

## Supplementation with $\alpha$ -tocopherol or a combination of $\alpha$ -tocopherol and ascorbic acid protects the gastrointestinal tract of iron-deficient rats against iron-induced oxidative damage during iron repletion

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*(Received 21 April 1999 – Revised 11 October 1999 – Accepted 9 December 1999)*

Recently we have shown the susceptibility of Fe-deficient rat intestine to oxidative damage during Fe repletion. The role of dietary antioxidants like ascorbic acid,  $\alpha$ -tocopherol and a combination of both in counteracting the oxidative stress was tested in this study. Five groups of thirteen weanling WKY female rats were fed with an Fe-deficient diet for a period of 5 weeks. Another set of thirteen rats received an Fe-sufficient diet and served as the control group (Con). Oral administration of either vehicle (D), 8 mg Fe alone ( $D^+$ ) or in the presence of 24 mg ascorbic acid ( $D^+$ +C), 40 mg  $\alpha$ -tocopherol ( $D^+$ +E) or a combination of both ( $D^+$ +C+E) per d for 15 d was carried out in Fe-depleted rats. The impact of this treatment protocol on Fe status, oxidative stress and antioxidant status at the site of Fe absorption was assessed. It was observed that though the indicators of Fe status were normalised on Fe supplementation, the oxidative stress as reflected by the levels of both thiobarbituric-acid reactive substances (TBARS) and protein carbonyls were significantly greater in  $D^+$  and  $D^+$ +C compared to  $D^+$ +E,  $D^+$ +C+E and Con groups. The mucosal cell DNA damage was seen in  $D^+$ ,  $D^+$ +C and  $D^+$ +E groups on electrophoresis. Functional integrity as assessed by the activities of alkaline phosphatase and lys-ala-dipeptidyl aminopeptidase were normalized in all the groups treated with the anti-oxidant(s). There were significant positive alterations in some of the endogenous antiperoxidative systems and in serum caeruloplasmin activity in  $D^+$ +E and  $D^+$ +C+E groups. Paradoxically, serum ascorbate levels were significantly lower in  $D^+$ +C than in  $D^+$ +E and  $D^+$ +C+E groups. This could be due to the protection offered by  $\alpha$ -tocopherol in the presence of Fe. It is concluded that supplementation of  $\alpha$ -tocopherol alone or in combination with ascorbic acid protects the gastrointestinal tract of Fe-deficient rats against Fe-mediated oxidative damage during Fe repletion. However, ascorbic acid alone does not protect the gastrointestinal tract against Fe-induced oxidative stress.

### Iron: Oxidative stress: Ascorbic acid: $\alpha$ -Tocopherol

Fe-deficiency anaemia is a major public health problem in many developing countries. Therapeutic supplementation with Fe is one of the short-term measures suggested to control Fe deficiency in the vulnerable segments of the population, namely pregnant women and children (Horn, 1988; Indian Council of Medical Research Task Force Studies 1989, 1992). Absorption of Fe is a highly regulated process and therefore a large fraction of the administered dose remains unabsorbed in the small intestine of the gastrointestinal tract. Cells of all forms of life require Fe and the physico-chemical properties which enable Fe to be an essential factor also allow Fe to be toxic when not carefully handled by proteins and shielded from surrounding

media (Rayan & Aust, 1992; McCord, 1998). It is expected that exposing the intestine continuously to Fe may cause free radical-mediated reactions at the site of absorption. This in turn can bring about a variety of dysfunctions in the intestine. Recently, we have shown that the Fe-deficient rat intestine is more susceptible to peroxidative damage of biomolecules like lipids and proteins during Fe supplementation in rats (Srigiridhar & Nair, 1998).

$\alpha$ -Tocopherol, which is a critical component of the antioxidant system in all tissues (Machlin & Bendich, 1987), has recently received attention as a potential therapeutic agent to reduce clinical disease states associated with

**Abbreviations:** Gpx, glutathione peroxidase; SOD, superoxide dismutase; TBARS, thiobarbituric-acid reactive substances.

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Table 1. Experimental design

| Feeding duration                            | Treatment |     |                |                   |                   |                     |
|---|-----------|-----|----------------|-------------------|-------------------|---------------------|
|   | Con       | D   | D <sup>+</sup> | D <sup>+</sup> +C | D <sup>+</sup> +E | D <sup>+</sup> +C+E |
| Depletion (5 weeks)                         | +Fe       | -Fe | -Fe            | -Fe               | -Fe               | -Fe                 |
| Repletion (15 d)                            |           |     |                |                   |                   |                     |
| Iron (8 mg) in vehicle                      | -         | -   | +              | +                 | +                 | +                   |
| Vehicle*                                    | +         | +   | -              | -                 | -                 | -                   |
| Ascorbic acid (24 mg) in 0.2ml water        | -         | -   | -              | +                 | -                 | +                   |
| DL- $\alpha$ -Tocopherol (40 mg) in vehicle | -         | -   | -              | -                 | +                 | +                   |
| Vehicle†                                    | +         | +   | +              | +                 | -                 | -                   |

Con, control group; D, deficient group; D<sup>+</sup>, deficient group repleted with iron; D<sup>+</sup>+C, deficient group repleted with iron and ascorbic acid; D<sup>+</sup>+E, deficient group repleted with iron and DL- $\alpha$ -tocopherol; D<sup>+</sup>+C+E, deficient group repleted with iron, DL- $\alpha$ -tocopherol and ascorbic acid; +Fe, control, adequate-iron diet (35 mg/kg diet); -Fe, iron-deficient diet (8 mg iron/kg diet); +, given by gavage; -, not given.

\* 1 ml 0.01 M-HCl.

† 0.2 ml arachis oil.

excess free radical production (Packer & Landvik, 1989; Horwitt, 1991). However, the antioxidant activity of vitamin C has been questioned recently (Halliwell, 1996). The predominance of the pro-oxidant properties of vitamin C in the presence of Fe has been recently demonstrated (Herbert *et al.* 1996). Inclusion of ascorbic acid in Fe-fortified infant cereals and formulas was shown to enhance hydroxyl radical formation (Almaas *et al.* 1997). Although ascorbic acid is known to increase Fe absorption (Wienk *et al.* 1997), there are no studies demonstrating the antioxidant or pro-oxidant actions of vitamin C during Fe treatment under *in vivo* conditions.

In the present study, the antioxidant effects of  $\alpha$ -tocopherol and ascorbic acid were examined both independently and in combination in preventing the Fe-mediated peroxidative damage during oral repletion in Fe-deficient rats.

## Materials and methods

### Chemicals

Malondialdehyde, 2-thiobarbituric acid, pyrogallol, glutathione reductase, GSH, lys-ala-7-amido 4-methyl coumarin, bovine serum albumin, NADPH, cumene hydroperoxide,  $\beta$ -glycerophosphate, butylated hydroxytoluene, *O*-dianisidine, DL- $\alpha$ -tocopherol, hyaluronidase, RPMI-1640 medium, sodium lauryl sarcosinate and bromophenol blue sodium salt were purchased from Sigma Chemical Company (St. Louis, MO, USA). RNase and proteinase K were from Boehringer Mannheim (Mannheim, Germany). Diethylene triamine pentacetic acid and ascorbic acid were from Loba Chemi (Mumbai, India) and HPLC grade methanol was from Qualigens (Mumbai, India). All other chemicals used were of analytical grade and procured locally.

### Animals, diet and study design

Female WKY rats (21 d old) with a mean body weight of 34 g (*n* 78) were obtained from the National Centre for Laboratory Animal Science at the National Institute of Nutrition, Hyderabad, India. The Institution's guide for

the care and use of laboratory animals was used. The rats were randomly divided into six groups (*n* 13 per group) and housed individually in polypropylene cages in a temperature- (24  $\pm$  2°C) and humidity- (50%) controlled room with a 12 h light-dark cycle. Dietary Fe deficiency was induced in five groups by placing them on diet with low Fe content (Srigiridhar & Nair, 1998). The sixth group received an Fe-sufficient diet (35 mg Fe/kg) throughout the study and served as control (Con). Rats were allowed free access to these diets and water for a period of 5 weeks.

The different groups of depleted rats while on the Fe-deficient diet were then treated with vehicle (D), 8 mg of Fe in 1 ml 0.01 M-HCl alone (D<sup>+</sup>) or together with 24 mg ascorbic acid in 0.2 ml distilled water (D<sup>+</sup>+C) or 40 mg DL- $\alpha$ -tocopherol in 0.2 ml arachis oil (D<sup>+</sup>+E) or a combination of both (D<sup>+</sup>+C+E) for 15 d. The antioxidant supplement was given 30 min before the administration of Fe (Table 1). The dose of 8 mg was prepared by dissolving 39.7 mg of FeSO<sub>4</sub>.7H<sub>2</sub>O and this is the amount of estimated Fe required per day to regenerate haemoglobin from 80 g/l to 140 g/l in 15 d assuming 2% absorption and a blood volume of 8 ml/100 g body weight. Groups D and Con received only vehicle and served as negative and positive controls respectively. Food was withheld overnight after the last dose and animals were killed by cervical dislocation to obtain duodenum (10 cm) and proximal jejunum (10 cm) and liver. To minimise experimental variation, rats from all groups were sacrificed on each day. Serum was separated and stored at -80°C until further analysis.

### Tissue processing

The intestinal segments collected from each rat were homogenised in 0.2 mol KCl/l containing 0.5 mM-butylated hydroxytoluene and the mucosal suspension was prepared according to the method described earlier (Srigiridhar & Nair, 1998). The homogenate was subjected to differential centrifugation (Sorvall RC 5B, Du Pont Company, Wilmington, DE, USA) at 800 g for 30 min, 12 000 g for 30 min, and 100 000 g for 1 h at 4°C and the respective supernatants were collected and stored at -20°C for further analysis. The protein content in these supernatants was determined by the

method of Bradford (1976) using bovine serum albumin as the standard.

#### *Markers of functional integrity*

The activities of alkaline phosphatase and lys-ala-dipeptidyl aminopeptidase are maximal towards the upper half of the villus and the basal villus respectively (Eloy *et al.* 1978). The activities of these enzymes were measured in the supernatant centrifuged at 12 000 *g* to assess the functional integrity of the small intestine.

*Alkaline phosphatase.* The method of Bodansky (1932), using  $\beta$ -glycerophosphate as the substrate, was employed. The amount of inorganic phosphate liberated was estimated by the method of Chen *et al.* (1956).

*Lys-ala-dipeptidyl aminopeptidase.* The activity of this enzyme was measured according to the method of Imai *et al.* (1983) using lys-ala-7-amido-4-methyl coumarin as substrate and 7-amido-4-methyl coumarin as the standard.

#### *Markers of oxidative stress*

*Lipid peroxidation.* The lipid peroxide content in the 12 000 *g* supernatant was measured as thiobarbituric-acid reactive substances (TBARS) (Balasubramanian *et al.* 1988). Supernatant (1 ml, 400  $\mu$ g protein) was combined with an equal volume of TCA (100 g/l) and thiobarbituric acid solution (6.7 g/l). The solution was heated for 20 min in a water-bath at 100°C to precipitate the proteins. The absorbance was determined in the supernatant fraction at 532 nm against a blank containing all reagents except the supernatant. The concentration of TBARS was quantified against a standard curve derived from a freshly prepared solution of malondialdehyde (0.1–5 nmol/l).

*Protein oxidation by protein carbonyl levels.* This was estimated by the method of Reznick & Packer (1993). Briefly, the method involves incubation of 1 ml 100 000 *g* supernatant (1 mg protein) with 4 ml 10 mmol/l 2,4-dinitrophenylhydrazine in 2.5 M-HCl for 1 h in the dark. Then, 5 ml TCA (200 g/l) was added and the protein precipitate washed three times with ethanol–ethylacetate (1 : 1, v/v) and dissolved in 2 ml of 6 M-guanidinium-HCl. A spectral scan at 355–390 nm was performed to obtain the peak corresponding to protein hydrazone. The recovery of protein in the above solution was quantitated by reading the absorbance at 280 nm using bovine serum albumin in 6 M-guanidinium-HCl as standard. The molar absorption coefficient of 2,4-dinitrophenolhydrazone of 22 000 cm<sup>2</sup>/mol was used to calculate the concentration of carbonyls formed.

#### *Antioxidant enzymes*

*Catalase.* The activity of catalase was measured in 12 000 *g* supernatant (100  $\mu$ g protein) by following the decomposition of H<sub>2</sub>O<sub>2</sub> at 240 nm for 1 min (Aebi, 1983).

*Superoxide dismutase.* Cu,Zn-superoxide dismutase (SOD) activity was assayed in 100 000 *g* supernatant (150  $\mu$ g protein) by following the inhibition of auto-oxidation of pyrogallol at 420 nm for 1 min (Marklund & Marklund, 1974).

*Glutathione peroxidase.* Glutathione peroxidase (Gpx)

activity was assayed in 100 000 *g* supernatant (125  $\mu$ g protein) using cumene hydroperoxide as the substrate. Activity was expressed as the amount of NADPH oxidised for 1 min at 340 nm (Paglia & Valentine, 1967).

#### *Serum and liver iron*

The Fe content was estimated by using bathophenanthroline as the chromogen (International Committee for Standardization in Haematology, 1971). Mineral solutions of the liver samples were prepared after dry-ashing at 550°C.

#### *Serum caeruloplasmin*

The ferroxidase activity of serum caeruloplasmin was measured by using *O*-dianisidine dihydrochloride as the substrate (Roxborough *et al.* 1995). The activity of caeruloplasmin was calculated using the extinction coefficient of *O*-dianisidine (Johnson *et al.* 1967).

#### *Serum ascorbic acid and $\alpha$ -tocopherol levels*

*Ascorbic acid.* The method of Zannoni *et al.* (1974) was used to measure serum ascorbic acid levels. The serum (200  $\mu$ l) was deproteinized with 12  $\mu$ l TCA (400 g/l). To the supernatant obtained was added 10  $\mu$ l *O*-H<sub>3</sub>PO<sub>4</sub> (85%); 80  $\mu$ l aqueous  $\alpha,\alpha'$ -dipyridyl (10 g/l) and 10  $\mu$ l aqueous ferric chloride (30 g/l) in that order. The final volume was made upto 250  $\mu$ l with TCA (50 g/l) and the developed colour read at 525 nm.

*$\alpha$ -tocopherol.* HPLC methodology according to Miller *et al.* (1984) was adopted. To 200  $\mu$ l serum was added an equal volume of 100% ethanol and extracted with 400  $\mu$ l hexane and 300  $\mu$ l of it collected. The hexane phase was dried under N<sub>2</sub> and reconstituted in 35  $\mu$ l ethanol. Of this, 20  $\mu$ l was immediately analysed by reverse-phase HPLC. The mobile phase was methanol–water (95 : 5, v/v) with a flow rate of 2 ml/min. The HPLC system consisted of a 15 cm C<sub>18</sub> reversed-phase analytical stainless-steel column (Shodex, Shoko. Co. Ltd, Showa Denko K.K., Japan) with Shimadzu LC 6A (Nakagyo-ku, Kyoto, Japan) equipped with system controller SCL6A, wavelength detector SPD-6AV. The DL- $\alpha$ -tocopherol was identified by the retention time (8.2 min) and quantitated by measuring the peak areas using dual channel Shimadzu C-R3A chromatopack integrator system. Calibration was done daily with known quantities of standard DL- $\alpha$ -tocopherol. The recovery of added DL- $\alpha$ -tocopherol was found to be 100% using this procedure.

#### *Isolation of mucosal cells*

Mucosal cells were isolated by the method described by Kimmich (1970). Pieces (1 cm) of everted segments of intestine were incubated for 30 min in an isolation medium containing bovine serum albumin 1 g/l and 1 g hyaluronidase/l at 37°C in a shaking water-bath. The suspension produced by mechanical agitation was then filtered and centrifuged at 300 *g* for 5 min at room temperature. The pellet was then washed and suspended in 0.8 ml RPMI-1640 medium. The viability of cells by Trypan Blue-exclusion test was more than 85%.

### DNA extraction and electrophoresis

DNA was isolated according to the method described by McGowan *et al.* (1994). About  $2.0\text{--}2.5 \times 10^5$  cells were lysed and digested with RNase A and proteinase K. Electrophoresis was carried out on 2% agarose gels in 10 mM-tris borate and 1 mM EDTA, pH 8 (TBE) using a submerged electrophoresis system (Broviga Co., Chennai, India) at 30 V for 5 h. A gel documentation system (Mitsubishi UVP, Image store 5000; UVP International UV Products Ltd, Cambridge, Cambs., UK) was used to obtain the electrophoresis pattern.

### Statistical analysis

The results were analysed using a statistical Programme for Social Sciences: SPSS/PC+, version 5.0 (SPSS Inc., Chicago, IL, USA). A one-way ANOVA was employed for comparison among the six groups. Post-hoc multiple comparison test of significant differences among groups were determined. The values were considered significantly different if  $P$  values were  $< 0.05$ .

## Results

### Iron status

At the end of 5 weeks of Fe depletion, groups fed on the low-Fe diet exhibited Fe deficiency. There was a significant lowering of haemoglobin levels from 152 (SD 1.5) g/l in control to 79 (SD 2.9) g/l in deficient groups. Oral administration of Fe to deficient groups resulted in significantly higher levels of haemoglobin, serum and liver Fe concentrations compared to group D which received vehicle (Table 2). A significant lowering in haemoglobin levels was found in group  $D^+$ +E compared to groups Con and  $D^+$ +C. Liver Fe was significantly greater in all the Fe-supplemented groups compared to Con group. In group  $D^+$ +C liver Fe content was significantly higher compared to the rest of the Fe-supplemented groups  $D^+$ ,  $D^+$ +E and  $D^+$ +C+E. Serum Fe was significantly reduced in groups  $D^+$ +E and  $D^+$ +C+E compared to groups Con,  $D^+$  and  $D^+$ +C.

### Serum caeruloplasmin activity

The ferroxidase activity of serum caeruloplasmin was significantly lowered in  $D^+$  compared to all other groups (Table 2). The activity of caeruloplasmin was greatest in D. Its activity was significantly greater in  $D^+$ +C,  $D^+$ +E and  $D^+$ +C+E compared to  $D^+$  group.

### Intestinal lipid peroxidation, protein carbonyls and DNA degradation

TBARS formation in the intestine of Fe-deficient rats was lower than in the other groups. Significantly higher TBARS levels were observed in  $D^+$  and  $D^+$ +C compared to all other groups ( $P < 0.05$ ). In  $D^+$ +E and  $D^+$ +C+E the TBARS levels were higher compared to Con. Protein carbonyl levels were also significantly elevated in groups  $D^+$  and  $D^+$ +C compared to other groups (Fig. 1).

In  $D^+$  and  $D^+$ +C groups the intestinal mucosal DNA was observed to be completely degraded on agarose gel electrophoresis (Fig. 2, lanes 3 and 4). Although there was degraded mucosal DNA in  $D^+$ +E group the extent of degradation was less than in  $D^+$ +C group. Intact mucosal DNA (lane 6) was similar in Con, D and  $D^+$ +C+E groups.

### Marker enzymes

The activities of both the marker enzymes, alkaline phosphatase and lys-ala-dipeptidyl aminopeptidase were significantly lower in group  $D^+$  than in other groups (Table 3). Fe deficiency *per se* has significantly lowered the activity of peptidase to levels similar to  $D^+$ . Whereas in group  $D^+$ +C+E its activity was significantly greater than the Con group.

### Intestinal antiperoxidative system

The activity of SOD was significantly greater in group  $D^+$  compared to groups  $D^+$ +C and  $D^+$ +E with comparable activity among the other groups (Fig. 3). The activity of catalase was lowest in group D compared to all other groups. It was significantly lower in group  $D^+$ +C compared to groups  $D^+$ ,  $D^+$ +E and  $D^+$ +C+E ( $P < 0.05$ ). A significantly higher catalase activity was observed in groups  $D^+$

**Table 2.** Haemoglobin, liver and serum concentration of iron and serum caeruloplasmin level in iron-deficient rats fed iron with or without vitamins C and E\*

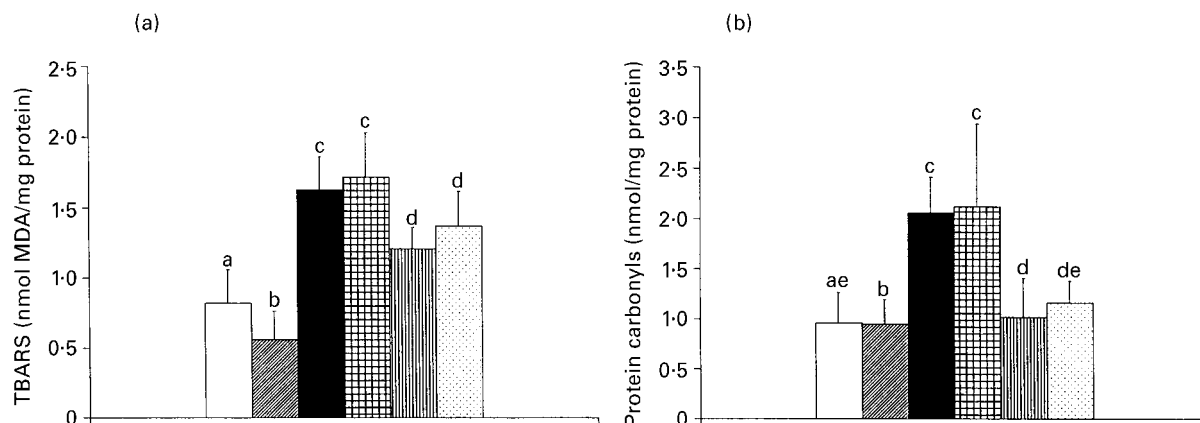
(Mean values and standard deviations)

| Treatment  | Haemoglobin (g/l)  |     |          | Liver iron ( $\mu\text{mol/g}$ tissue) |      |          | Serum iron ( $\mu\text{mol/l}$ ) |      |          | Serum caeruloplasmin (nmol/mg protein/min) |     |          |
|------------|--------------------|-----|----------|--|------|----------|----------------------------------|------|----------|--|-----|----------|
|            | Mean               | SD  | <i>n</i> | Mean                                   | SD   | <i>n</i> | Mean                             | SD   | <i>n</i> | Mean                                       | SD  | <i>n</i> |
| Con        | 152 <sup>a</sup>   | 4.5 | 9        | 32 <sup>a</sup>                        | 6.6  | 6        | 69 <sup>a</sup>                  | 14.3 | 5        | 18.1 <sup>a</sup>                          | 2.3 | 8        |
| D          | 79 <sup>b</sup>    | 7.2 | 9        | 15 <sup>b</sup>                        | 4.7  | 6        | 31 <sup>b</sup>                  | 5.5  | 5        | 22.8 <sup>c</sup>                          | 0.6 | 8        |
| $D^+$      | 147 <sup>ce</sup>  | 3.3 | 9        | 65 <sup>c</sup>                        | 3.1  | 5        | 80 <sup>a</sup>                  | 6.4  | 5        | 10.1 <sup>b</sup>                          | 2.9 | 7        |
| $D^+$ +C   | 151 <sup>cd</sup>  | 5.1 | 9        | 85 <sup>d</sup>                        | 12.2 | 7        | 72 <sup>a</sup>                  | 10.0 | 5        | 21.1 <sup>ac</sup>                         | 5.0 | 7        |
| $D^+$ +E   | 146 <sup>e</sup>   | 2.5 | 10       | 68 <sup>c</sup>                        | 18.4 | 6        | 46 <sup>c</sup>                  | 10.2 | 5        | 22.8 <sup>c</sup>                          | 5.0 | 7        |
| $D^+$ +C+E | 148 <sup>ace</sup> | 4.2 | 9        | 66 <sup>c</sup>                        | 15.7 | 6        | 50 <sup>c</sup>                  | 11.2 | 5        | 21.0 <sup>ac</sup>                         | 7.7 | 7        |

Con, control; D, deficient group;  $D^+$ , deficient group repleted with iron;  $D^+$ +C, deficient group repleted with iron and ascorbic acid;  $D^+$ +E, deficient group repleted with iron and DL- $\alpha$ -tocopherol;  $D^+$ +C+E, deficient group repleted with iron, DL- $\alpha$ -tocopherol and ascorbic acid.

<sup>a,b,c,d,e</sup> Mean values within a column with unlike superscript letters were significantly different:  $P < 0.05$ .

\* For details of animals, diets and procedures, see Table 1.



**Fig. 1.** Concentration of (a) thiobarbituric-acid reactive substances (TBARS) and (b) protein carbonyls in the small intestinal mucosa of rats during iron repletion with or without ascorbic acid and DL- $\alpha$ -tocopherol. MDA, malondialdehyde. (□), Control, Con; (▨), deficient group, D; (■), deficient group repleted with iron, D<sup>+</sup>; (▩), deficient group repleted with iron and ascorbic acid, D<sup>+</sup>+C; (▧), deficient group repleted with iron and DL- $\alpha$ -tocopherol; D<sup>+</sup>+E; (▦), deficient group repleted with iron, DL- $\alpha$ -tocopherol and ascorbic acid, D<sup>+</sup>+C+E. Values are means for 7–8 rats per group with standard deviations represented by vertical bars. <sup>a,b,c,d,e</sup> Mean values with unlike superscript letters were significantly different:  $P < 0.05$ . For details of animals, diets and procedures see Table 1.

and D<sup>+</sup>+C+E compared to Con group ( $P < 0.05$ ). The activity of Gpx was significantly greater in D compared to the rest of the groups ( $P < 0.05$ ). Also the activity of this enzyme was significantly lowered in group D<sup>+</sup>+C+E compared to groups Con, D, D<sup>+</sup>+C and D<sup>+</sup>+E. The levels of mucosal GSH were significantly lowered in D<sup>+</sup> compared to groups Con, D<sup>+</sup>+C and D<sup>+</sup>+C+E ( $P < 0.05$ ).

#### Serum ascorbic acid and $\alpha$ -tocopherol levels

Serum ascorbate levels were low in D<sup>+</sup> and D<sup>+</sup>+C groups compared to D<sup>+</sup>+E and D<sup>+</sup>+C+E groups (Table 4). The highest concentration of serum ascorbate was observed in D<sup>+</sup>+C+E which was significantly different from all other groups ( $P < 0.05$ ) except D<sup>+</sup>+E group. Serum  $\alpha$ -tocopherol levels were significantly higher in D<sup>+</sup>, D<sup>+</sup>+E and D<sup>+</sup>+C+E compared to Con, D and D<sup>+</sup>+C ( $P < 0.05$ ). In group D<sup>+</sup>+C serum  $\alpha$ -tocopherol level was significantly lower compared to all the other groups.

## Discussion

### Effects of iron supplementation with ascorbic acid and $\alpha$ -tocopherol on iron status

The results of this present study indicate that supplementation of Fe alone or in combination with antioxidants

**Table 3.** Changes in marker enzyme activities in the small intestinal mucosa in iron-deficient rats during iron repletion with or without vitamins C and E\*

(Mean values and standard deviations)

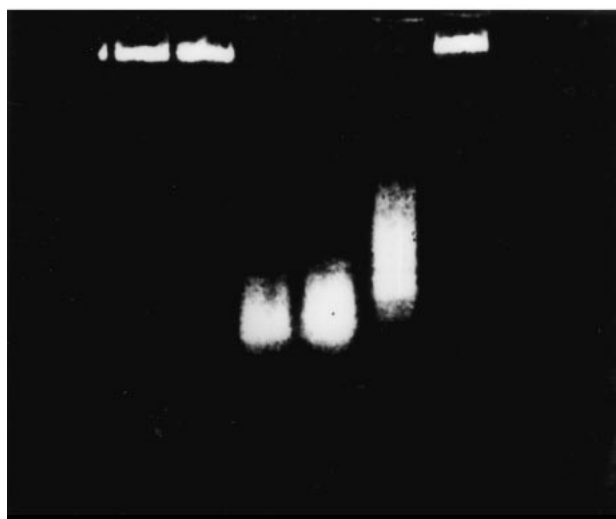
| Treatment           | Alkaline phosphatase<br>( $\mu$ mol Pi/mg protein/min) |      |          | Lys-ala-dipeptidyl<br>amino peptidase<br>(pmol/mg protein/min) |     |          |
|---------------------|--|------|----------|--|-----|----------|
|                     | Mean   | SD   | <i>n</i> | Mean   | SD  | <i>n</i> |
| Con                 | 0.36 <sup>a</sup>                                      | 0.13 | 8        | 12.1 <sup>a</sup>  | 2.5 | 8        |
| D                   | 0.38 <sup>a</sup>                                      | 0.09 | 8        | 8.8 <sup>b</sup>   | 0.7 | 8        |
| D <sup>+</sup>      | 0.23 <sup>b</sup>                                      | 0.03 | 7        | 10.3 <sup>b</sup>  | 0.3 | 7        |
| D <sup>+</sup> +C   | 0.33 <sup>a</sup>                                      | 0.08 | 7        | 13.1 <sup>ac</sup>   | 1.3 | 7        |
| D <sup>+</sup> +E   | 0.32 <sup>a</sup>                                      | 0.02 | 7        | 12.6 <sup>bc</sup>   | 1.9 | 7        |
| D <sup>+</sup> +C+E | 0.32 <sup>a</sup>                                      | 0.06 | 7        | 14.2 <sup>c</sup>  | 1.5 | 7        |

Pi, inorganic phosphate; Con, control; D, deficient group; D<sup>+</sup>, deficient group repleted with iron; D<sup>+</sup>+C, deficient group repleted with iron and ascorbic acid; D<sup>+</sup>+E, deficient group repleted with iron and DL- $\alpha$ -tocopherol; D<sup>+</sup>+C+E, deficient group repleted with iron, DL- $\alpha$ -tocopherol and ascorbic acid.

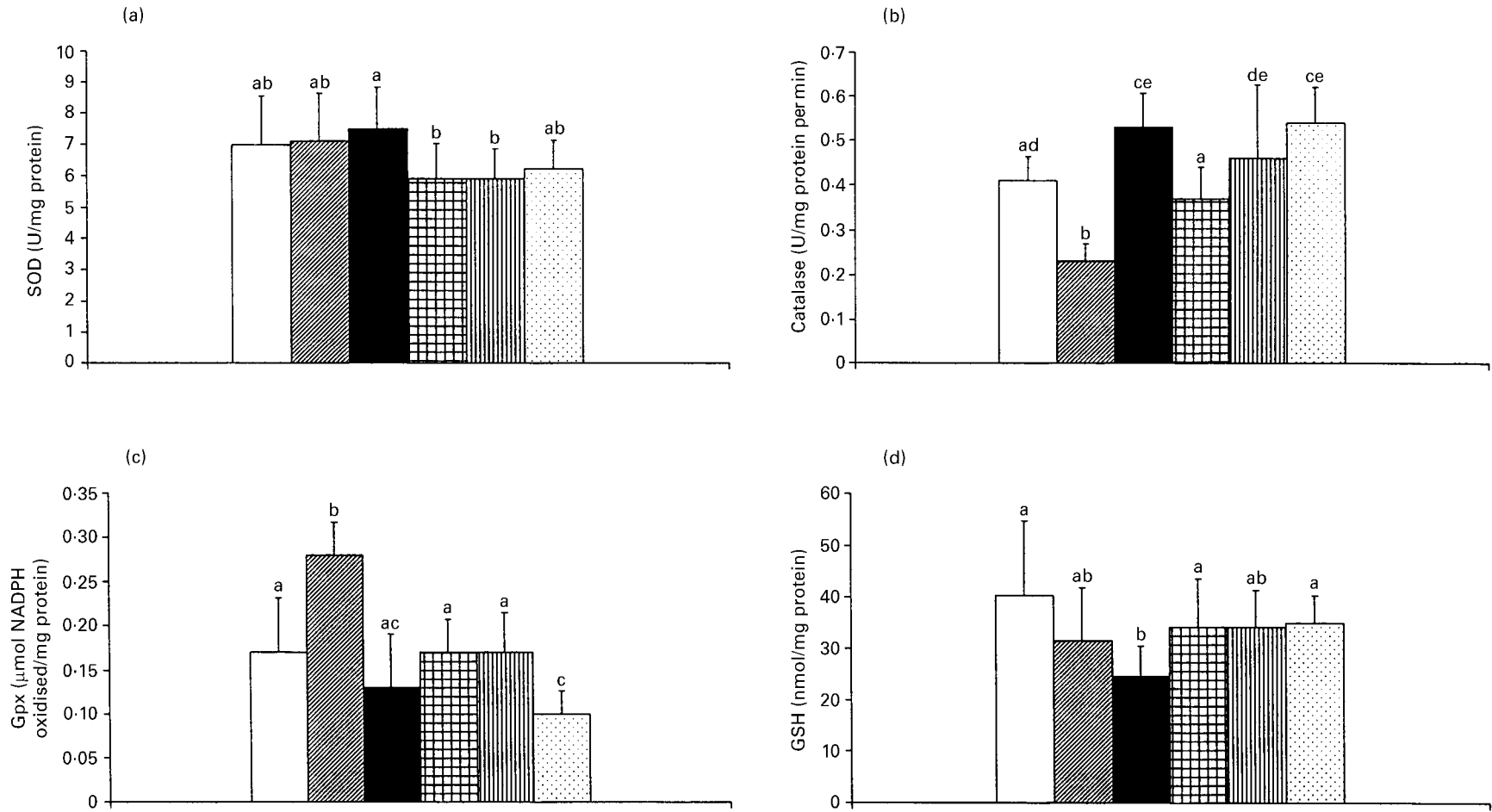
<sup>a,b,c</sup> Mean values within a column with unlike superscript letters were significantly different:  $P < 0.05$ .

\* For details of animals, diets and procedures, see Table 1.

1 2 3 4 5 6



**Fig. 2.** Electrophoretic pattern of intestinal mucosal DNA obtained from iron-deficient rats during oral repletion of iron with or without ascorbic acid and DL- $\alpha$ -tocopherol. Lane 1, control (Con); lane 2, deficient group (D), lane 3, deficient group repleted with iron (D<sup>+</sup>); lane 4, deficient group repleted with iron and ascorbic acid (D<sup>+</sup>+C); lane 5, deficient group repleted with iron and DL- $\alpha$ -tocopherol (D<sup>+</sup>+E); lane 6, deficient group repleted with iron, DL- $\alpha$ -tocopherol and ascorbic acid (D<sup>+</sup>+C+E). For details of animals, diets and procedures see Table 1.



**Fig. 3.** Levels of (a) Superoxide dismutase (SOD), (b) catalase, (c) glutathione peroxidase (Gpx) and (d) reduced glutathione (GSH) in the small intestinal mucosa of rats during iron repletion with or without ascorbic acid and DL- $\alpha$ -tocopherol. (□), Control, con; (▨), deficient group, D; (■), deficient group repleted with iron, D<sup>+</sup>; (▩), deficient group repleted with iron and ascorbic acid, D<sup>+</sup>+C; (▧), deficient group repleted with iron and DL- $\alpha$ -tocopherol, D<sup>+</sup>+E; (▤), deficient group repleted with iron, DL- $\alpha$ -tocopherol and ascorbic acid, D<sup>+</sup>+E+C. Values are means for 7–8 rats per group with standard deviations represented by vertical bars. <sup>a,b,c,d,e</sup> Mean values with unlike superscript letters were significantly different:  $P < 0.05$ . For details of animals, diets and procedures see Table 1.

vitamin C or E orally for a period of 15 d to Fe-depleted rats normalized all the indicators of Fe status. Higher levels of liver and serum Fe were only observed in D<sup>+</sup>+C group and not in D<sup>+</sup>+E and D<sup>+</sup>+C+E groups. This may be due to the well known Fe-absorption promoting effects of ascorbic acid (Cook & Monsen, 1977) or may be as a consequence of lowered uptake of Fe by the small intestine exerted by the exogenously given vitamin E. Vitamin E is known to have beneficial effects against Fe toxicity and Fe induced hepatic vitamin E depletion in mice (Omara & Blakley, 1993).

*Effects of iron supplementation with ascorbic acid and  $\alpha$ -tocopherol on oxidative stress*

Repletion of deficient rats with oral Fe increased the intestinal oxidative stress as reflected by lipid peroxidation, protein oxidation and DNA damage. Similar findings on lipid peroxidation and protein oxidation were reported by us in an earlier study (Srigiridhar & Nair, 1998). Repletion with Fe and vitamin C also produced similar effect in the gastrointestinal tract mucosa. This was an unexpected observation and contrary to the antioxidant property of ascorbic acid. On the other hand, supplementation with  $\alpha$ -tocopherol (D<sup>+</sup>+E), was able to reduce the Fe-mediated oxidative damage to a certain extent. Neither supplementation with vitamin C nor E could protect the mucosal DNA from oxidative damage caused during Fe repletion. A combination of these two vitamins (D<sup>+</sup>+C+E) could offer a significant level of protection to the gastrointestinal tract mucosa. Thus, the beneficial role of vitamin C and E in combination would neutralize the Fe-mediated oxidative damage during Fe repletion, though the mechanism is not clear.

The greater oxidative stress observed in D<sup>+</sup>- and in D<sup>+</sup>+C-supplemented groups may be because of the depletion of endogenous vitamin E levels in the intestinal tissue. Physiologically,  $\alpha$ -tocopherol is a more potent antioxidant than ascorbic acid in reducing the oxidative damage (Herbert *et al.* 1996). It was shown earlier that in Fe-loaded patients with thalassaemia major, excess lipid peroxidation in erythrocytes, is associated with reduced serum vitamin E concentrations (Rachmilewitz *et al.* 1976). Consumption of excess dietary Fe was also shown to have a negative impact on vitamin E status (Omara & Blakley, 1993). In addition to the above reasons, perhaps vitamin C in the presence of Fe has no antioxidant property and may be acting as a pro-oxidant. A recent study conducted by Podmore *et al.* (1998) provides evidence for the pro-oxidant properties of ascorbic acid in human subjects. They have shown that vitamin C significantly increased the modified bases of DNA (8-oxoadenine) when 500 mg ascorbic acid/d was administered as a dietary supplement to healthy human subjects for 6 weeks. In the present study, in spite of greater oxidative stress at the site of absorption, D<sup>+</sup>+C group registered better Fe status compared to all the other groups. The reason for this effect needs to be investigated. Thus, the protection offered in the group D<sup>+</sup>+C+E could be due to the masking of the oxidant effect by  $\alpha$ -tocopherol over ascorbic acid.

*Effects of iron supplementation with ascorbic acid and  $\alpha$ -tocopherol on serum ascorbate and  $\alpha$ -tocopherol levels*

It is observed that serum ascorbate levels were significantly lower in D<sup>+</sup> and D<sup>+</sup>+C groups compared to vitamin E-supplemented groups, which were able to maintain the level of serum ascorbate. This could be due to the following mechanisms. Depletion of ascorbate in D<sup>+</sup>+C group could be due to the generation of hydroxyl radicals following the administration of oral Fe and ascorbic acid as suggested earlier by Slivka *et al.* (1986). In addition, the hydroxyl radical produced in the microenvironment may be causing depletion of endogenous  $\alpha$ -tocopherol levels which otherwise can protect ascorbic acid. This argument is supported by the striking observation of depletion of  $\alpha$ -tocopherol in D<sup>+</sup>+C. Further evidence for the role of continuous dietary Fe intake in the generation of hydroxyl radical *in vivo* has been provided by Kadiiska *et al.* (1995). It is, therefore, possible that these radicals would be responsible for the depletion of endogenous vitamin E levels. Also, the exogenous supplementation of vitamin E along with vitamin C is able to protect the ascorbic acid directly.

*Effects of iron supplementation with ascorbic acid and  $\alpha$ -tocopherol on intestinal functional integrity*

The lowered functional integrity similar to that reported earlier by us (Srigiridhar & Nair, 1998) and restoration of it upon supplementation with antioxidants vitamins C and E were observed. These observations suggest the protective effects of these antioxidants on Fe-induced functional damage.

*Effects of iron supplementation with ascorbic acid and  $\alpha$ -tocopherol on antiperoxidative status*

Fe repletion with or without ascorbic acid and  $\alpha$ -tocopherol for a period of 15 d has caused certain alterations in both enzymatic and non-enzymatic antiperoxidative system. The activity of SOD lowered significantly ( $P < 0.05$ ) when Fe was supplemented either with vitamin C or E. The reasons for the lowering of SOD activity in the antioxidant supplemented rats are not clear at present. Catalase activity was again shown to respond positively with better Fe status as shown earlier (Srigiridhar & Nair, 1998). The activity of this enzyme was found to be the highest in groups D<sup>+</sup>, D<sup>+</sup>+E and D<sup>+</sup>+C+E and the lowest in D. However, there was a significant lowering in the activity of catalase in group D<sup>+</sup>+C. This could be due to the lowered mucosal Fe concentration consequent to the action of vitamin C in enhancing absorption of Fe by the mucosal cells and its subsequent transportation to the storage sites. The above possibility is supported by the higher concentration of liver Fe in D<sup>+</sup>+C compared to all the other groups.

The biological importance of Gpx in combating the oxidative stress over catalase has been shown by Halliwell & Gutteridge (1986). The activity of Gpx was found to have opposite effects during Fe depletion (higher) and repletion (lower). Also, there was an inverse relationship between the activities of catalase and Gpx, the greatest ratio of 5.4:1 which was observed in D<sup>+</sup>+C+E group. This confirms our

**Table 4.** Serum ascorbate and  $\alpha$ -tocopherol levels in iron-deficient rats during iron repletion with or without vitamins C and E\* (Mean values and standard deviations)

| Treatment           | Serum ascorbate ( $\mu\text{mol/l}$ ) |      |          | Serum $\alpha$ -tocopherol ( $\mu\text{mol/l}$ ) |      |          |
|---------------------|---------------------------------------|------|----------|--|------|----------|
|                     | Mean                                  | SD   | <i>n</i> | Mean   | SD   | <i>n</i> |
| Con                 | 34 <sup>ac</sup>                      | 16.4 | 7        | 2.4 <sup>ac</sup>                                | 0.86 | 4        |
| D                   | 25 <sup>ab</sup>                      | 9.0  | 7        | 2.9 <sup>a</sup>                                 | 0.82 | 4        |
| D <sup>+</sup>      | 17 <sup>b</sup>                       | 4.5  | 7        | 4.9 <sup>bd</sup>                                | 0.74 | 4        |
| D <sup>+</sup> +C   | 26 <sup>ab</sup>                      | 6.5  | 8        | 0.72 <sup>c</sup>                                | 0.28 | 4        |
| D <sup>+</sup> +E   | 47 <sup>cd</sup>                      | 17.5 | 9        | 6.1 <sup>d</sup>                                 | 1.66 | 4        |
| D <sup>+</sup> +C+E | 53 <sup>d</sup>                       | 24.1 | 7        | 5.6 <sup>d</sup>                                 | 1.48 | 4        |

Con, control; D, deficient group; D<sup>+</sup>, deficient group repleted with iron; D<sup>+</sup>+C, deficient group repleted with iron and ascorbic acid; D<sup>+</sup>+E, deficient group repleted with iron and DL- $\alpha$ -tocopherol; D<sup>+</sup>+C+E, deficient group repleted with iron, DL- $\alpha$ -tocopherol and ascorbic acid.

<sup>a,b,c,d</sup> Mean values within a column with unlike superscript letters were significantly different:  $P < 0.05$ .

\*For details of animals, diets and procedures, see Table 1.

earlier observations (Srigiridhar & Nair, 1998). As the substrate for both these enzymes is the same, i.e. hydroperoxides, the higher activity of one would compensate the other. The depletion in GSH level in D<sup>+</sup> indicates the occurrence of oxidative stress in the mucosal cells. But in the presence of exogenous antioxidants, the levels of GSH did not alter, thus offering protection to the gastrointestinal tract mucosa.

#### *Effects of iron supplementation with ascorbic acid and $\alpha$ -tocopherol on serum caeruloplasmin*

Caeruloplasmin acts as a powerful ferroxidase, thereby catalysing the oxidation of ferrous Fe to the less reactive ferric state (Gutteridge & Quinlan, 1992). Plasma caeruloplasmin activity was the highest in Fe-depleted group and the lowest in Fe-repleted group (D<sup>+</sup>). These observations are consistent with the findings reported earlier (Yu *et al.* 1993; Mukhopadhyay *et al.* 1998; Srigiridhar & Nair, 1998). Caeruloplasmin activity remained close to the normality when Fe was given along with exogenous antioxidants vitamin C and E. This might be a trigger of defence against oxidative stress (Kresk-Staples & Webster, 1993) operating in vitamin E-supplemented groups. However, in D<sup>+</sup>+C there was greater oxidative stress which cannot be attributed to caeruloplasmin activity.

Thus, it can be concluded from the above studies that supplementation of vitamin C along with Fe improves Fe status but does not prevent the Fe-mediated peroxidative damage of rat intestine. Supplementation of vitamin E alone or a combination of both the vitamins E and C normalises Fe status and protects the intestine against Fe-mediated oxidative damage. The results of this study thus provide simple antidotes to alleviate the gastrointestinal tract side-effects associated with oral administration of Fe in supplementation programmes. To what extent the concern of Fe-induced toxicity causing the gastrointestinal tract discomfort could be due to this oxidative stress cannot be ascertained from the present study. Daily supplementation of therapeutic doses of Fe for correction of Fe deficiency should be accompanied by either  $\alpha$ -tocopherol or a combination of  $\alpha$ -tocopherol and

ascorbic acid to minimise the oxidative stress and maintain functional integrity of gastrointestinal tract.

#### Acknowledgements

The authors thank Kamala Krishnaswamy, Director and B. Sivakumar, Deputy Director (Sr.Gr.), National Institute of Nutrition for their valuable suggestions. The authors also thank N. Balakrishna for his help in statistical analysis. A research fellowship to Mr K. Srigiridhar by the Indian Council of Medical Research, New Delhi, India is gratefully acknowledged.

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