CHAPTER 5

BIOAVAILABILITY STUDIES OF CAROTENOIDS USING HUMAN EXFOLIATED COLONIC EPITHELIAL CELLS*

5.1 Introduction

As described before, a number of factors affect the bioavailability of vitamin A and provitamin A carotenoids such as their conversion to vitamin A, rate of absorption, transport, chemical nature of the provitamin A, and also the fat content in the diet. The type of food matrix in which carotenoids are located is a major factor affecting the bioavailability of provitamin A carotenoids; bioavailability of β-carotene from vegetable in particular has been shown to be low compared to that of purified β-carotene added to a simple matrix, whereas for lutein the difference is much less (Van het Hof et al., 2000).

As indicated before, vitamin A status is assessed at different levels; however routine measurement of plasma vitamin A may not be a good indicator as vitamin A level in the plasma is often ‘buffered’ and the best indicator is the liver level of vitamin A. Invasive sampling of blood and liver biopsies are not practically feasible in field investigations and there are no non-invasive methods to assess the provitamin A / vitamin A status in a target population.

Stool is one of the major excretory products of all higher animals and it consists of a mixture of undigested food residues, microflora, endogenous secretions and cellular components exfoliated from the gastro intestinal tract (Iyengar et al., 1991); this excretory product is generated with predictable regularity. It is reported that about 55% of the stool mass on dry weight basis is contributed by microbial flora (Stephen et al., 1980). Recovery and characterisation of exfoliated intestinal tract cells from human stools has been reported by Albaugh et al. (1992) using countercurrent centrifugal elutriation. The gastro intestinal tract sheds about 2 x 10^{11} cells per day as estimated from DNA-loss-

rate studies (Snyder et al., 1974). Exfoliated cells represent an important source of informational macromolecules providing a picture of the immediate past history of the colonic epithelium (Albaugh et al., 1992). Recent advances in the biology of the gut made it possible to obtain pure exfoliated viable cells from human stools for metabolic studies (Nair et al., 1996). The possibility of using human exfoliated colonic epithelial cells as an indicator for provitamin A/vitamin A bioavailability was examined and the results are presented in this chapter.

5.2 Materials and Methods

5.2.1 Separation of colonic epithelial cells

Exfoliated colonic epithelial cells were isolated by a modified procedure of Albaugh et al. (1992) as described in the chapter 2.

5.2.2 Extraction of $\beta$-carotene from colonic epithelial cells and HPLC analysis

Colonic epithelial cells were extracted and HPLC analysis was done using a SPD 10A Shimadzu liquid chromatograph operating at room temperature. A Whatman Partisil-5 ODS 3 column was used. Freshly prepared degassed mobile phase (acetonitrile: chloroform: isopropanol: water in 780: 160: 35: 25, v/v) was used. Flow rate was adjusted to 2.0 ml/minute. $\beta$-carotene was measured at 450 nm and retinol at 325 nm. Authentic samples of $\beta$-carotene, lutein and retinol and other carotenoids were used to identify the respective retention time. The peak area measurements were used for calculation. The carotenoids extracted from cells from 500 mg stool sample was sufficient for eight to ten replicate analysis and the detection limit at high resolution setting was 10 ng (in injected sample). Duplicate analysis of each sample was done.

5.2.3 Extraction and analysis of supplements

Carotenoids were extracted from Spirulina and Agathi and analysed by HPLC.

5.2.4 Culture of colonic epithelial cells

Freshly isolated cells were washed with PBS and suspended in the culture medium (MEM). 1.5 ml medium was taken in 35 mm culture dishes. Cultures were
Bioavailability studies of carotenoids using human exfoliated colonic epithelial cells

incubated in a Forma CO₂ incubator maintained in 95% air, 5% CO₂ at 37°C. After required time of incubations culture dishes were observed under microscope. Growth and viability were assessed at regular intervals.

5.2.5 Protein estimation

Protein content was estimated using Lowry’s (1951) method as described earlier.

5.2.6 Separation and analysis of ¹⁴[C] carotenoids

¹⁴[C] carotenoid and retinol present in the colonic epithelial cells were extracted, separated by TLC and the radioactivity present in carotenoids and retinol was measured as described in the Chapter 2.

5.2.7 Subjects

Subjects were selected based on their ability to control different dietary regimes and obtained informed consent from them. Volunteers from the laboratory provided stool samples for isolation of colonic epithelial cells. Daily diet diary on food intake was maintained and caloric values (Gopalan et al., 1995) were computed. β-carotene, fat and protein content and intake of energy from the diet were also determined.

5.2.8 Study design

Subjects (n=9) were advised to take β-carotene poor diets for 3 weeks. Then the same subjects were divided in two groups. One group (group II, n=3+3) was asked to take diets with high β-carotene content and others with β-carotene poor diet (group I). Subsequently on the 7th day, subjects in group II (n=3+3) were divided into two groups (group II a and II b) of three each and were again administered a bolus of high β-carotene diet with supplements as cooked Agathi (Sesbania grandiflora) and Spirulina pellets for one day and continued β-carotene free diet for another one week as shown in the study design (Figure 5.1). Stool samples were collected from the subjects daily. Colonic epithelial cells were isolated, extracted, and subjected to HPLC analysis for β-carotene, lutein and retinol contents.

Details of the analysis have been given in the Chapter 2.
5.3 Results

5.3.1 Isolation of colonic epithelial cells

Colonic epithelial cells were isolated from the different fecal samples. Using Trypan-blue exclusion method viability of the isolated cells was checked. Quantity of viable cells obtained from a unit mass of feces was also recorded. Results of cell yield and its viability are shown in Table 5.1. Cells were sedimented in different fractions, based on the buoyant density of the cells. Higher rate of recovery of viable cells was obtained from the interphase fraction. About 15-20 x 10^6 cells/g stool were recovered from the interphase fraction, of which about 70-80% of the cells, appeared to be viable and about 65% of the cells in cushion fraction was also found to be viable. Only 35% cells in the pellet fraction were viable.
Table 5.1
Distribution of colonic epithelial cells

<table>
<thead>
<tr>
<th>Fractions</th>
<th>No. of cells/g stool (x 10⁶)</th>
<th>% viability</th>
<th>β-carotene content (ng/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interphase</td>
<td>15-20</td>
<td>70-80</td>
<td>50.0</td>
</tr>
<tr>
<td>Cushion</td>
<td>10-15</td>
<td>60-70</td>
<td>25.8</td>
</tr>
<tr>
<td>Pellet</td>
<td>5-10</td>
<td>30-40</td>
<td>20.7</td>
</tr>
</tbody>
</table>

Exfoliated colonic epithelial cells were isolated from the stool samples, distribution of cells present in the different fractions were determined. Viability of the cells was assessed by Trypan-blue exclusion. Cells were extracted and β-carotene content was estimated by HPLC. Values are from three different samples.

5.3.2 Carotenoid and retinol content of colonic epithelial cells

The content of vitamin A and β-carotene in exfoliated colonic epithelial cells was analysed. Cells were isolated from the stool samples of the subjects. Nutrient intake and vitamin A and β-carotene intake of the 24 hour diet was recorded. Table 5.2 shows the results of analysis of colonic epithelial cells.

Table 5.2
Change in β-carotene and retinol content in exfoliated colonic epithelial cells with change in the β-carotene content in the diets

<table>
<thead>
<tr>
<th>Group</th>
<th>Type of diet</th>
<th>Average nutrient intake (24 hrs.)</th>
<th>β-carotene and retinol content (ng/mg cell protein) in colonic epithelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Protein (g) Fat (g) Energy (k.cal.)</td>
<td>Interphase Pellet Interphase Pellet</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β-car (μg) mean ± SE</td>
<td>mean ± SE</td>
</tr>
<tr>
<td>I</td>
<td>β-carotene poor</td>
<td>59.2 42.4 2156 95</td>
<td>14.3 ± 2.1 9.3 ± 2.0</td>
</tr>
<tr>
<td>II a</td>
<td>β-carotene rich</td>
<td>60.8 45.7 2805 1500</td>
<td>38.1 ± 4.5 30.0 ± 4.9</td>
</tr>
<tr>
<td>II b</td>
<td>β-carotene rich</td>
<td>62.0 40.6 2605 1800</td>
<td>50.6 ± 5.6 43.0 ± 4.3</td>
</tr>
</tbody>
</table>

Subjects of group II a and II b received carotenoid sufficient diet and those of group I received carotenoid poor diet for a period of 7 days after a preparatory phase of 3 weeks as indicated in Fig. 5.1. The daily intake of protein, fat and β-carotene was calculated from the diet diary. Exfoliated colonic epithelial cells were isolated from the subjects (3 each) after 7 days (day 0 in flow chart) and the distribution of β-carotene and retinol in different fractions of the cells were analysed in duplicate, using HPLC. The results are given as mean ±SE (n=3).

Relationship between dietary intake of provitamin A and retinol level in the colonic epithelial cells was further studied by allowing subjects to take carotenoid rich diet.
Subjects were allowed to take diet containing carotenoid rich *Spirulina* or Agathi. The colonic epithelial cells were isolated and subjected to analysis of β-carotene and retinol. *Spirulina* and Agathi were selected as the supplements to enrich carotenoid content in the diet. Analysis of the carotenoids of *Spirulina* and Agathi were done and the results are shown in Table 5.3. Although both were carotenoid rich, the relative content of provitamin A β-carotene was more in *Spirulina*. Other than carotenoids such as α-carotene, β-carotene, cryptoxanthin and zeaxanthin, *Spirulina* contained significant amount of lutein; but no lutein could be detected in Agathi extracts.

Analysis of the β-carotene and retinol content in the cells in different fractions was done and the results are shown in Table 5.2. β-carotene and retinol were present in both interphase and pellet fraction of the cell isolate. However cells in the interphase fractions contained significantly higher amount of β-carotene, than the pellet fraction. Average β-carotene content of 27.4 ng/mg protein was present in the pellet fraction and that in the interphase fraction was 34.3 ng/mg protein. While in the case of retinol, cells isolated from the interphase fractions showed greater values, 72.6 ng/mg than that of pellet fraction, which contained about 33.1 ng retinol/mg cell protein.

### Table 5.3

<table>
<thead>
<tr>
<th>Carotenoids</th>
<th>Agathi Mean ± SD</th>
<th>Spirulina Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>trans-β-carotene</td>
<td>184 ± 5.7</td>
<td>225 ± 8.7</td>
</tr>
<tr>
<td>α-carotene</td>
<td>47 ± 3.2</td>
<td>4.5 ± 5.0</td>
</tr>
<tr>
<td>9-cis-β-carotene</td>
<td>83 ± 8.1</td>
<td>36 ± 3.4</td>
</tr>
<tr>
<td>13-cis-β-carotene</td>
<td>35 ± 5.0</td>
<td>8.9 ± 3.0</td>
</tr>
<tr>
<td>Lutein</td>
<td>- -</td>
<td>180 ± 2.5</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>22 ± 3.5</td>
<td>89.8 ± 3.8</td>
</tr>
<tr>
<td>Cryptoxanthin</td>
<td>80 ± 7.6</td>
<td>18 ± 5.3</td>
</tr>
<tr>
<td>Lycopene</td>
<td>2 ± 0.3</td>
<td>- -</td>
</tr>
</tbody>
</table>

*values are expressed as μg/gm dry weight of the supplement.

Carotenoids of *Spirulina* and Agathi, which were used as supplements, were extracted and analysed by HPLC and the amount of different carotenoids are given. Values are mean of triplicate analysis.

β-carotene content of cells isolated from subjects of group I was 14.3ng in interphase fraction whereas the pellet fraction contained 9.3 ng; and that of group II a recorded values 38.1 and 30.0 respectively. Cells from interphase and pellet fraction of subjects of group II b contained 50.6 ng and 43.0 ng β-carotene in the pellet fraction. Among the three groups, retinol content in the interphase fraction did not show any
significant variation (range of 63.0-80.0). Retinol content in the pellet fraction was in the range of 23.0-42.5 ng/mg cell protein. Average nutrient intake from these diets were also shown in the Table 5.2 where, average protein content was in the range of 59-62 g; fat (41-46g) and energy content ranged from 2156- 2805 k cal. Subjects of group I ingested 95 µg of β-carotene and that of group II a and group II b ingested 1500 µg and 1800 µg respectively.

5.3.3 Relation between changes in the dietary carotenoid status and levels of β-carotene in exfoliated colonic epithelial cells

Dependence of the provitamin A content in colonic epithelial cells to the dietary content of carotenoids and its bioavailability was studied. After 7 days when the β-carotene content in cells attained a peak level of 50 ng, the subjects were asked to withdraw all carotenoid containing food from the diet and considered this day as first day of the cycle (Figure 5.2). Subjects took three types of diet (a) Spirulina diet, group

Fig.5.2
Kinetics of distribution of β-carotene in exfoliated human colonic epithelial cells

Subjects were divided into three groups of three each and those in group Ila and group IIb received β-carotene containing diet while those of group I received β-carotene poor diet as described in the text (0 day). Subjects in all the groups were then allowed to take β-carotene poor diet for 7 days. At the end of the wash out period subjects of group IIa (-•-) were allowed to take a bolus of cooked Agathi (1500 µg β-carotene) and those of group II b (-○-) were allowed to take a bolus of Spirulina (1800 µg β-carotene) while those of group I (-▲-) served as control with no supplements. Followed by supplementation, (▼) subjects of group Ila and group IIb took β-carotene poor diet for another 7 days. Stool samples were collected daily from all subjects, cells were isolated and subjected to analysis. β-carotene values are expressed as mean ± SE (n=3). On comparison of values of group I with II a and II b, p<0.01 on days 1 – 4 and on days 10 – 15 and not significant on day 7.
II b (b) *Sesbania* diet, group II a and (c) low β-carotene diet, group I, that contained about a total of 1800, 1500 and 90 mg of β-carotene respectively. They ingested these supplements on 7th day after complete withdrawal of carotenoids from the diet, as single dose (bolus). Then the β-carotene status of exfoliated cells was assessed daily, for 7 days, starting from the day of withdrawal. Details of the experiment are given in the study design (Figure 5.1). After HPLC analysis the β-carotene values were plotted, as shown in Figure 5.1. Results show that in group II a and II b, β-carotene content in the colonic cells ranged from 20 ng to 50 ng. Exfoliated cells from subjects who took diet devoid of any carotenoids (group I) showed an average baseline level of 10 to 18 ng β-carotene per mg cell protein.

### 5.3.4 Kinetics of accumulation of retinol and lutein in colonic epithelial cells

In order to confirm whether the changes in the carotenoid content in colonic cells were related to changes in status of carotenoids in the diet, the analysis of total carotenoids and lutein, a carotenoid which was present only in *Spirulina* and not in Agath leaves were done.

**Fig. 5.3**

HPLC Profile of carotenoids present in the colonic epithelial cells

Colonic epithelial cells isolated form the subjects who took Agathi (group IIa) and *Spirulina* (group IIb) were extracted for the carotenoids and analysed by HPLC. Lutein and β-carotene were detected at 2.5 and 3.8 minutes respectively for subjects who took *Spirulina* (B) and for subjects who took Agath only (A), β-carotene (A) was detected. [↓] indicates elution pattern of authentic standards of lutein and β-carotene (C) at 445 nm.
Subjects in group IIa and IIb who took bolus of Agathi and *Spirulina* were examined for the changes in total carotenoid, lutein and retinol content in colonic epithelial cells. HPLC analysis of the carotenoids from exfoliated cells of subjects who took *Spirulina* or Agathi supplements were done and the HPLC profile is given in Figures 5.3. HPLC analysis indicated that, the cells from subjects who took *Spirulina* contained significant amount of lutein in addition to β-carotene and other carotenoids while that for subjects who took Agathi, did not contain lutein. This was further analysed by measuring the changes in the levels of total carotenoids and retinol in colonic cells at different days and the results are shown in Figure 5.4 and 5.5

**Fig. 5.4**

*Changes in total carotenoids, lutein and retinol content of Colonic epithelial cells from subjects who took *Spirulina* diet*

Colonic epithelial cells of subjects in group II b who took *Spirulina* as supplement, were isolated and analysed for total carotenoid, lutein and retinol contents using HPLC. Values are mean ± SD (n=3)

Retinol level was found almost unchanged up to 3rd day and subsequently increased to 100 ng/mg cell protein, and then decreased to base line level of 62 ng/mg cell protein. Total carotenoid level increased from 10 ng on the first day to 45 ng on the 5th day after supplementation, and then remained at constant levels of 45 ng/mg cell protein on subsequent days.

Subjects who took *Spirulina* as supplement, showed presence of lutein in their colonic epithelial cells. On the first day, 9 ng lutein was found and it increased up to 30 ng
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on 7th day. About 1000 μg of lutein was present in the single dose of ingested food, which contained Spirulina as supplement.

Similarly, exfoliated colonic cells from subjects who took Sesbania (Agath) as food supplement was also analysed. After a washout period and subsequent feeding with a diet containing Agath, β-carotene status was analysed. Subjects in group II a ingested about 1500 μg β-carotene. Colonic epithelial cells were analysed for carotenoids and retinol. Details of its kinetics are shown in the Figure. 5.5. Retinol content ranged from 55 ng to 80 ng, whereas the β-carotene level showed a gradual increase from the first day to 6th day. Moderate level of β-carotene retained up to 3rd day and subsequently an increase was noted. Peak value of 50 ng was attained on 6th day and thereafter β-carotene level showed a decrease. Of the different samples analysed, none of the cell extracts showed any significant level of lutein, unlike in subject who took Spirulina as supplement.

![Fig.5.5](image-url)

Changes in total carotenoids and retinol content of colonic epithelial cells from subjects who took Agathi diet

Colonic epithelial cells were isolated from subjects in group IIa who took Agath as supplement, analysed for total carotenoids, and retinol. Lutein was not detected in any of the samples. Values are mean ± SD (n=3).

5.3.5 Relation between carotenoid level in plasma and exfoliated colonic cells

In order to examine whether, there was any correlation between the plasma level of carotenoids and that of exfoliated colonic cells, blood samples were collected on
different days from the subjects after the ingestion of a single dose of β-carotene rich Spirulina supplement and the plasma level of β-carotene and retinol were analysed and the results are shown in Table 5.4. β-carotene content increased steadily from a basal level (day 0 on flow chart) of 3.7 to 4.7 mg/L on 6th day and then decreased to basal level on 10th day while there was no significant change in the retinol level in the plasma during this period. These results suggest that the exfoliated colonic cells may be loaded with β-carotene provided by the blood and not by any direct transfer from carotenoid supplements.

### Table 5.4

**Changes in levels of β-carotene and retinol in plasma**

<table>
<thead>
<tr>
<th>Components</th>
<th>Time after ingestion</th>
<th>Components</th>
<th>Time after ingestion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-carotene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/L)</td>
<td>mean ± SE</td>
<td>(mg/L)</td>
<td>mean ± SE</td>
</tr>
<tr>
<td>I</td>
<td>3.67 ± 0.25</td>
<td>II</td>
<td>3.76 ± 0.28</td>
</tr>
<tr>
<td>II</td>
<td>3.88 ± 0.25</td>
<td>III</td>
<td>4.34 ± 0.27</td>
</tr>
<tr>
<td>III</td>
<td>4.66 ± 0.24</td>
<td>IV</td>
<td>3.70 ± 0.28</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>V</td>
<td>2.80 ± 0.15</td>
</tr>
<tr>
<td>V</td>
<td></td>
<td>VI</td>
<td>2.80 ± 0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Blood was collected at regular intervals from subjects (n=3) of group IIB who received a single dose of Spirulina as described in legends to Figure 5.2. Plasma was separated and β-carotene and retinol were analysed. Time after ingestion I, II, III, IV, V and VI corresponds to days 7, 9, 10, 11, 13 and 15 respectively in the Figure 5.2. Each sample was analysed in duplicate. Results are expressed as mean ± SE (n=3). p<0.05 on comparing basal value (I) with that of V for β-carotene; not significant for retinol.

5.3.6 Uptake of β-carotene by exfoliated human colonic epithelial cells in vitro

In order to understand the mechanism of absorption of carotenoids, in vitro studies using isolated colonic cells in culture were carried out. Cells in MEM seeded on to collagen coated plastic dishes appeared as monolayer culture (Figure 5.6). Isolated colonic epithelial cells in culture were used to study the uptake of β-carotene.

5.3.6.1 Uptake of 14[C]-labeled β-carotene by the colonic epithelial cells in culture

14[C]-labeled β-carotene was used for the uptake studies. Metabolically labeled 14[C]-β-carotene produced using Spirulina system (Chapter 3) was used for the study. Purified 14[C]-β-carotene (4550 cpm/µg) was supplemented to the culture medium. Time course analysis of β-carotene uptake was studied at different intervals, and the results are shown in Figure 5.7. There was an increase in percentage of radioactivity in
Legends to Figure 5.6
Exfoliated human colonic epithelial cells under phase contrast microscope

Colonic cells were isolated washed with PBS and cells in Eagle's MEM were seeded on to collagen coated plastic dishes and incubated the culture at 37°C in a CO₂ incubator for 16 hours. Cells appeared as monolayer culture (x 300)
Fig. 5.6
the β-carotene fraction extracted from cells with increase in duration of the culture. Maximum percentage of radioactivity of 4.3% in the β-carotene fraction was obtained when incubated for 6 hours which was reduced to 3% at 12 hours.

![Graph](image)

Colonic epithelial cells were incubated in medium containing $^{14}$C-labeled β-carotene with specific activity (4550 cpm/ml medium). Cells were harvested at 3, 6 and 12 hrs intervals. Extracted the cells and β-carotene analysed using TLC, radioactivity in the β-carotene fraction was estimated. Values given are mean of triplicate analysis (mean ± SD).

5.3.6.2 Effect of bile salts

Uptake of β-carotene by the colonic epithelial cells in cultures the presence of different concentrations of deoxy cholic acid was studied. The results are shown in the Figure 5.8. A significant increase in the amount of β-carotene uptake by cells was observed in the presence of deoxy cholic acid. Maximum uptake was observed at a concentration of 12 µM. No further increase in β-carotene uptake was found when higher concentration of bile salts was used.
Colonic epithelial cells (5 x 10⁶ cells/ml) were seeded on culture plates and incubated in a CO₂ incubator at 5% CO₂ and 95% air at 37°C. After 4 hours unattached cells were removed and fresh medium was added. Cells were supplied with 0.08 µg β-carotene/ml medium for 12 hours in the presence of different concentrations of deoxy cholic acid. Cells without deoxy cholate served as control. At the end of the experiment, medium was removed, cells were harvested, extracted and the β-carotene content was determined in cells by HPLC as described under methods. Values given are mean of triplicate analysis (mean ± SD).

The uptake of β-carotene from the medium in the presence of deoxy cholic acid was also studied at different time intervals. Incubated the cells for different times, viz, 3, 6, and 12 hours, in presence 12 µM cholic acid. Figure 5.8a show the kinetics of uptake of β-carotene from the medium. With increase in the incubation time, there was an increase in the uptake of β-carotene. β-carotene concentration in the cells increased from 8 ng to a maximum of 32 ng at 6th hour, while in the control where no cholic acid was added in the medium, showed only an average of 18 ng. After 6th hour, its content decreased to 15 ng.
Colonic epithelial cells (5 x 10⁶ cells/ml) were seeded on culture plates and incubated in a CO₂ incubator at 5% CO₂ and 95% air at 37°C. After 4 hours unattached cells were removed and added fresh medium. Cells were supplied with 0.08 µg β-carotene/ml medium in the presence 12 µM of deoxy cholic acid. Cells without deoxy cholate served as control. At different time intervals cells were harvested, extracted and the β-carotene content was determined by HPLC as described under methods. Values given are mean of triplicate analysis (mean ± SD).

5.3.6.3 Effect of lecithin

Effect of lecithin on the uptake of β-carotene by the colonic epithelial cells in culture was studied. Cells were maintained in culture and the medium was supplemented with different concentration of lecithin in presence of β-carotene. Amount of β-carotene taken up by cells after 12 hours of incubation was studied. Cells were harvested, extracted and analysed by HPLC and the results are shown in Figure 5.9. Presence of lecithin in the medium caused an increase in the uptake of β-carotene. Maximum uptake of β-carotene was found when 4 mM lecithin was present in the medium.
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Fig 5.9
Effect of change in concentration of lecithin on the uptake of β-carotene by colonic epithelial cells *in vitro*

Colonic epithelial cells (5 x 10⁶ cells/ml) were seeded on culture plates and incubated in CO₂ incubator at 5% CO₂ and 95% air at 37°C. After 4 hours unattached cells were removed and added fresh medium. Cells were supplied with 0.08 µg β-carotene/ml medium for 12 hours in the presence of different concentration of lecithin viz., 0.0 mM, 2.0 mM, 4mM and 10 mM. Cells without lecithin served as control. At the end of the experiment, medium was removed, cells were harvested, extracted and the β-carotene content was determined by HPLC as described under methods. Values given are mean of triplicate analysis (mean ± SD).

Fig. 5.10
Effect of lecithin on the uptake of β-carotene by colonic epithelial cells: time course analysis

Colonic epithelial cells (5 x 10⁶ cells/ml) were seeded on culture plates and incubated in CO₂ incubator at 5% CO₂ and air at 37°C. After 4 hours unattached cells were removed and added fresh medium. Cells were supplied with 0.08 µg β-carotene/ml medium in the presence of 4 mM of lecithin. Cells without lecithin served as control. At different time intervals cells were harvested, extracted and the β-carotene content was determined by HPLC as described under methods. Values given are mean of triplicate analysis (mean ± SD).
Kinetic analysis of the effect of the lecithin was further studied. Cells were maintained in cultures, in the presence of lecithin (4 mM) for different time intervals and the amount of β-carotene taken up by the cells was studied and the results are shown in Figure 5.10. β-carotene concentration increased with the time and maximum uptake was noted at 12th hr; about 4 fold increase in uptake of β-carotene was observed when compared to the control cultures where no lecithin was added.

5.3.6.4 Effect of Triolein

Effect of triolein on the uptake of β-carotene by the colonic epithelial cells in culture was studied by supplementing different concentrations of Triolein to the medium and the amount of β-carotene taken up by the cells was analysed. The results are shown in the Figure 5.11. Addition of triolein caused an increase in the uptake of β-carotene. Maximum uptake was observed when 10 mM triolein was used. About 68.5 ng β-carotene / mg cell protein was detected in the cells. About 7% of the β-carotene in the medium was taken up by the cells in presence of triolein, whereas in control the uptake was less than 1% of the β-carotene in the medium. No further increase in β-carotene uptake was observed when higher concentration of triolein was used.

Fig 5.11
Effect of triolein on the uptake of β-carotene from the medium by colonic exfoliated cells

Colonic epithelial cells (5 x 10⁴ cells/ ml) were seeded on culture pates and incubated in CO₂ incubator at 5% CO₂ and 95% air at 37°C. After 4 hours unattached cells were removed and added fresh medium. Cells were supplied with 0.08 μg β-carotene/ ml medium for 12 hours in the presence of different concentration of triolein viz., 2.0 mM, 5.0 mM, 10.0 mM and 15.0 mM. Cells without triolein served as control. At the end of the experiment, medium was removed, cells were harvested, extracted and the β-carotene content was determined by HPLC as described under methods. Values given are mean of triplicate analysis (mean ± SD).
Effect of triolein on the uptake of \( \beta \)-carotene was also studied at different time intervals by maintaining the cells in culture for different time intervals in the presence of triolein (10 mM). The results are shown in Figure. 5.12 The amount of \( \beta \)-carotene taken up by cells increased with time and maximum uptake of 78 ng was observed when cultures were incubated for 6 hrs.

**Fig. 5.12**

Effect of Triolein on the uptake of \( \beta \)-carotene by colonic epithelial cells: time course

![Graph showing the effect of triolein on the uptake of \( \beta \)-carotene by colonic epithelial cells over time.]

Colonic epithelial cells (5 x 10^6 cells/ml) were seeded on culture plates and incubated in CO\(_2\) incubator at 5% CO\(_2\) and air at 37°C. After 4 hours unattached cells were removed and added fresh medium. Cells were supplied with 0.08 µg \( \beta \)-carotene/ml medium in the presence 10 mM of triolein. Cells without triolein served as control. At different time intervals cells were harvested, extracted and the \( \beta \)-carotene content was determined by HPLC as described under methods. Values given are mean of triplicate analysis (mean ± SD).

### 5.4 Discussion

Vitamin A or provitamin A status is assessed at different levels like plasma analysis. Routine measurement of plasma vitamin A may not always give the actual status due to the buffering action of liver. Invasive sampling of liver and tissues are not feasible in field investigations involving human beings. Human excreta contain viable exfoliated colonic epithelial cells and now it is possible to obtain pure exfoliated viable cells from the stool. Isolation of viable exfoliated colonic cells from stool samples of human beings is a crucial step wherein early studies used centrifugal elutriation (Iyengar, 1991) for...
isolation. A novel and simple approach for the isolation of the cells has been demonstrated
(Albaugh et al., 1992), where the fresh human stools dispersed in buffered saline solution
can be fractionated over percol/BSA gradient to yield 9 discrete bands of the cells in the
density range of 1.033 to 1.139 which could be further purified over Histopaque 1077.
These cells are of colonic origin and they express characteristic cellular markers, like
cytokeratins, colon specific antigens etc. A host of biomedical utilities of this technique
have been illustrated, particularly because it would provide readily available source of
colonic epithelial cells for culture. Usually colonic epithelial cells are terminally differentiated
and are devoid of proliferatory activity. It is easy to understand the aneuploidic or
otherwise aberrant cells collected from the stools. Nair et al. (1996) reported that the
cells in lighter fraction retained micronutrients such as tocopherols, retinol and the
carotenoids, when subjects are in diet rich in vegetables. Results of experiment using
exfoliated colonic epithelial cells isolated from subjects who took carotenoid free and
rich diets presented in this chapter indicate that measurement of provitamin A carotenoid
level of these cells is a useful non-invasive technique to assess the bioavailability of
provitamin A carotenoids.

The colonic epithelial cells isolated from different stool samples were analysed
for their β-carotene and vitamin A content and the results showed that β-carotene and
retinol were present in both interphase and pellet fraction of the cells. However, the cells
in the interphase fraction contained significantly higher amounts of β-carotene than the
pellet and it ranged from 0.0207 to 0.050 μg/mg cell protein. More than 70% of these
cells were viable as evidenced by Trypan-blue exclusion test. These cells were
metabolically active as evidenced by their ability to uptake radiolabeled carotenoids as
well as their ability to attach to matrix proteins and remain in culture. The degree of
viability obtained in this study was similar to that reported by other investigators. Other
reports also indicate that these cells expressed CD markers and are metabolically active
(Nair et al., 2003).

In order to examine whether the levels of β-carotene in the diet has any effect on
the level of β-carotene in these cells, subjects were allowed to be on a preparatory
phase for three weeks whereby the provitamin A carotenoid could be brought down to
very low level. Analysis of nutrient intake from diet diary indicated that all the subjects in
both carotenoid free and carotenoid rich diet took adequate amount of major nutrients
and that any variation was not due to variation in the intake of other nutrients, particularly
fat in the diet. The β-carotene content was significantly lower in cells from stool samples of subjects who took β-carotene poor diet than those on β-carotene rich diet. When the subjects, who were taking carotenoid rich diet, switched over to carotenoid free diet, there was a steady decrease in the β-carotene content in the cells and was lowest on 7th day. Subsequently, when these subjects were allowed to take a single spell of Spirulina/Agathi supplemented diet, the β-carotene level in the exfoliated cells increased progressively and attained maximum after 6 days and declined thereafter. However there was no significant difference in the retinol content in the cells from subjects of different groups. The retinol content was in the range of 20-85 ng/mg cell protein. No decrease in retinol content was observed because the subjects were not vitamin A deficient and apparently had adequate tissue reserves of vitamin A.

It appears that the levels of β-carotene in exfoliated colonic epithelial cells can be used as an indicator of the availability of provitamin A carotenoids of dietary origin. Analysis of carotenoids from the exfoliated colonic epithelial cells indicates that there is a significant relationship between the intake of dietary carotenoids and β-carotene content of colonic epithelial cells. This is based on the following observations. (a) β-carotene content in exfoliated colonic epithelial cells from subjects who were taking diets poor in provitamin A carotenoids was very low. (b) Intake of β-carotene rich diet resulted in an increase in the β-carotene content in exfoliated colonic epithelial cells. Variability in the viability and corresponding β-carotene content in the different fractions of the cells under different dietary regimes is a reflection of its origin and age in the gut as most senescent population is present in the lighter fraction, which appeared in the interphase fraction. Our results confirm the earlier report (Nair et al., 1996) wherein various sub populations of colonic epithelial cells have been identified. Analysis of β-carotene content in the colonic epithelial cells from subjects, who took β-carotene rich diet after the washout period when β-carotene poor diet was taken, revealed that a lag period of 5-7 days was required for the changes in carotenoid content in the exfoliated colonic epithelial cells. It coincides with the reported turnover rate of the epithelium and its migration from proliferatory zone to luminal surface (Lepkin et al., 1973). Withdrawal of β-carotene from the diet caused a decrease in the β-carotene content in the cell and was minimum on 7th day after the withdrawal of β-carotene. However, retinol content in the cells remained almost at the same level during this period. When β-carotene poor diet was taken by the subject for a period up to 2 weeks, the level of β-carotene in these cells
Bioavailability studies of carotenoids using human exfoliated colonic epithelial cells

remained as low as 0.010 to 0.0173 μg / mg cell protein, while the retinol level was 0.070 to 0.0976 μg / mg cell protein which was similar to that of subjects receiving β-carotene rich diet. The amount of vitamin A that is metabolically derived from specific carotene containing food is largely unknown (Edwards et al., 2001).

Lutein is another carotenoid present in Spirulina, while it is absent in Agathi. Analysis of carotenoids of colonic epithelial cells showed that, cells from subjects who took Spirulina containing diet, contained significant amount of lutein, and its level increased from 0.0100 μg /mg cell protein to 0.0470 μg /mg cell protein during a period of 6 days after supplementation; the pattern of change was similar to the changes in the level of β-carotene. Lutein was not detected in cells from subjects receiving Agathi. Increase in lutein content of colonic epithelial cells seemed to serve as an internal marker suggesting that the changes in β-carotene content in colonic epithelial cell was due to increase in its bioavailability.

In order to examine whether, there was any correlation between the plasma level of carotenoids and that of exfoliated colonic cells, blood was collected on different days from the subjects after the ingestion of a single dose of β-carotene rich Spirulina supplements and the plasma level of β-carotene and retinol were analysed and the results are shown in Table 5.4. β-carotene content increased steadily from a basal level of 3.7 to 4.7 mg/L on 6th day and then decreased to basal level on 10th day while there was no significant change in the retinol level in the plasma during this period. These results suggest that the exfoliated colonic cells may be loaded with β-carotene provided by the blood and not by any direct transfer from carotenoid supplements.

Not much information is available in the literature about the use of colonic epithelial cell cultures for understanding the absorption of β-carotene. Therefore the present study was also aimed at to evaluate the in vitro uptake of β-carotene by the colonic epithelial cells. Colonic epithelial cells resemble the human intestinal epithelial cells that absorb nutrients from the diet. In order to understand the modulatory mechanism of absorption of carotenoids by the colonic epithelial cells, influence of different factors on the uptake of β-carotene were studied in in vitro culture system. Bile salt is one of the factors which involves in the emulsification and increased solubilisation of lipophilic carotenoids (Hollander and Ruble, 1978). In the present experiments, addition of deoxy cholic acid, a primary bile acid increased absorption of β-carotene by the colonic cells from the medium in a concentration dependent manner. Optimum absorption obtained in presence
of 12 μM deoxy cholic acid when compared to the control where no bile salts were present in the medium. At higher concentrations, uptake of β-carotene decreased, which probably may be due to its effect on cell viability. The effect of deoxy cholic acid on the absorption appeared to be time dependent, about four fold increase in β-carotene content was observed in the colonic cells incubated for 6 hours when compared to the control where no primary bile acids was supplied. Primary bile acids through the enterohepatic recirculation, aids the emulsification and absorption of fat and fat soluble compounds of the diet. These *in vitro* studies indicate that bile acids promote uptake of carotenoids and suggest that secretion of bile acids through the enterohepatic recirculation is a critical factor in determining the uptake/bioavailability of carotenoids. Supplementing lecithin, a major phospholipid and triolein, a simple triglyceride increased uptake of β-carotene by colonic epithelial cells in culture. At high concentrations of phospholipids and triolein a decrease in the uptake of carotenoids by cells in culture was found, which may be due to their effects on cells, particularly on the fluidity of cell membrane.

Although it is generally believed that the uptake of fat soluble molecules like carotenoids, vitamin A and E by the cells is facilitated by phospholipids and triglycerides mainly due to their ability to disperse carotenoids in the medium and facilitate their binding and or movement across the cell membrane, specific mechanisms of such effect are not clearly known. These *in vitro* results indicate that the uptake of provitamin A carotenoids by colonic epithelial cells and therefore the bioavailability of provitamin A carotenoids, depend on the availability of fat in the diet. These *in vitro* results support the results on the role of fat in the diet on the absorption of carotenoids by experimental animals reported in the previous chapter. The oral bioavailability of β-carotene is reportedly variable and dependent on the presence of fat in the diet and bile in the intestine (Nirenberg *et al.*, 1991). Secretion of bile salt is found to be related with fat in the diet, and the experiments with animal models also suggest a modified pattern of β-carotene bioavailability in the plasma in the presence of fat along with carotenoid rich supplements.

Usual methods for the bioavailability studies of vitamin A involve invasive sampling of blood or tissues which may not be practical in the case of a large target population. These studies indicate that the micronutrient analysis, particularly β-carotene of colonic epithelial cell is a useful non-invasive method to assess the bioavailability of provitamin A carotenoids and the efficacy of intervention studies involving dietary supplementation. It requires further validation using large number of samples particularly children at different stages of growth.