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Original paper

DUODENAL MUCOSAL AND PLASMA ASCORBATE LEVELS OF PATIENTS WITH IRON DEFICIENCY

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Summary: Iron is a vital element for almost all living organisms. In mammals iron is taken by the intestinal epithelium, primarily in the duodenum. The initial step of absorption involves the reduction of ferric to ferrous iron both in gastric lumen and at the brush-border apical membrane. Reductase activity is increased by factors physiologically stimulating iron absorption, such as iron deficiency and chronic hypoxia. Ascorbic acid (Vitamin C) has long been known to enhance absorption of dietary iron in humans as shown by several nutritional/dietetic studies. This effect has been ascribed to lumenal reduction and solubilization of iron. Recent molecular cloning of the mammalian duodenal brush-border reductase activity has provided evidence that ascorbate may play an intracellular role in determining iron absorption rates. Previously, ascorbate concentrations have been determined in duodenum, but only in normal subjects and there is no evidence on how duodenal ascorbate alters in relation to intestinal iron absorption. The aim of this study is to examine mucosal and plasma levels of ascorbate and dehydroascorbate in normal subjects and patients with iron deficiency that is known to be a stimulator for iron absorption. Duodenal biopsies were homogenized in 5% metaphosphoric acid using single burst homogeniser. Tissue and plasma ascorbate levels were assayed by ferrozine spectrophotometric method. Blood was taken from each subject to assess the iron status. The analyses of human samples revealed increased duodenal ($p < 0.001$, $n = 20$) and plasma ($p < 0.001$, $n = 6$) ascorbate levels in patients with iron deficiency. These findings support an important intracellular role of ascorbic acid in human dietary iron absorption.

Key words: iron absorption, duodenal brush-border reductase activity, duodenal mucosal ascorbate levels, plasma ascorbate levels

Introduction

Iron is one of the most abundant metals in the earth's crest and is an essential element for almost all living organisms. However, excess of iron is highly toxic because of its ability to promote free radical formation via Fenton's reaction. Because of this dual nature, body iron levels are held within narrow limits. Iron has no normal pathway for excretion from the body. Therefore the positive iron balance is maintained by regulating predominantly the intestinal absorption of the element.

In man dietary iron intake consists of two components: haem iron (red meat) and non-haem iron (vegetables, cereals). Non-haem iron exists predominantly in the highly insoluble ferric form Fe(III) and therefore is poorly bioavailable. It is the reason for the high percent of iron deficiency anaemia in the developing countries, where the combination of poor iron supplies and parasitic infections reduces iron stores.

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Abbreviations used: AA-ascorbic acid; DHA-dehydroascorbic acid; MPA-metaphosphoric acid; DTT-dithiothreitol; NEM-N-ethylmaleimide; TCA-trichloracetic acid; Dcytb-duodenal cytochrome b

Non-haem iron is absorbed early in the duodenum, where the low pH favours the solubility of iron. Low molecular iron complexes and Fe^{2+} are well absorbed. Iron absorption occurs mainly in the proximal intestine with the maximal rate in the duodenum and the lowest in the ileum (1).

It is now known that ferrous iron is transported across the apical membrane of the duodenal cell by a divalent metal transporter (DMT1), also called Nramp2 (2). DMT1 must be presented with the ferrous form of the metal and the ability of intestine to take up ferric iron is attributed to the presence of a reductase activity. The duodenal ferri-reducing activity displays adaptive response to iron status; it is increased by physiological stimuli of iron absorption such as chronic hypoxia and iron deficiency (3). In addition it is found that the activity has a profile in the small intestine, similar to that of iron absorption-highest in the duodenum and lowest in the ileum.

The gene, responsible for duodenal reducing activity, Dcytb (duodenal cytochrome b) was identified with a subtractive cloning strategy (4). Identification of the protein sequence revealed that Dcytb is homologous to cytochrome b561 (41% identical, 54% similar). Cytb561 acts as a transmembrane electron shuttle between the cytoplasm (using cytoplasmic ascorbate as an electron donor) and the inside of chromaffin adrenal granules, where the electron is accepted by semi-dehydroascorbic acid (5). Putative binding sites for the cytb561 (AA semi-DHA) are conserved in Dcytb, suggesting that Dcytb might react with these compounds.

The aim of the present study is to determine if duodenal and plasma ascorbate levels in man are altered in iron deficiency that is known to be a stimulator of iron absorption.

Material and Methods

AA and DHA assay

A spectrophotometric assay for determination of ascorbic and dehydroascorbic acid in human tissues was used. The spectrophotometric determination is modified from an assay described by Kampfenkel (6) and based on the reduction of Fe^{3+} to Fe^{2+} by ascorbic acid and spectrophotometric detection of Fe^{2+} complexed with Ferrozine. Dehydroascorbic acid was reduced to ascorbic acid by preincubation of the samples with dithiothreitol (DTT). Excess DTT was removed with N-ethylmaleimide (NEM) and total ascorbic acid was determined by the same procedure. The concentration of dehydroascorbic acid was then calculated from the difference of total ascorbic and ascorbic acid. Plasma AA and DHA levels were analysed according to modification of the method of Okamura (7) (*Table I*).

Table I Determination of AA and DHA in plasma

Component (mL)	AA	Total AA	Blank
Plasma	0.3	0.3	0.150 deion. H_2O
10 mmol/L DTT	–	0.1	
40 mmol/L NEM	–	0.1	
DTT-NEM	0.2	–	0.1
0.6 mol/L TCA	0.5	0.5	0.250
Supernatant	0.5	0.5	–
4.2 mol/L H_3PO_4	0.2	0.2	0.2
0.2 mol/L $FeCl_3$	0.2	0.2	0.2
30 mmol/L ferrozine	0.1	0.1	0.1

Table II Iron Deficiency Parameters

Laboratory Measurements	Iron Deficiency
Serum Ferritin	< 20 μg/L
TIBC	> 70 μmol/L
Transferrin Saturation	< 20%
CRP	< 5 mg/L
Haemoglobin	
men	< 130 g/L
women	< 120 g/L
Mean Cell Volume	< 84 fL

Human samples

Prior to the study, ethical approval was obtained from St. Thomas' Hospital and King's College Hospital ethics committees and informed consent was obtained from all human subjects. Human duodenal biopsies were taken by endoscopy during routine gastrointestinal examination for suspected malabsorption, peptic ulceration, non-ulcer dyspepsia. For every human subject 3 biopsies were taken from the second part of the duodenum. One biopsy was sent for routine histology and other two were homogenised in 5% ice-cold MPA using high speed Ultra Turrax homogeniser and analysed for AA and DHA levels. Plasma was obtained by whole blood centrifugation using EDTA as an anticoagulant. Iron status of each subject was unknown to the investigators until all analyses were completed. The criteria for diagnosing of iron deficiency are presented in *Table II*.

All results are expressed as mean values ± standard deviation (SD). Data for the human samples were analysed using Student's t-test.

Results and Discussion

The analyses of human samples revealed significantly increased duodenal (*Figure 1*) and plasma ascorbate (*Figure 2*) concentrations related to iron deficiency. No detectable DHA was found in human duodenal and plasma samples.

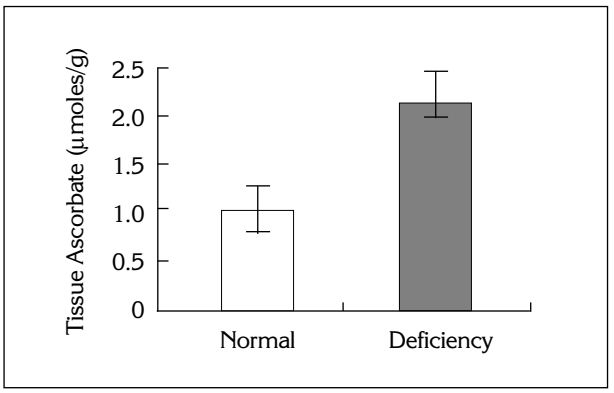

Figure 1. Duodenal ascorbate concentrations in human biopsies of normal subjects and patients with iron deficiency
Data are mean values ± SD. Statistical analysis: Student's t-test $p < 0.001$, $n = 20$

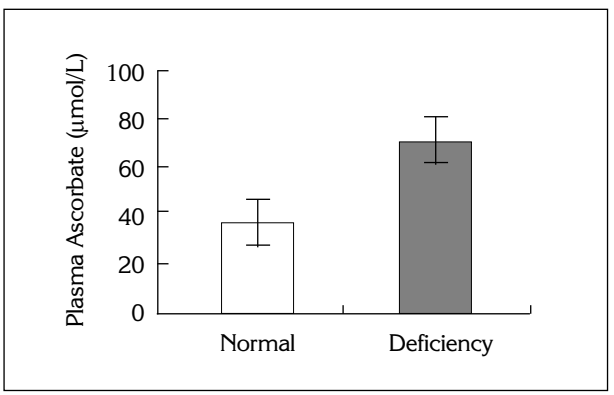

Figure 2. Plasma ascorbate concentrations in normal subjects and patients with iron deficiency
Data are mean values ± SD. Statistical analysis: Student's t-test $p < 0.001$, $n = 6$

The duodenal ascorbate concentrations in normal subjects, obtained by the spectrophotometric assay, are in full agreement to those, obtained by HPLC with electrochemical detection (8). The developed ferrozine assay could present reliable results for AA and DHA, analysed in intestinal and plasma human samples.

Ascorbic acid has long been known to enhance iron absorption from test meals (9). This effect has been ascribed to lumenal reduction and solubilization of iron. More recent work has shown that ascorbate levels are correlated with iron absorption in a range of typical American meals (10) although it seems that ascorbate is more important enhancer when meat is absent from the diet (11). Effects of ascorbate are diminished in complex meals and over long periods of study (12). Epidemiological studies have also found correlation between ascorbate and iron stores (13). Importantly, ascorbate has its biggest effect in the absence of meat, i.e. in those circumstances where iron deficiency is most likely to occur. Structural analysis and work with cultured cells expressing Dcytb (4, 14) suggest that the intracellular electron source for this reductase is cytosolic ascorbate. This could provide one mechanism for molecular explanation of interaction between intracellular ascorbate levels and the rate of iron absorption.

One study has suggested that tissue ascorbate levels are increased in iron deficiency and they are decreased in iron overload (15). Previously, ascorbate levels have been determined in duodenum but only in normal subjects (8) and there is no information on how duodenal ascorbate alters in relation to intestinal iron absorption. Our observations are a new finding in humans, consistent with suggestion that proximal intestinal ascorbate could be involved in iron absorption as iron absorption occurs mainly in the proximal intestine (3). Human ascorbate levels changed in the same direction as change in iron absorption. This finding is consistent with our previous observations for mouse models of altered iron metabolism (iron deficiency, iron overload, hypoxia and genetic iron overload secondary to hypotransferrinaemia with chronic anaemia).

Since synthesis of AA does not occur in human tissues another origin has to be sought for the increased duodenal levels. One possibility is increased retention either from dietary sources or plasma. Another possibility is that increased dietary iron absorption leads to decreased oxidation of ascorbate. These possibilities require further investigation.

The data suggest an important role of intracellular duodenal AA in human dietary iron absorption in addition to the well known effect of ascorbate seen in previous dietary studies. The findings support ascorbate as the intracellular electron donor for Dcytb activity.

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NIVOI ASKORBATA U DUODENALNOJ MUKOZI I PLAZMI PACIJENATA S DEFICITOM GVOŽĐA

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Kratak sadržaj: Gvožđe je vitalni element za skoro sve žive organizme. U sisara gvožđe se unosi preko intestinalnog epitela, primarno u duodenumu. Početni stupanj apsorpcije uključuje redukciju feri do fero jona kako u gastričnom lumenu tako i u četkastom pokrovu apikalne membrane. Reduktaznu aktivnost povećavaju faktori koji fiziološki stimulišu apsorpciju gvožđa, kako u slučaju deficita gvožđa, tako i pri hroničnoj hipoksiji. Dugo je poznato da askorbinska kiselina (Vitamin C) potstiče apsorciju gvožđa koje se unosi hranom kod ljudi što je dokazano u više nutricionih studija. Ovaj efekt je pripisan luminalnoj redukciji i solubilizaciji gvožđa. Nedavna molekularna kloniranja dudonalne reduktazne aktivnosti četkastog dela su potvrdila da askorbat može imati ulogu u određivanju brzine apsorpcije gvožđa. Ranije, koncentracije askorbata su određivane u duodenumu, i to samo kod zdravih osoba i nisu postojali dokazi kako se duodenalni askorbat menja u odnosu na intestinalnu apsorpciju gvožđa. Cilj ovih proučavanja je bio da se ispitaju nivoi askorbata u mukozi i u plazmi i dihidroksiaskorbata kod zdravih osoba i pacijenata sa deficitom gvožđa. Duodenalne biopsije su homogenizovane u 5% metafosfornoj kiselini. Nivoi tkivnog i plazmatskog askorbata su određivani spektrofotometrijski sa ferozinom. Krv je uzimana od svakog pacijenta kako bi se procenio status gvožđa. Ispitivanja humanih uzoraka su potvrdila povećanje duodenalnog ($p < 0,001$, $n = 20$) i plazmatskog ($p < 0,001$, $n = 6$) nivoa akskorbata u pacijenata sa deficitom gvožđa. Ovi nalazi su potvrdili značaj intracelularne uloge askorbinske kiseline u humanoj apsorpociji gvožđa koje se unosi hranom.

Ključne reči: apsorpcija gvožđa, aktivnost duodenalne reduktaze, nivo askorbata duodenalne mukoze, nivo askorbata u plazmi

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