



## ORIGINAL ARTICLE

## Provitamin A Carotenoids Reduce Consequences of Radiation Stress Apoptosis: Its Implications in Cancer Radiotherapy

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### ABSTRACT

Carotenoids are widespread group of naturally occurring red, yellow and orange pigmented micronutrients & fat-soluble colorants. Carotenoids are precursors of vitamin A and have antioxidant effects. While over 600 carotenoids have been found, the most common forms are alpha-carotene, beta-carotene, lycopene, crocetin, canthaxanthin, and fucoxanthin. Beta-carotene is the most widely studied. It is composed of two molecules of vitamin A (retinol) joined together. Beta-carotene is converted to retinol at the level of the intestinal mucosa. The antioxidant function of beta-carotene is due to its ability to quench singlet oxygen, scavenge free radicals and protect the cell membrane lipids from the harmful effects of oxidative degradation. oxidative stress and reactive oxygen species can be either the initiating stimulus or mediators of apoptosis. It is also clear from the findings that the antioxidants like beta carotene can protect the normal cell against radiation induced apoptosis at the regulatory gene level. The data is indicative of the future prospects and ensures exploitation of oral administration of beta-carotene against radiation in clinical application and radiotherapy for cancer. Taking jejunal crypt cell as model for a fast proliferating tissue, present work has been undertaken to evaluate the radioprotective efficacy of beta-carotene against radiation induced apoptosis in jejunal epithelium.

**Key Words:** Carotenoids, beta-carotene, radioprotection, Apoptosis, Cancer

### INTRODUCTION

Radiation induced intracellular oxidation may play a central role in apoptotic cell death. The antioxidants like beta carotene protect the cell against apoptosis from radiation at the regulatory gene level. The data will be indicative of the future prospects and ensures exploitation of oral administration of beta-carotene against radiation in clinical application and radiotherapy for cancer. Apoptosis is a form of regulated cell death that has attracted a lot of interest in the recent year. Radiation damages DNA, the genetic code contained in all cells. Multiplying cells are particularly vulnerable to DNA damage by radiation. The uncontrolled multiplication of the cells that make up benign and malignant tumors therefore makes them vulnerable to the effects of radiation. Radiation does also damage the DNA of multiplying normal cells, but normal cells also usually have a greater capacity to repair DNA damage than cancer in tumor cells do. Radiation also triggers apoptosis. Apoptosis is programmed cell death - cells have an internal mechanism that causes cell death when activated, and radiation can trigger that mechanism. Apoptosis is sometimes referred to as programmed cell death (PCD) because it is an integral part of developmental programme and is frequently the result of temporal course of cellular events.

Recently radiobiologists have begun to acknowledge the importance of apoptosis or programmed cell death in radiation response. The process of apoptosis was originally describe more than 20 years ago but its importance in a number of biological functions has been identified only in recent years. It can be induced by a variety of stimuli such as gluco-corticoids, chemotherapeutic agents, various oxidants and ionizing radiations.

Earlier work carried out in our laboratory has shown a profound on neurons, a non-renewal cell. The fat soluble beta carotene is converted to Vitamin A in the body as it is needed, for the most part this conversion take place in the small Intestine. The present work has been undertaken to evaluate the radio-protective efficacy of beta-carotene against radiation induced apoptosis. The importance of radiobiological studies could be highlighted as there in no environment which is absolutely devoid of radiations and there is always a regular intake of radiation by our body. Most

of body tissues are radio-vulnerable. However, all cells do not respond to radiation in the same way. The response of a tissue or organ to a dose of ionizing radiation depends primarily upon two factors – the inherent sensitivity of the individual cells and the kinetics of the population as a whole of which the cells are a part.

Among the numerous problems pertaining to the biological effects of ionizing radiation, radiation injury to the digestive system occupies a special place. Small Intestine is a cell renewal system. Crypts of Lieberkuhn constitute its proliferative compartment, consisting of undifferentiated cells. The function of which is produce cells for another population.

Regulation of crypt epithelial apoptosis and stem cell fate after potentially mutagenic or carcinogenic injury may also be an important factor in the initiation of gastrointestinal neoplasia. Gastrointestinal adenomas and carcinomas arise through the acquisition of multiple, independent genetic mutations and subsequent clonal expansion of mutated epithelial stem cells or other long-lived progenitor cells that reside within the crypt (Fearon and Vogelstein, 1990; Hautt et al 1992.). Programmed cell death, or apoptosis, is the predominant biological response of crypt epithelial cells to low levels of genotoxic and cytotoxic damage that occur chronically in the small intestine and colon (Potten et al, 1997 B; Potten and Grant, 1998). This process results in the removal of individual genetically damaged cells from the crypt epithelium. Thus it has been suggested that apoptosis is an effective cellular strategy for decreasing the probability that any particular crypt epithelial cell will survive injury and acquire the set of multiple mutations necessary for malignancy to occur (Potten, et al., 1992).

It is essential in relation to radiation studies that biologists have investigated the protective effect of natural and synthetic compounds, which influence at the level of genetic regulators along with its quenching power of the reactive energy of the free radicals and eliminating property of oxygen. Radiation-induced acceleration in apoptosis has been found associated with greater adaptability which declines with age. The intracellular oxidation due to the higher metabolic rate in suckling and weaning and young age groups (1 to 4 weeks) might play a major role in different apoptotic response. Oxidative stress promotes damage to the cell structure including proteins, lipids, membranes and DNA, that plays a key role in the development of cancer. (Khandrika, et al., 2009 Fearon and Faux, 2009 Valko, et al., 2006). The high proliferating capacity and a feedback mechanism which can speed up or slow down the production of new cells according to demand should easily involve age associated regulatory genes, which warrants careful evaluation for an effective radiotherapy with a due consideration of the age of subject in conjunction with medicine like NSAIDs, the selective cyclooxygenase-2 (COX-2) inhibitor celecoxib and many associated with one or more known candidate tumour suppressor genes.

The chemoprevention and anti-cancer effects of retinoid may play an important role in the preservation of organs.

$\beta$  carotene one of the carotenoids has been thought of value to human and other species not only as precursor to vitamin A but also having excellent antioxidant properties.  $\beta$ - carotene is highly effective quencher of singlet oxygen and a direct scavenger of free radicals.  $\beta$ - carotene survives the process of absorbing singlet oxygen intact. Therefore, a single molecule can arrest 1000 molecules of singlet oxygen. The fat soluble  $\beta$  carotene is converted to Vit A in the body as it is needed, for the most part this conversion take place in the small Intestine. The present work has been undertaken to evaluate the radio-protective efficacy of  $\beta$  carotene against radiation induced apoptosis. The fat soluble  $\beta$  carotene is converted to Vitamin A in the body as it is needed, for the most part this conversion take place in the small Intestine. Hence the present work will verify the above hypothesis and evaluate the radio-protective efficacy of  $\beta$  - carotene against radiation induced apoptosis.

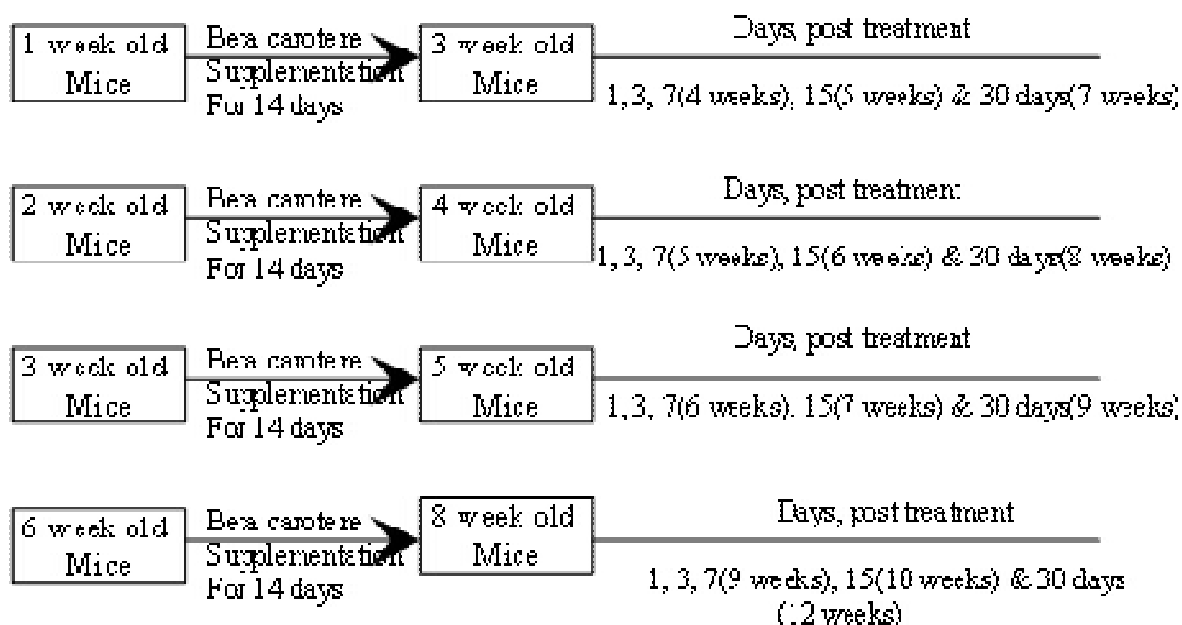
## **MATERIAL AND METHOD**

The present investigation has been designed in two phases:

The first phase of experiments was conducted to observe and evaluate the effect of  $\beta$  -carotene administration in developing (1-3 weeks) and adult (6 weeks old) mice. In the second phase of experiments, the protective efficacy of  $\beta$  carotene has been examined in the Intestine.

## EXPERIMENTAL DESIGN

Swiss albino mice of 1, 2, 3 and 6 weeks age were selected from the colony and supplemented beta carotene for 14 days to animals of each age group. Due to this experimental protocol-



### ANIMALS:

Swiss albino mice (*Mus musculus*) originally procured from AIIMS, New Delhi were maintained and bred in ventilated and constant temperature (18-22°C) housing and were fed with balanced food manufactured by Hindustan Lever Ltd. Water was given *ad libitum*.

### SOURCE OF IRRADIATION:

Mice were irradiated at the Radiotherapy Unit of S.M.S. Hospital, Jaipur, India. by a Theratron model B 60Co beam therapy unit supplied by the AER, Canada Ltd., Canada. Mice were exposed to a total dose of 4.5 Gy.

### RADIOPROTECTIVE DRUG - $\beta$ -CAROTENE:

$\beta$  carotene was procured from Sigma Chemicals, USA in powder form. It was dissolved in olive oil and solution containing 1 mg  $\beta$ - carotene /ml of olive oil is prepared. This solution was given to animals orally for two weeks at the dose of 30 mg/kg of their body weight.

### QUALITATIVE AND QUANTITATIVE STUDIES:

The jejunum (a portion of small intestine next to duodenum) was removed carefully and fixed in Bouin's fluid. After routine dehydration and paraffin embedding, blocks were prepared. 5 m thick sections were cut, fixed and stained with Eosin and Harris Haematoxylin. Stained sections of jejunum were observed for the qualitative and quantitative histopathological variations. Quantitatively, the following parameters were studied in crypts and villi in all the sets of experiments.

### CRYPT CELLULARITY PARAMETERS:

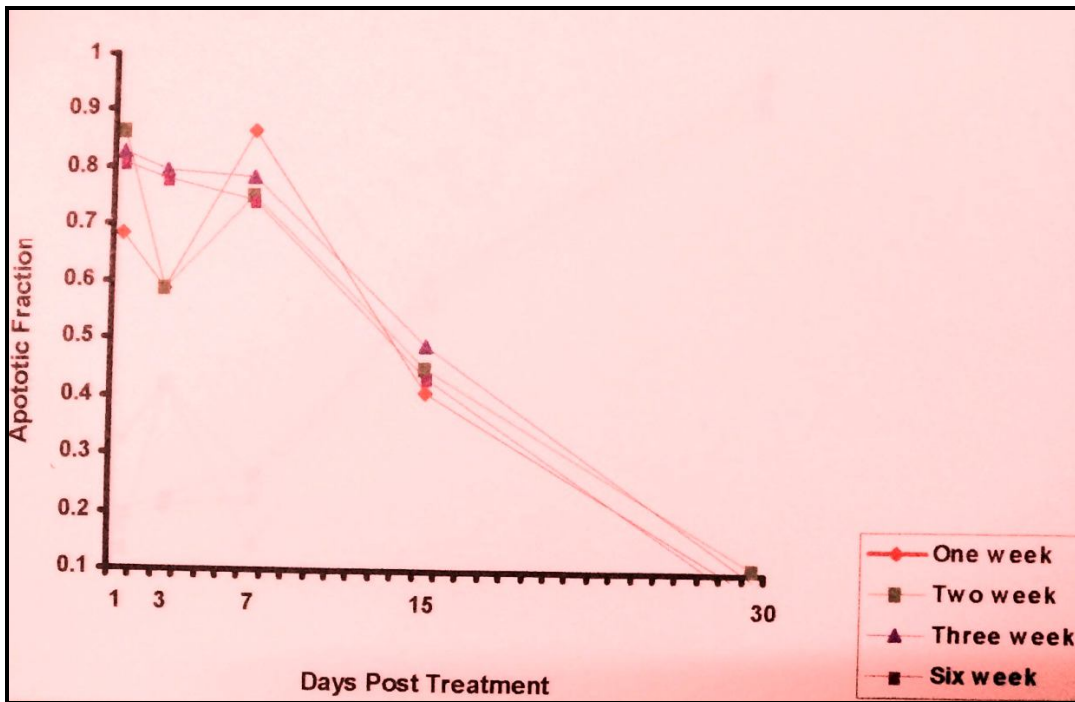
Quantitative Histopathological variations of Intestine-

1. Apoptotic Index
2. Post exposure survival fraction of jejunal crypt cells spared from apoptotic death.
3. Number of apoptotic cells /crypt section

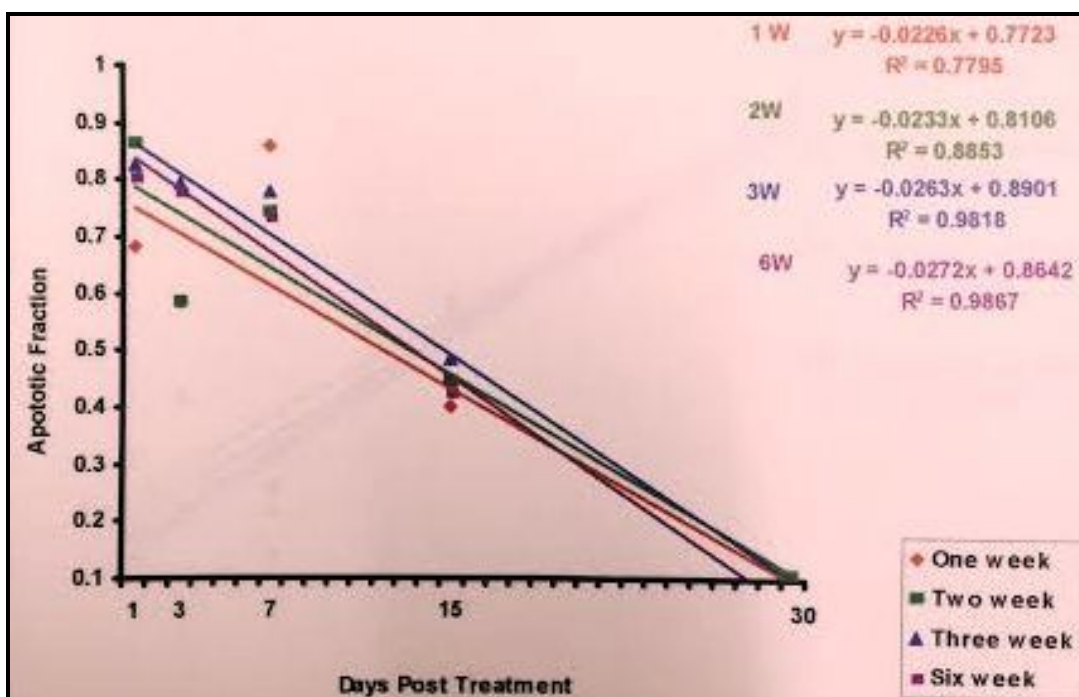
4. Number of mitotic figures /crypt section
5. Number of total cell population /crypt section

Tunel Method of Detection was used. This technique uses the enzyme terminal deoxynucleotidyl transferase (TdT) to label cells that have oligonucleosomal nicks/strand breaks in their DNA. In Situ Cell Death Detection Kit.

**Graph A:** Variation in the Apoptotic fraction of Mice in Different age groups at Various Post Treatment Days, in the presence and Absence of  $\beta$ - Carotene



**Graph B:** Variation in the Apoptotic fraction of Mice in Different age groups at Various Post Treatment Days, in the presence and Absence of  $\beta$ - Carotene



## RESULTS

Evaluation of Apoptotic Cell In terms of Survival Fraction of Jejunal Crypt Cells: Survival fraction of jejunal crypt cells exhibit an inverse relationship with apoptotic index. At all the intervals, when crypt cellularity registers a decrease, the apoptotic cells exhibit an increase. Minimum survivability at post exposure day 1 has been shown by mice in which  $\beta$  carotene treatment initiated from 2 weeks of age. Survivability of crypt cells was found minimum at this interval attributed to greatest sensitivity at this period. This age group shows maximum apoptotic index at the same interval. Hence, it is clearly shown that there exists an inverse relationship between these two factors. An inverse relationship between survival fraction of crypt cells and apoptotic index has been noticed, as evident by survival curve of jejunal crypt cells. An inverse relationship between the number of apoptotic cells and mitotic figures noticed as radiation insult induces a decrease in crypt cellularity, mitotic activity and villus cellularity on one hand and an increase in apoptotic bodies on the other hand. The biphasic spurts of apoptotic index at day 1 and 7 was notice in early age groups which was much lower in beta carotene treated group which is suggestive of subside radiation stress on inherent programmed death. Pattern of variation in total cells of crypt and villus clearly supports the fact that the cells to villi supplied from the crypt in such groups got rid of the influence of radiation.  $\beta$ -carotene administration prior to irradiation reduces the stress on developing jejunal epithelium and augments the process of recovery and reparation.  $\beta$ -carotene offers higher protection in experimental animals of one week age group as compared to other ages. In one week age group animals, the maximum susceptibility to apoptotic death was noticed at post exposure days 1 and 7 (Table A,B & Graph A,B)

## DISCUSSION

Cells can respond to stress in various ways ranging from the activation of survival pathways to the initiation of cell death that eventually eliminates damaged cells.

The rate of repair failing will depend on the stress experienced by the cell. This experience will depend on 2 factors, inherent and exogenous. The possibility cannot be ruled out that the inherent factor, concerned gene could be influenced by the external factors like food etc. A feedback mechanism is also expected which could speed up or slow down the production of new cells according to stress. As crypts posses all characteristic required for a tissue to be radiosensitive. Any radiation exposure will disrupt the normal equilibrium of intestinal mucosa, which in turn will have direct bearing on its functional aspect like digestion and absorption of food.

Jejunal crypt cells undergo apoptosis in response to ionizing radiation exposure. In mice the number of cells deleted by apoptosis is determined by several factors including the dose of radiation, the time of day the apoptosis level is quantified, and the strain of mouse irradiated. In an *in vivo* micro colony survival assay, pre-irradiation administration of beta-carotene increased the number of surviving crypts in the jejunum by a factor upto 2.0 ( $P < 0.05$ ) and villi cellularity by 1.8 ( $P < 0.05$ ) fold in comparison to irradiated control. Pre-irradiation administration of carotene reduced the incidence of apoptotic bodies in the crypts ( $P < 0.05$ ) in a time dependent manner and depicted a mitotic arrest till 30<sup>th</sup> day interval; the percentage of mitosis was observed to be nearly similar to that of unirradiated control. This study suggests that arrest of cell division may help in protecting the clonogenic cells against radiation. It would be interesting to investigate further the role of beta carotene in influencing various cell cycle regulators like p53, bcl-2, TGF-beta, Cyclin-E etc. The *in vivo* response to radiotherapy is not well understood but appears to involve the p53 tumor suppressor protein.

The difference in radiation-induced apoptosis levels between C57BL/6J (B6) and C3Hf/Kam (C3H) mice is claimed to be controlled by multiple genes by Weil and collaborators (2001). This set of genes is distinct from that controlling thymocyte apoptosis levels in the same strain combination. A new quantitative trait locus on chromosome 15, Rapop5, partly accounts for the murine strain difference in susceptibility to radiation-induced jejunal crypt cell apoptosis. A sexual dimorphism in the extent of radiation-induced jejunal crypt cell apoptosis, with female mice having higher levels has also been reported (Weil et al., 2001). The expression of apoptosis-inducing p53 target genes during gamma-irradiation-induced cell death in p53(+/+) or p53(-/-) mouse tissues using in situ hybridization reveal striking tissue specificity with distinct regulation of target p53-induced

genes in different cells and tissue compartments (Fei et al., 2002). Their findings indicate that the selectivity of p53 in transactivation following DNA damage *in vivo* results in unique tissue and cell type specificity, which may correlate with growth arrest or variable sensitivity to gamma-irradiation. IGF-I regulates cell number by stimulating crypt cell proliferation and decreasing apoptosis preferentially within the stem cell compartment. (Wilkins et al., 2002) Our data tend to support the hypothesis that DNA synthesis inhibition and cell cycle redistribution are surrogates for radiosensitization. More data points are however required before a definite conclusion can be drawn.

### **INFLUENCE OF BETA CAROTENE**

The effects of various carotenoids on the proliferation, cell cycle, apoptosis and  $\beta$ -expression of bcl-2 gene in breast cancer cell MCF-7 was investigated by Li and co-workers. The effects of individual carotenoids on cell cycle and the apoptosis observed by flow cytometry showed that 4 tested carotenoids inhibited the proliferation of MCF-7 cell line, but with different potencies. Beta-carotene and lycopene were the most active inhibitors (inhibition rate 88.2% and 87.8%, respectively) followed by zeaxanthin and astaxanthin. All 4 carotenoids did not induce cell apoptosis. Cell cycle progression was blocked at G (2)/M phase with 60 micromol/L lycopene and at G (0)/G (1) phase with 60 micromol/L zeaxanthin dipalmitate. Carotenoids down regulated bcl-2 gene expression (Li et al., 2002). The induction of apoptosis in transformed cells by carotenoids may explain their protective effect against cancer formation in humans (Muller et al., 2002).

Retinoid is one of the most promising substances for chemoprevention and anti-cancer effect. Retinoid has the following reported actions: induction of cell differentiation, control of cancer growth, repair of the precancerous lesion, prevention of the secondary carcinogenesis, control of angiogenesis and prevention of metastasis, and immunostimulation. In one of the studies, retinoid modified the cell cycle, reinforced the G1 check mechanism which is lost in cancer cells and induced apoptosis. Retinoid augmented the membrane permeability of anti-cancer drugs such as 5-FU, and reduced the exocytosis of anti-cancer drugs by suppressing the  $\beta$ -expression of the transport protein cMOAT. Retinoid also suppressed the invasive growth of the cancer cells. With the FAR therapy regimen (5-FU and retinil palmitate with radiation) and subsequent surgery, the disease-specific five-year survival rate was close to 50% in various head and neck cancers. Thus, the chemoprevention and anti-cancer effects of retinoid may also play an important role in the preservation of organs (Yamamoto, 2001).

### **CONCLUSION**

Intracellular oxidation may play a central role in apoptotic cell death. It is also clear from the findings that the antioxidants like beta carotene protect the cell against apoptosis from radiation at the regulatory gene level. The data will be indicative of the future prospects and ensures exploitation of oral administration of  $\beta$ -carotene against radiation in clinical application and radiotherapy for cancer.

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