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IRON ENHANCEMENT OF ASCORBATE TOXICITY

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Iron has been shown to enhance ascorbate-induced damage to both acetylcholine esterase and *E. coli* B in a manner analogous to previous studies with ascorbate and copper ions. It is suggested that the mechanism of damage entails interaction of iron with biological macromolecules, followed by its reduction by ascorbate. Subsequently, the iron (II) could participate in generating hydroxyl radicals from hydrogen peroxide via the Fenton reaction, which in turn, could damage biomolecules in a site-specific and multiple hit fashion. The high abundance of iron in biological systems, especially in certain storage disorders, may indicate an important toxicological role of the combination of iron and ascorbate.

KEY WORDS: Vitamin C, iron toxicity, hydrogen peroxide, free radicals, acetylcholine esterase, enzyme inactivation, cell killing.

INTRODUCTION

Earlier studies concerning deleterious biological processes have established that low levels of copper and iron ions sensitize damage to isolated enzymes,[1] DNA,[2] bacterial cells[3] and coliphages[4] that is induced by superoxide, ascorbate, paraquat and other reducing agents. The proposed mechanism for the processes suggest that metal ions are reduced by the reducing agent, and subsequently react with hydrogen peroxide to generate the highly reactive hydroxyl radicals OH·.[1] When these metals interact with macromolecules,[5] the free radicals will react with the target, most probably the same macromolecules, in site-specific and multiple hit modes.[6]

The abundance of iron in living systems,[7] and the ability of ferrous ions to increase ascorbate oxidation *in vitro*,[8] raises the possibility that iron, like copper, can enhance superoxide or ascorbate-induced damage to a variety of biological targets. Indeed, we have shown that iron could enhance the toxicity of paraquat in an analogous mode to the effect of copper.[9–11]

While a major pathway for iron utilization in mammalian systems is for haemoglobin synthesis in the bone marrow and young red blood cells,[12] all other cells have an active iron metabolism.[13] Iron is stored as ferritin[14] and forms complexes with proteins, amino acids, nucleotides and other molecules,[15] and a small (approximately 1 μM) component of iron is also loosely bound and detachable.[16] Accumulation of iron in cells, is particularly prevalent in disorders of mucosal absorption such as idiopathic haemochromatosis or thalassemia major[17] or when there is an exceptional dietary overload, e.g. among Bantu adults of Southern Africa.[18] Repeated blood transfusions to individuals with chronic anaemias can also lead to iron overload.[19] Thus, iron abundance and its mobilization between stores and end-utilization sites combined with its ability to undergo cyclic oxidation-reduction reactions suggest that iron is a likely physiological catalyst of the production of hydroxyl radicals from

ascorbate or superoxide via the Fenton reaction. It has already been shown that low molecular weight iron-complexes, present in sera of patients with idiopathic haemochromatosis, stimulate both the peroxidation of membrane lipids and the formation of hydroxyl radicals.[20] Consequently, iron could play a synergistic role in sensitizing free radical-induced damage to biomolecules.

METHODS

Inactivation of acetylcholine esterase

Stock of acetylcholine esterase from Sigma (1 mg/ml) was prepared by dissolving the protein in Tris-HCl buffer (0.01 M, pH 7.0). Inactivation reactions were performed on the solutions containing 0.1 mg/ml enzyme and other additives as indicated in the reaction mixture. The pH of the reaction mixture was lowered using 0.01 M HCl to pH 3.8 and then iron was added, in order to avoid its precipitation. After five minutes of pre-incubation, allowing the iron to interact with the enzyme, the pH was slowly raised to 7.0 by addition of 0.01 M NaOH. The initiation of enzyme inactivation of acetylcholine esterase was by the introduction of ascorbate. The time course of enzyme inactivation was determined using 50 μl samples taken at pre-determined time intervals and assayed for esterase activity using a procedure identical to that by Shinar *et al.*[1]

E. coli inactivation

E. coli B (SR-9) was used throughout. The cells were cultured at 37°C using a shaking incubator (G-24 Environmental incubator shaker, New Brunswick Scientific Crop.) at 200 rpm in a medium which contained KH_2PO_4, 0.05 M (Merck), K_2HPO_4, 0.017 M (Merck), sodium citrate, 0.017 M (AR-Mallinckrodt), $(NH_4)_2SO_4$, 0.007 M (Baker analytical reagent) at pH 7.0. $MgSO_4 \cdot 7H_2O$, 0.004 M (Merck), was added after sterilization in an autoclave, and glycerol 0.100 M (Frutarom, Israel) was added as carbon source. The washing was carried out by centrifugation (Sorval RC-5) 5 min at 0°C using a total volume of 5 ml/wash. The washed cells were suspended in phosphate buffer (0.001 M, pH 7.4) containing glucose (0.027 M) and $MgSO_4$ (0.001 M) to a density of 1×10^9 cells/ml. Inactivation of cells was determined according to the following procedure: The total volume of each system was 1 ml, containing 1×10^7 cells. Samples from the reaction mixture were taken at various times and were diluted with a phosphate buffer (0.001 M, pH 7.4) containing diethylenetriamine pentaacetic acid (Detapac) (10 μM) and gelatin 0.5% w/v (Sigma) in order to stop the reaction. The samples were additionally diluted in this manner two more times to reach a factor of 10^3–10^6 and were then plated in quadruplicate on agar dishes containing agar, 2% bacto-tryptone, 1% (Difco Laboratories), and sodium chloride 0.085 M (Frutarom). The survival curves were evaluated from colony count after overnight incubation at 37 °C. Anaerobic conditions were obtained by bubbling high purity nitrogen (> 99.999%) through the reaction buffer for 20 min before the reaction and flushing over the bacteria suspension during the reaction.

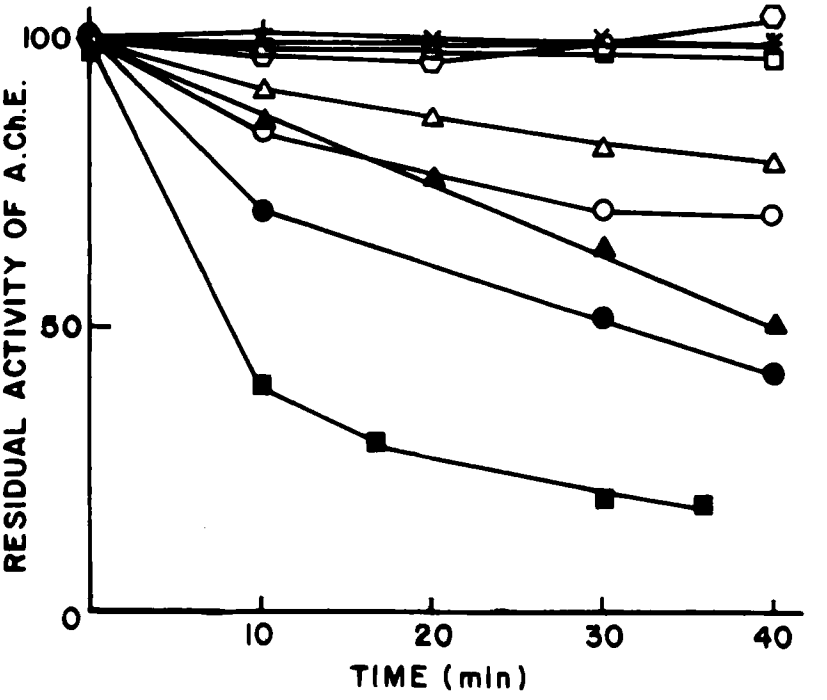

FIGURE 1 The time course of inactivation of acetylcholine esterase ascorbate (2 mM) and iron as indicated below:
○ ferric ammonium sulphate (5 μM) loaded at pH 7.0
■ ferric ammonium sulphate (5 μM) loaded at pH 4.0
△ ferrous sulphate (2 μM) loaded at pH 7.0
▲ ferrous sulphate (5 μM) loaded at pH 7.0
○ ferrous sulphate (2 μM) loaded at pH 3.8
● ferrous sulphate (5 μM) loaded at pH 3.8
+ no iron added, in the presence of Detapac (0.1 mM)
□ control with ferric (5 μM) loaded at pH 3.8 without ascorbate
x control with ferrous (5 μM) loaded at pH 3.8 without ascorbate.

RESULTS

A. Inactivation of acetylcholine esterase:

Figure 1 shows the time-course of acetylcholine esterase inactivation at pH 7.0. When the enzyme is allowed to incubate with ferrous ions (Fe(II)) for 5 min, the rate of ascorbate-induced inactivation is greatly enhanced. This effect is even more pronounced when the enzyme is pre-incubated with ferrous ions under acidic conditions (pH 3.8) in order to avoid iron precipitation, and the pH is then restored to 7.0 and the inactivation initiated. No loss of enzymatic activity was recorded subsequent to the addition of ferrous ions in the absence of ascorbate. Similarly, the addition of ascorbate *together with* the chelator Detapac, resulted in maintaining full enzymatic activity.

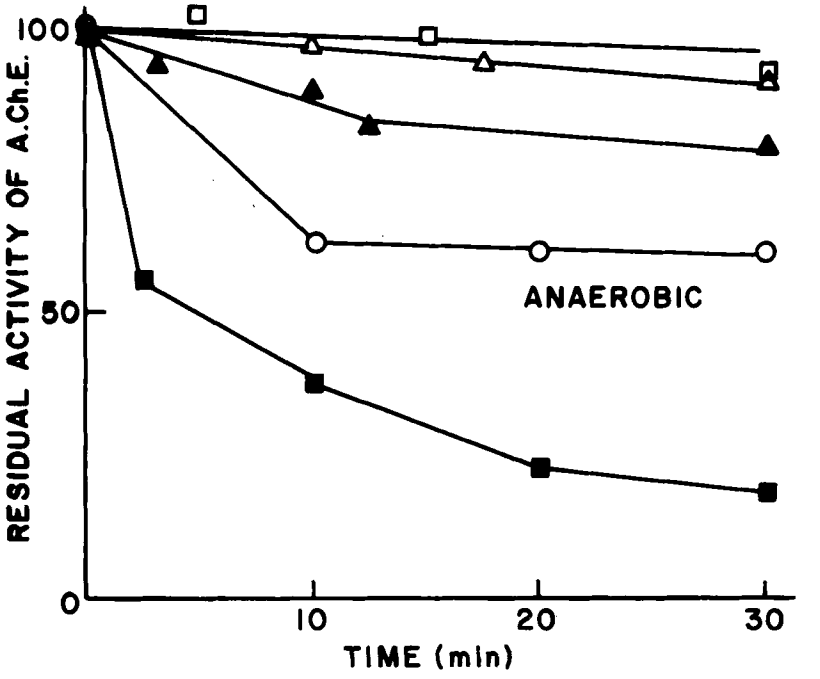

FIGURE 2 The time course of inactivation of acetylcholine esterase when exposed to the combination of ascorbate and iron under *anaerobic conditions*. Samples contained acetylcholine esterase (0.1 mg/ml) in Tris buffer (0.010 M, pH 7). Aqueous solutions of ferrous sulphate (100 μM) were quickly diluted into the enzyme solution to a final concentration of 5 μM. The enzyme solution remained at pH 7.0. The reaction was initiated by the addition of ascorbate (0.002 M).

△ no additions
▲ H_2O_2 (0.044 mM)
■ H_2O_2 (2.2 mM)
○ control without ascorbate and iron, containing H_2O_2 (2.2 mM)
□ control with ascorbate (containing iron, 5 μM) and H_2O_2 (2.2 mM).

The effect of ferric ions (Fe(III)) is also depicted in Figure 1. Similarly, ferric ions are shown to enhance ascorbate-induced inactivation. When iron was introduced at pH 4.0, the effect of Fe(III) is greater than Fe(II). Applying of ferric ions at pH 7.0 did not result in any inactivation. Also, there is no loss of enzymatic activity in the absence of ascorbate or, when ascorbate is added together with Detapac.

Tris buffer had no effect on the enzyme inactivation, i.e. the addition of 10 μM ferrous ions and 2 mM ascorbate (pH 7.0) resulted in a loss of activity similar to the inactivation in the presence of varying concentrations of Tris buffer (0.5 mM, 1 mM, 3 mM or 10 mM), (results not shown).

Ascorbate alone (without iron or detapac) caused only a marginal inactivation of the acetylcholine esterase, at a rate which did not exceed 10–20% of that in the presence of iron. This could be attributed to residual iron (or copper) contamination of the reaction mixtures.[1]

Figure 2 shows that the inactivation of the enzyme upon the addition of ferrous (5 μM) and ascorbate is greatly reduced under anaerobic conditions. However, after

the addition of hydrogen peroxide (0.044 mM) partial inactivation is restored and continues to intensify with increasing concentrations of hydrogen peroxide (2.2 mM). Inactivation is greatest when induced by the combination of ferrous, hydrogen peroxide *and* ascorbate rather than ferrous ions and hydrogen peroxide alone.

The protective effect of catalase (100 μg/ml) has been shown in that it markedly reduces ferrous/ascorbate inactivation when present prior to the addition of iron under acidic conditions. In contrast, if catalase was added *following* the addition of iron to the reaction mixture, a partial protection was recorded. This is in accord with a previous study where we have used denatured catalase and have shown that the protective effect of catalase arises mostly from its catalytic activity, and is due only marginally, to its metal chelating properties.[1] Thus, enzyme activity remains at 80%, 73%, 77% and 79% following 10, 20, 30 and 40 min of incubation, respectively.

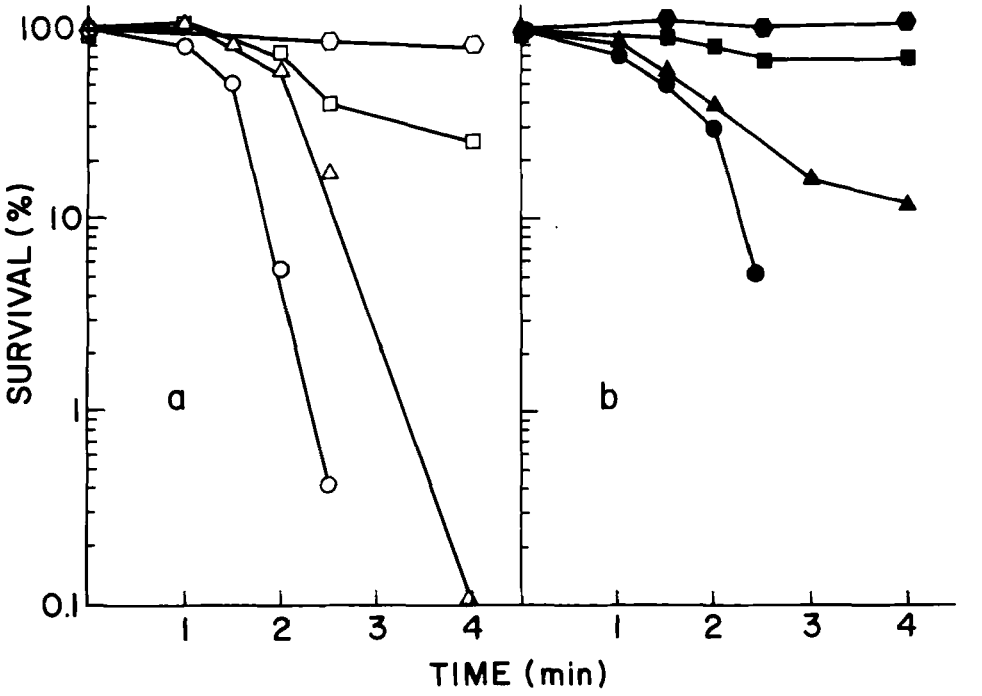

FIGURE 3 Inactivation of *E. coli* B when exposed to the combination of ascorbate and iron. Cell suspension contained ascorbate (0.5 mM, *E. coli* B (2×10^7 cells/ml), phosphate buffer (1 mM, pH 7.4) and magnesium sulphate (1 mM).

a. The effect of ferrous sulphate (2 μM)

○ Preincubation of the cells (5 min) with iron (pH 4.0), followed by addition of ascorbate at time zero.
△ Preincubation of the cells (5 min) with iron (pH 5.0), followed by addition of ascorbate at time zero.
□ Preincubation of the cells (5 min) with iron (pH 7.0), followed by addition of ascorbate at time zero.
⬡ Control without iron, containing Detapac (0.1 μM) and ascorbate (2 mM).

b. The effect of ferric ammonium sulphate (2 μM)

● Preincubation of the cells (5 min) with iron (pH 4.0), followed by addition of ascorbate at time zero.
▲ Preincubation of the cells (5 min) with iron (pH 5.0), followed by addition of ascorbate at time zero.
■ Preincubation of the cells (5 min) with iron (pH 5.7), followed by addition of ascorbate at time zero.
⬢ Control without ascorbate containing ferric (2 μM) loaded pH 4.

B. Killing of E. coli B cells

Bacterial inactivation curves are biphasic. Phase I is a lag period characterised by a low slope of inactivation. The second phase, which begins approximately at 2 min is steeper with slope k.

Figure 3 demonstrated that ascorbate-induced *E. coli* inactivation is greatly enhanced by ferrous and ferric ions when iron is introduced at an acidic pH. Panel A shows the maximal sensitizing effect of the addition of ferrous ions at acidic pH's. This effect rapidly decreases as the pH is increased above 5.0. Panel B shows the effect ferric ions exert. This is similar to that of ferrous ions, at pH 4.0 and 7.0; while at pH 5.7, ferrous ions showed greater effect. Cell viability is not changed by the addition of ascorbate alone or iron by itself.

Figure 4a shows that the anaerobic combination of ascorbate and iron is not sufficient to cause cellular killing. The addition of hydrogen peroxide to that system resulted in the expected high rate of cell inactivation. Hydrogen peroxide alone (0.1–0.3 mM) proved innocuous to the cells both under anaerobic and aerobic conditions. However, the combination of ferrous iron and hydrogen peroxide yielded considerable cellular inactivation.

Addition of hydrogen peroxide to the bacterial suspension in the complete reaction mixture led to an increase in the rate of inactivation (k), and a shortening of the initial lag phase. Similarly, in the presence of 2 μM of ferrous ions and 0.50 mM ascorbate, the addition of 0.25 mM H_2O_2 resulted in a 5-fold increase in k, and a 2-fold shortening of the lag phase (not shown).

Figure 4b shows that the combination of ferric ions with hydrogen peroxide is not sufficient to cause bacterial inactivation. When ascorbate was introduced to the reaction mixture containing ferric ions preloaded to the cells, only a moderate inactivation of the cells occurred. Addition of hydrogen peroxide to the bacterial suspension containing ascorbate and ferric ions led to a marked increase in the rate of inactivation.

Discussion

This study indicates that iron can sensitise biological damage induced by ascorbate in both systems: isolated enzyme and bacterial cells. This phenomenon is in agreement with earlier studies using the combination of iron and ascorbate or other xenobiotics in viruses and bacterial cells.[21–26] These are also analogous to other results obtained when using the combination of copper and ascorbate, copper and superoxide radical, copper and hydrogen peroxide,[11,26–35] copper or iron and paraquat[9,36,37] and copper and isouramil.[34] The results of this study are in accord with the site-specific mechanism of free radical-induced damage.[38] According to this mechanism, ascorbic acid reduces Fe(III) and also serves as a source for H_2O_2 via its oxidation by molecular oxygen. The chemical solubility, and ligand properties of iron[39] as well as studies by others,[40,41] suggest that no free iron can be found under our conditions. Rather iron would form complexes with various biomolecules.[42] This is in accord with current findings that loading of ferric iron at pH 7.0 did not sensitise the inactivation of both bacterial cells and isolated enzyme in solution. On the other hand, ferric ions could enhance ascorbate-induced inactivation of both the cells and the isolated protein when the loading was carried out at acidic pH.

The role of iron and copper in free radical-induced biological damage has been

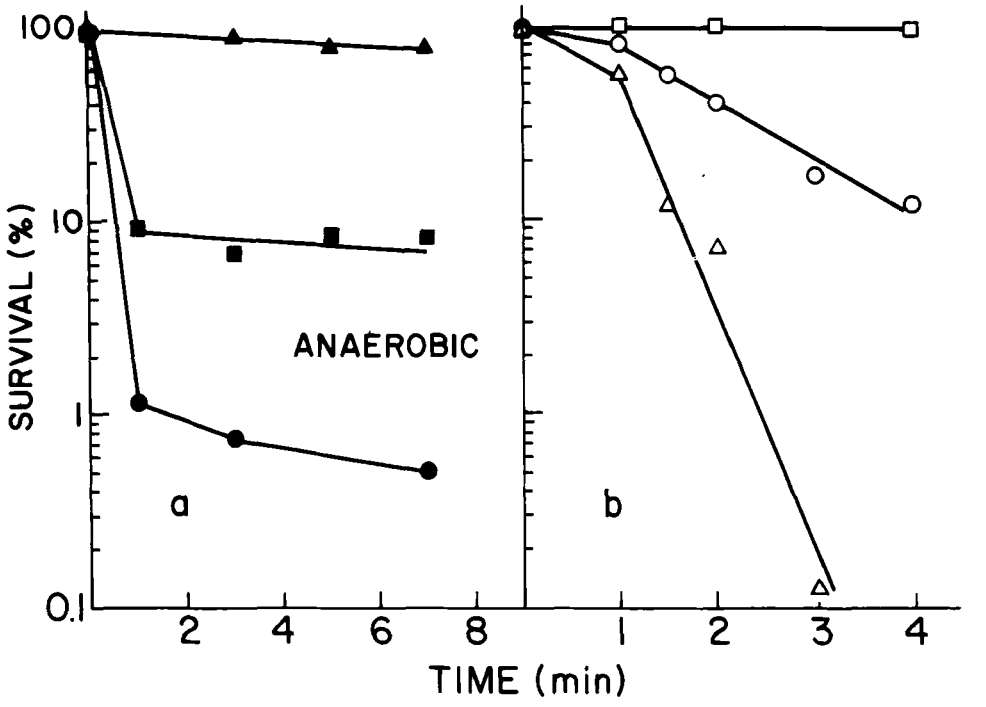

FIGURE 4 The effect of hydrogen peroxide on bacterial inactivation. a. Effect induced by the combination of ferrous and ascorbate under anaerobic conditions. Samples contained *E. coli* B cells (2×10^7 cells/ml), phosphate buffer (1 mM, pH 7.4), and magnesium sulphate (1 mM).
▲ ferrous sulphate (5 μm), ascorbate (0.5 mM)
■ ferrous sulphate (5 μM), H_2O_2 (0.25 mM)
● ferrous sulphate (5 μM), H_2O_2 (0.25 mM), ascorbate (0.5 mM)
b. The effect induced by the combination of ferric and ascorbate under aerobic conditions. Samples were loaded with iron by preincubation (5 min) with ferric ammonium sulphate (2 μM, pH 5), followed by adjusting the pH to 7.4 and various additions at zero time.
□ H_2O_2 (0.25 mM)
○ ascorbate (0.5 mM)
△ ascorbate (0.5 mM) + H_2O_2 (0.25 mM).

widely studied (for recent review, see[38]). Paraquat toxicity, being an extensively studied model system of free radical-induced damage, also served for studies of the role of iron and copper.[9,34,36,37] In paraquat toxicity in *E. coli* cells, the proposed role for iron[9] is identical to that presented in this communication. According to this proposed mechanism, iron is reduced by either paraquat radical or the secondary superoxide radical and produced OH radicals in a recurrent and multi-hit mode at the iron binding site.

The reduced iron within its complex reacts with hydrogen peroxide (originating from ascorbate or superoxide radicals) in a Fenton reaction. In this reaction the highly reactive secondary OH radicals are generated and react with the target biological molecule (BM) at the site of their formation:

$$\text{Ascorbate} + \text{Fe(III)}---\text{BM} \xrightarrow{O_2} \text{Fe(II)}---\text{BM} + H_2O_2$$

$$H_2O_2 + \text{Fe(II)}---\text{BM} \longrightarrow OH^{\cdot} + OH^{-} + \text{Fe(III)}---\text{BM}$$

According to this proposal $OH^{\cdot}$ radicals are produced at the metal-binding site on biological molecules, resulting in site-specific damage at the site of metal binding, rather than a statistically-random injury. In the case of DNA-iron complex, irreversible damage could occur if both strands are broken, similar to the case when bleomycin or adriamycin is applied in the presence of iron and reducing agents,[43,44] or when copper and ascorbate are applied to DNA.[45]

The possible sensitization of ascorbate-induced damage by iron may imply that the ingestion of massive doses of vitamin C is inadvisable for individuals with an abnormally large pool of stored iron.[46] Likewise, ascorbate may prove clinically deleterious under specific circumstances, especially in patients where the concentration of redox-active iron-complexes ("free-iron") is abnormally high. For instance the level of labile iron may reach a few micromolar in synovial fluids and in some rheumatoid patients.[41,47] In such cases iron can catalyze the production of hydroxyl radicals from ascorbate or superoxide ions *and* hydrogen peroxide. These oxygen metabolites could have been formed in the inflamed rheumatoid joint by invading phagocytes. The hydroxyl radical thus formed could attack the hyaluronic acid, cause its degradation and consequently lead to a loss in its lubrication activity.[48]

In conclusion, this study indicates some specific reactions involving the mediatory role of iron in free radical-induced damage. These reactions, might be relevant to clinical cases and could explain some of the deleterious side-effects of iron overload.

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