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The Mechanism of Iron (III) Stimulation of Lipid Peroxidation

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A study conducted on Fe^{2+} autoxidation showed that its rate was extremely slow at acidic pH values and increased by increasing the pH; it was stimulated by Fe^{3+} addition but the stimulation did not present a maximum at a Fe^{2+}/Fe^{3+} ratio approaching 1:1. The species generated during Fe^{3+}-catalyzed Fe^{2+} autoxidation was able to oxidize deoxyribose; the increased Fe^{2+} oxidation observed at higher pHs was paralleled by increased deoxyribose degradation. The species generated during Fe^{3+}-catalyzed Fe^{2+} autoxidation could not initiate lipid peroxidation in phosphatidylcholine liposomes from which lipid hydroperoxides (LOOH) had been removed by treatment with triphenylphosphine. Neither Fe^{2+} oxidation nor changes in the oxidation index of the liposomes due to lipid peroxidation were observed at pHs where the Fe^{3+} effect on Fe^{2+} autoxidation and on deoxyribose degradation was evident. In our experimental system, a Fe^{2+}/Fe^{3+} ratio ranging from 1:3 to 2:1 was unable to initiate lipid peroxidation in LOOH-free phosphatidylcholine liposomes. By contrast Fe^{3+} stimulated the peroxidation of liposomes where increasing amounts of cumene hydroperoxide were incorporated. These results argue against the participation of Fe^{3+} in the initiation of LOOH-independent lipid peroxidation and suggest its possible involvement in LOOH-dependent lipid peroxidation.

Keywords: Fe(III), lipid peroxidation, phosphatidylcholine liposomes, inorganic initiator, organic initiator

Abbreviations: Cumene-OOH: cumene hydroperoxides, LOOH: lipid hydroperoxides, Mes: morpholinoethanesulphonic acid, PC: egg yolk phosphatidylcholine, TBAR: thiobarbituric acid reactive material, TPP: triphenylphosphine.

INTRODUCTION

Lipid peroxidation is generally thought to be a major mechanism of cell injury in aerobic organisms subjected to oxidative stress. It is strongly affected by transition metals, such as iron, that catalyze many of the reactions involved in this process[1–3]. In particular, a stringent requirement for Fe^{2+} in several *in vitro* lipid peroxidation systems suggests that a metal-driven reduction of oxygen is the source of some type of "initiator" able to overcome the dissociation energy of an allyl bond and to cause hydrogen abstraction and formation of a lipid alkyl radical. The series of

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reactions triggered by this inorganic "initiator" are known as Fe^{2+} catalyzed LOOH-independent initiation of lipid peroxidation.

Transition metals can substantially enhance lipid peroxidation also by the reductive cleavage of lipid hydroperoxides (LOOH) to the alkoxyl radicals; these species rearrange into epoxyallylic radicals which couple with oxygen to form the peroxyl radicals able to abstract hydrogen from lipid to form new lipid alkyl radicals[4,5]. These reactions are referred to as LOOH-dependent lipid peroxidation.

Reports by many investigators, showing that the rate and extent of lipid peroxidation depends on the simultaneous availability of Fe^{2+} and Fe^{3+}, led to the hypothesis that Fe^{3+} was necessary for the maximal rate of generation of the oxygen derived "initiator" and, thus, for the stimulation of the LOOH-independent lipid peroxidation. Goddard and Sweeney[6] proposed perferryl ion, formed during Fe^{2+} autoxidation, as the likely initiator able to abstract hydrogen from a methylene carbon of an unsaturated fatty acid. Fe^{3+} would favor the perferryl formation and the maximal rate of lipid peroxidation by inhibiting the addition of an electron to the perferryl radical by excess Fe^{2+}[7]. Other authors[8,9] attributed the enhanced initiation of lipid peroxidation caused by Fe^{3+} to the Fe^{3+} itself; the oxidized form of the metal would constitute a specific Fe^{2+}-Fe^{3+}-O_2 complex active in initiation. This hypothesis was further supported by the results obtained in many other experimental systems[10–13]: invariably a Fe^{2+}/Fe^{3+} ratio of about 1:1 was reported to elicit an optimal initial rate of lipid peroxidation. Although the body of evidences reiterating Fe^{3+} stimulation of lipid peroxidation is constantly increasing, the mechanism of Fe^{3+} effect remains a matter of controversy[14,15]. In the present report we studied the effect of Fe^{3+} on different reactions that require Fe^{2+} and are involved, either directly or indirectly, in lipid peroxidation. In particular, in this report we investigated: a) Fe^{2+} autoxidation; b) generation of oxidizing species during Fe^{2+} autoxidation; c) Fe^{2+}-catalyzed LOOH-independent initiation of lipid peroxidation; d) reductive cleavage of cumene hydroperoxide by Fe^{2+}; e) Fe^{2+}-catalyzed cumene hydroperoxide-dependent initiation of lipid peroxidation.

MATERIALS AND METHODS

Chemicals

Morpholineethanesulphonic acid (Mes), triphenylphosphine (TPP), 2-deoxyribose and all other chemicals, of the highest grade available, were purchased from Sigma Chemical Co. (St. Louis, MO. USA). Egg phosphatidylcholine (PC) was from Lipid Products (Redhill, U.K.) and 1,10-phenanthroline was from Merck (Darmstadt, Germany). All reagents were prepared in Chelex resin-treated distilled water.

Fe^{2+} Determination

Measurement of Fe^{2+} concentration was made by the o-phenanthroline method according to Mahler and Elowe[16]. All incubations were carried out in 1 ml 5 mM Good type buffers. The concentrations of the components of the reaction mixtures and the incubation conditions are given in the table and figure legends. The reactions initiated by $FeCl_2$ addition, were incubated at room temperature. At the time stated, the reactions were stopped by addition of 0.2 ml 25 mM 1,10 phenanthroline and A_{515} was immediately read.

Deoxyribose Degradation

Damage to deoxyribose was detected by the thiobarbituric acid reactive material (TBAR) generated[17]. The reactions were set up as described above for Fe^{2+} determination except that 2.8 mM deoxyribose was present in the assay. At the time stated, the reactions were stopped by the addition of 1.5 ml 1% thiobarbituric acid containing 10 μL of 2% butylated hydroxytoluene (BHT) and of 1.5 ml 20% acetic acid/Na acetate, pH 3.5. The $FeCl_3$ concentration of both samples and controls, set by stopping the reactions at zero time, was brought to

0.2 mM (final concentration in the original assay) by the addition of suitable amounts of $FeCl_3$. The tubes were heated for 10 min at 100°C to develop the color. When cool, the tube contents were extracted with 4 ml butan-1-ol. Phases were separated by centrifugation at 3000 rpm for 10 min and the upper organic layer, containing the chromogen, was measured at 532 nm relative to appropriate blanks.

Liposome Preparation

The standard vortex mixed egg yolk phosphatidylcholine liposomes containing triphenylphosphine (PC-TPP) were prepared by minor modifications of a previously described method[18]. The phospholipids (about 18 mg), added with TPP in chloroform (0.6 μMoles)[19] were dried under nitrogen, added with 6 ml distilled water, vortex mixed for 10 min and stored at 4°C for 1 h. Similar procedures were used to prepare the liposomes containing cumene hydroperoxide (cumene-OOH). The phospholipid content of the liposome suspensions was determined by the method of Marinetti[20].

Oxidation Index

Lipid peroxidation was measured by determining the oxidation index of the liposomes[21]. At the time stated, the reaction mixtures containing 150 μg phospholipid were extracted with 1 ml butan-1-ol. Phases were separated by centrifugation at 3000 rpm for 10 min and the 200–300 nm ultraviolet spectrum of the upper organic phase was recorded against appropriate blanks containing all reagents but liposomes. The oxidation index (A_{233nm}/A_{215nm}) was determined.

LOOH Determination

The LOOH content of the liposomes was determined with the thiocyanate method[22] as described by Cavallini *et al.*[23]. The liposomes (150 μg phospholipid) were dissolved in 3 ml of glacial acetic acid/chloroform (3:2 v/v); 5μl of 3.6% $FeSO_4$ (in 3.6% HCl) were added followed after 30s by 250 μl of 20% KSCN. Determination of lipid hydroperoxide content was made by using a calibration curve, obtained under the same conditions with cumene hydroperoxide as a standard.

RESULTS

$FeCl_2$ 100 μM incubated in 5 mM Good type buffer autoxidizes; the rate of Fe^{2+} autoxidation increases by increasing the pH (Figure 1A). When the reactions are conducted in the presence of 25 μM $FeCl_3$, the rate of Fe^{2+} autoxidation is significantly increased (Figure 1B). The rate of autoxidation of $FeCl_2$ 100 μM at pH 6.1, 6.5 and 6.8 depends on the Fe^{2+}/Fe^{3+} ratio (Figure 2).

When the autoxidation of $FeCl_2$ 100 μM in Mes buffer at pH 6.1, 6.5 and 6.8 was conducted for 45 min in the presence of deoxyribose 2.8 mM, no generation of TBAR was observed. However, when Fe^{2+} autoxidation was stimulated by $FeCl_3$ 100 μM, some deoxyribose was oxidized; the amount of TBAR detected, although very low, was higher at higher pH values (Table I).

Vortex mixed PC-TPP liposomes were prepared in the presence of TPP 100 μM to reduce to LOH the LOOH present in the liposomes. No LOOH were detected in this liposome preparation. The incubation of PC-TPP liposomes with $FeCl_2$ 100 μM in the absence and presence of $FeCl_3$ 100 μM does not result in any Fe^{2+} oxidation (Figure 3) besides the spontaneous or Fe^{3+} stimulated ones due to Fe^{2+} autoxidation in Mes buffer, pH 6.5. When the liposomes were incubated with increasing $FeCl_2$ concentrations in 5 mM Mes buffer pH 6.8 in the presence or absence of $FeCl_3$ 75 μM, neither Fe^{2+} oxidation by the liposomes (Figure 4A) nor alteration of the oxidation index of the liposomes (Figure 4B) were observed at all $FeCl_2$ concentrations tested.

PC-TPP liposomes prepared in the presence of increasing concentrations of cumene-OOH contained detectable amounts of hydroperoxides

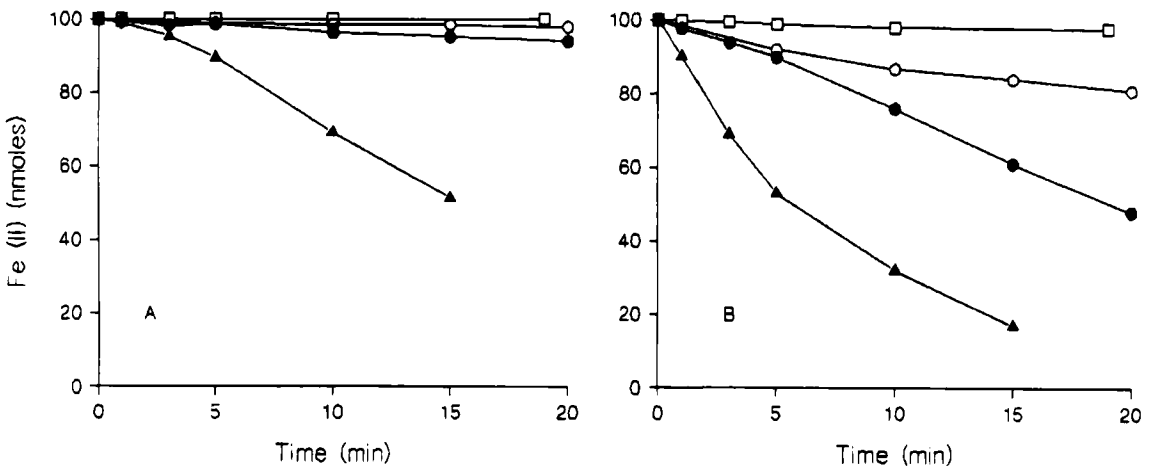

FIGURE 1 Effect of pH on the time course of Fe^{2+} autoxidation. The disappearance of 100 μM Fe^{2+} from 5 mM Mes buffer, pH 6.1 (□), 6.5 (○), 6.8 (●) and from Mops buffer, 7.2 (▲) in the absence (panel A) and presence (panel B) of 25 μM $FeCl_3$ was measured.

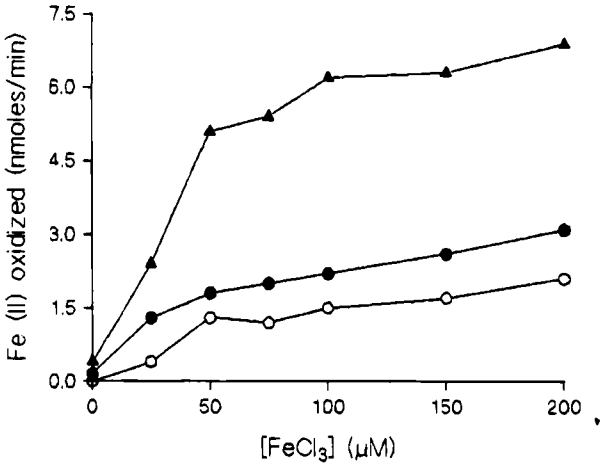

FIGURE 2 Effect of the Fe^{2+}/Fe^{3+} ratio on the rate of Fe^{2+} autoxidation. $FeCl_2$ (100 μM) was incubated with increasing concentrations of $FeCl_3$ in 5 mM Mes buffer, pH 6.1 (○), 6.5 (●) and 6.8 (▲). The Fe^{2+} oxidized after 3 min incubation was measured.

TABLE I Deoxyribose degradation by Fe^{2+}Autoxidation at Different pHs

Assay Conditions	Fe^{2+} oxidized (nmol)	TBAR generated (nmol)
pH 6.1 standard assay	2 ± 0.2	n.d.
+ $FeCl_3$ 100 μM	26 ± 0.3	0.4 ± 0.2
pH 6.5 standard assay	4 ± 0.2	n.d.
+ $FeCl_3$ 100 μM	47 ± 0.4	1.4 ± 0.2
pH 6.8 standard assay	7 ± 0.3	n.d.
+ $FeCl_3$ 100 μM	65 ± 0.4	2.8 ± 0.3

Reaction mixtures for Fe^{2+} oxidation assay contained 100 μM $FeCl_2$ in 5 mM Mes buffer; reaction mixtures for deoxyribose degradation assay contained also 2.8 mM deoxyribose. Incubations were at 25°C for 45 min in the absence or presence of $FeCl_3$ 100 μM. Data represent mean ± S.E. for three determinations in triplicate. n.d., none detected.

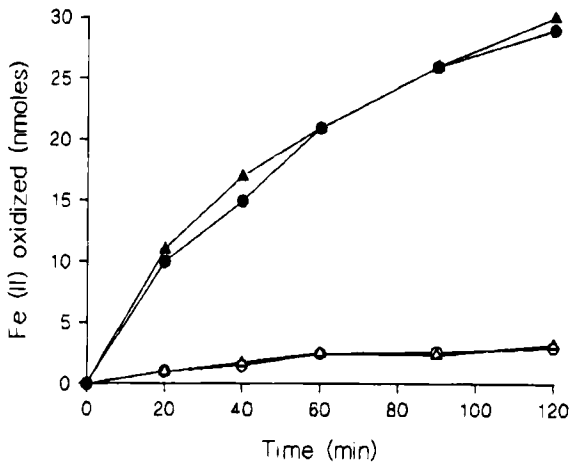

FIGURE 3 Effect of PC-TPP liposomes on the time course of Fe^{2+} oxidation. PC-TPP liposomes (150 µg phospholipid, 5 nmol TPP) were incubated in 5 mM Mes buffer, pH 6.5, with $FeCl_2$ 100 µM in the absence (○) and presence (●) of $FeCl_3$ 100 µM; control assays, without liposomes, containing $FeCl_2$ 100 µM in the absence (△) and presence (▲) of $FeCl_3$ 100 µM were also run. The Fe^{2+} oxidized was measured.

(Figure 5A). When these liposomes were incubated with increasing concentrations of $FeCl_2$, the metal was oxidized and the extent of oxidation correlates positively with the hydroperoxide content (Figure 5B). The maximal amount of Fe^{2+} oxidized exceeded the amount of hydroperoxides present in the liposomes. PC liposomes prepared in the presence of TPP 100 µM, cumene-OOH 250 µM and incubated in Mes buffer, pH 6.1, in the presence of increasing $FeCl_2$ concentrations, show a stimulation of Fe^{2+} oxidation and an increase of the oxidation index by $FeCl_3$ addition (Figures 4A, 4B).

By contrast, cumene-OOH (50 µM) oxidizes a stoichiometric amount of Fe^{2+} when incubated with 100 µM $FeCl_2$ in 5 mM Mes buffer, pH 6.1 and the reaction is not affected by the addition of $FeCl_3$ 50 µM (results not shown).

DISCUSSION

The results obtained in our experimental system, namely a) the rate of Fe^{2+} autoxidation in Good type buffers is extremely slow at acidic pH values (Figure 1A); b) it increases by increasing the pH (Figure 1A); c) it is stimulated by Fe^{3+} addition (Figure 1B) and d) the stimulation is not maximal at a Fe^{2+}/Fe^{3+} ratio approaching 1:1 (Figure 2), are consistent with the mechanism of Fe^{2+} autox-

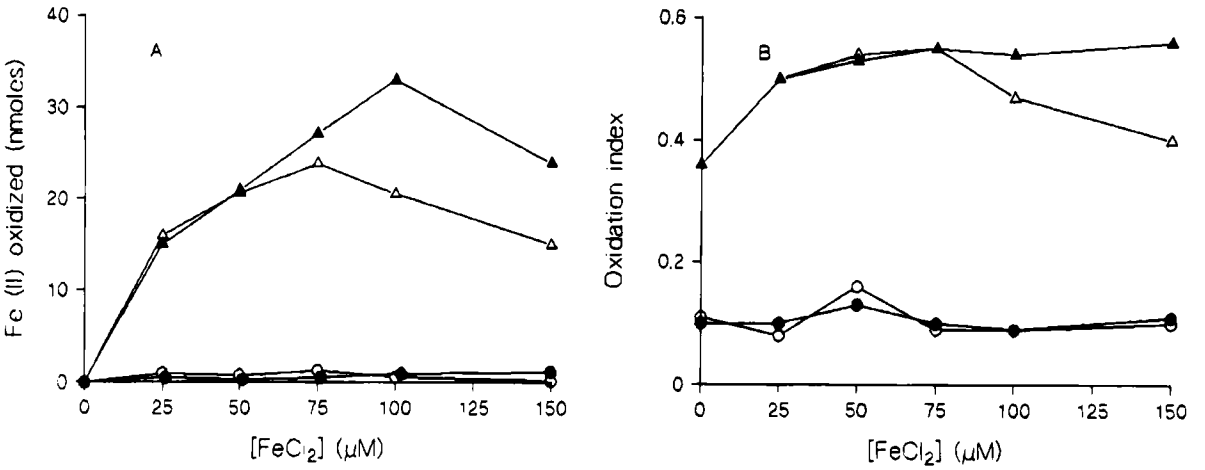

FIGURE 4 Effect of $FeCl_3$ on the peroxidation of PC-TPP and PC-TPP-Cumene-OOH liposomes. Increasing concentrations of $FeCl_2$ were incubated with PC-TPP liposomes (150 µg phospholipid, 5 nmol TPP) in 5 mM Mes buffer, pH 6.8 (circle) and with PC-TPP-Cumene-OOH liposomes (150 µg phospholipid, 5 nmol TPP, 12.5 nmol Cumene-OOH) in 5 mM Mes buffer, pH 6.1 (triangle) in the absence (open symbols) and presence (closed symbols) of $FeCl_3$ 100 µM. The Fe^{2+} oxidized by the liposomes after 10 min incubation (panel A) and the oxidation index of the liposomes (panel B) were measured.

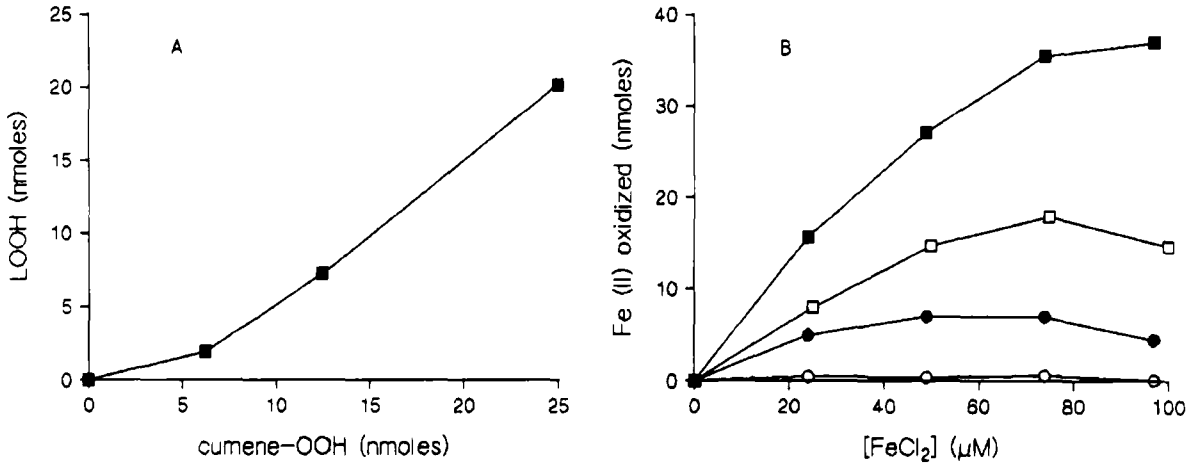

FIGURE 5 Effect of Cumene-OOH in PC-TPP liposomes on lipid peroxidation. *Panel A.* PC-TPP liposomes were prepared in the presence of increasing amounts of Cumene-OOH. The LOOH content of the liposomes was determined. *Panel B.* PC-TTP (150 μg phospholipid, 5 nmol TPP) liposomes containing 0 (○), 2 (●), 7 (□) and 20 (■) nmol LOOH were incubated with increasing concentrations of $FeCl_2$ in 5 mM Mes buffer, pH 6.5. The Fe^{2+} oxidized after 10 min incubation was measured.

idation originally proposed by Weiss[24] and Hochstein *et al.*[25] who suggested the formation of perferryl ion. The species generated during Fe^{3+}-catalyzed Fe^{2+} autoxidation was able, in our experimental conditions, to oxidize deoxyribose to TBAR (Table I); the increased Fe^{2+} oxidation observed at higher pHs was paralleled by increased TBAR generation. However, the capacity of the oxidant species to degrade deoxyribose was modest as detectable amounts of TBAR were observed only when a large amount of Fe^{2+} was oxidized (Table I). Besides, the species generated during Fe^{3+}-catalyzed Fe^{2+} autoxidation at pH 6.5 and 6.8 could not initiate lipid peroxidation in PC liposomes from which LOOH had been removed by treatment with TPP (Figures 3, 4). These observations contrast with the suggestion that a perferryl species, formed during Fe^{2+} autoxidation, is responsible for initiation; it supports the opinion expressed by Koppenol[26] who, on thermodynamic grounds, predicted a poor capacity of this ion to abstract the allyl hydrogen from a methylene carbon.

In our experimental system, a Fe^{2+}/Fe^{3+} ratio ranging from 1:3 to 2:1 was unable to initiate lipid peroxidation in PC-TPP liposomes. In fact, neither Fe^{2+} oxidation, due to lipid peroxidation, nor increase in the oxidation index of the liposomes were observed at all Fe^{2+}/Fe^{3+} ratio tested (Figure 4). Our results, thus, do not validate the hypothesis that an iron/oxygen complex comprised of both Fe^{2+} and Fe^{3+} might initiate lipid peroxidation.

PC-TPP liposomes containing detectable amounts of cumene-OOH oxidize an amount of Fe^{2+} exceeding that expected by the Fenton-like reaction (Fe^{2+} + cumene-OOH → Fe^{3+} + cumene-O· + OH^-); this result suggests that a Fe^{2+}-catalyzed LOOH-dependent lipid peroxidation occurs within these liposomes.

The demonstration that Fe^{3+} can stimulate the peroxidation of PC-TPP-Cumene-OOH liposomes (Figure 5), at a pH (6.1) where Fe^{2+} autoxidation is negligible, suggests that the oxidized form of the metal enhances the Fe^{2+}-catalyzed LOOH-dependent lipid peroxidation. This result might account also for the contradiction between our data and those presented by groups sustaining the hypothesis that Fe^{3+} affects the initiation of lipid peroxidation. The different results might be due to the LOOH content of the lipid substrates studied. The presence of LOOH would

not be surprising as they were reported to be present in commercial lipid preparations[27] and to be generated during the sonication of lipids containing polyunsaturated fatty acids[18,21]. Only chemical[19] or enzymatic[28] treatments of the lipids effectively remove them. As none of such treatments was reported in the investigations conducted by the authors claiming a Fe^{3+} effect on initiation, it seems likely that the discrepancies observed are due to the fact that they were not actually studying the LOOH-independent, but the LOOH-dependent lipid peroxidation.

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