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Acute iron overload and oxidative stress in brain

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ABSTRACT

An *in vivo* model in rat was developed by intraperitoneally administration of Fe-dextran to study oxidative stress triggered by Fe-overload in rat brain. Total Fe levels, as well as the labile iron pool (LIP) concentration, in brain from rats subjected to Fe-overload were markedly increased over control values, 6 h after Fe administration. In this *in vivo* Fe overload model, the ascorbyl ($A^{\bullet}$)/ascorbate (AH^{-}) ratio, taken as oxidative stress index, was assessed. The $A^{\bullet}/AH^{-}$ ratio in brain was significantly higher in Fe-dextran group, in relation to values in control rats. Brain lipid peroxidation indexes, thiobarbituric acid reactive substances (TBARS) generation rate and lipid radical ($LR^{\bullet}$) content detected by Electron Paramagnetic Resonance (EPR), in Fe-dextran supplemented rats were similar to control values. However, values of nuclear factor-kappaB deoxyribonucleic acid (NFκB DNA) binding activity were significantly increased (30%) after 8 h of Fe administration, and catalase (CAT) activity was significantly enhanced (62%) 21 h after Fe administration. Significant enhancements in Fe content in cortex (2.4 fold), hippocampus (1.6 fold) and striatum (2.9 fold), were found at 6 h after Fe administration. CAT activity was significantly increased after 8 h of Fe administration in cortex, hippocampus and striatum (1.4 fold, 86, and 47%, respectively). Fe response in the whole brain seems to lead to enhanced NF-κB DNA binding activity, which may contribute to limit oxygen reactive species-dependent damage by effects on the antioxidant enzyme CAT activity. Moreover, data shown here clearly indicate that even though Fe increased in several isolated brain areas, this parameter was more drastically enhanced in striatum than in cortex and hippocampus. However, comparison among the net increase in $LR^{\bullet}$ generation rate, in different brain areas, showed enhancements in cortex lipid peroxidation, without changes in striatum and hippocampus $LR^{\bullet}$ generation rate after 6 h of Fe overload. This information has potential clinical relevance, as it could be the key to understand specific brain damage occurring in conditions of Fe overload.

1. Introduction

Several pathological conditions are currently associated with iron (Fe) overload. Clinical and epidemiologic observations indicate that increased Fe storage status is a risk factor in several diseases, such as porphyria cutanea tarda and the sudden infant death syndrome (Puntarulo, 2005). Hepatotoxicity is the most consistent finding in patients with Fe overload, followed by cardiac disease, endocrine abnormalities, arthropathy, osteoporosis, and skin pigmentation (Galleano and Puntarulo, 1994). Fe excess generates oxidative stress. Superoxide anion (O_2^{-}) and hydrogen peroxide (H_2O_2) toxicity arises from their Fe-dependent conversion into the extremely reactive hydroxyl radical ($^{\bullet}OH$) (Haber–Weiss reaction) generating severe damage to membranes, proteins, and deoxyribonucleic acid (DNA) (Halliwell and Gutteridge, 1984). Different protocols of Fe treatments in the rat, supplemented either in the diet or intraperitoneally (ip) injected, lead to a specific profiles in Fe content deposition in several tissues and plasma (Piloni and Puntarulo, 2010). Fe-dextran treatment, primarily affecting the liver, constitutes a good model for Fe toxicity evaluation, as it leads to similar pathological and clinical consequences observed after acute Fe overload in humans (Puntarulo, 2005). In the rat, we have developed an *in vivo* model consisting of ip Fe-dextran administration, followed by excision of the organ after 6 h (Galleano and Puntarulo, 1992). The effect of increasing Fe content in rat brain under this protocol of Fe administration has not been described yet, however, it has been shown to increase liver, kidney and circulating

Abbreviations: NF-κB, nuclear factor kappa B; DNA, deoxyribonucleic acid; $A^{\bullet}$, ascorbyl radical; AH^{-}, ascorbate; EPR, Electron Paramagnetic Resonance; $LR^{\bullet}$, lipid derived radicals; TBARS, thiobarbituric acid reactive substances; CAT, catalase; O_2^{-}, superoxide anion; O_2, oxygen; H_2O_2, hydrogen peroxide; $^{\bullet}OH$, hydroxyl radical; ROS, reactive oxygen species; LIP, labile iron pool; CA, calcein; TGA, thioglycolique acid; DMSO, dimethyl sulfoxide; TEMPO, 2,2,5,5-tetramethyl piperidine-1-oxyl; PBN, N-t-butyl-α-phenyl nitrone; α-T, alpha tocopherol; DHA, dehydroascorbic acid; SOD, superoxide dismutase; DF, deferoxamine mesylate; Ft, ferritin.

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Table 1
Fe content and lipid peroxidation after Fe-dextran treatment.

	Control	Fe-dextran	References
Liver			
Total Fe content (μg/g tissue)	45 ± 10	201 ± 28* (+446%)	(1)
TBARS (pmol/min/mg prot)	40 ± 1	110 ± 30* (+175%)	(2)
Kidney			
Fe content (μg/g DW)	14 ± 3	113 ± 15* (+707%)	(3)
TBARS (pmol/min/mg prot)	29 ± 2	37 ± 3* (+27%)	(3)
Plasma			
Fe content (μg/dl)	126 ± 20	1538 ± 158* (+1220%)	(4)
TBARS (nmol/ml)	0.7 ± 0.1	2.7 ± 0.1* (+285%)	(4)
$A^{\bullet}/AH^{-}$ ratio ($\times 10^{-4}$ AU)	4 ± 1	11 ± 1* (+275%)	(5)

Acute Fe overload was developed by injecting male Wistar rats with Fe-dextran (500 mg/body wt). Control animals were injected with dextran. Data in liver were taken after 6 h of the Fe administration and data in kidney and plasma after 20 h of Fe supplementation. (1) Galleano et al., 2004; (2) Puntarulo, 2005; (3) Galleano and Puntarulo, 1994; (4) Galleano and Puntarulo, 1995; (5) Galleano et al., 2002.
* Significantly different from control values ($p < 0.01$).

total Fe content, in concomitance with thiobarbituric acid reactive substances (TBARS) generation in those compartments (Table 1). It was suggested that Fe supplied as Fe-dextran is initially taken up by Kupffer cells in the liver, and when their storage capacity is exceeded the metal is accumulated by parenchymal cells producing a mild Fe overload. Interestingly, acute Fe overload alters the Kupffer cell functional status by inducing a progressive enhancement in macrophage-dependent respiratory burst activity at early times after treatment, a process associated with significant production of reactive oxygen species (ROS) (Tapia et al., 1998).

In order to enter the brain, Fe needs to cross two distinct barriers, the blood–brain barrier and the blood–cerebrospinal barrier (Pardridge et al., 1987; Zheng and Monnot, 2012). Fe-related neurodegenerative disorders can result from both Fe accumulation or defects in its metabolism and/or homeostasis (Batista-Nascimento et al., 2012). Brain tissue is thought to be more susceptible to ROS-dependent damage than other organs, a feature associated with (i) the fact that neurons are enriched in mitochondria and possess a rather high aerobic metabolism; (ii) the low levels of some antioxidant enzymes; (iii) the high content of polyunsaturated fatty acids in brain membranes; and (iv) the high Fe content, which may combine their effects to make the brain a preferential target for oxidative stress-related degeneration (Halliwell, 2006). Under physiological conditions, neuronal Fe is not reactive despite its high level, probably because its absorption, transport, and storage are tightly regulated; however, Fe-induced oxidative stress is likely to occur if an excess of ionic Fe is achieved (Floyd and Carney, 1992). Abnormal Fe metabolism can result in human neurologic disorders such as Alzheimer's, Parkinson's, and Huntigton disease (Gerlach et al., 1994). This assumption is supported by reports from Yu et al. (2011) showing that Fe-overload-induced oxidative stress in rats strengthen the complexity of risk assessment of psychological stress, as well as those from Chen et al. (2013) stating that increased Fe^{2+} in the brain, attained by intracerebral ventricular injection of Fe^{2+} induced autophagic cell death. Papanastasiou et al. (2000) reported that Fe uptake by the brain of rats chronically supplemented with Fe-carbonyl or intravenously injected with Fe polymaltose, was not significantly affected. More recently, Maaroufi et al. (2009) developed a chronic Fe overload model consisting in 3 mg Fe/kg, daily administrated to adult rats during 5 days. These treatments resulted in a late (16 days after treatment) and significant Fe accumulation in the hippocampus, the cerebellum, and the basal ganglia. Maaroufi et al. (2011) studies in rats, receiving daily Fe ip injections (3 mg $FeSO_4$/kg) or 0.9% sodium chloride (vehicle) during 21 consecutive days, showed that Fe accumulation correlated with behavioral deficits. However, TBARS content was not increased in different brain areas. This effect was accompanied by enhanced superoxide dismutase (SOD) and catalase (CAT) activities, suggesting that chronic Fe administration may have induced adaptive responses involving stimulation of the antioxidant defense systems. The underlying mechanisms of tissue damage are not clear, and 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.

The aim of the present study was to contribute to the understanding of the complex mechanism(s) triggered by Fe-overload in rat brain by assaying oxidative stress parameters. The kinetics of total Fe content and the labile Fe pool (LIP) were described. Oxidative stress status was estimated by assaying the ascorbyl radical ($A^{\bullet}$) content/ascorbate (AH^{-}) ratio and lipid damage as the rate of TBARS generation. EPR detection of lipid radicals ($LR^{\bullet}$) generation, CAT activity and nuclear factor-kappaB deoxyribonucleic acid (NF-κB DNA) binding activity were also evaluated.

2. Materials and methods

2.1. Animal preparation

Male Sprague-Dawley rats (180 ± 10 g, 45 ± 5 days old), from the Animal Facility of the School of Pharmacy and Biochemistry of the University of Buenos Aires, were used. The animals were housed under standard conditions of light, temperature and humidity with unlimited access to water and food. A single dose of 500 mg/kg body weight Fe-dextran was ip injected. Control rats were sham-injected ip with physiological saline solution. At 1, 2, 4, 6, 8, 21 and 29 h after Fe treatment or some of this time points specified in each experiment, brain was removed from the anesthetized animals in a CO_2 chamber. Depending on the protocol, brain cortex, hippocampus and striatum or the whole brain were rapidly removed and immediately frozen and stored under liquid N_2. Dissection was performed as described by Czerniczyniec et al. (2011). Experimental animal protocols and animal procedures complied with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, NIH Publication 6-23, revised 1985) and according to the principles and directives of the European Communities Council Directives (86/609/EEC). The procedures also received approval from the local ethics committee.

2.2. Total Fe content

Brain cortex, hippocampus and striatum and the whole brain were dried in an oven at 60 °C until constant weight, were mineralized in HNO_3 according to Laurie et al. (1991). Fe concentration was spectrophotometrically determined after reduction with thioglycolic acid measuring the absorbance at λ = 535 nm in the presence of bathophenanthroline according to Brumby and Massey (1967).

2.3. Labile iron pool (LIP)

The LIP was determined by a fluorescence technique with the Fe sensor calcein (CA) according to Darbari et al. (2003) with modifications. Brain samples were homogenized in 2 volumes of 40 mM potassium phosphate buffer, 120 mM KCl, pH 7.4. The homogenate was centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant was passed through filters with 30,000 nominal molecular weight limit. The filtered solution was then reduced for 10 min with 1 volume of 8% thioglycolic acid (TGA). Fe in the reduced solution was measured using 1 mM CA solution in 40 mM potassium phosphate buffer, 120 mM KCl, pH 7.4. When Fe is added to CA solution a fraction of the dye binds free Fe^{2+} leading to the generation of the Fe-bound (quenched) form [CA-Fe], while another fraction remains free as unbound calcein [CA] and provides the basal fluorescence. The fluorescence (λ_{exc} = 485 nm, λ_{em} = 535 nm) was monitored until stabilization of the signal and then deferoxamine mesylate (DF) salt was added to a final concentration of 800 mM. The fluorescence was monitored until a new stabilization of the signal. Then Fe^{2+} + Fe^{3+} concentration was assessed according to Robello et al. (2007).

2.4. Detection of $A^{\bullet}$ by Electron Paramagnetic Resonance (EPR)

Over the past decade the EPR detectable concentration of $A^{\bullet}$ has been interpreted either as an index of the transient changes in AH^{-} status (Pietri et al., 1994) or as a reflection of the ongoing free radical flux in the studied system (Jurkiewicz and Buettner, 1994; Galleano et al., 2002). Measurements were performed at room temperature in a Brucker (Karlsruhe, Germany) espectrometer EMX plus Banda X. Brain tissue was homogenized in dimethyl sulfoxide (DMSO) and immediately transferred to a Pasteur pipette for $A^{\bullet}$ detection. Instrument settings were as follows: modulation frequency 500 kHz, microwave power 10 mW, microwave frequency 9.75 GHz, centered field 3520 G, time constant 40.96 ms, modulation amplitude 1 G and sweep