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**Effects of vitamin antioxidant supplementation on cell kinetics of patients with adenomatous polyps**

Department of Gastroenterology, Meath and Adelaide Hospitals, Trinity College, Dublin, Ireland.

R.J. Cahill, K.R. O'Sullivan, P.M. Mathias, S. Beattie, H. Hamilton, C.O'Morain

Correspondence to: Prof. C O'Morain, Dept. Gastroenterology, Meath Hospital, Heytsbury St., Dublin, Ireland.

**Abstract**

Colonic crypt cell proliferation is used as an indicator of risk of colorectal carcinoma. Subjects with adenomatous polyps and cancer have an increased cell proliferation and a shift of the proliferative zone towards the apex of the crypt. Epidemiological and *in vitro* studies have confirmed a link between vitamins A, E, C,  $\beta$ -carotene and colorectal cancer. *In vitro* bromodeoxyuridine immunohistochemical technique was used to assess the effect of daily oral supplementation with vitamin E (160 mg), vitamin C (750 mg) or  $\beta$ -carotene (9 mg) on the colonic crypt cell proliferation in patients with adenomatous polyps ( $n = 40$ ) compared with normal subjects with no colonic disease ( $n = 20$ ). The patients were given supplementation for one month and colonic biopsy specimens were taken before and at the end of the trial. Patients with adenomatous polyps had a significantly higher mean labelling index per cent than controls ( $p < 0.001$ ). Vitamin C or  $\beta$ -carotene supplementation, however, significantly reduced the total proliferation ( $p < 0.005$ ) whereas vitamin E supplementation had no effect on the colonic crypt cell proliferation.  $\beta$ -carotene reduced cell proliferation at the base of the crypt only. Vitamin C reduced cell proliferation in all the crypt compartments from the apex to the base to those values seen in age and sex matched controls. These findings indicate that prolonged supplementation with vitamin C may reduce the recurrence of adenomatous polyps.

**Introduction**

Colorectal cancer develops through a complex process from hyperproliferative mucosa to adenoma and eventually to carcinoma [1]. The pathogenesis of this sequence of events is largely unknown. It is generally accepted however, that hyperproliferation of the colorectal mucosa with a shift of the proliferative zone to the upper portion of the crypt is the first step of the sequence [2].

One of the primary insults to the colonic mucosa in the complex adenoma-carcinoma sequence may be an increase in the free radical activity, which has been shown to cause DNA damage [3] and gene mutations. Antioxidants are a defence against free radical activity and epidemiological studies confirm that populations with a low level of antioxidants may have a higher risk of colorectal cancer [4, 5].

Vitamins A, E and C are potent antioxidants and the serum concentrations of these antioxidants have been shown to inversely correlate with the risk of developing colorectal cancer [4-6]. Clinical trials [9-12] have assessed the effect of these antioxidant vitamins as supplements on the risk of colorectal cancer.

The aim of this study was to assess the effect of oral supplementation with vitamin C, vitamin E or  $\beta$ -carotene on colonic crypt cell proliferation of patients with adenomatous polyps.

**Effects of vitamin antioxidant supplementation on cell kinetics of patients with adenomatous polyps**

**Patients and Methods**

Sixty nine subjects undergoing colonoscopy were recruited for this study. These were aged between 34-80, had a complete colonoscopic examination of the large bowel, were capable of following the study protocol, and gave written informed consent.

Subjects were excluded from the study if they suffered from any debilitating or life threatening disease, including invasive cancer. Patients with familial polyposis coli, inflammatory bowel disease, malabsorption syndrome, or a personal history of colonic resection were excluded. Subjects with an incomplete examination of the large bowel, severe dysplasia, or carcinoma in situ were excluded. Any subjects taking vitamin supplements within a year of entering the trial were not included in this study.

Forty subjects with adenomatous polyps proved histologically of diameter greater than 5 mm were included in the final analysis. Twenty subjects with normal colonoscopy and histologically normal colonic mucosa were included as a control group. Table 1 shows the demographic profile of these subjects.

Table 1: Demographic profile of the study groups

Study group	(n)	Age mean (SD)	Age range (y)	Men : Women
No Supplement	10	60 (2.0)	41-77	5:5
Vitamin E	10	62 (2.8)	43-73	7:3
Vitamin C	10	60 (2.5)	53-73	7:3
$\beta$ -carotene	10	63 (4.5)	34-80	3:7
Normal Subjects	20	61 (3.8)	34-80	10:10

The patients with adenomatous polyps were assigned to one of four study groups using the random sampling quota technique allowing for 10 subjects in each study group. Group 1: no supplementation. Group 2: 160 mg of vitamin E daily. Group 3: 750 mg of vitamin C daily. Group 4: 9 mg of  $\beta$ -carotene daily. The supplementation dose was given for 1 month, in two or three tablets with meals. Vitamin supplements were kindly donated by Nature's Own, West Malvern, United Kingdom.

Colonic biopsy specimens were taken before and at the end of the supplementation period. The colonoscopies were carried out between 1100 and 1300 to allow for diurnal variation in proliferation rates [13]. Four pinch biopsy specimens were taken for cell proliferation analysis from macroscopically normal mucosa in the sigmoid colon, 20 cm from the anus, to control for the anatomical difference in cell proliferation [14]. These were immediately placed in modified Waymouth's medium. Twenty mls of whole blood was also collected into empty vacutainers before and at the end of the one month trial period for analysis of serum vitamins, as an assessment of compliance to the supplementation. Compliance was also confirmed by a tablet count.

**Cell Proliferation Technique [15]**

*In vitro* incorporation of bromodeoxyuridine was achieved by culturing the biopsy specimens in Waymouth's medium (Flow Labs, Scotland) supplemented with 10% fetal calf serum, 1mM glutamine and gentamicin, 50  $\mu$ M bromodeoxyuridine (Sigma Co, Poole, UK), and 5  $\mu$ M fluorodeoxyuridine (Sigma Co, Poole, UK). The biopsy specimens were incubated in the medium for one hour at 101.3 kPa of pressure in a sealed modular incubation chamber (Flow Labs,

*Gut* 1993, 34, 963-967.

### **Effects of vitamin antioxidant supplementation on cell kinetics of patients with adenomatous polyps**

Hertfordshire, UK) previously infused with 95% oxygen and 5% carbon dioxide at a thermoregulated temperature of 37°C. The biopsy specimens were fixed in Carnoy's fixative overnight and embedded in paraffin wax.

Immunohistochemical detection of the bromodeoxyuridine incorporation was carried out on 4 µm sections. After deparaffinisation and rehydration through alcohol to phosphate buffered saline, endogenous peroxidase activity was blocked by immersing the sections in 5% H<sub>2</sub>O<sub>2</sub> in Methanol. The biopsy DNA was denatured by incubation in 1M HCL at 60°C for eight minutes. The sections were incubated in 5% normal rabbit serum to reduce background staining and then incubated with the monoclonal antibody to bromodeoxyuridine (Dakopatts, Denmark) diluted 1:100 with phosphate buffer for one hour. The slides were then washed in phosphate buffered saline and incubated with a 1:300 dilution of biotinylated anti-mouse antibody (Dakopatts, Denmark). After further washings in phosphate buffered saline the slides were incubated with peroxidase conjugated strepavidin diluted 1:400, washed in phosphate buffered saline and the labelled cells were visualised by the diaminobenzidine reaction and lightly counterstained with haematoxylin.

#### ***Immunohistochemical Analysis***

A mean of 20 sections were examined for each subject. Only crypts longitudinally sectioned and visible in their entire length were analysed. A mean number of 10 well orientated crypts were examined for each specimen.

Labelling index per cent (LI%) was measured by counting the number of bromodeoxyuridine positive cells and expressing the result as a percentage of the total number of cells in a crypt. The relative positions of the positive cells in the crypt were noted. For cell kinetics evaluation, each crypt was divided into five compartments of equal size. The compartments were referred to by ordinal numbers from 1 (apex) to 5 (base) and the labelling index calculated for each compartment. The samples were analysed blind by a single observer.

#### ***Serum Analysis of Vitamins***

Serum analysis of vitamins was performed as a method of assessing compliance. The serum was stored at -20°C until all samples were collected. retinol, β-carotene, vitamin C and α-tocopherol were analysed using high performance liquid chromatography. Plasma cholesterol was measured by a Boehringer Mannheim GmbH kit (Mannheim, Germany). The α-tocopherol values were expressed as a ratio of α-tocopherol to cholesterol values.

#### ***Statistical Analysis***

The total LI% and the LI% per compartment was compared before and after supplementation in each group. Serum vitamin values were compared before and after the supplementation period. The significance was analysed using the Signed Rank Sum test.

#### **Results**

Forty nine patients with adenomatous polyps were recruited into this study, nine were not included in the final analysis (five were lost to follow up, two were diagnosed with upper gastrointestinal cancer, one died of myocardial infarction and one patient in the β-carotene group was not compliant). Patients were deemed compliant for this study on the basis of a significant rise in serum concentrations of the supplemented vitamin and a correct tablet count.

**Effects of vitamin antioxidant supplementation on cell kinetics of patients with adenomatous polyps**

On analysis of the total labelling index per cent (Fig. 1) patients with adenomatous polyps had significantly higher mean (SEM) LI% (6.2 (0.3)) compared with normal controls (3.2 (0.3)),  $p < 0.001$ . When the LI% was analysed before and after the trial (Table 2) it was found that the total LI% or compartment LI% did not change over the one month period in the absence of supplementation (Figs. 1 and 2).

Table 2: Cell kinetics of macroscopically normal mucosa of all study groups before and after supplementation.

	No Supplement		Vitamin C		Vitamin E		β-carotene	
	Before trial	After trial	Before	After	Before	After	Before	After
No. of subjects	10	10	10	10	10	10	10	10
Crypts per subject	12 (0.4)	12 (0.6)	11 (0.2)	10 (0.2)	11 (0.3)	12 (0.7)	10 (0.3)	10 (0.2)
Total cells counted per subject	1190 (46)	1193 (39)	1279 (37)	1466 (74)	1247 (63)	1376 (61)	1256 (65)	1326 (47)
Total labelled cells per subject	82 (6.9)	77 (8.3)	68 (5.6)	39 (4.3)	68 (6.1)	76 (5.7)	88 (7.3)	50 (4.5)
Total Cells per crypt	99 (4.5)	99 (4.7)	121 (4.7)	149 (5.6)	115 (4.4)	114 (4.5)	127 (7.9)	129 (5.1)
LI% total	6.9 (0.7)	6.6 (0.8)	5.4 (0.3)	2.5 (0.2)*	5.4 (0.3)	5.7 (0.4)	7.1 (0.7)	3.9 (0.4)*
LI% per compartment								
LI% 1	0.5 (0.2)	0.5 (0.2)	0.08 (0.1)	0.03 (0.03)	0.05 (0.1)	0.2 (0.1)	0.2 (0.1)	0.2 (0.1)
LI% 2	2.4 (0.5)	2.0 (0.5)	1.7 (0.5)	0.3 (0.1)+	1.7 (0.4)	2.0 (0.6)	1.8 (0.3)	1.3 (0.2)
LI% 3	6.8 (0.8)	6.5 (0.6)	6.5 (0.6)	2.2 (0.4)*	4.8 (0.7)	7.4 (0.8)	5.7 (0.6)	4.4 (0.8)**
LI% 4	12.5 (0.9)	10.5 (1.0)	10.3 (0.8)	4.5 (0.4)*	9.9 (0.9)	11.0 (1.1)	13.8 (1.5)	7.2 (3.0)*
LI% 5	12.5 (1.3)	12.0 (1.4)	8.2 (1.3)	5.7 (0.5)*	9.4 (0.7)	7.4 (0.8)	13.5 (1.5)	6.7 (2.1)**

All results expressed as mean (SEM); \* $p < 0.005$ ,  $p < 0.05$ , \*\* $p < 0.01$ , when compared with values before supplementation.

LI% = labelling index per cent.

β-carotene supplementation significantly reduced the total LI% ( $p < 0.005$ ) (Figs. 1 and 3). On compartmental analysis (Table 2) the proliferation at the base of the crypt (compartments 3, 4 and 5) was significantly reduced, however, there was no significant reduction at the apex of the crypt (compartments. 1 and 2). When the total LI% was compared with that of age and sex matched controls (Table 3), it was found that although β-carotene reduced the total LI% it did not reduce it to control values (Fig. 3).

Table 3: Comparison of cell proliferation indices of β-carotene and vitamin C study groups after trial to age and sex matched controls.

	Vitamin C		β-carotene	
	Normal Subjects	After trial	Normal Subjects	After trial
No. of subjects	10	10	10	10
Age	60 (2.0)	60 (2.5)	63 (5.0)	63 (4.5)
Men : Women	7:3	7:3	3:7	3:7
Total cells counted	1652 (68)	1466 (4.3)	1474 (72)	1326 (47)
Total labelled cells	54 (6.8)	39 (4.3)	44 (3.2)	50 (4.5)
Total LI%	3.1 (0.2)	2.5 (0.2)	3.0 (0.3)	3.9 (0.4)*
LI% per compartment				
LI% 1 (apex)	0	0.03 (0.03)	0.08 (0.1)	0.15 (0.1)
LI% 2	0.4 (0.2)	0.3 (0.1)	0.3 (0.2)	1.3 (0.2)+
LI% 3	2.7 (0.4)	2.2 (0.4)	2.2 (0.5)	4.4 (0.8)+
LI% 4	5.3 (0.7)	4.5 (0.4)	5.7 (0.5)	7.2 (0.1)*
LI% 5 (base)	5.9 (0.5)	5.7 (0.5)	7.0 (0.7)	6.7 (0.7)*

All results expressed as mean (SEM); \* $p < 0.005$ , + $p < 0.05$  when compared with age and sex matched controls.

LI% = labelling index per cent.

Vitamin C supplementation significantly reduced the total LI% ( $p < 0.005$ ) (Figs. 1 and 2). On compartmental analysis, proliferation in all compartments was significantly reduced (Table 2). When these were compared with age and sex matched controls (Table 3), no significant difference was seen in any of the labelling indices (Fig. 4).

### Effects of vitamin antioxidant supplementation on cell kinetics of patients with adenomatous polyps

Vitamin E supplementation had no effect on the increased proliferation found in patients with adenomatous polyps.

Figure 1: Comparison of the total labelling index per cent (LI%) of all the study groups before and at the end of the trial (mean, SEM). \*p < 0.005 when compared with values before supplementation. +p < 0.001 when compared with patients with a history of adenomatous polyps.

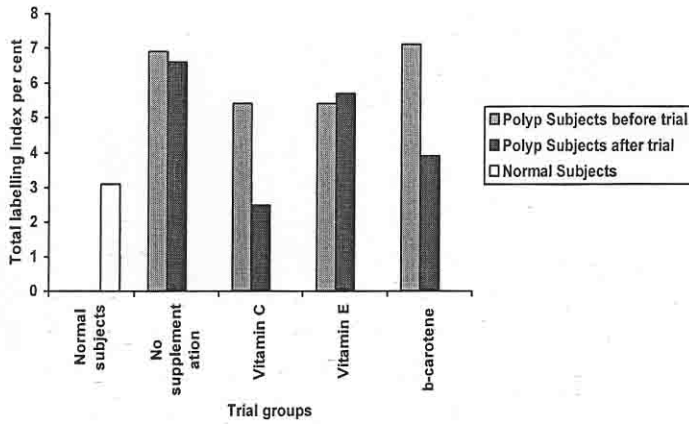
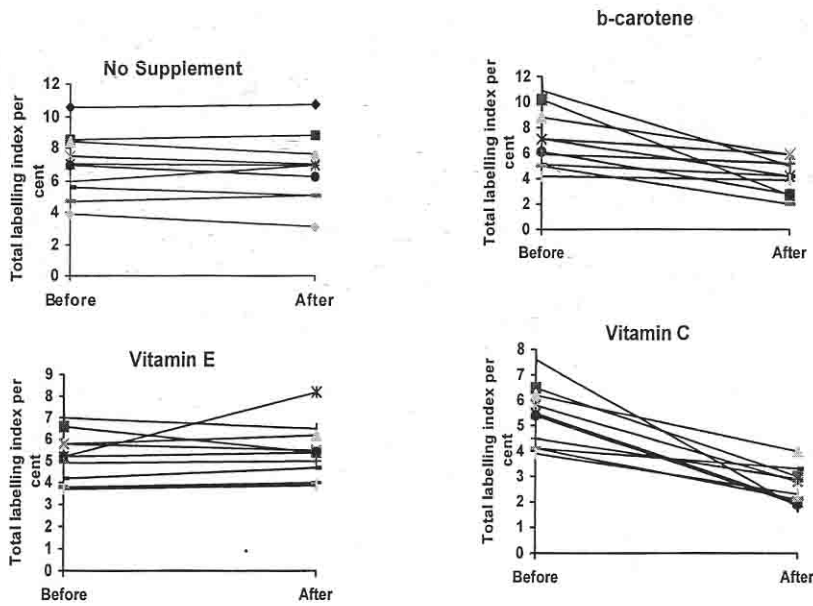


Figure 2: Individual LI% of each adenomatous polyp subject. \*p < 0.005 compared with values before the trials.



### Effects of vitamin antioxidant supplementation on cell kinetics of patients with adenomatous polyps

Figure 3: Comparison of the compartmental LI% before and after supplementation of the  $\beta$ -carotene group and age and sex matched controls (mean, SEM).

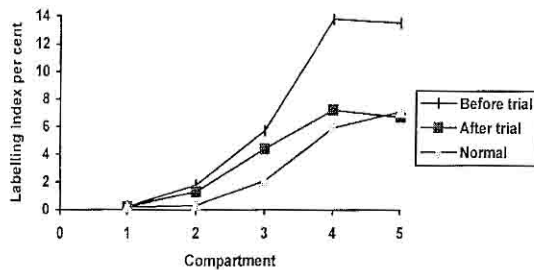
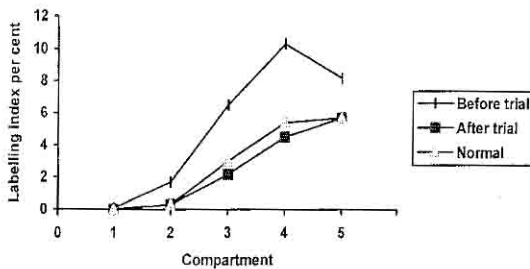


Figure 4: Comparison of the compartmental LI% before and after supplementation of the vitamin C group and age and sex matched controls (mean, SEM).



#### Discussion

This study assessed the effect of antioxidant vitamin supplementation on colonic crypt cell proliferation [16] in patients with adenomatous polyps of the colon. In contrast with other vitamin supplementation studies [9-12] this study assessed the effect of single vitamin supplements only, the study was prospective with a control group, subjects with familial polyposis coli were excluded and subjects were sequentially assigned to each group.

Subjects with adenomatous polyps had an increased cell proliferation with an increasing trend of proliferating cells towards the apex of the crypt as has been found by others [14-17]. We found that vitamin E supplementation had no effect on this abnormal proliferation.  $\beta$ -carotene and vitamin C supplementation significantly reduced the total cell proliferation.  $\beta$ -carotene did not change the shift of the proliferative zone towards the apex of the crypt and the indices of proliferation taken after supplementation were not reduced to those found in age and sex matched controls. Vitamin C reduced the cell proliferation to that found in age and sex matched controls, it also reduced the upward shift in the proliferative zone.

Although the mean LI% of subjects with adenomatous polyps was significantly higher than those with no colonic disease ( $p < 0.001$ ), there was an overlap in the total LI% of both groups (Fig. 2). It is possible that the younger control subjects with a high LI% may develop an adenomatous polyps as an ageing phenomenon. Paganelli et al [18] found that there was no significant difference in the LI% between subjects with a small adenoma (< 1 cm), single

*Gut* 1993, 34, 963-967.

### Effects of vitamin antioxidant supplementation on cell kinetics of patients with adenomatous polyps

large adenoma, multiple small adenomas, multiple adenomas including at least 1 large, colorectal cancer and no colorectal disease. Our results agree with studies carried out by Roncucci et al [17], Bleiberg et al [16] and Terpestra et al [14], however, who have all found that subjects with adenomatous polyps had a significantly higher labelling index than those with no colonic disease.

The increased proliferation and the significant upwards trend of the proliferating cells towards the apex of the crypt, seen in patients with adenomatous polyps, is a product of the increased S-phase duration of the pre-malignant cells and may be specifically related to cancer [16]. A mucosa without histological signs of malignancy but with an increase S-phase duration, would indicate that the malignant process has started [16].

Most studies assessing cell proliferation have used the tritiated thymidine immunohistochemical technique. The bromodeoxyuridine immunohistochemical technique has overcome many of the problems associated with the tritiated thymidine technique and the accuracy of this technique has been verified by Lacy et al [19]. They found it to be as accurate and reliable as the tritiated thymidine autoradiography technique, the gold standard for the histological study of cell proliferation when examining mucosa of the digestive tract. The bromodeoxyuridine technique has many advantages over this technique as it is completed in 3-4 days compared with the 14 days for tritiated thymidine and furthermore the reaction is found in the plane of the tissue section facilitating rapid cell counting. Proliferating cell nuclear antigen immunohistochemical technique although a promising method of assessing cell proliferation has yet to be fully standardised.

Evidence linking vitamin antioxidants and cancer obtained from epidemiological studies is conflicting [5, 6, 20-22]. We recently assessed serum concentrations of vitamin A and E in Irish subjects with adenomatous polyps and colorectal cancer [4]. We found lower values of vitamin E in these subjects compared with healthy controls (12.5  $\mu\text{M}$  v 16.4  $\mu\text{M}$ ,  $p < 0.005$ ) and lower vitamin A values in patients with colorectal carcinoma compared with controls (1.33  $\mu\text{M}$  v 1.5  $\mu\text{M}$ ,  $p < 0.005$ ). Stronger evidence provided by *in vitro* studies has confirmed the link between vitamins A, E, C and  $\beta$ -carotene and colonic cancer [23-25].

A large prospective study by Wald et al [20] found that serum vitamin E values in subjects who subsequently developed colorectal cancer were no different to those of age and sex matched controls. *In vitro* work has also shown that vitamin E has no effect on the regression of induced tumours, which would validate our findings in this study.

Few studies have been carried out assessing the effect of vitamin supplementation on adenomatous polyps or colorectal cancer. De Cosse et al [11] found vitamin C and vitamin E supplementation had no effect on polyp recurrence, but in that study the supplements were taken in combination with fibre supplementation. Bussey et al [9] examined the effect of vitamin C supplementation on cell proliferation using the tritiated thymidine technique. They found that supplementation reduced the total cell proliferation although in contrast with our results, vitamin C did not change the upward shift of the proliferative zone.

In conclusion this study has shown that both  $\beta$ -carotene and vitamin C supplementation reduce the abnormal colonic crypt cell proliferation found in subjects with adenomatous polyps. Short term supplementation with vitamin C reduces the cell proliferation to normal values possibly by reducing the S-phase duration. This may reduce the risk of progression to carcinoma and the recurrence of adenomatous polyps. Long term studies will be needed to assess if this effect is maintained.

Effects of vitamin antioxidant supplementation on cell kinetics of patients with adenomatous polyps

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