Pergamon

Biochemical Pharmacology, Vol. 47, No. 5, pp. 795–799, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(93)E0028-6

MECHANISM OF INHIBITORY EFFECTS OF CHELATING DRUGS ON LIPID PEROXIDATION IN RAT BRAIN HOMOGENATES

ANDREI B. KOZLOV, ELENA A. OSTRACHOVITCH and IGOR B. AFANAS'EV*†

*Vitamin Research Institute, Nauchny pr. 14A, GSP-7, 117820, Moscow, and State Medical University, Ostrovityanova 1, 117513, Moscow, Russia

(*Received* 23 *March* 1993; *accepted* 26 October 1993)

Abstract—The mechanism of the inhibitory effects of chelators (desferrioxamine, EDTA, rutin, phenanthroline and ADP) on the production of oxygen radicals in the Fenton reaction and on lipid peroxidation of rat brain homogenates has been studied. It was found that the inhibitory effects of the chelators correlated well with their abilities to oxidize ferrous ions in solution and brain homogenates. On these grounds, it was concluded that the oxidation of Fe^{2+} ions inside a ferrous ion–chelator complex is a major mechanism of inhibitory effects of these chelators on free radical processes. It is proposed that this mechanism is also realized during therapeutic treatment with chelators of patients with "free radical" pathologies such as Fanconi anemia, β-thalassemia and Diamond–Blackfan anemia.

Key words: chelators; lipid peroxidation; oxygen radicals

Compounds possessing chelating properties can be used in the treatment of many "free radical" pathologies including atherosclerosis [1], Fanconi anemia [2, 3], and especially diseases associated with iron overload such as, thalassemia, Down's syndrome, etc. [4–7]. Since many of these diseases are associated with disturbances of iron metabolism, one may propose that the chelators manifest their therapeutic activity by removing or inactivating iron ions. It is usually accepted that the toxic effects of iron ions is a consequence of catalytic decomposition by ferrous ions of hydrogen peroxide or hydroperoxides to form active hydroxyl and oxyl radicals (reactions 1 and 2):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + HO^{-} \quad (1)$$

(The Fenton reaction)

$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + RO^{\cdot} + HO^{-}. \quad (2)$$

These reactions are believed to be the initiation steps of many free radical damaging processes (lipid peroxidation, oxidative modification of proteins and lipoproteins, an increase in chromosome aberrations, etc.).

In this investigation we studied the inhibitory effects of five iron chelators: desferrioxamine, rutin (a bioflavonoid, vitamin B), EDTA, ADP and phenanthroline on lipid peroxidation of rat brain homogenates and compared them with the effects of these compounds on oxygen radical production in the Fenton reaction and on the oxidation of ferrous ions in solution and of brain homogenates. Peroxidation of brain homogenates may be considered a model of free radical destructive processes initiated by endogenous iron. We found that the inhibitory activities of chelators correlated more closely with their ability to oxidize ferrous ions; this process could be an important factor in their therapeutic activities.

† Corresponding author.

‡ Abbreviations: CL, chemiluminescence; ESR, electron spin resonance; TBA, 2-thiobarbituric acid; AOA, antioxidant activity

MATERIALS AND METHODS

Chemicals

Desferrioxamine B was from CIBA-Geigy (Basel, Switzerland), *o*-phenanthroline was from Serva (Heidelberg, F.R.G.), ADP was from Reanal (Budapest, Hungary). TBA‡ was from Fluka (Buchs, Switzerland), SDS and phosphotungstic acid were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Rutin and EDTA were of U.S.S.R. production.

Preparation of rat brain homogenates

The whole rat brain (2.5–3.0 g) was homogenized in 10 mM Tris–HCl buffer (15 mL, pH 7.4) at 5° and centrifuged at 1000 *g* for 15 min. The supernatant obtained was then diluted three times with Tris–HCl buffer and used immediately for the determination of AOA. For determination of iron content, brain samples were homogenized in 10 mM Tris–HCl buffer (10 mL) and used without preliminary centrifugation.

Measurement of AOA

(a) Optical spectrophotometric technique [8]. Two 10% supernatant samples (6 mL each) in Tris–HCl buffer obtained from brain homogenate were incubated at 37° and continuously mixed for 2 hr with or without a chelator (70 μM). Peroxidation was terminated by adding 1.5% H_3PO_4 (3 mL) and EDTA (200 μM) to the incubation mixture (0.25 mL). Then, 0.5% solution of TBA (1 mL) was added,

and the solution was heated for 45 min. After centrifugation of precipitated proteins at 1800 g, the content of TBA reactive substances was determined by measuring the absorbance at 532 nm. AOA of chelators was calculated with Eqn 3:

$$AOA = \frac{A_{ch} - A_o}{A_{cont} - A_o} \quad (3)$$

Here, A_{ch} is an absorbance at 532 nm in the presence of a chelator, A_{cont} is a control value (without a chelator), and A_o is an absorbance of supernatant sample stored without any treatment.

(b) Fluorescence spectrophotometric technique [9]. A 10% homogenate solution in 0.9% NaCl was incubated with and without chelator (70 μM) at 37° and continuously mixed for 2 hr. Then, 7% SDS solution (0.2 mL), 0.1 N HCl (2 mL), 10% phosphotungstic acid (0.3 mL), and twice distilled water (0.4 mL) were added to the incubation mixture (0.1 mL) at room temperature. After 5 min, 0.67% 2-TBA solution [solved in acetic acid (1:1), 1 mL] was added, and the mixture was heated at 90° for 45 min. After cooling, butanol (5 mL) was added and after centrifugation the content of TBA reactive substances was determined on a Perkin Elmer Fluorimeter (515 nm for excitation and 553 nm for emission). AOA was calculated as described above.

CL measurements

(a) The Fenton reaction. Luminol (50 μM), hydrogen peroxide (1.3 mM), and a chelator (0–70 μM) were mixed in Tris–HCl buffer (pH 7.4) in the 1-mL cell of a luminometer. CL was registered continuously, after starting the reaction by the addition of $FeSO_4$ (10 μM).

(b) Autoxidation of ferrous ions in solution. Luminol (200 μM) or lucigenin (80 μM) were added to 10 mM Tris–HCl buffer (pH 7.4) containing $FeSO_4$ (50 μM) and a chelator (200 μM) at 25°, and CL was registered continuously.

Determination of endogenous free iron in rat brain homogenates by the ESR method

The content of free iron ions in brain homogenate was determined by measuring the amplitude of an ESR signal of the Fe^{3+}–nitroxide complex with the g factor of 2.03 [10]. The samples of brain homogenate (1.0 mL) and a chelator in 100 μM Tris–HCl buffer (0.5 mL) were mixed at 25°. Then reaction mixture (0.9 mL) was incubated with 40% $NaNO_2$ solution (0.1 mL) for 10 min and frozen at the temperature of liquid nitrogen, and ESR spectra were recorded.

Determination of ferrous ions in solution

Fe^{2+} concentration in solution was determined by a phenanthroline method as described earlier [11]. Optical spectra were recorded on a Beckman DU-7 spectrophotometer, and fluorescence spectra were recorded on a Perkin Elmer fluorimeter. CL was measured on a LKB 1251 luminometer (Wallach Oy, Finland) and ESR spectra were recorded on a Varian E-4 ESR spectrometer.

RESULTS

The addition of ferrous ions to hydrogen peroxide in Tris–HCl buffer almost immediately induced luminol-dependent CL (Fig. 1A), which was effectively inhibited by rutin, phenanthroline, desferrioxamine and EDTA (Fig. 2) (I_{50} values equal to 6, 8, 12 and 23 μM, respectively). Contrary to that, ADP only slightly inhibited CL at low concentrations (up to 20 μM) and practically did not affect it at concentrations greater than 60–70 μM.

The formation of TBA reactive substances during the autoxidation of brain homogenates was measured

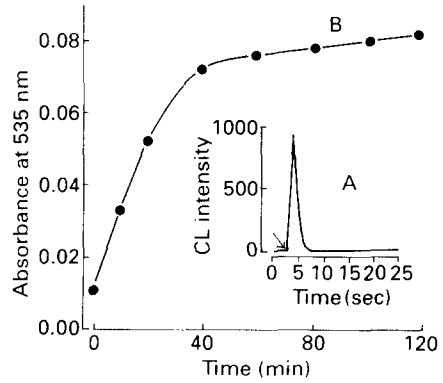

Fig. 1. (A) Luminol-amplified CL in the Fenton reaction. Luminol (50 μM) and hydrogen peroxide (1.3 mM) were mixed in Tris–HCl buffer (pH 7.4), and the reaction was started by adding $FeSO_4$ (10 μM). (B) Kinetics of the formation of TBA reactive substances in the peroxidation of rat brain homogenate. A 10% supernatant sample of brain homogenate in Tris–HCl buffer (6 mL) was incubated at 37° and continuously mixed. The reaction was terminated by the addition of 1.5% H_2PO_4 and EDTA, and TBA reactive substances were measured.

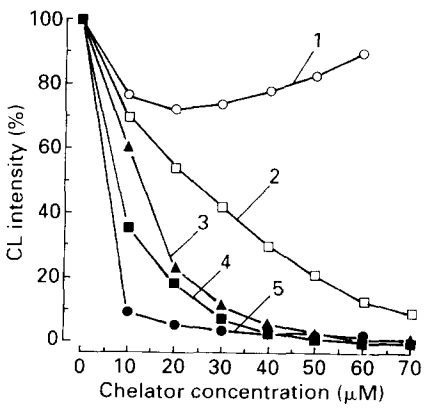

Fig. 2. The effects of chelators on luminol-amplified CL in the Fenton reaction. Experimental conditions are the same as in Fig. 1A. CL intensity without chelators is taken as 100%. Each point is an average of four experimental results, SD ± 2%. 1, ADP; 2, EDTA; 3, desferrioxamine; 4, phenanthroline; 5, rutin.

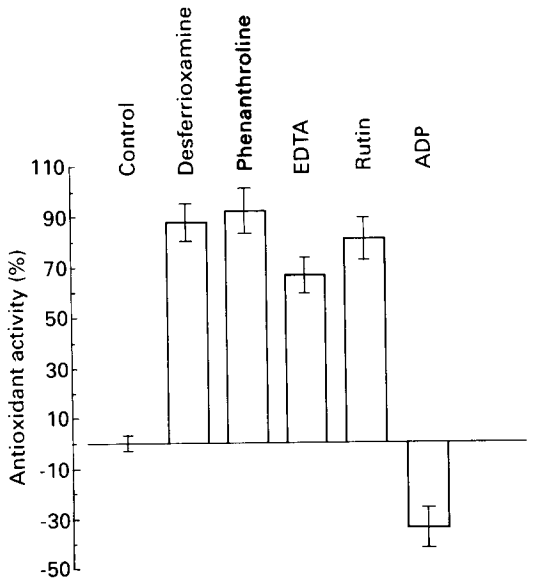

Fig. 3. Average values of AOA of chelators (N = 9, ± SD) in lipid peroxidation of rat brain homogenates.

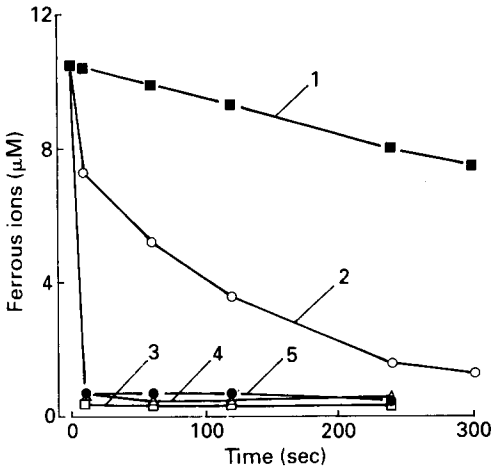

Fig. 4. Kinetics of ferrous ion autoxidation in solution in the presence and absence of chelators. $FeSO_4$ (200 μM) and a chelator (70 μM) were incubated in Tris–HCl buffer (pH 7.4) and then *o*-phenanthroline (1 mM) was added. The content of Fe^{2+}–phenanthroline complex was determined spectrophotometrically [11]. 1, Without chelators; 2, with ADP; 3, with desferrioxamine; 4, with rutin; 5, with EDTA.

spectrophotometrically as described by Stocks *et al.* [8] (Fig. 1B). Used at concentrations which completely inhibit luminol-dependent CL in the Fenton reaction (70 μM), rutin, phenanthroline and desferrioxamine effectively inhibited lipid peroxidation of brain homogenates (values of AOA were about 75–85%); a value of AOA for EDTA was slightly smaller (about 65%) (Fig. 3). Again, the effect of ADP was quite different: it caused lipid peroxidation (the AOA was −35%). Similar results were obtained when the reaction was carried out in accord with the experimental technique described by Niwa *et al.* [9]. The values of AOAs for rutin, *o*-phenanthroline, desferrioxamine and EDTA were 65 ± 10% (data not shown).

Ferrous ions were slowly oxidized at pH 7.4 in Tris–HCl buffer (Fig. 4, Curve 1). The addition of all chelators sharply increased the rate of autoxidation: rutin, EDTA and desferrioxamine immediately oxidized ferrous ions into ferric ions (Fig. 4, curves 3, 4 and 5), while ADP gave a smaller effect (Fig. 4, curve 2). Autoxidation of ferrous ions in the presence of desferrioxamine and EDTA was accompanied by luminol-amplified CL, but no CL was observed in the presence of rutin, ADP and phenanthroline (Fig. 5). (Desferrioxamine and rutin also induced a very weak lucigenin-dependent CL, data not shown.) Desferrioxamine, phenanthroline, rutin and EDTA also sharply decreased (by 70–85%) the level of "free" ferrous ions in brain homogenates (Fig. 6), while ADP did not change or even increase it. (The content of ferrous ions in the presence of ADP was 120 ± 20.)

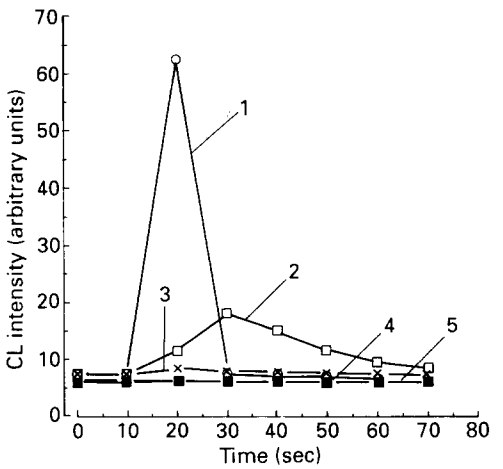

Fig. 5. Stimulation by chelators of luminol-amplified CL. $FeSO_4$ (50 μM) was added to the solution of luminol (100 μM) and a chelator (200 μM) in 10 mM Tris–HCl buffer and CL was registered continuously.

DISCUSSION

Of all the chelators used only the effects of EDTA and ADP on free radical production in the *in vitro* systems have been previously studied. EDTA was found to be the most active catalyst of hydroxyl radical production from H_2O_2 decomposition (the Fenton reaction, Eqn 1) [12, 13]. ADP appears also to be able to catalyse the Fenton reaction, although possessing a smaller catalytic activity [14]. Catalytic effects of EDTA and ADP might be explained by a decrease in the reduction potential of the Fe^{3+}/Fe^{2+} couple or by lessening the rate of oxidation of active ferrous ions into inactive ferric ions [14].

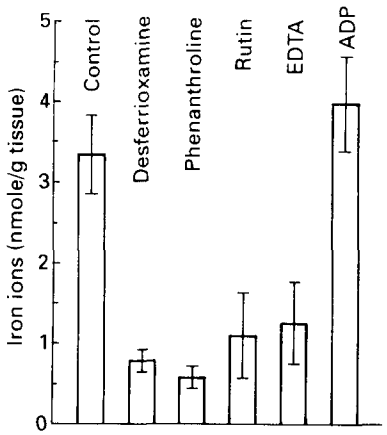

Fig. 6. Average concentrations (N = 4, ± SD) of endogenous ferrous ions in rat brain homogenates in the absence and presence of chelators. The samples of brain homogenate (1 mL) and a chelator [100 μM in Tris–HCl buffer (0.5 mL)] were mixed at 25°. Then the incubation mixture (0.9 mL) was mixed with 40% $NaNO_2$ solution (0.1 mL), and the ESR spectrum of Fe^{3+}-nitroxide was recorded at liquid nitrogen temperature.

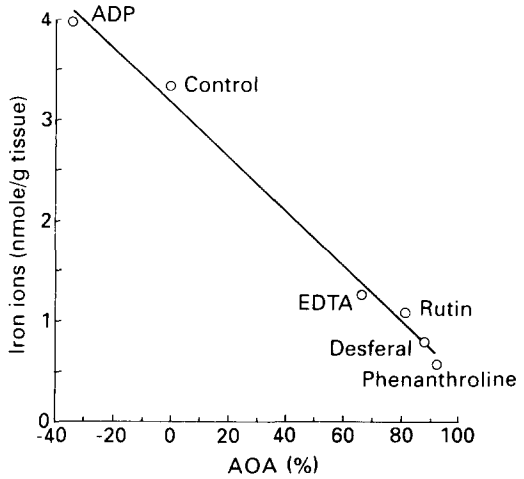

Fig. 7. Correlation between the ferrous ion content in rat brain homogenate in the presence of chelators and the values for the AOAs of chelators.

In contrast, ADP and EDTA usually exhibit opposite effects in lipid peroxidation: ADP enhances the rate of lipid peroxidation, while EDTA inhibits [15]. From these observations, some authors concluded that the formation of hydroxyl radicals is of small importance for the initiation of lipid peroxidation [16–18]. However, there are exceptions; thus, EDTA accelerated the rate of linoleate peroxidation [19] and exhibited a biphasic effect on the peroxidation of brain microsomes, enhancing the peroxidation rate at $Fe^{2+}/EDTA > 1$ and diminishing it at $Fe^{2+}/EDTA < 1$ [20].

Our data suggest a new mechanism for the inhibitory action of chelators in free radical processes. As is seen from Fig. 4, EDTA, desferrioxamine and rutin almost immediately oxidized Fe^{2+} ions, while ADP exhibited a significantly smaller effect. This suggests that the acceleration of Fe^{2+} oxidation inside a Fe^{2+}–chelator complex is a major cause of the inhibitory effects of chelators on the processes catalysed by ferrous ions. Indeed, the chelators, which induced rapid oxidation of ferrous ions in solution (for example, rutin and desferrioxamine), also exhibited the strongest inhibitory effects on the generation of active oxygen species capable of stimulating luminol-dependent CL in the Fenton reaction (Fig. 2). On the other hand, ADP has the weakest effects on both the oxidation of ferrous ions and luminol-dependent CL in the Fenton reaction.

In some ways EDTA is an exception to this rule since it rapidly oxidized Fe^{2+} ions but was a less effective inhibitor of luminol-dependent CL in the Fenton reaction than rutin, desferrioxamine or *o*-phenanthroline (Fig. 2). This can possibly be explained by a strong catalytic effect of the Fe^{2+}–EDTA complex on hydroxyl radical production in this reaction [12, 13]. Thus, the total effect of chelators apparently depends on competition between the Fenton reaction (Eqn 5) catalysed by an Fe^{2+}–chelator complex and the autoxidation of this complex (Eqn 6):

$$Fe^{2+} + \text{chelator} \leftrightarrows Fe^{2+}\text{–chelator} \quad (4)$$

$$Fe^{2+}\text{–chelator} + H_2O_2 \rightarrow Fe^{3+}\text{–chelator} + HO^{\cdot} + HO^{-} \quad (5)$$

$$Fe^{2+}\text{–chelator} + O_2 \rightarrow Fe^{3+}\text{–chelator} + O_2^{\cdot-}. \quad (6)$$

As the complex formation of ferrous ions by EDTA increases the rate of the Fenton reaction by approx. 1000 times (Ref. 15, p. 201), EDTA seems to be the only chelator for which the rate of reaction (Eqn 5) becomes comparable with that of reaction 6. In addition, there may be other pathways, by which chelators inhibit oxygen radical formation in the Fenton reaction. One of them is that chelators also scavenge superoxide ion participating in the redox cycling of Fe^{3+}/Fe^{2+} pair. Indeed, we have shown earlier [21, 22] that rutin and the Fe^{3+}–rutin complex are able to react with superoxide ion. However, in our case this mechanism seems to be unimportant because the ability of the chelators studied to catalyse ferrous ion oxidation corresponded well with their ability to suppress oxygen radical production by the Fenton reaction.

We suggest that the oxidative mechanism of the inhibitory effects of chelators also occurs in the peroxidation of brain homogenates. It can be seen (Fig. 6) that the addition of rutin, desferrioxamine, phenanthroline and EDTA to brain homogenates induced rapid oxidation of "free" ferrous ions, while ADP was inactive. The same tendency was observed for the inhibitory effects of chelators on brain homogenate peroxidation where ADP even acquired prooxidant activity (Fig. 3). Consequently, a good correlation was observed between the efficiency of Fe^{2+} oxidation in brain homogenates and the values of AOAs for the chelators studied (Fig. 7). We also used another and possibly a more sensitive experimental technique [9] to check the values of

AOA of chelators using the method developed by Stocks *et al.* [8]. There was no significant difference in the results obtained.

Reaction 6 indicates that superoxide may be formed during the oxidation of Fe^{2+}–chelator complexes. As mentioned above, superoxide ions may participate in redox cycling of Fe^{3+}/Fe^{2+} pair and some other damaging free radical processes. To evaluate these potentially toxic effects, we measured the production of active oxygen species during the autoxidation of ferrous ions at physiological pH in the presence of chelators (Fig. 5). It is seen that desferrioxamine and to a lesser extent EDTA stimulated luminol-amplified CL, while rutin, phenanthroline and ADP were inactive. Similar but smaller CL responses were observed when lucigenin was used instead of luminol (data not shown). Both luminol- and lucigenin-dependent CL responses were completely inhibited by superoxide dismutase (SOD), confirming the formation of superoxide ions during the oxidation of ferrous ion–chelator complexes. Thus, the known toxic effects of EDTA and desferrioxamine could be, at least partially, explained by the generation of superoxide ions in the oxidation of their ferrous complexes.

Our data suggest that the therapeutic effects of such chelating drugs as desferrioxamine, rutin and EDTA may be a consequence of their ability to oxidize ferrous ions in the Fe^{2+}–chelator complexes. In this way the chelators may inhibit ferrous ion-catalysed free radical processes and suppress the initiation of free radical pathologies. Our findings may explain the benefits of long-term administration of the bioflavonoid rutin for the treatment of such pathologies as Fanconi anemia, β-thalassemia and Diamond-Blackfan anemia [2, 3, 7].

REFERENCES

1. Cranton EM and Frackelton JP, Free radical pathology in age associated diseases: treatment with EDTA chelation, nutrition and antioxidants. *J Holistic Med* **6**: 6–37, 1984.
2. Korkina LG, Samochatova EV, Maschan AA, Suslova TB, Cheremisina ZP and Afanas'ev IB, Release of active oxygen radicals by leukocytes of Fanconi anemia patients. *J Leukocyte Biol* **52**: 357–362, 1992.
3. Korkina LG, Samochatova EV, Maschan AA, Suslova TB, Cheremisina ZP and Afanas'ev IB, The use of rutin for treatment of Fanconi anemia patients. *Drugs Today* **28** (Suppl A): 165–169, 1992.
4. Callender ST and Weatherall DJ, Iron chelation with oral desferrioxamine. *Lancet* **2**: 689, 1980.
5. Schafer AI, Rabinowe S and Le Boff MS, Long-term efficacy of deferriosamine iron chelation in adults with acquired transfusional iron overload. *Arch Intern Med* **145**: 1217–1221, 1985.
6. Kontoghiorghes GJ, Aldouri MA, Hoffbrand AV, Barr J, Wonke B, Kourouclaris Th and Sheppard L, Effective chelation of iron in β thalassemia with the oral chelator 1,2-dimethyl-3-hydroxypyrid-4-one. *Br Med J* **295**: 1509–1512, 1987.
7. Korkina LG, Afanas'ev IB, Deeva IB, Suslova TB, Cheremisina ZP, Maschan AA, Samochatova EV and Rumyanzev AG, Free radical status of blood of the patients with iron overload: the effect of chelating treatment. *Drugs Today* **28** (Suppl A): 137–141, 1992.
8. Stocks J, Gutteridge JMC, Sharp RI and Dormandy TL, Assay using brain homogenate for measuring the antioxidant activity of biological fluids. *Clin Sci Mol Med* **47**: 215–222, 1974.
9. Niwa Y, Kanoh T, Sakane T, Soh H, Kawai S and Miyachi Y, The ratio of lipid peroxides to SOD activity in the skin lesions of patients with severe skin diseases: an accurate prognostic indicator. *Life Sci* **40**: 921–927, 1987.
10. Tarasova NI, Kovalenko OA and Vanin AF, Mechanism of iron incorporation in living cells. *Biofizika* **26**: 678–683, 1981.
11. Kozlov AV, Egorov DYu, Vladimirov YuA and Azizova OA, The formation of iron–ascorbic acid complexes under *in vitro* physiological conditions and in tissue. *Biofizika* **35**: 513–517, 1990.
12. Morehouse LA, Bucher JR, Tien M and Aust SD, The promotion of "Fenton's chemistry" by EDTA-Iron. In: *Oxy Radicals and their Scavenger Systems* (Eds. Cohen G and Greenwald RA), Vol. I, pp. 288–291. Elsevier, Amsterdam, 1983.
13. Winterbourn CC, Sutton HC and Vile G, Catalysts of the Haber–Weiss reaction. Studies with enzymatically and radiolytically generated superoxide. In: *Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine* (Ed. Rotilio G), pp. 9–15. Elsevier, Amsterdam, 1986.
14. Floyd RA, Direct demonstration that ferrous ion complexes of di- and triphosphate nucleotides catalyze hydroxyl free radical formation from hydrogen peroxide. *Arch Biochem Biophys* **225**: 263–270, 1983.
15. Afanas'ev IB, The interaction of oxygen radicals with natural and biologically active compounds, cellular components and cells. In: *Superoxide Ion: Chemistry and Biological Implications, Vol. 2*, p. 149. CRC Press, Boca Raton, FL, 1991.
16. Bast A and Steeghs MHM, Hydroxyl radicals in NADPH dependent microsomal liquid peroxidation. *Experientia* **42**: 555–556, 1986.
17. Vile GF and Winterbourn CC, Iron binding to microsomes and liposomes in relation to lipid peroxidation. *FEBS Lett* **215**: 151–154, 1987.
18. Puntarulo S and Cederbaum AI, Comparison of the ability of ferric complexes to catalyze microsomal chemiluminescence, lipid peroxidation, and hydroxyl radical generation. *Arch Biochem Biophys* **264**: 482–487, 1988.
19. Bucher JR, Tien M, Morehouse LA and Aust SD, Influence of superoxide dismutase and catalase on strong oxidant formation during autoxidation of ferrous chelates. In: *Oxy Radicals and their Scavenger Systems* (Eds. Cohen G and Greenwald RA), Vol. I, pp. 292–295, Elsevier, Amsterdam, 1983.
20. Marton A, Sukosd-Rozlosnik N, Vertes A and Horvath I, The effect of EDTA–Fe(III) complexes with different chemical structure on the lipid peroxidation in brain microsomes. *Biochem Biophys Res Commun* **145**: 211–217, 1987.
21. Afanas'ev IB, Dorozhko AI, Brodskii AVC, Kostyuk VA and Potapovitch AI, Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem Pharmacol* **38**: 1763–1769, 1989.
22. Afanas'ev IB, Dorozhko AI, Polozova NI, Kuprianova NS, Brodskii AV, Ostrachovitch EA and Korkina LG, Is superoxide an initiator of microsomal lipid peroxidation? *Arch Biochem Biophys* **302**: 200–205, 1993.