Aliment Pharmacol Ther 2000; 14: 353–358.

Plasma antioxidant levels in chronic cholestatic liver diseases

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Accepted for publication 8 December 1999

SUMMARY

Background: A predictable consequence of cholestasis is malabsorption of fat-soluble factors, (vitamins A, D, E, K) and other free radical scavengers, such as carotenoids. It has been suggested that oxygen-derived free radicals may be involved in the pathogenesis of chronic liver damage.

Aims: (i) To evaluate retinol, α-tocopherol and carotenoid plasma levels in two groups of patients with chronic cholestatic liver disease (primary biliary cirrhosis and primary sclerosing cholangitis); (ii) to compare the respective plasma levels with those of the general population; (iii) to correlate the plasma levels with disease severity.

Methods: A total of 105 patients with chronic cholestasis were included in the study: 86 with primary biliary cirrhosis (81 female, five male, mean age 55.5 ± 11 years), 19 with primary sclerosing cholangitis (seven female, 12 male, mean age 35 ± 11 years; six patients had associated inflammatory bowel disease); 105 sex- and age-matched subjects from the general population in the same geographical area (88 female, 17 male, mean age 51.3.5 ± 10 years) served as controls. Carotenoids (lutein zeaxanthin, lycopene, β-carotene, α-carotene, β-cryptoxanthin), retinol and α-tocopherol were assayed by high-pressure liquid chromatography. A food frequency questionnaire was administered to each subject to evaluate the quality and the quantity of dietary compounds. Data were processed by analysis of variance and linear regression analysis, as appropriate.

Results: Both primary biliary cirrhosis and primary sclerosing cholangitis patients had significantly lower levels of retinol, α-tocopherol, total carotenoids, lutein, zeaxanthin, lycopene, α- and β-carotene than controls ($P < 0.0001$). Among the cholestatic patients, no significant difference in the concentration of antioxidants was observed between primary biliary cirrhosis and primary sclerosing cholangitis subjects. Anti-oxidant plasma levels were not affected by the severity of the histological stage in primary biliary cirrhosis, but a negative correlation was found between total carotenoids and both alkaline phosphatase (ALP) and gammaglutamyl transpeptidase (GGT) ($P < 0.013$ and $P < 0.018$, respectively). Within the primary sclerosing cholangitis group, no correlation was found between total carotenoids and cholestatic enzymes. Nutritional intake in cholestatic patients was comparable to controls, including fruit and vegetable intake.

Conclusions: Although no clinical sign of deficiency is evident, plasma levels of antioxidants are low in cholestatic patients even in early stages of the disease. This is probably due to malabsorption of fat-soluble vitamins, as well as other mechanisms of hepatic release, suggesting the need for dietary supplementation.

INTRODUCTION

A predictable consequence of cholestasis is malabsorption of fat-soluble factors (vitamins A, D, E, K) and other free radical scavengers, such as carotenoids. Low serum levels of vitamin A are frequently reported in primary biliary cirrhosis and seem to be correlated with the histological stage of the disease.[1–4] It is also a common experience that the clinical consequences of this vitamin deficiency, such as impaired adaptation to darkness, are extremely rare in primary biliary cirrhosis patients. This

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may be explained by the fact that low serum vitamin A levels may reflect a defective mobilization of vitamin A from the liver, instead of a consequence of vitamin A deficiency.[5]

Carotenoids are a family of at least 600 substances including β-carotene, lycopene, β-criptoxantine, zeaxanthin and lutein.[6] Their absorption depends on bioavailability from the food matrix and solubility in micelles. After absorption through passive diffusion, carotenoids undergo chylomicron metabolism.[7] Carotenoids with no substituted β-ionone cycles (α and β-carotene and β-cryptoxanthin) have pro-vitamin A activity.[7] Moreover, carotenoids have several biological functions, including immunomodulation and regulation of enzyme activity involved in carcinogenesis.[8] Moreover, there is some clinical evidence that polyprenoid acid, an acyclic retinoid, prevents second primary hepatomas after surgical resection of the original tumour or the percutaneous injection of ethanol.[9] On the other hand, chronic cholestatic liver disease, namely primary biliary cirrhosis, and primary sclerosing cholangitis have been shown to be associated with a risk of hepatic cancer, hepatocellular carcinoma (HCC) and cholangiocarcinoma, respectively.[10–13] Moreover, recent studies suggest that primary biliary cirrhosis is associated with a high risk of extrahepatic malignancies.[11, 14, 15] However, there is some controversy concerning the possible risk of breast cancer in primary biliary cirrhosis patients from North America[16] and Scotland,[17] while these data have not been confirmed in recent epidemiological studies from Europe.[11, 14] Both environmental and alimentary factors have been suggested to explain these discrepancies, including the dietary intake of antioxidants.

The aims of the present study were therefore: (i) to evaluate retinol, α-tocopherol and carotenoid plasma levels in two groups of patients with chronic cholestatic liver disease (primary biliary cirrhosis and primary sclerosing cholangitis); (ii) to compare their antioxidant plasma levels with those of the general population; (iii) to correlate the respective plasma levels with disease severity.

MATERIALS AND METHODS

Patients

The study included 105 patients with chronic cholestatic liver disease:

- Group A: 86 subjects with primary biliary cirrhosis (81 female, five male) with a mean age of 55.5 ± 11 years (range 29–81 years); 42 subjects had histological stage I–II, 23 stage III, and 21 stage IV, according to Scheuer's classification.[18] The diagnosis of primary biliary cirrhosis was obtained on the basis of clinical immunological (anti-mitochondrial antibodies (AMA) positivity at titre >1:40) and histological criteria.
- Group B: 19 subjects with primary sclerosing cholangitis (seven female, 12 male) with a mean age of 31 ± 11 years (range 17–68 years); six patients had an associated inflammatory bowel disease (five ulcerative colitis, and one Crohn's disease). The diagnosis of primary sclerosing cholangitis was obtained according to the clinical, immunological and radiological (ERCP) criteria.

Controls

A total of 105 controls were selected to match the patients with respect to age and sex. These subjects (17 male, 88 female) with a mean age 51.3 ± 10 years (range 17–81 years) belonged to the general population of the same geographical area. This sample population was recruited from a generating-computer list of the resident population listed on the electoral register.

Design of the study

The study was approved by the local ethical committee and all subjects gave their informed consent. Venosection was performed on each subject after an overnight fasting and plasma samples were stored at – 80 °C until tested.

Carotenoids (lutein, zeaxanthin, lycopene, β-carotene, α-carotene, β-cryptoxanthine) retinol and α-tocopherol were tested from each serum.

A food frequency questionnaire was administered to each subject to evaluate the quality and the quantity of dietary compounds. The food questionnaire has been previously validated for the Italian general population under the auspices of the National Research Council as a part of the 1995 FATMA project (Prevention and control of illness risk factors N.95.00869.PF41). The questionnaire was administered by the same dedicated research fellow; each interview lasted approximately 40 min. The questionnaire reports a list of 99 foods and

Table 1. Plasma values (mean ± s.d.) of antioxidants in primary biliary cirrhosis and primary sclerosing cholangitis patients and in controls

Antioxidants	Primary biliary cirrhosis $n = 86$	Primary sclerosing cholangitis $n = 19$	Controls $n = 105$
Retinol (μmol/litre)*	1.59 ± 1.36	1.57 ± 0.49	4.016 ± 0.06
α-tocopherol (μmol/litre)*	18.16 ± 7.38	15.55 ± 4.36	40.41 ± 14.25
Total carotenoids (μmol/litre)*	2.23 ± 1.18	2.21 ± 0.70	3.81 ± 1.75
β-carotene (μmol/litre)*	0.738 ± 0.47	0.574 ± 0.28	1.449 ± 0.98
α-carotene (μmol/litre)*	0.14 ± 0.10	0.18 ± 0.13	0.26 ± 0.22
Zeaxanthin (μmol/litre)*	0.128 ± 0.08	0.119 ± 0.06	0.297 ± 0.18
β-criptoxantine (μmol/litre)	0.384 ± 0.31	0.401 ± 0.24	0.31 ± 0.25
Lutein (μmol/litre)*	0.444 ± 0.24	0.420 ± 0.19	0.744 ± 0.43
Lycopene (μmol/litre)*	0.480 ± 0.32	0.519 ± 0.23	0.803 ± 0.44

$P < 0.0001$ (primary biliary cirrhosis/primary sclerosing cholangitis vs. controls).

meals, with six items for each, three representing different helpings in terms of size and weight on the basis of a visual scale (from a colour atlas) and three for recording daily/weekly consumption including sauces, dressing and flavouring.

Methods

Plasma levels of lutein, zeaxanthin, lycopene, β-carotene, α-carotene, β-cryptoxanthine, retinol and α-tocopherol were measured by high-pressure liquid chromatography. Samples were analysed with a Spherisorb S5 ODS column (250 × 4.6 mm) for simultaneous UV detection at 450 nm (for carotenoids), 325 nm (for retinol), and 292 nm (for α-tocopherol) (Uvicon Spectrophotometer 922, Kontron Instruments). An internal standard for each antioxidant has been added to the serum sample, hydrolysed in KOH-ethanol at – 80 °C and extracted with hexane. The evaporated extract was dissolved in methanol, and the aliquot was measured with a HP-1090 liquid chromatograph equipped with a diode-array spectrophotometric detector (Beckman Instruments).

Statistical analysis

Data were analysed by one- and two-way analysis of variance (ANOVA) and linear regression analysis, as appropriate.

RESULTS

Both primary biliary cirrhosis and primary sclerosing cholangitis patients exhibited significantly lower levels of retinol, α-tocopherol, total carotenoids, lutein, zeaxanthin, lycopene, α-and β-carotene than controls ($P < 0.0001$); B-cryptoxanthine plasma levels were similar in cholestatic liver diseases compared to controls (Table 1). Among cholestatic patients, no significant difference in the concentration of antioxidants was observed between primary biliary cirrhosis and primary sclerosing cholangitis subjects (Table 1).

In the primary biliary cirrhosis group no significant difference was noticed between plasma levels of antioxidants and the histological stage; in other words, a greater severity of liver disease failed to coincide with a significant reduction in retinol, α-tocopherol and carotenoids. However, a weak negative correlation was found between total carotenoids and both alkaline phosphatase (ALP) ($r = –0.27$, $P < 0.013$) and gammaglutamyl transpeptidase (GGT) ($r = –0.26$, $P < 0.018$).

In the primary sclerosing cholangitis group no correlation was found between total carotenoids and cholestatic enzymes.

Alimentary intake

The quality and the quantity of alimentary intake was comparable in patients with cholestasis and in sex- and age-matched controls (Table 2). In particular, the mean calorie intake was 1995 ± 570 kcal/day in patients with cholestasis and 2141 ± 518 kcal/day in controls; the difference was not statistically significant. The percentage of proteins, lipids and carbohydrates was similar in the two groups. Moreover, the quantity of total carotenoids and foods containing retinols and carotenoids (fruits and vegetables) was comparable in the two groups.

Daily intake	Cholestatic patients	Controls	*P*
kcal	1995 ± 570	2141 ± 518	N.S.
Proteins (%)	19.6 ± 3.2	15.5 + 3.6	N.S.
Lipids (%)	30.7 ± 6.3	28.8 + 5.0	N.S.
Carbohydrates (%)	52.0 ± 6.7	54.1 + 5.9	N.S.
Fruit (g/day)	193 ± 132	201 ± 14	N.S.
Vegetables (g/day)	171 ± 97	166 ± 10	N.S.
Total fibre (g/day)	17.1 ± 5.9	20.1 ± 7.5	N.S.
Total carotenoids (mg/day)	5.73 ± 5.5	5.1 ± 1.9	N.S.

Table 2. Estimated daily intake (mean ± s.d.) of macronutrients in patients with cholestasis and in controls

DISCUSSION

Our study demonstrates that plasma levels of retinol, α-tocopherol and carotenoids (with the exception of β-cryptoxanthine) are lower in cholestatic patients than in age-matched controls from the general population of the same geographical area. This difference is found even in early stages of the disease and is unaffected by the end-stage histological stage. Moreover, the results of the food frequency questionnaire, administered to a sample of the study population evidenced that the nutritional intake in cholestatic patients was no different from the general population. These findings raise several points of discussion.

First of all, this is the first clinical study to consider a large sample of chronic cholestatic patients receiving no vitamin supplements by comparison with sex- and age-matched controls. Our patients were being followed-up (by A.F.). with no specific dietary prescription, apart from being asked to avoid alcoholic drinks. None of the patients was drinkers and none complained of impaired night vision or had signs of decompensated cirrhosis.

Secondly, we performed the nutritional survey to verify whether cholestatic patients had a similar calorie intake to sex- and age-matched controls from the general population. Analysis of the food questionnaire failed to reveal any statistical difference between patients and healthy subjects. In particular, the intake of foods containing retinols and carotenoids (fruit and vegetables) was adequate and similar in the two groups. (Table 2).

Since we found a negative correlation between total carotenoids and both ALP and GGT in primary biliary cirrhosis, it may be that the lower antioxidant levels are due to malabsorption of fat-soluble vitamins. We found no such correlation in primary sclerosing cholangitis patients, but the lack of statistical significance might be due to a small sample size. However, the correlation between total carotenoids and both ALP and GGT is relatively poor and GGT is not considered a particularly good marker of cholestasis. Nevertheless, our data show that total carotenoid levels were significantly lower in patients than in controls, as well as tocopherol plasma levels. In a human model of extrahepatic cholestasis Leo *et al.*,[19] showed that retinol and tocopherol were not significantly affected by biliary tract disease, suggesting no impairment in their absorption. On the other hand, β-carotene was significantly decreased in both plasma and bile in subjects with bile duct stones. However, the consequences of intrahepatic cholestasis are completely different from those of extra-hepatic cholestasis. An interesting observation comes from Nyberg *et al.*,[5] who showed an abnormal staining pattern of cellular retinol-binding protein, believed to be involved in the intrahepatic transport of vitamin A in patients with primary biliary cirrhosis. The number, size and cellular retinol-binding protein staining intensity of fat-storing (Ito) cells were clearly higher in primary biliary cirrhosis patients than in controls with normal liver biopsy. This result suggests that low vitamin A levels in primary biliary cirrhosis are due to defective mobilization of vitamin A from liver. Moreover, it poses a serious problem from the therapeutic point of view. In fact, we found low levels of plasma antioxidants even in early stages of the disease, so vitamin A supplementation might be useful in cholestatic patients. However, high-dose vitamin A therapy might by-pass the serum retinol-binding protein-controlled transport, thus inducing tissue deposition. The investigation of tissue deposition of vitamin A in our study has not been performed, but since dietary intake of this vitamin is adequate, we feel that that further studies are warranted in order to guide decisions on vitamin A supplementation. Another interesting mechanism of action of alpha-tocopherol may be the prevention of fibrogenesis cascade, mediated by the oxidative stress.[20] Nevertheless, the future guidelines on vitamin A supplementation should consider the still unresolved public health concern relative

to the excessive vitamin A intake, possibly causing toxicity, including birth defects not only in animals but also in man.[21–23]

The last point is the protective role of carotenoids against carcinogenesis. A factor that has not been adequately studied is vitamin A deficiency, which has been shown to induce metaplasia in epithelial cells in mice, together with an increased susceptibility to malignancy.[24] The above-mentioned discrepancy between North American and European studies on the association of breast cancer in primary biliary cirrhosis, might also be explained by different eating habits. Nevertheless, dietary factors are not the most relevant predisposing factors in the development of cancer: sex hormone imbalance may account for the development of cancer in primary biliary cirrhosis females, as previously reported.[25, 26] Our study failed to demonstrate an inadequate dietary intake in both macro- and micronutrients. Thus, similar nutritional studies would have to be performed in other countries, too, in order to investigate this important point.

Unfortunately, our study does not include non-cholestatic liver diseases as controls, thus the importance of cholestasis *per se* cannot really be assessed. Despite this limitation, we can conclude that plasma levels of tocopherols and some carotenoid products are low in cholestatic liver disease, even in early stages; this peculiar antioxidant status is not due to an impairment of dietary intake. In addition to the impairment of liver mobilization, a malabsorption of fat-soluble vitamins might explain the tocopherols deficiency. Due to this dual mechanism, dietary supplementation is probably needed in chronic intrahepatic cholestasis. Further studies on retinol and carotenoid metabolism are warranted to establish standard guidelines for dietary supplementation.

ACKNOWLEDGEMENTS

This work was partially supported by a University grant (MURST 60%).

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