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# Iron Overload and Lipid Peroxidation in Biological Systems

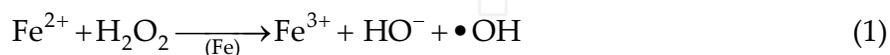
Paula M. González, Natacha E. Piloni and Susana Puntarulo

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## 1. Introduction

Fe is an essential element for the growth and well-being of almost all living organisms, except for some strains of lactobacillus, where the role of Fe may be assumed by another metal [1]. It is involved in many biological functions since by varying the ligands to which it is coordinated, Fe has access to a wide range of redox potentials and can participate in many electron transfer reactions, spanning the standard redox potential range. It is also involved in O<sub>2</sub> transport, activation, and detoxification, in N<sub>2</sub> fixation and in several of the reactions of photosynthesis [2]. However, there are problems in the physiological management of Fe, since in spite of its overall abundance, usable Fe is in short supply because at physiological pH under oxidizing conditions, Fe is extremely insoluble. Anytime Fe exceeds the metabolic needs of the cell it may form a low molecular weight pool, referred to as the labile iron pool (LIP), which catalyzed the conversion of normal by-products of cell respiration, like superoxide anion (O<sub>2</sub><sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), into highly damaging hydroxyl radical (•OH) through the Fenton reaction (reaction 1) or by the Fe<sup>2+</sup> catalyzed Haber-Weiss reaction (reaction 2), or into equally aggressive ferryl ions or oxygen-bridged Fe<sup>2+</sup>/Fe<sup>3+</sup> complexes. Fe<sup>3+</sup> can be reduced either by O<sub>2</sub><sup>-</sup> (reaction 3) or by ascorbate leading to further radical production.



Defense against the toxic effect of Fe and O<sub>2</sub> mixtures is provided by two specialized Fe-binding proteins: the extracellular transferrin (Tf) and the intracellular ferritin (Ft). Both

retain Fe in the form of  $\text{Fe}^{3+}$  which unless mobilized will not be able to efficiently catalyze the production of free radicals. Fe is stored mainly intracellularly, where its potentially damaging effects are greatest.

The marine ecosystem can be seen as an integrative system with many factors that interact with the biota. Natural variables such as temperature, winds, precipitations, tide flows, currents, human activities, affect metal deposition into the sea. Once metals become bioavailable, they can enter the food web starting with the primary producers, and also in heterotrophic organisms at the bottom of the marine food chain, such as benthic filter feeders. Metals follow a bioaccumulation process inside the animals, depending on the animal's detoxification capacities and on exogenous Fe availability.

In plants, Fe concentrations increased during seed maturation, and by immunodetection experiments it was indicated that Ft concentration of seeds also increased with maturity, containing up to 1800 atoms of Fe per molecule [3]. This seed Fe could be stored for future use during seedling growth, as has been proposed by Hyde et al. [4], avoiding toxicity. Over growth, the oxidative stress depends upon a wide array of factors related to an enhanced radical production due to several metabolic pathways activated during the initial water uptake, including mitochondrial  $\text{O}_2$  consumption. On the other hand, excess Fe effects seem to be limited mostly to the hydrophobic domain of the cell following different profile than during physiological development.

In the last decade or so, important advances have been made in the knowledge of conditions that involve Fe-overload in humans. Those conditions would include short term processes, as organ or tissue ischemia-reperfusion and local inflammation, as well as progressive pathologies essentially affecting the central nervous system. In the first case, the de-compartmentalization of Fe would lead to the expansion of the LIP and the increase of the oxidative damage. In the second case, it has been described an increase in Fe levels in the substantia nigra of Parkinsonian brains [5], Hallervorden-Spatz syndrome [6] and in mitochondria of Friedrich's ataxia cerebella [7]. Hereditary hemochromatosis is a very common genetic defect in the Caucasian population, with an autosomal recessive inheritance. It is characterized by inappropriately increased Fe absorption from the duodena and upper intestine, with consequent deposition in various parenchymal organs, notably the liver, pancreas, heart, pituitary gland and skin [8]. Fe overload is characterized by the presence of several clinical manifestations such as: increased susceptibility to infections, hepatic dysfunction, tumors, joint diseases, myocardiopathy, and endocrine alterations. Fe overload has been also observed (a) if dietary Fe is excessive, such as in the severe Bantu siderosis, reported in the Bantu tribe of Africa who drink acidic beer out of Fe pots, (b) in other inherited diseases, such as congenital atransferrinemia (lacking circulating Tf), and (c) during the medical treatment of thalassemia. Moreover, clinical and epidemiologic observations indicated that increased Fe storage status is a risk factor in several diseases such as porphyria cutanea tarda and sudden infant death syndrome, among others.

Oxidative damage to lipids had been studied over several decades, and it had been characterized in terms of the nature of the oxidant, the type of lipid, and the severity of the

oxidation. Many stable products are formed during the process and accordingly, the assays developed to assess these products to evaluate lipid peroxidation include many techniques. The most currently used assay is the determination of malondialdehyde (MDA) formation with the thiobarbituric acid reactive substances test (TBARS). However, electron paramagnetic resonance (EPR) spectroscopy has shown the capacity of detecting, in the presence of exogenous traps, the presence of the lipid radical formed during peroxidation, by yielding unique and stable products. EPR, also known as Electron Spin Resonance (ESR) is at present the only analytical approach that permits the direct detection of free radicals. This technique reports on the magnetic properties of unpaired electrons and their molecular environment [9].

This chapter will be dedicated to overview the Fe-related alterations in oxidative metabolism in photosynthetic and non-photosynthetic organisms after experimental exposure to excess Fe employing different protocols of administration. Data assessing lipid peroxidation post-treatment both, as TBARS generation and/or EPR detection of lipid radicals, are reviewed in a wide range of biological systems.

## 2. Fe overload in aquatic organisms

Fe content in the upper earth's crust is around 6% [10]. The Fe concentration in sediments influences the Fe concentration in the associated surrounding seawater. However, the concentration of dissolved Fe (defined as Fe that can diffuse through a membrane of less than 0.45  $\mu\text{m}$ ) in open-oceanic waters is extremely low ( $< 56 \text{ ng/l}$ ) [11]. Natural parameters that augment the Fe levels in coastal and central oceanic areas are: aeolian deposition of dust, river discharge, washout of dust particles in the atmosphere by rainfall, ground water discharge, glacial melting, volcanic sediments, coastal erosion and up-welling of Fe-rich deep waters over hydrothermal vents [12]. Human activities also have a great impact on Fe levels, especially around coastal areas. Chemical and mining industries, disposal of waste metal, ports, aeolian deposition of atmospheric dust from polluted areas, are some of the human activities bringing Fe and other metals to the marine ecosystem. Therefore waters from different regions may have different Fe concentrations. Fe was recognized as a bioactive element [13] and a deficiency in Fe had been suggested to limit primary productivity in some ocean regions [14,15]. Fe uptake is strictly required for phytoplankton development since the photosynthetic apparatus contains numerous loci for Fe. Moreover, it was pointed out that it is critical to avoid Fe overload in water with low organic matter content under aquarium conditions to prevent Fe-dependent toxicity [16].

Over a decade ago, Estévez et al. [17] studied the effect of *in vivo* Fe supplementation to the green algae *Chlorella vulgaris* in terms of the establishment of oxidative stress conditions. Growth under laboratory conditions increased with Fe availability up to 90  $\mu\text{M}$  with increases in biomass, suggesting that Fe supply at concentrations lower than 90  $\mu\text{M}$  could be considered limiting for algal growth. However, Kolber et al. [18] pointed out that in their field experiments in the equatorial Pacific, 2 days following Fe enrichment, photosynthetic energy conversion efficiency began to decline. It was also indicated that some algal cultures

showed deleterious effects if exceeding an Fe threshold (14-28  $\mu\text{M}$ ) in unpolluted freshwater [16]. Between 90 and 200  $\mu\text{M}$  Fe in *C. vulgaris* cultures, there was no effect on growth with increased Fe additions and further increases on Fe availability led to a drastic decrease in the growth of the cultures (Table 1). The increase of Fe at the intracellular level showed a linear dependence with the concentration of added Fe below 200  $\mu\text{M}$  Fe, however concentrations between 200 and 500  $\mu\text{M}$  Fe added to the medium led to a less active increase in intracellular Fe (Table 1), suggesting an intracellular control for Fe uptake. Thus, the data presented by Estévez et al. [17] under laboratory conditions suggested the possibility that excess Fe could be responsible for the decrease in *C. vulgaris* growth by inducing oxidative stress. Accordingly, when *C. vulgaris* cells were incubated with an EPR-spin trapping for lipid radicals ( $\alpha$ -(4-pyridyl 1-oxide)-*N*-*t*-butyl nitron, POBN), a POBN-spin adduct was observed. The spin adduct EPR spectra exhibit hyperfine splitting that were characteristic for POBN/lipid radicals,  $a_N = 15.56$  G and  $a_H = 2.79$  G, possibly generated from membrane lipids as a result of  $\beta$ -scission of lipid-alkoxyl radicals [19,20]. Quantification of lipid radical EPR signals in algal cells indicated that Fe supplementation significantly increased radical content in the membranes supplemented with the higher Fe dose, as compared to cells supplemented with 90  $\mu\text{M}$  Fe (Table 1). These results indicate that lipid peroxidation was increased by Fe availability. In this context, even though an increased content of antioxidants has been detected in *C. vulgaris* cells exposed to increased Fe, the damaging potential of Fe excess in the cell did not seem to be efficiently controlled by the activity of the antioxidants [17].

Fe added ( $\mu\text{M}$ )	Chlorophyll content ( $\mu\text{M}$ )	Intracellular Fe (nmol ( $10^7$ cell) $^{-1}$ )	Lipid radicals (pmol ( $10^7$ cell) $^{-1}$ )
0	1.2	4 $\pm$ 1	nd
50	4.5*	18 $\pm$ 2*	nd
90	7.0*	28 $\pm$ 3*	6 $\pm$ 2
200	6.8*	62 $\pm$ 3*	nd
300	6.0*	65 $\pm$ 7*	nd
500	1.2	85 $\pm$ 10*	36 $\pm$ 9**

<sup>1</sup>Taken from [17]. nd stands for not determined.

*C. vulgaris* cultures were supplemented with up to 500  $\mu\text{M}$  Fe (EDTA:Fe, 2:1). Development was followed measuring chlorophyll content and each experimental value represents chlorophyll content of the cultures after 12 days of development. Intracellular Fe content as a function of the Fe addition to the incubation medium was spectrophotometrically measured. Data are expressed as means  $\pm$  SE of 4-6 independent experiments, with two replicates in each experiment. Lipid radicals were detected and quantify by EPR.

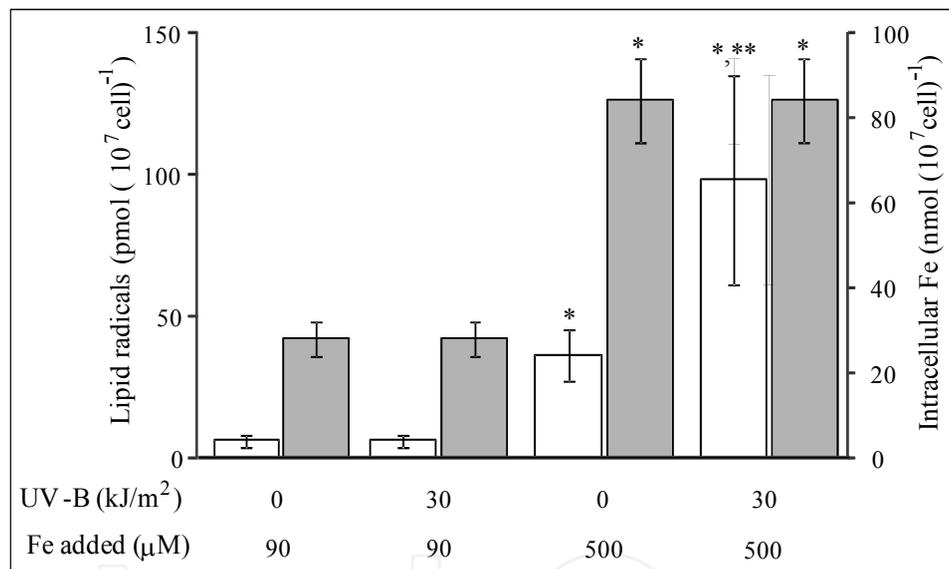
\*significantly different from value without Fe added,  $p \leq 0.05$ . ANOVA.

\*\*significantly different from value in the presence of 90  $\mu\text{M}$  Fe added,  $p \leq 0.05$ . ANOVA.

**Table 1.** Fe supplementation effect on *C. vulgaris* culture after 12 days of development<sup>1</sup>

It has been postulated that if as a result of ozone loss, UV-B flux at the surface of the earth increases, negative impacts on biological organisms will be inevitable since UV-B radiation causes a multitude of physiological and biochemical changes in photosynthetic organisms, probably related to oxidative stress [21,22]. Estévez et al. [17] exposed to 30 kJ/m<sup>2</sup> UV-B C.

*vulgaris* cells grown at up to 500  $\mu\text{M}$  Fe. They observed that either 50 or 90  $\mu\text{M}$  Fe did not alter significantly cell morphology. However, 30  $\text{kJ}/\text{m}^2$  UV-B exposure of algal cultures grown at 500  $\mu\text{M}$  Fe affected cellular internal structure and there were no signs of cellular division. Exposure of *C. vulgaris* cells to 30  $\text{kJ}/\text{m}^2$  UV-B during lag phase did not significantly affect the content of lipid radicals in log phase of development under conditions of standard supplementation of Fe (90  $\mu\text{M}$ ) (Figure 1). This parameter was significantly increased by the addition of 500  $\mu\text{M}$  Fe during development of the cultures in the absence of UV-B irradiation. Exposure of the cultures grown at 500  $\mu\text{M}$  Fe to 30  $\text{kJ}/\text{m}^2$  UV-B during log phase led to a further increase in the content of lipid radicals in the membranes. In conclusion, even though exposure of *C. vulgaris* cells to UV-B under Fe standard concentration did not lead to cellular oxidative alterations, increase in Fe availability (500  $\mu\text{M}$  Fe) was responsible for a substantial increase in lipid deterioration in the membranes by oxidative stress. These data strongly suggest that oxidative stress triggered by an excess content of Fe could affect cellular growth and have a negative biogeoimpact to phytoplankton when exposed to other environmental conditions.



Algal cultures ( $2 \times 10^5$  cells  $\text{ml}^{-1}$ ) were added with Fe starting day 0 of growth, and were assayed on day 12 (log phase). Data are expressed as means  $\pm$  SE of 4-6 independent experiments, with 2 replicates in each experiment.

\*significantly different from values for cells grown in the presence of 90  $\mu\text{M}$  Fe without exposure to UV-B,  $p \leq 0.05$ . ANOVA.

\*\*significantly different from values for cells added with 500  $\mu\text{M}$  Fe without exposure to UV-B,  $p \leq 0.05$ . ANOVA. (Statview SE+, v 1.03, Abacus Concepts Inc, Berkeley, CA).

Lipid radicals were detected and quantified by EPR and intracellular Fe by the use of acid solutions to digest the cells which were measured spectrophotometrically after reduction with thioglycolic acid followed by the addition of bathophenanthroline.

**Figure 1.** Effect of Fe addition on UV-B-dependent lipid radical ( $\square$ ) and intracellular Fe content ( $\blacksquare$ ) in algae cells. Taken from [17].

Marine animals incorporate Fe bound to inorganic particles or to organic matter during food ingestion. Further, dissolved Fe is absorbed over the respiratory surfaces and mantle tissue in filter-feeding molluscs. The extrapallial water around these tissues is constantly

exchanged with the surrounding seawater. Marine invertebrates are less tolerant of metal accumulation than vertebrates and can be affected at lower metal concentrations. Bivalves are widely used as sentinel organisms in marine pollution monitoring programs, due to their sessile and filtering habits, and their ability to bioaccumulate organic pollutants and metals in their tissues [23]. The exposure of marine molluscs to metals has been shown to induce oxidative stress through the formation of reactive O<sub>2</sub> species (ROS) and reactive nitrogen species (RNS), leading to lipid peroxidation. Bivalves have also been used as models for the study of the effect of Fe supplementation. Viarengo et al. [24] treated the mussel *Mytilus galloprovincialis* with 300-600 µg Fe/l (as FeCl<sub>3</sub>) and observed a significantly Fe accumulation in the digestive gland (DG) (190 ± 25, 394 ± 131 and 412 ± 146 µg Fe/l in 0, 300 and 600 µg Fe/l supplemented animals, respectively). The TBARS content was measured in animals treated with 600 µg Fe/l, and a significant increase was observed among control and treated mussels. Lately, Alves de Almeida et al. [25] exposed mussels from *Perna perna* species to 500 µg/l Fe (as FeSO<sub>4</sub>) and it was reported that mussels exposed to Fe for 12, 24 and 72 h presented increased phospholipid hydroperoxide glutathione peroxidase (PHGPx) activity, and no differences in MDA levels. However, at 120 h of Fe exposure both, MDA and PHGPx were significantly higher than control. Such increased MDA levels agree with previous findings by Viarengo et al. [24]. The negative correlation observed between PHGPx activity and MDA levels after Fe exposure, supports an interpretation that PHGPx protects tissues from lipid peroxidation. Thus, the exposure of mussels to Fe along with a concomitant increase in •OH formation would be involved in the modulation of PHGPx activity, however the precise mechanism remains unclear. Also, exposure of mussels to 500 µg/l of Fe caused no changes in other antioxidant enzymes such as glutathione S-transferase and glutathione peroxidase. These data suggest that PHGPx have a role in the susceptibility of DG of mussels against lipid peroxidation, and that exposure to transition metals such as Fe could lead mussels to stimulate PHGPx in order to prevent lipid peroxidation. Thus, the authors postulated that the evaluation of MDA levels in parallel with antioxidant defenses, such as PHGPx, could be considered as a potential new biomarker of toxicity associated with contaminant exposure in marine organisms.

Recently, González et al. [26] investigated the oxidative effects produced by the *in vivo* Fe exposure of the bivalve *Mya arenaria*. The soft shell clams were collected on an intertidal sand flat near Bremerhaven, Germany, and the bivalves were placed in small aquaria containing 500 µM Fe (EDTA:Fe, 2:1). Exposure to 500 µM Fe in natural seawater resulted in a significant increase in DG total Fe content (Table 2). After 2 days of exposure to Fe, TBARS content showed a significant increase by approximately 3.8-fold as compared to control values. This increase was followed by a decrease to control values at treatment day 7 and afterwards TBARS concentration increased constantly until day 17 (Table 2). The LIP in DG tissue increased on day 7 of exposure to high dissolved Fe concentration. By day 9, the LIP increase was accompanied by a significant induction of the oxidative stress signals, ROS and ascorbyl radical content and correlated with the final increase of TBARS content in tissues. Once the LIP has increased, the catalytically active Fe is able to efficiently catalyze Fenton [27,28] and Haber-Weiss reactions [29,30] and consistently and drastically accelerated

accumulation of TBARS. Contrary, oxidative stress effects measured on day 2 of treatment cannot be attributed to a significant increase of the LIP, since neither total Fe content nor the LIP were enhanced over the initial values in the 0 day exposure group. However, the H<sub>2</sub>O<sub>2</sub> scavenging antioxidant, catalase (CAT), increased after 2 days of treatment compared to controls (day 0) but the activity went back to control level on day 7 of exposure. Catalase activity was, however, increased again on day 9 of exposure compared to controls [26]. It was postulated that the initial phase of elevated oxidative stress, occurring before significant Fe accumulation could be attributed to indirect effects under the experimental exposure conditions. Metabolic rates were not measured, but it is possible that Fe exposure triggers an initial stress response including accelerated respiration as the animals are pumping to rid themselves of the inflowing Fe enriched seawater. H<sub>2</sub>O<sub>2</sub> is a good candidate for triggering cellular responses since it is a stable species [27]. H<sub>2</sub>O<sub>2</sub> diffuses freely into the tissue and leads the oxidative stress, and further increases causes oxidative damage, assessed as TBARS content. H<sub>2</sub>O<sub>2</sub> induced oxidative stress may have triggered the endogenous antioxidant system in such a manner that by day 7 of exposure to Fe excess the TBARS content was reduced to the starting values. Even though the superoxide dismutase (SOD) activity was not changed, induction of other protective mechanisms, such as metallothioneins, might act as effective transient control of heavy metal effects during the initial phase of exposure [24,25].

Time (days)	Total Fe content (ng Fe/mg FW)	TBARS (pmol/mg FW)	LIP (ng LIP/mg FW)
0	39 ± 4	57 ± 8	3.8 ± 0.4
2	48 ± 8	218 ± 14***	5.3 ± 1.3
7	42 ± 6	75 ± 13	7.2 ± 0.3*
9	66 ± 4**	157 ± 14***	14.2 ± 1.1*
17	106 ± 3**	226 ± 20***	10.4 ± 0.7*

<sup>2</sup>Taken from [26].

\*significantly different from the value at day 0 with  $p < 0.05$ ,

\*\* $p < 0.01$  and

\*\*\* $p < 0.001$ . ANOVA.

Experimental bivalves were placed in small aquaria containing 13 l (1 l/animal) of natural seawater of 23-26‰ at 10°C, and 500 μM Fe (Fe:EDTA, 1:2).

**Table 2.** Fe supplementation effect on lipid peroxidation in *Mya arenaria*<sup>2</sup>

Other studies evaluate the impact of nutritional Fe on Fe level and concentrations of MDA in tissues. Baker et al. [31] analyzed the Fe in the diet of the African catfish, *Clurims gariqinus*. This fish model is of particular relevance when considering that *C. gariqinus* is typically cultured in earth-ponds, and these may be high in dissolved Fe content. Additionally, catfish may consume mud-burrowing organisms to supplement their diet, with incidental associated silt consumption, and therefore further metal loading. After 5 weeks of feeding the animals with a diet supplemented with Fe (6354.4 mg Fe/kg), the total

Fe content was measured in muscle, liver and blood-plasma and no significant differences with control animals were found, suggesting the possibility of efficient regulation of Fe status by the fish. MDA determination in tissues revealed that there was significantly more MDA in livers and hearts of fish fed high Fe diets than in controls, and no significant difference was found in skeletal-muscle. Values of MDA concentration were higher in Fe-stressed liver tissue comparative to other tissues, possible because hepatic tissue is lipid-rich making the liver a target organ for lipid peroxidation. The relative lack of response in skeletal muscle may have resulted from decreased abundance of polyunsaturated fatty acids within this tissue, and these findings are consistent with those of Desjardins et al. [32].

All together these data show that Fe in aquatic ecosystems could be a major stressor having a main role in lipid peroxidation not only in unicellular species, such as algae, but also in higher organisms, such as invertebrates and vertebrates. These kind of analyses should be performed before consider ecological strategies which may involved Fe fertilization in seawater [33-35], to increase primary production in the oceans as an answer to global temperature increments. These actions may drastically modify marine communities in ocean layers triggering oxidative reactions, which should be properly considered due to the fact that Fe may be profitable or unfavorable, depending of its usefulness as a micronutrient or as a catalyzer of free radical reactions.

### 3. Fe overload in soybean seeds

Plants have developed several mechanisms to maintain fairly constant internal concentrations of mineral nutrients over a wide range of external concentrations. To avoid Fe-dependent oxidative cellular damage, Fe<sup>2+</sup> is either incorporated into the mineral core of Ft [36] which is located exclusively in the plastids [37] or reoxidized by O<sub>2</sub> and chelated by organic acids [38]. Bienfait et al. [39] reported that plants grown on Fe-EDTA formed a substantial pool of free space Fe in the roots and that Fe could be mobilized upon Fe-free growth in order to be transferred to the leaves. During growth in water culture at pH 5 to 6, a free space pool of 500 to 1000 nmol/g FW was formed in roots of bean grown in the presence of Fe-EDTA 20 μM and a pool of 20 to 50 nmol/g FW in roots without Fe supplementation. Like Ft in the cell, the free space Fe<sup>3+</sup> precipitate is not only an immobile result of a defensive action against an excessive Fe supply; the plant may also use it as storage form of Fe that can be mobilized [39]. Even more, Caro and Puntarulo [40] indicated that O<sub>2</sub> radical generation depends on total Fe content, however it could mostly reflect Fe content in the free space. In soybean, Fe<sup>3+</sup> reduction is an obligatory step in Fe uptake, and this is probably true for all strategy I plants [41]. Both total Fe content and the *in vitro* rate of Fe reduction were higher in roots grown in the presence of exogenously added Fe (up to 500 μM) than in roots grown in absence of supplemented Fe (Table 3). However, no visual differences (e.g. evidence of damage) between any of the roots or growth (assessed as the fresh weight of the roots, 0.21 ± 0.01 g/root) have been observed at the studied range of Fe supplementation. Total Fe content in soybean roots exposed to 50 and 500 μM Fe-EDTA, was higher than in roots grown in absence of supplemented Fe

(Table 3) and lipid oxidation, assessed as the content of TBARS, were not significantly affected by Fe supplementation up to 500  $\mu\text{M}$ , to the incubation medium (Table 3). However, Fe supplementation to the roots did affect  $\alpha$ -tocopherol content that was significantly decreased in the homogenates and the microsomes isolated from roots supplemented with Fe, as compared with values in roots developed in absence of Fe [40]. These data suggest that *in vivo* Fe supplementation could increase  $\text{O}_2$  radical generation in soybean roots that was adequately control.

	No added Fe	500 $\mu\text{M}$ added Fe	Ref
<b>Soybean roots</b>			
Total Fe content ( $\mu\text{g/g}$ FW)	$0.07 \pm 0.01$	$0.15 \pm 0.02^*$	[40]
Fe-EDTA reduction rate (nmol/min/mg prot)	$1.4 \pm 0.1$	$3.1 \pm 0.6^*$	[40]
TBARS (nmol MDA eq/mg)	$5.7 \pm 0.7$	$5.7 \pm 0.7$	[40]
<b>Soybean embryonic axes</b>			
Total Fe content (nmol/mg DW)	$1.3 \pm 0.2$	$3.9 \pm 0.8^*$	[42]
Fe-EDTA reduction rate (nmol/min/mg DW)	$15 \pm 1$	$22 \pm 2$	[42]
Ft ( $\mu\text{g}$ Ft/ g DW)	$34 \pm 11$	$27 \pm 10$	[42]
Ft Fe content (Fe atoms/molec Ft)	$1054 \pm 111$	$494 \pm 103^*$	[42]
LIP (pmol/mg DW)	$50 \pm 10$	$310 \pm 50^*$	[42]
TBARS (nmol MDA eq/mg)	$0.4 \pm 0.1$	$0.3 \pm 0.1$	[75]

\*significantly different from values without Fe addition,  $p \leq 0.05$ . ANOVA.

**Table 3.** Fe supplementation effects in soybean after 24 h of incubation

Robello et al. [42] reported that total Fe content in soybean embryonic axes exposed to 500  $\mu\text{M}$  Fe-EDTA was higher than in axes grown in absence of supplemented Fe after 24 h of incubation. However, neither Fe reduction rate nor growth assessed, either as the fresh weight or the dry weight of the embryonic axes, were significantly affected by Fe supplementation to the incubation medium. Membrane integrity was no affected by the supplementation with 50 and 500  $\mu\text{M}$  Fe:EDTA (1:2) since electrolyte leakage at 24 h and 48 h of imbibition was not significantly different from electrolyte leakage found in non-supplemented Fe axes ( $15.3 \pm 0.7$  and  $8.0 \pm 0.3\%$ , after 24 h and 48 h of incubation with 500  $\mu\text{M}$  Fe, as compared to  $12.4 \pm 0.4$  and  $8.6 \pm 0.6\%$ , after 24 h and 48 h of incubation in the absence of added Fe, respectively). Moreover, as it was previously reported in soybean

roots [43], Fe accumulation was not followed by Ft accumulation in soybean embryonic axes upon growth. Without any significant change in the content of Ft in the embryonic axes incubated for 24 h upon Fe supplementation, a 53% decrease in the Fe content per molecule of Ft was observed in the presence of 500  $\mu\text{M}$  Fe (Table 3). These data differed from previous observations showing Fe induction of Ft synthesis and accumulation in soybean [44], however, the nature of the model employed by Lescure et al. [44], cells in suspension grown heterotrophically, could alter the kinetic of the response. In this regard, it should not be discarded that a transient increase in Ft content could occur under these experimental conditions before 24 h of imbibition. The observed rapid decrease in Fe content per molecule of Ft, as compared to non-added Fe conditions, could reflect an early loosing of Ft molecules altered by free radicals, or a reduction of its capacity of binding Fe, or both. The increase in the protein sensitivity to proteases would lead to an early degradation, as compared to axes grown in a non-added Fe medium. The increased rate of ROS generation could be due to the significant increase in the LIP under conditions of Fe supplementation. However, it is important to point out that the substantial increase in the total Fe content in axes grown in the presence of 500  $\mu\text{M}$  Fe for 24 h, as compared to seeds grown in non-added Fe medium, could not be allocated as the measured increase in the LIP that would represent only the 10% of the increase in the total Fe content. Besides the LIP critical importance as initiator of free radical reactions and the decisive requirement of keeping Fe concentration as low as possible to minimize cellular deterioration, the role of other soluble and insoluble Fe-storage proteins, the formation and contribution of Fe-nitrosyl complexes, glutathione, nitric oxide, etc. should be considered among other non-protein agents, as possible candidates to handle Fe transport and storage under stress conditions since TBARS content was not significantly affected in Fe overloaded soybean embryonic axes (Table 3). Beside the apoplastic space [45], Lanquar et al. [46] identified the vacuole as a major compartment for Fe storage in plant seeds and showed that retrieval of the Fe stored in vacuoles is an essential step for successful germination in a wide range of environments.

On the other hand, recently Simontacchi et al. [47] summarized assays performed to characterize lipid radical-dependent oxidation in photosynthetic organisms where EPR was successfully employed to evaluate not only lipidperoxidation but also to analyze the relative scavenging capacity of plant extracts, the effects of both, natural environmental challenges and oxidative stress situations, in several model and biological systems. Further studies should be oriented in this direction to explore the critical effect of Fe overload on radical-dependent pathways that play a major role in plant metabolism.

#### **4. Fe overload in mammals**

Fe overload in mammals has been often associated with injury, fibrosis, and cirrhosis in the liver followed by cardiac disease, endocrine abnormalities, arthropathy, osteoporosis and skin pigmentation [48]. Several mechanisms has been proposed whereby excess hepatic Fe causes cellular injury, but Fe-induced peroxidative injury to phospholipids of organelle membranes is a potential unifying mechanisms underlying the major theories of cellular

injury in Fe overload [49]. With progressively increasing Fe deposition, the capacity to maintain Fe in storage forms is exceeded resulting in a transient increase in the hepatic LIP [50]. Moreover, Fe-catalyzed generation of ROS has been implicated in the pathogenesis of many disorders including atherosclerosis [51,52], cancer [53], ischaemia reperfusion injury [54,55] besides in Fe overload [56], such as haemochromatosis [57].

Several experimental models of Fe overload have been developed. In the dietary model used by Dabbagh et al. [58] rats were fed for 10 weeks a chow diet enriched with 3% (w/w) reduced pentacarbonyl Fe (a 99%, w/w, pure form of elemental Fe). Dietary Fe overload resulted in significant increases in hepatic Fe levels; with no difference in Fe content in serum (Table 4). Lipid peroxidation was assessed by measuring TBARS and F2-isoprostanes. The latter are a series of prostaglandin-F2-like compounds derived from the free-radical-catalyzed, non-enzymic peroxidation of arachidonic acid [59] and the *in vivo* levels of F2-isoprostanes have been shown to increase dramatically in acute hepatotoxicity [60]. Direct evidence for moderately increased lipid peroxidation products in liver was reported after dietary Fe overload. In addition to hepatic oxidative damage, Fe overload also caused changes in the plasma lipid profile. These data suggest that in this rat model of Fe overload, oxidative stress is associated with depletion of endogenous antioxidants in plasma and liver, and although no conclusive evidence for lipid peroxidation in plasma was found, hepatic F2-isoprostane levels were significantly increased in treated rats.

Experimental Fe overload in rats using dietary supplementation with carbonyl Fe is a well established model, where Fe deposition results mainly in the hepatocytes in a periportal distribution, as observed in idiopathic hemochromatosis [48]. Galleano and Puntarulo [61] used the dietary carbonyl-Fe model carried out on male Wistar rats that were fed during 6 weeks with either a) control chow diet, or b) control chow diet supplemented with 2.5% (w/w) carbonyl-Fe. Both, Fe and TBARS content, were increased in liver (Table 4). However, mild dietary Fe overload increased Fe content in plasma but did not lead to a significant increase in TBARS probably because Fe content after dietary Fe supplementation was increased less dramatically in plasma than in liver (88% and 15-fold, respectively), suggesting that plasma mechanisms for sequestering catalytically active Fe were fully operative (Table 4). Under these conditions, TBARS content in plasma does not seem to be a good indicator of oxidative stress conditions in the liver, and more sensitive techniques should be used in plasma to assess Fe-dependent oxidative stress.

Cockell et al. [62] used sucrose-based modified AIN-93G diets formulated to differ in Fe (35 mg/kg and 1500 mg/kg for control and Fe overloaded diets). Weanling male Long-Evans rats were fed these diets for 4 weeks and killed. Fe content was measured in plasma and liver. No differences in plasma between control and treated groups were found, meanwhile a significantly increase in liver between control and treated groups was observed. Since TBARS content in livers was significantly increased in Fe overloaded animals, hepatic Fe concentrations in this study were correlated positively with increases in TBARS. However, Fischer et al. [63] showed that Fe overloaded diets did not significantly alter other oxidative stress indices, such as DNA double-strand breaks or NF- $\kappa$ B activation despite observed increases in hepatic lipid peroxidation.

	Fe content		TBARS	
	control	Fe-overload	control	Fe-overload
<b>Pentacarbonyl Fe, diet 3% (w/w)</b>				
Liver [58]	104 ± 15(a)	1391 ± 242*(a)	-	-
Plasma [58]	134 ± 55(c)	124 ± 46(c)	nd	nd
<b>Carbonyl Fe, diet 2.5% (w/w)</b>				
Liver [61]	69 ± 16(a)	1091 ± 178*(a)	0.45 ± 0.05(b)	0.58 ± 0.01*(b)
Plasma [61]	179 ± 43(c)	336 ± 57*(c)	0.6 ± 0.1(d)	0.6 ± 0.2(d)
<b>Sucrose-basemodified AIN-93G, diet 1500 mg/kg</b>				
Liver[62]	218 ± 46(e)	895 ± 376**(e)	0.54 ± 0.07(b)	0.78 ± 0.19**(b)
Plasma [62]	2.72 ± 1.74(i)	3.82 ± 1.21(i)	-	-
<b>Fe-dextran, ip 500 mg/kg</b>				
Liver [68]	257 ± 11(e)	1837 ± 205*(e)	40 ± 1(f)	110 ± 30*(f)
Plasma [70]	126 ± 20(g)	1538 ± 158*(g)	0.7 ± 0.1(h)	2.7 ± 0.1*(h)
Kidney [49]	14 ± 3(e)	113 ± 15*(e)	29 ± 2(f)	37 ± 3*(f)

Letters indicate the units for each parameter as follows: (a) µg Fe/g FW; (b) nmol/mg prot; (c) µg/dl; (d) nmol/ml; (e) µg Fe/g DW; (f) pmol/min/mg prot; (g) µg Fe/dl; (h) nmol/l; (i) mg/l.

\*significantly different from control values  $p < 0.01$ ,

\*\* $p < 0.001$ , ANOVA.

nd stands for not-detectable.

**Table 4.** Fe effects in different organs and plasma employing several models of Fe overload

Fe-dextran treatment seems as a good model for the study of Fe toxicity resembling the pathological and clinical consequences of acute Fe overload in humans [48]. Fe supplied as Fe-dextran, is initially taken up by Kupffer cells, and when their storage capacity is exceeded the metal is accumulated by parenchymal cells producing a mild Fe overload. The increased Fe content alters the Kupffer cell functional status by inducing a progressive increase in macrophage-dependent respiration at earlier times after treatment. The effect is sensitive to macrophage inactivation by  $GdCl_3$  pretreatment, decreases the respiratory response of the Kupffer cell to particle stimulation, plays a role in the development of liver injury, and seems to condition the impairment of hepatic respiration observed at later times after Fe overload [64]. Other pathological situations that increase oxidative conditions in the cell, could enhance Fe-dependent damage. As an example, hyperthyroidism increases the susceptibility of the liver to the toxic effects of Fe, which seems to be related to the development of a severe oxidative stress status in the tissue, thus contributing to the concomitant liver injury and impairment of Kupffer cell phagocytosis and particle-induced respiratory burst activity [65]. It was also shown that acute Fe overload was responsible for oxidative stress in rat testes with a concurrent decrease of antioxidant content [66,67]. The oxidative stress has been developed using Fe-dextran intra peritoneal (ip) administration as 500 mg/kg body weight and killed after 20 h.

Spontaneous organ chemiluminescence (CL) reflects the rate of lipid peroxidation reactions through the detection of the steady-state level of excited species and is considered to be an

useful technique to evaluate oxidative stress *in vivo*. Galleano and Puntarulo [68] reported an association between Fe content and light emission in rats exposed to Fe-dextran after 2-6 h. Presumably, with progressively increasing Fe deposition, the capacity of maintaining Fe in storage forms is exceeded resulting in a transient increase in the hepatic LIP. However, at longer times (20 h) the significant increase in cytosolic Fe is limited, and CL goes back to control values. Moreover, cytochrome P<sub>450</sub> inactivation is an early event and precedes other enzyme inactivation [68]. Data included in Table 4 show that liver Fe content was increased by 7-fold after 8 h of Fe-dextran administration, and TBARS generation rate was enhanced by 3-fold (6 h after ip) suggesting that liver is deeply affected by acute Fe-overload.

Mammalian red blood cells are particularly susceptible to oxidative damage because (i) being an O<sub>2</sub> carrier, they are exposed uninterruptedly to high O<sub>2</sub> tension, (ii) they have no capacity to repair their damaged components, and (iii) the haemoglobin is susceptible to autoxidation and their membrane components to lipid peroxidation. Red blood cells, however, are protected by a variety of antioxidant systems which are capable of preventing most of the adverse effects of oxidative stress, under normal conditions [69]. Galleano and Puntarulo [70] reported, employing the ip Fe-dextran model of Fe overload, that 20 h after Fe-dextran injection Fe concentration in plasma of treated rats showed approximately 12-fold increase, and TBARS content in plasma showed a 285% increase as compared to control values (Table 4). On the other hand, *in vitro* studies showed that Fe can stimulate the peroxidation of erythrocytes membrane lipids. Since red blood cells from Fe overloaded rats are continuously being exposed to an increase Fe content, no differences in TBARS content were detected in red blood cells from control rats as compared to erythrocytes from Fe overloaded rats, suggesting high resistance to oxidative stress of these cells.

Galleano et al. [71] also employed this model to comparatively studying Fe overload in kidney. Fe content in whole kidney was 8-fold increased (Table 4), and 5-fold increased in kidney mitochondria (16 ± 5 to 78 ± 1 nmol/mg prot for control and treated animals, respectively). Even though TBARS content showed no significant differences after Fe administration, in Fe-treated rats TBARS production rate by kidney homogenates was higher in treated animals than in kidneys from control rats (Table 4). The authors suggested that Fe-dextran treatment does not affect kidney integrity, even though increases in lipid peroxidation rate occurs.  $\alpha$ -tocopherol, one of the most efficient antioxidant in the hydrophobic phase, appeared to be effective in controlling Fe-dextran dependent damage in kidney.

Brain tissue is thought to be very sensitive to oxidative stress. Neurons are enriched in mitochondria and possess a very high aerobic metabolism, which makes these tissues susceptible to ROS-dependent damage than other organs. Moreover, low levels of some antioxidant enzymes, high contents of polyunsaturated fatty acids in brain membranes, and high Fe content may combine their effects to make the brain a preferential target for oxidative stress-related degeneration [72]. Maaroufi et al. [73] developed a chronic Fe overload model consisting in a daily 3 mg Fe/kg administrated in adult rats during 5 days. These treatments resulted, 16 days after treatment, in a significant Fe accumulation in the

hippocampus, cerebellum, and basal ganglia. Lately, Maaroufi et al. [74] studied rats which received daily one ip injection of 3 mg FeSO<sub>4</sub>/kg dissolved in sodium chloride 0.9% (or vehicle) during 21 consecutive days, and this accumulation was correlated to behavioral deficits. No increase levels of the TBARS content in different brain structures were observed in any brain region investigated. This observation suggested that chronic Fe administration had induced adaptive responses involving stimulation of the antioxidant defenses since, both SOD and CAT activities, were increased after treatment.

Thus, different forms and quantities of Fe administrated to rats, supplemented either as diets or ip, lead to an increase in Fe content in several tissues and plasma. This Fe increase seems to be associated with an increase in lipid peroxidation. The underlying mechanisms of tissue damage are unclear, but they probably depend on the Fe administration protocol. Even though lipid damage was observed in many cases after Fe overload, antioxidant capacity seems to play a crucial role in controlling the impairment mechanisms.

## 5. Concluding remarks

Fe metabolism is very complex since Fe is both, an essential element and a toxic compound that has to be carefully kept under a regulated concentration in a living cell. Toxic Fe activity is due to its ability of catalyzing free radical reactions. The most efficient Fe fraction to act as a free radical promoter is that forming the LIP. LIP content is the resultant of multiple dynamic equilibrium between the Fe incorporated to the cell, utilized and intracellularly stored. We have briefly reviewed the role of Fe on the oxidative damage to lipid membranes employing both *in vitro* and *in vivo* models of Fe overload in several biological systems. Much progress has still to be made in order to understand the nature and function of the LIP, the mechanisms of the Fe-catalyzed reactions *in vivo*, the contribution of Fe to oxidative stress and disease, and the development of appropriate chemotherapeutic strategies. Thus, alterations in Fe metabolism should be carefully analyzed before evaluating cellular responses to either damaging agents or xenobiotics of biomedical or ecological impact since Fe is a double-faced element that can be either good or bad to the cell, depending on whether it serves as a micronutrient or as a catalyst of free radical reactions.

Since a tight metabolic organization is required to successfully face oxidative external conditions in invertebrates, anthropogenic contamination with Fe could be toxic for animals that are adapted to their natural environment. As it could be understood from the data presented here, it is strongly suggested that natural habitats should be strictly preserved even though absolute Fe content did not seem to reach critical values to avoid cellular deterioration.

Mobilization of Fe stored in plant seeds is an essential step for germination in a wide range of environments. The analysis of these aspects would provide information that could be the key to understand Fe nutrition in plants, and will allow the designing and engineering of crop plants requiring minimal fertilizer input, contributing to a more ecological agricultural practice under optimal and sub-optimal environmental conditions avoiding reaching Fe overload conditions that would jeopardize successful plant development.

Moreover, therapeutic strategies should be designed to chelate either Fe from the LIP or Fe loosely bound to Ft to avoid Fe-related oxidative damage. Focus in chemical-related aspects of the Fe-chelator complexes should help to fulfill the new drugs designing expectances to control Fe toxicity in humans that through promoting lipid peroxidation could severely affect human health.

## Author details

Paula M. González, Natacha E. Piloni and Susana Puntarulo\*  
*Physical Chemistry-PRALIB, School of Pharmacy and Biochemistry, University of Buenos Aires, Junín, Buenos Aires, Argentina*

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\* Corresponding Author

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