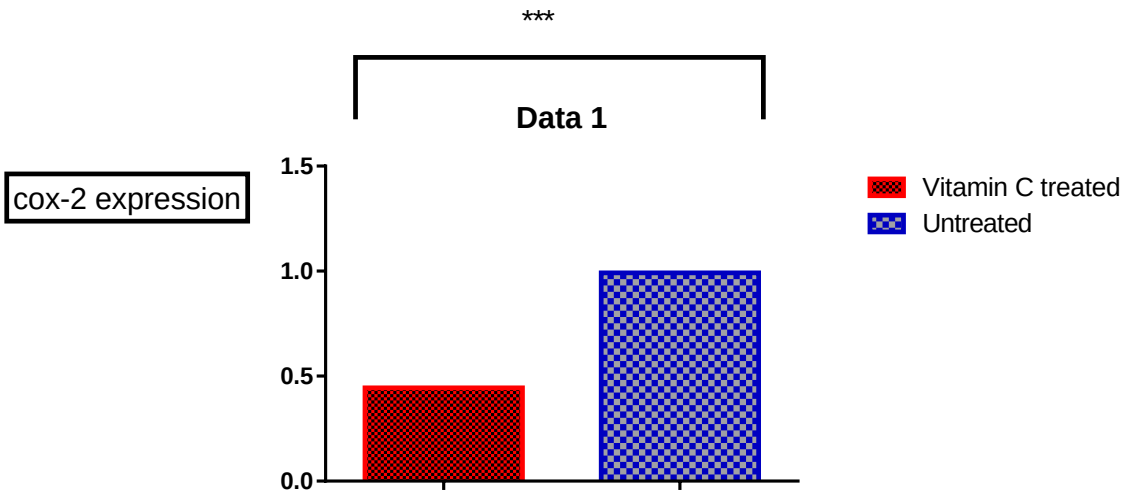


Figure.4.11. **Expression analysis of COX-2.** Expression of coz2 in Huh-7 cell line treated with increasing Vitamin C compared to untreated Huh-7 cells. Expression of COX-2 in cells treated ascorbate is significantly lower as compared to untreated cells.

DISCUSSION

Hepatocellular carcinoma is a very common disease ranking fifth worldwide and is third major cause of death. Main causes of liver cancer are HBV, HCV, chronic alcohol consumption and liver damage that might be a result of all the above factors. The tumor develops slowly and gradually accumulating many genetic and epigenetic changes in the process which ultimately leads to increased cell proliferation (Farazi *et al.,* 2006).

Inflammation play a crucial part in initiation, promotion and metastatic steps of the tumorigenic process (Grivennikov et al., 2010). It is observed that persistent chronic inflammation caused by infection by a parasite (e.g. HCV or HCV) make the grounds for development of cancer (Coussens et al., 2002; Karin et al., 2005).

Several types of inflammations can cause initiation and promotion of cancer. Infections like hepatitis B or C and liver damage due to alcoholism cause prolonged activation of immune cells which lead to chronic inflammation. Chronic inflammation provide suitable microenvironment for the development of hepatocellular carcinoma (HCC) (Karin et al, 2006, Wu et al 2009a).

Abnormal expression of any factor involved in control of inflammation leads to altered expression of cancer related genes and genes involved in apoptosis and cell proliferation (Eiro *et al.,* 2012). There is also evidence that inflammatory cells and immune modulators promote tumor development and metastasis (Eiro *et al.,* 2012). Inflammatory microenvironment and tumor development. Inflammatory cells are believed to enhance tumorigenesis by virtue of non-specific pro-inflammatory cytokines including, tumor

necrosis factor (TNF), (Aggarwal *et al.,* 2006; Robinson *et al.,* 2005). Activated nuclear factor-kappa b (NF-kb) is a transcription factor that initiates transcription of cell proliferative and anti-apoptotic genes, and is believed to be one of the main factors involved in inflammation related tumorigenesis. It initiates transcription of many inflammatory, anti-apoptotic and proliferatory genes including cox-2, which is an important inflammation inducing and proliferative agent. All of these factors collectively act as initiators of proliferation and inhibitors of apoptosis and thus promote tumorigenesis.

Tumor necrosis factor-alpha acts as a pro-inflammatory cytokine, cell proliferation activator as well as pro-apoptotic factor. In absence or low expression of NF-κB, TNFα function is shifted from proliferative to apoptotic pathway (Chu *et al.,* 1997).

Quantitative polymerase chain reaction was performed to assess the expression level of inflammation genes in hepatocellular carcinoma cell line huh-7 after 24-hour exposure to ascorbic acid. The expression level of three genes including TNFα, NF-κB and cox-2 was measured. We observed that Vitamin C exposure at all concentrations caused significant decrease in expression level of cox-2 with p value being 0.0002. a significant decrease (p value=0.0009) in expression level of NF-κB was also observed at 10 mM concentration of Vitamin C. at the other concentrations, i.e. 2,4,6 and 8 mM, the expression of NF-κB remained constant. On the other hand, ascorbic acid caused a significant increase (p value=0.015) in the expression level of TNFα.

Tumor necrosis factor-alpha is known to be involved in two independent pathway which diverge at the very early stage of TNFα signaling. One pathway causes apoptosis whereas the other is involved in inflammation. The inflammation pathway is mediated by NF-κB which is the key link of inflammation and cancer. Once activated, NF-κB signals

TNFα to stop TNFα mediated apoptosis (Daniel et al., 1996). NF-κB promotes cell survival by initiating transcription of anti-apoptotic and proliferative genes (Lin et al., 2007) it also initiates transcription of COX-2 which is a very important gene involved in inflammation and cancer (Eiro et al., 2012; Koga et al., 1999).

In absence of NF-κB, TNFα is considered as a potent anti-tumor gene and has been proposed to be used in therapy against many cancers.

In the present study, we observed that ascorbic acid upregulates expression of TNFα whilst keeping expression of NF-κB relatively constant, and ultimately downregulating it at 10 mM concentration. A study carried out by Bowie et al. in 2000 reported that Vitamin C inhibits activation of NFκB by inhibiting blocking of IκBa. IκB activation is blocked by IκB Kinase (IKK) which in turn is mediated by p38. Vitamin C affects expression of p38, causes its constitutive expression thus inhibiting blockage of IκB by IKK. Thus allowing IκB to inhibit activity of NF-κB.

The MTT cell viability assay have shown strong anti-tumorigenic potential of Vitamin C against Huh7 cell line with EC50 being 2.16. The present study suggest two possible mechanisms for Vitamin C anti-tumorigenic activity. One is through TNFα mediated apoptosis, and other is through anti-inflammatory pathway. TNFα initiates activation of two independent pathways. One being of cell proliferation and other of apoptosis (Takada *et al.,* 2003; Degterev *et al.,* 2003). NF-κB is activated through cell proliferative pathway of TNFα and this is the main factor involved in inhibiting apoptotic activity of TNFα and promoting inflammation (Daniel et al., 1996).as current study have shown that Vitamin upregulates TNFα keeping the level of NF-κB constant, and a previous study carried out by Bowie et al. has revealed that Vitamin C prevents activation of NF-κB

by affecting IKK. Based on these facts we can conclude that Vitamin C has shifted TNFα towards apoptotic pathway by blocking cell proliferative pathway and thus kills tumor cells through TNFα mediated apoptosis. The present study has also revealed that Vitamin C causes a significant decrease in expression of cox-2, an inflammatory mediator that is involved in development of inflammation in tumorigenesis by production of prostaglandins in inflammatory and tumor conditions (Kujubu *et al.,* 1991; Hla *et al.,* 1992) Cox-2 is a one of the genes whose expression is initiated by transcription factor NF-κB (Ke et al., 2006). This observation provides a supportive evidence in the favor of above conclusion that Vitamin C shifts TNFα from inflammatory and proliferative pathway towards apoptotic pathways. Anti-inflammatory role of Vitamin C have also been demonstrated by various studies before (Korantzopolous *et al.,* 2004 and Peters *et al.,* 2001).

Conclusions

The above discussion leads to the conclusion that Vitamin C induces TNF-α mediated apoptosis in huh7 cells. Vitamin C upregulates TNFα, keeping the level of NF-κB constant.

Thus shifting TNF-α from inflammatory and proliferative pathway to apoptotic pathway. Downregulation of cox-2 by Vitamin C shows that TNF-α is no longer active as a pro-inflammatory cytokine and is inducing apoptotic cell death in cancerous cells.

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