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Effects of Co-Supplementation of Iron with Ascorbic Acid on Antioxidant—Pro-Oxidant Balance in the Guinea Pig

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The relationship between intake of iron with ascorbic acid and their uptake into the plasma and liver of guinea pigs was studied. The influence on the antioxidant/pro-oxidant balance of liver microsomes was also determined. Animals were fed a standard pelleted diet low in iron and ascorbic acid for 35 days. The pellet diet was supplemented by oral dosing with a solution containing either maintenance dietary levels of ascorbic acid and iron, or one of three regimens that increased the dosage of these substances ten fold. There were no significant differences in animal growth rate or food intake between these regimens. Liver and plasma total ascorbate levels were significantly increased ($p < 0.05$) in animals receiving either ascorbic acid alone (liver 126 ± 36 μg/g tissue wet wt. and plasma 51.7 ± 17.0 μM; n = 9) or ascorbic acid and iron (105 ± 18 μg/g and 40.3 ± 15.3.0 μM; n = 8) compared to controls (84 ± 36 μg/g and 15.3 ± 8.5 μM; n = 11). Total iron levels in the liver (76.7 ± 7.3 μg/g; control; n = 6) and plasma (2.4 ± 0.03 mg/l; control) were not significantly raised in animals under these conditions of iron or ascorbate intake. Liver microsomes isolated from animals receiving iron had a greater susceptibility to oxidative stress in terms of malondialdehyde production during auto-oxidation compared to those from control animals under the same conditions. This effect was eliminated on combining ascorbic acid with the iron supplementation, suggesting that oral administration of vitamin C has a protective rather than a pro-oxidant effect under these circumstances.

Keywords: Oxidative stress, iron, ascorbic acid, antioxidant supplementation

INTRODUCTION

The ability of iron to exist in several redox states and also to bind oxygen has given this transition metal a critical status in living systems. However, the redox activity of iron is also a potential source of toxicity since iron non-specifically bound to low-molecular-mass ligands is capable of generating free radicals.[1] Under normal physiological conditions it is unlikely that much, if any free iron is available *in vivo* for such reactions since iron levels are tightly controlled

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and iron is usually specifically bound to proteins for transport or storage.[2,3] However, there are situations where the body iron status can change which, in turn may induce pathophysiological symptoms. In man, prolonged iron overload as a result of untreated haemochromatosis has been shown to induce multiple pathologies including cancer of the liver[4] and, in a prospective population study, both elevated body iron (serum ferritin ≥ 200 μg/l) and high dietary iron intake were associated with an increased risk of myocardial infarction.[5] The possibility that increased iron intake may be harmful has led to concern over human dietary supplementation, especially when iron is co-administered with substances, such as ascorbic acid, which may increase the bioavailability of iron.[6–8] However, other authors have disputed that ascorbic acid supplementation has adverse effects in the normal population.[9,10] Little is known about the *in vivo* effects of a mild dietary challenge of iron and ascorbic acid under non-deficient non-overload conditions, similar to those that might be attained during a vitamin/mineral supplement regimen. The question that this study addresses is, whether a mild dietary challenge to guinea pigs by oral dosing with iron and ascorbic acid alters their uptake into the plasma and liver, and influences oxidative susceptibility.

MATERIALS AND METHODS

Animals and Diet

Male Dunkin-Hartley guinea pigs approximately 3 months old with an initial weight of 350–410 g were randomly allocated to the experimental diets and kept in groups of 3–5 in polypropylene cages with stainless steel grids. All animals were initially fed a standard guinea-pig diet (Special Diet Services) for approximately 10 days. The diet was then changed to a rabbit diet that was low in iron (supplemented as ferrous sulphate, 194 mg/kg diet) and vitamin C (as ascorbyl polyphosphate, 73 mg/kg) compared to the guinea-pig diet (537 and 300 mg/kg diet respectively). In addition, animals in the control group received a daily oral dose of iron (as ferrous sulphate) and ascorbic acid to give a maintenance level of intake of these substances (Table I). The supplements were prepared separately, stored as preweighed solid and dissolved in deionised water immediately before use. The required volume of each supplement solution was applied by syringe to the back of the throat of the animal and swallowed reflexively. For example, a 350 g animal eating 25 g of diet a day at the start of the experiment, and with oral dosing would receive approximately 4 mg of Fe and 10 mg of vitamin C a day. Animals in the experimental groups received a similar regimen as the controls except that the oral dose of the supplement/s being studied was at a concentration ten fold higher than that of the controls. Thus four separate dietary regimens were set up: I. control, II. raised iron, III. raised ascorbic acid and IV. raised iron and ascorbic acid. (Table I). For example, a 350 g animal eating 25 g diet a day at the start of the experiment, and with maximum levels of oral dosing would receive approximately 32 g of Fe and 85 mg of vitamin C daily. Food and purified

TABLE I Oral dosing regimens

Oral Dosing Treatment	Fe (mg/100g body wt)	Ascorbic Acid (mg/100g body wt)
Control	0.84	2.4
Raised Iron	8.4	2.4
Raised Ascorbic Acid	0.84	24
Raised Iron and Ascorbic Acid	8.4	24

Iron as $FeSO_4.7H_2O$ and ascorbic acid as L-ascorbic acid; dissolved in purified water and administered daily by oral dosing.

water were supplied *ad libitum*. The animals were maintained on the regimen for 35 days. Their individual body weights, group food and water consumption were determined three times a week. Overall during the experiments, one animal was removed from a control group for health reasons, all other guinea pigs showed no adverse effects due to the different dietary regimens. At the end of the experimental period the animals were heparinised (100 IU/ 100g body weight) and killed by an intraperitoneal injection of sodium pentabarbitone (Sagital). Samples of blood were taken for analysis. Also the liver was thoroughly flushed with saline via the hepatic portal vein and samples taken for further analysis or for preparation of microsomes (see below).

Total Iron

Samples of plasma and liver were stored at –70°C for iron analysis. The samples were digested with nitric acid and total iron levels determined by inductively coupled plasma emission spectrometry using a low flow microconcentric nebuliser with a conventional Scott-type double pass spray chamber (Jobin Yvon 24).

Ascorbic Acid

Samples of plasma and liver homogenate were treated with 0.3 M trichloroacetic acid (TCA; 1:1) and stored at –70°C. Total ascorbic acid levels were determined using a modification of the fluorimetric method of Deutsch and Weeks.[11] In brief the samples plus TCA were oxidised with iodine, complexed with orthophenylenediamine (OPD) and the fluorescence read on a Shimadzu RF-1501 fluorimeter (excitation 348 nm, emission 423 nm). The concentration of ascorbic acid in the incubation mixtures was determined by comparison with previously prepared standards.

α-Tocopherol

α–Tocopherol concentrations in the plasma and liver homogenate samples were determined by high performance liquid chromatography (HPLC).[12] The samples were first deproteinised with methanol (1 ml:1 ml of plasma and 2 ml:1 ml of liver homogenate) and the α-tocopherol extracted in 1ml of hexane. The α-tocopherol content was measured by normal phase HPLC (Novapak silica column: 4 μm, 3.9 × 156 mm). The mobile phase consisted of 92% hexane and 8% methyl *tert*-butyl ether pumped at a flow of 1ml/min. Detection and quantification were performed fluorimetrically (295 nm excitation, 340 mm emission). Concentration was obtained by comparison with standard concentrations of α-tocopherol and δ-tocopherol was used as the internal standard.

Liver Microsomes

Liver microsomes were prepared by differential centrifugation (138,000 g for 45 min in 0.25 M sucrose), washed twice in 25 mM 3-[N-morpholino] propanesulphonic acid (MOPS; Sigma)-150 mM KCl, pH 7.4 and were stored at -70°C until required.[13] All solutions were metal chelated with washed beads coated with chelating resin (iminodiacetic acid, Chelex 100; Sigma; 2 g Chelex/l of solution); the solution was decanted from the resin coated beads and stored in plastic containers. Protein levels in the microsomes were determined using the Bradford assay.[14] Aliquots of liver microsomes (0.25 mg/ml protein) were incubated in air at 37°C and lipid autoxidation occurred. After the required time (0 min, 30 min or 60 min) peroxidation was stopped by the addition of 300 μM butylated hydroxytoluene (BHT; final concentration). Bound malondialdehyde (MDA) was released by a modification of the alkaline hydrolysis system.[15] Total MDA (free and bound) was measured with an HPLC assay,[16] using a Spherisorb amino acid phase column (250 × 4.8 mm, Hichrom U.K.). The mobile phase consisted of 10% acetonitrile and 90% tris buffer (0.03 M, pH 7.0) at a flow rate of 1 ml/min. MDA containing samples were injected using a Rheodyne syringe loading injector with a loop volume of 20 μl. MDA

was detected and quantified by reference to standards at 267 nm using a Varian UV-50 variable wavelength detector. The MDA standard solutions were prepared by hydrolysis of 1,1,3,3,-tetraethoxypropane and the concentration confirmed by u.v. spectroscopy at 270 nm ($\varepsilon = 34{,}000$).

Analysis of Data

Data are presented as means ± s.d. Results were compared using a Students t test, p values <0.05 were considered significant.

RESULTS

Body Growth and Dietary Intake

Animals receiving maintenance levels of iron and ascorbic acid had an initial mean body weight of 379 ± 27 g (n = 11, pooled from 3 experiments). After 35 days the body weight of animals on this control diet had increased by 47 ± 12%. In the experimental groups, animals subjected to oral dosing with iron and ascorbic acid either separately or in combination (Table I) were not significantly different in final body weight nor was the estimated daily food intake different from the control animals or each other. This shows that oral dosing with the various supplementary regimens had no significant effect on growth or appetite.

Total Iron

Determination of the total iron content of either the plasma or liver samples showed no significant differences between the levels found in control animals and any of those on the increased level dietary regimens, even in animals receiving iron supplementation (Fig. 1).

Antioxidant Levels

Ascorbic Acid

The mean ascorbic acid content of the liver and plasma in control animals was 84 ± 36 μg/g tissue wet wt and 15.3 ± 8.5 μM (2.7 ± 1.5 mg/l) respectively (Fig. 2). Ascorbic acid levels were significantly increased in those animals receiving either ascorbic acid alone, 126 ± 36 μg/g liver tissue and 51.7 ± 17.0 μM (9.1 ± 3.0 mg/l plasma), or ascorbic acid co-supplemented with iron, 105 ± 18 μg/g liver tissue and 40.3 ± 15.3 μM (7.1 ± 2.7 mg/l plasma; Fig 2). It is difficult to make direct comparisons with the levels of ascorbic acid quoted by other workers. In the guinea-pig there is a total dependency on dietary intake, therefore the concentrations in the food and/or water, the methods of delivery, duration of feeding and the method of analysis all play a part in the final concentration determined, and ascorbic acid levels for guinea-pig liver ranging from 0.01–99 μmol/g tissue have been quoted.[17]

α-Tocopherol

Oral dosing of iron and ascorbic acid either separately or in combination produced no significant differences in the levels of α-tocopherol in the plasma or liver. The α-tocopherol levels in the plasma and liver of control animals was 2.09 ± 1.16 μM (0.9 ± 0.5 mg/l) and 4.2 ± 1.5 μg/g tissue wet weight which is comparable to the range of 2–3 nmol/ml for guinea plasma[18] and 5.9–31.9 μg/g wet weight for liver.[19]

Oxidative Stress in Liver Microsomes

There was a significant elevation in the susceptibility to oxidative stress in the animals receiving iron supplementation (Fig. 3). This was seen as an increase in MDA level in microsomes from these animals, incubated in the absence of a prooxidant, compared to the levels seen from controls under the same conditions. The microsomes (0.25 mg protein/ml) were subjected to autooxidation by incubating at 37°C in air for 30 min and 60 min. A lesser and non-significant effect was found in microsomes from animals given the same level of iron with ascorbic acid, suggesting that combined ascorbic acid intake with

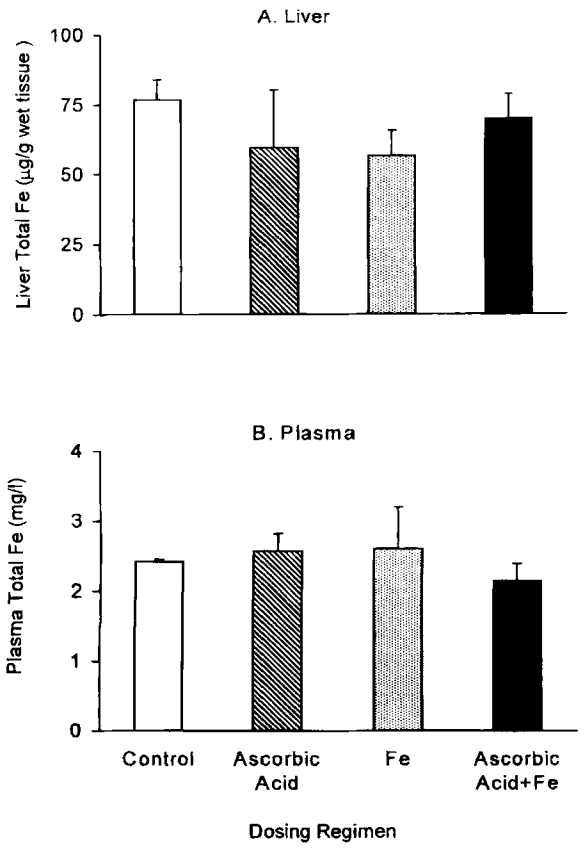

FIGURE 1 The effects of daily oral dosing for 35 days with iron (8.4 mg/100 g body weight) and ascorbic acid (24 mg/100 g body weight) separately and in combination on the total iron content of **A**). Liver and **B**). Plasma Fe, iron; control dosing levels were 0.84 mg/100g body weight iron and 2.4 mg/100 g body weight ascorbic acid. Mean ± s.d. are shown; n= 6, control; n= 7, ascorbic acid; n= 4, Fe and n= 4, ascorbic acid + Fe.

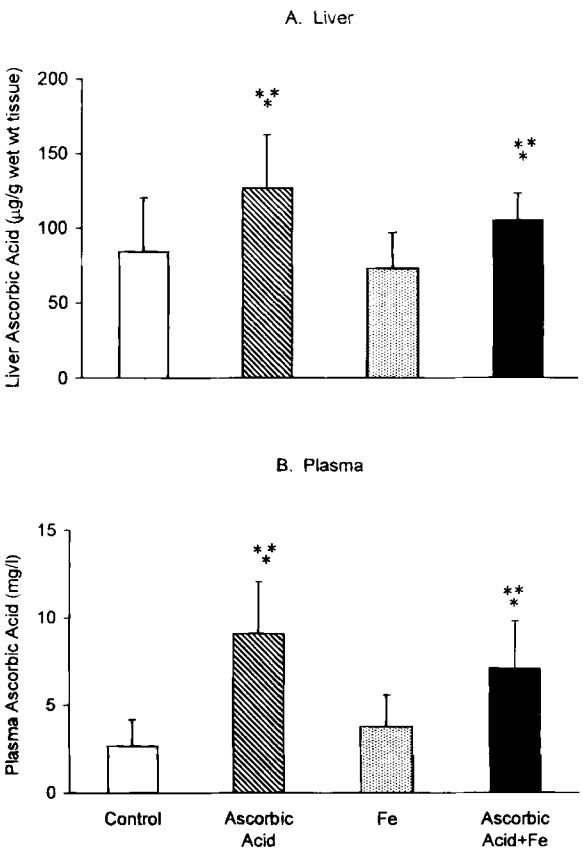

FIGURE 2 The effects of daily oral dosing for 35 days with iron (8.4 mg/100 g body weight) and ascorbic acid (24 mg/100 g body weight) separately and in combination on the ascorbic acid content of A). Liver and B). Plasma. Fe, iron; control dosing levels were 0.84 mg/100 g body weight iron and 2.4 mg/100 g body weight ascorbic acid. Mean ± s.d. shown; n= 11, control; n= 9, ascorbic acid; n= 9, Fe and n= 8 ascorbic acid + Fe. Students t test was used, $p < 0.05$ significantly different * = *vs* control; ** = *vs* Fe dosed animals.

iron may be protective in terms of susceptibility to oxidative stress.

DISCUSSION

Increased intake of iron, and iron with ascorbic acid at the levels used in this study did not alter the total iron levels in the plasma or liver. Although iron levels can be increased by dietary intake this is usually most successful in iron deficient subjects[20,21] and it has been suggested that with chronic iron intake in iron-replete subjects, absorption is down regulated in the gastrointestinal tract[22,23] which may have occurred in these experiments. Ascorbic acid has the potential to interact with iron in several ways. The reducing properties of ascorbic acid and the ability to convert Fe^{3+} to Fe^{2+} have been suggested as important factors in the gastrointestinal absorption of iron.[24,25] In man, nutritional studies have shown that ascorbic acid intake enhances the absorption of non-haem iron, as indicated by the incorporation of isotopic tracer into erythrocytes,[26] and increased levels of serum ferritin following a single meal.[27] In addition to this potential to raise

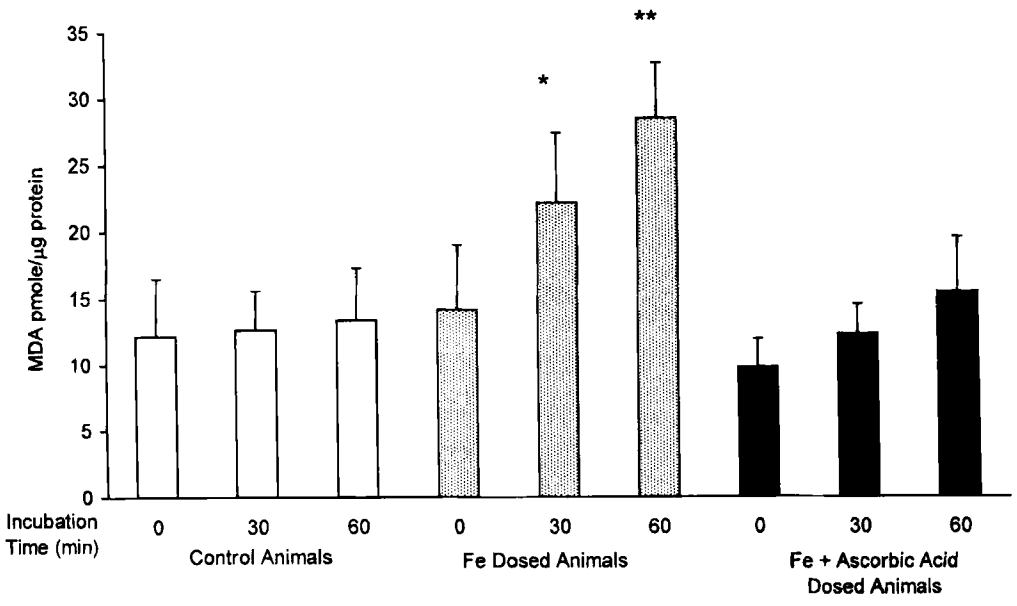

FIGURE 3 The effects of daily oral dosing for 35 days with iron (8.4 mg/100 g body weight) and ascorbic acid (24 mg/100 g body weight) separately and in combination on the susceptibility of liver microsomes to oxidative stress. The microsomes (0.25 mg protein/ml) were incubated in air at 37°C for 0 min, 30 min and 60min, the reaction was stopped with 300μM 2,[6]-di *tert* butyl-p-cresol, butylated hydroxytoluene (BHT) and total malondialdehyde (MDA) determined by HPLC. Fe, iron; control dosing levels 0.84 mg/100 g body weight iron and 2.4 mg/100 g body weight ascorbic acid. Mean ± s.d. from 4 incubations shown. Students t test was used, $p<0.05$ significantly different *vs* control at * = 30 min and ** = 60 min incubation times.

iron levels by an effect on iron absorption, tissue culture studies have shown that ascorbic acid may also increase iron levels by retarding ferritin degradation via its autophagic uptake into lysosomes.[28] However, although ascorbic acid can increase iron levels in these ways, it may have less effect on bioavailability in the longer term.[27,29]

Although total iron levels were not altered in the present study, increased dietary intake of iron did alter the endogenous susceptibility of liver microsomes to oxidative stress. This was seen as increased levels of peroxidation in the incubated microsomes from animals on iron supplementation. It is possible that iron supplementation resulted in the movement of iron between different pools[30,31] where it may have been more readily available to participate in peroxidation.

Total ascorbate levels in the liver and plasma were increased as a consequence of ascorbic acid supplementation. It is well known that, in vitro, ascorbic acid can provide a potent catalyst for free radical reactions through its redox cycling capacity,[32] although this effect may be concentration dependent, the pro-oxidant action being promoted by lower concentrations of ascorbic acid, while at higher concentrations free radical scavenging predominates.[33] *In vitro* experiments have shown that ascorbic acid can protect intact erythrocytes exposed to H_2O_2 but not red cells from patients with chronic haemolytic anaemia associated with glucose-6-phosphate dehydrogenase deficiency.[34] In rats, high levels of supplementation with ascorbic acid in animals on a marginal intake of vitamin E significantly increased *in vitro* erythrocyte hemolysis and liver lipid peroxidation and significantly lowered the erythrocyte level of reduced glutathione and the plasma level of vitamin E.[35] These effects could be counteracted by a small increase in vitamin E intake. Also in the streptozotocin diabetic rat, lipid peroxidation products were increased and antioxidant levels, including ascorbic acid, were lowered by diabetes.[36] High ascorbate supplementation in the

absence of insulin treatment restored plasma ascorbic acid levels to normal but did not decrease lipid peroxidation. The authors attributed this to an increase in iron availability as a result of the ascorbate treatment. In clinical studies, where there was evidence of increased levels of iron, ascorbic acid levels were depleted, for example, in haemochromatosis,[37] thalassemia[38] and iron overload in the Bantu.[39] Administering vitamin C these patients to relieve this deficiency has been reported to produce adverse clinical changes. These include deterioration in left ventricular function,[39] rapid progression of congestive cardiomyopathy and haemochromatosis[40] and potentiated haemolytic conditions such as paroxysmal nocturnal haemoglobinuria[41] and glucose-6-phosphate dehydrogenase deficiency.[42]

However, in the present experiments co-administration of ascorbic acid *with the same level of iron, that was shown to promote peroxidation*, significantly raised levels of plasma and liver ascorbic acid but did not produce any increase in the susceptibility of liver microsomes to peroxidation. On the contrary it had a protective effect. Previous experiments have shown that dietary modulation of ascorbic acid levels will alter its concentration in the blood and other tissues, influence glutathione peroxidase activity and the extent of endogenous lipid peroxidation. Reductions in blood and tissue levels of ascorbic acid induced by a low dietary intake of ascorbic acid have been associated with increased lipid peroxidation in guinea-pig liver microsomes[43] and guinea-pig liver and adrenal glands.[44] In the latter study, the authors reported a significant non-linear negative correlation between vitamin C levels and lipid peroxidation products found in these tissues. Conversely increasing tissue levels by dietary intake have increased glutathione peroxidase activity in guinea-pig liver as well as decreasing protein carbonyls and the level of endogenous MDA[45,46] in the absence of any additional applied oxidative stress. In mice administration of ascorbic acid by interperitoneal injection produced an increase in the concentration of this vitamin in the liver, spleen and lungs without any alteration in the MDA or conjugated diene content in these tissue, which the authors suggested provided evidence against increased levels of vitamin C having pro-oxidant effects.[47] Ascorbate has been reported as increasing ferritin mRNA translation in cultured cell systems,[48] although interestingly ascorbic acid failed to exacerbate or alleviate the inhibitory effect of raised iron levels on mouse 3T3 fibroblast proliferation.[49]

In conclusion, the results show that oral dosing of guinea-pigs with iron and ascorbic acid separately and together to provide a mild dietary challenge, increased liver and plasma levels of ascorbic acid but not total iron or α-tocopherol concentrations. This has the effect of increasing the susceptibility to peroxidation in the assay system used of liver microsomes from animals subjected to iron supplementation. Co-administration of ascorbic acid and the same level of iron did not produce any increase in the susceptibility to peroxidation, on the contrary it had a protective effect.

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