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Oxidative stress in the central nervous system of iron-deficient females

Estrés oxidativo en sistema nervioso central de hembras deficientes de hierro

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Abstract

Iron deficiency is a worldwide public health problem associated with behavioral and cognitive disturbances. These disturbances are irreversible if not treated during the perinatal period. Iron is a structural part of several proteins, such as the antioxidant enzyme catalase. It has been shown that females are less susceptible to oxidative stress. However, the levels of oxidative stress at the central system in the presence of chronic iron deficiency or post-weaning supplementation are unknown. Objective: to determine the levels of oxidative stress and antioxidant defense in females with chronic iron deficiency, untreated or treated with iron supplementation. Methodology: female Wistar rats with chronic iron deficiency and rats supplemented from weaning to adulthood (70 postnatal days) were euthanized to analyze brain tissue and determine oxidative stress through lipid peroxidation; and antioxidant effect by superoxide dismutase, catalase and total proteins. Contribution: in the presence of chronic iron deficiency, lipid peroxidation levels at the central system are so high that they cannot be counteracted by superoxide dismutase or catalase. However, postnatal supplementation prevents lipid peroxidation from being altered due to the high production of iron-induced antioxidant defense.

Iron, Iron deficiency, Sex

Resumen

La deficiencia de hierro es un problema mundial de salud pública asociado con perturbaciones conductuales y cognitivas. Dichas alteraciones son irreversibles si no son tratadas durante el periodo perinatal. El hierro es parte estructural de diversas proteínas, como la enzima antioxidante catalasa. Se ha demostrado que hembras son menos susceptibles a estrés oxidativo, sin embargo, se desconocen los niveles de estrés oxidativo a nivel central ante deficiencia de hierro crónica o suplementación posdestete. Objetivo: determinar los niveles de estrés oxidativo y defensa antioxidante en hembras con deficiencia de hierro crónica no tratada o tratada con suplemento férrico. Metodología: ratas Wistar hembras con deficiencia de hierro crónica y ratas suplementadas desde el destete hasta la edad adulta (70 días-posnatales) fueron eutanasiadas para analizar tejido cerebral y determinar estrés oxidativo a través de peroxidación lipídica; y efecto antioxidante por superóxido dismutasa, catalasa y proteínas totales. Contribución: ante deficiencia de hierro crónica, los niveles de peroxidación lipídica cerebral son tan elevados que no pueden ser contrarrestados por superóxido dismutasa o catalasa. Sin embargo, suplementar posnatalmente, impide que la peroxidación lipídica se altere debido a la alta producción de defensa antioxidante inducida gracias al hierro.

Hierro, Deficiencia de hierro, Sexo

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Introduction

Iron is a trace element involved in various functions such as: erythropoiesis, oxygen transport and storage, mitochondrial functions, synthesis and degradation of proteins, lipids and ribonucleic acids, cardiac and skeletal muscle metabolism, thyroid gland functions, central nervous system and immune system (Andrews & Schmidt, 2007). This micronutrient in its divalent or ferrous form (Fe^{2+}) can donate electrons, while in its trivalent or ferric form (Fe^{3+}) it can accept them. This is an essential characteristic for its function as an oxygen carrier, however, it can also have toxigenic potential by generating free radicals (Ganz & Nemeth, 2006). Iron is a structural part of antioxidant enzymes such as: cytochromes, catalases, peroxidases and oxygenases (Bresgen & Eckl, 2015; Casanueva & Viteri, 2003; Forrelat B M, 2000; MacKenzie *et al*., 2008; Toxqui *et al*., 2010), therefore, its deficiency can also lead to oxidative stress (Askar *et al*., 2017a).

Previous research shows that females are less susceptible to oxidative stress than males (Austad, 2006; Kander *et al*., 2017; Vina *et al*., 2011). This may be due to oestrogenic factors, including the antioxidant effect (Badeau *et al*., 2005; Kagan *et al*., 1992; Kagan & Tyurina, 1998; Packer *et al*., 1979) and the regulator of body iron levels (Borras, 1998) that favour splenic Fe stores and their serum levels (Haouari *et al*., 1993; Haouari *et al*., 1994).

When the amount of iron absorbed from the diet exceeds the demand required by the body, iron deficiency (DFe) occurs (Tussing-Humphreys et al., 2012). It particularly affects infants, young children, adolescents, older adults, those with chronic inflammatory diseases, and women; in the latter, menstruation and pregnancy are additional risk factors. Approximately 30-40% of women and preschool children in industrialised countries have DFe, while almost all individuals in these groups are affected in developing countries (Lundqvist & Sjoberg, 2007).

There are sex-specific and life-stage-specific increases in iron nutritional requirements. In the absence of dietary supplementation, DFe is reported in about 40% of preschool children, 30% of menstruating women and girls, and 38% of pregnant women (Kassebaum *et al*., 2014; Pasricha *et al*., 2013; Stevens *et al*., 2013). The causes of DFe in developing countries are typically: insufficient dietary intake and/or intestinal blood loss due to parasite colonisation. In contrast, in high-income countries, the main causes are: certain dietary habits (vegan diet or not consuming red meat) and certain pathological conditions (chronic blood loss or malabsorption) (Kassebaum *et al*., 2014).

During gestation, iron is obtained by the foetus through the placenta, with 80% of the transfer occurring during the third trimester of pregnancy. It is essential that the foetus acquires adequate iron stores from the mother to maintain its growth during the first 6 months of life, as the iron provided by breastfeeding is very low (Widdowson & Spray, 1951). Throughout pregnancy the prevalence of DFe is high; 43% of pregnant women worldwide are anaemic, with DFe being the cause in 50-75% of cases (Di Renzo et al., 2015). Even worldwide, the most common cause of DFe during the gestational and early postnatal period is maternal DFe (Lozoff *et al*., 1996). Other causes of DFe during the foetal and neonatal period are preterm birth and gestational complications (maternal diabetes, intrauterine growth restriction, maternal smoking, maternal obesity and inflammation) (Chang *et al*., 2011; Lukowski *et al*., 2010; Murray-Kolb & Beard, 2007).

In infants there are 3 dietary sources of iron: breast milk (with iron bound to lactoferrin), heme iron and non-heme iron. For neonates and young infants their only source of iron is in breast milk and/or formula (Siimes et al., 1979). At birth most term infants have normal to high Hb concentrations (15-17 g/dL) and thus remain iron saturated until 6 months of age. Infants born to mothers with DFe are at high risk of developing DFe at approximately 4-6 months of age, without being manifested at birth (Mills & Davies, 2012). During this stage iron is acquired entirely from the diet, in contrast to adults, for whom the diet provides only 5% of the daily requirement (DH, 2011).

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One of the major non-hematological problems associated with DFe is behavioural and cognitive disturbances, the underlying mechanisms of which involve dysfunctional myelination, altered neurotransmission and impaired brain development. Infants with DFe are particularly prone to cognitive problems, as well as auditory and visual dysfunction (Algarin *et al*., 2003). Unfortunately, the above problems appear in early childhood, when dopamine is the main neurotransmitter, so prevention of these impairments by late iron supplementation is uncertain (Algarin *et al*., 2013; Algarin *et al*., 2003). The strongest evidence for neurological impairment comes from studies on cognition in school-aged children and adolescents with DFe and DFe anaemia (Low *et al*., 2013). However, it is not known whether females suffering from chronic iron deficiency from foetal to adult age, as occurs in many people worldwide, or in their case, treated with iron supplementation during childhood, have altered levels of oxidative stress and antioxidant defence in the central nervous system, which would be related to behavioural and cognitive alterations such as those mentioned above, the reason for the present research.

Methodology

Ethical declaration

All studies were conducted according to approved institutional protocols in accordance with the Principles and Procedures outlined by the National Institutes of Health, National Institutes of Health Guide for the Care and Use of Laboratory Animals, in agreement with the Local Ethics Committee. For sample collection, sacrifice was induced with CO_2 to minimise distress.

Animals and diet

Wistar rats were used and maintained under standard vivarium conditions: a 12:12 light/dark cycle was used (light on at 5 o'clock), with a controlled temperature of approximately 22 ± 2°C and free access to food and water.

The study was conducted on female broods. Parents or offspring were subjected to the following conditions: 14 days prior to mating and during 25 days of gestation, 20 female rats (3 months old or 250 g) were fed an iron-deficient diet (10 ppm $FeSO_4$, Lab Diets AIN-76W / 10), "DFe group". Another 10 female rats received control diet (100 ppm FeSO4, Lab diets AIN-76W/100) "control group". 21 days after birth (DPN), the pups were weaned. Only females were selected for the present experiment, males were used in other projects. Female offspring were maintained on the same type of diet offered to their mothers until 70 DPN; with the exception of the "DFe+S" supplemented group, a set of ID female offspring, which received from 21 to 70 DPN control diet.

Sample collection

At 70 PND, study subjects were euthanised in a 100% saturated CO2 chamber in order to extract brain tissue and a blood sample for determination of haemoglobin-bound iron (Fe-Hb).

After collection, the brain was washed and immediately placed in PBS (pH 7.4) at a ratio of 1ml/3g sample weight. 1 mL of blood was obtained in test tubes with heparin.

The brain tissue was cut into small pieces and homogenised in a cold mortar and pestle and then in a Potter homogeniser, using a total volume of 4 mL of PBS (pH = 7.4). It was centrifuged at 12,000 rpm for 15 minutes at -4°C. The supernatant was taken and stored at -70°C until analysis.

To determine oxidative stress, the following markers were analysed: lipid peroxidation levels (LPOx), total protein (TP); and antioxidant activity of superoxide dismutase (SOD) and catalase (CAT).

Iron bound to haemoglobin (Fe-Hb)

Hb concentration was determined in triplicate by the cyanomethaemoglobin method using Drabkin's solution (Randox Mexico SA de CV) (Prohaska & Gybina, 2005; Unger *et al*., 2007).

Total body Fe-Hb concentrations were determined indirectly using the following formula (Hernandez *et al*., 2006; Wienk *et al*., 1999):

$$Fe - Hb(mg) = \frac{\left(\left(\frac{Hb}{L}\right) * (body\ weight) * 6.7 * 0.335\right)}{10000} \quad (1)$$

Where Hb (g) contains 0.335% iron. The blood volume in growing rats is 6.7% of body weight (g).

For the statistical analysis, SPSS 22® statistical software was used; initially a descriptive statistical analysis was performed for each variable considered. The results were presented as means ± standard deviation (mean±SD). To compare differences between two groups (e.g., "Control" and "DFe" or "DFe+S" groups), the Mann-Whitney U-test was used. Results of $p<0.05$ were considered statistically significant, with a 95 % confidence interval.%.

Determination of oxidative stress

Levels of lipid peroxidation

LPO levels were determined by the method of Buege and Aust (1978), where thiobarbituric acid reactive substances (TBARS) are quantified. The decomposition of unstable hydrogen peroxides derived from polyunsaturated fatty acids results in the formation of malondialdehyde which reacts with 2-thiobarbituric acid, giving a pink colour absorbing at 535 nm. The concentration of malondialdehyde was calculated with its molar extinction coefficient: 1.56x10-5cm^{-1}/M^{-1}.

Determination of the antioxidant effect

Super oxide dismutase "SOD" activity

SOD activity was determined by the method of Misra and Friodovich (1972) which is based on the measurement of the kinetics of oxidation of adrenaline by the superoxide radical in 5 minutes, the absorbance was measured at the wavelength of 480 nm in a Thermo Scientific Genesys 10S UV-Vis spectrophotometer with quartz cell. SOD activity was calculated using the molar extinction coefficient of epinephrine (0.021 mM^{-1} cm^{-1}).

'CAT' catalase activity

CAT activity was determined by the method of Radi *et al*., (1991), which is based on the measurement of the kinetics of hydrogen peroxide degradation over 2 min by CAT at a wavelength of 240 nm. The enzyme activity was calculated with the molar extinction coefficient of H2O2 (0.043 mM^{-1} cm^{-1}).

Total proteins

To specifically report enzyme activity, total proteins were measured by the method of Bradford (1976). Proteins bind to the dye Chromassie Blue G-250 in acidic medium achieving a blue colour, which has an absorbance at 595 nm wavelength. The concentration was calculated using a standard curve obtained with bovine serum albumin in the range of 50 to 500 ug/mL.

All experiments were performed in triplicate.

Results

Determination of iron bound to haemoglobin

When studying the ID group with respect to the control group, it was found that the former had 10.9% less Fe-Hb and 3.8% less Fe-Hb than the ID+S group, see Table 1.

Group	Fe - Hb
Control	3.71±0.11
Iron deficient+supplementation	3.47±0.11
Iron deficient	3.18±0.23*
* vs. Female control group (p≤0.05).	

Table 1 Haemoglobin-bound iron levels "Fe-Hb"

Determination of oxidative stress

Lipid peroxidation: As shown in figure 1a, DFe females presented higher levels of peroxidation, 17.6% higher in relation to control females and 14.1% to DFe+S.

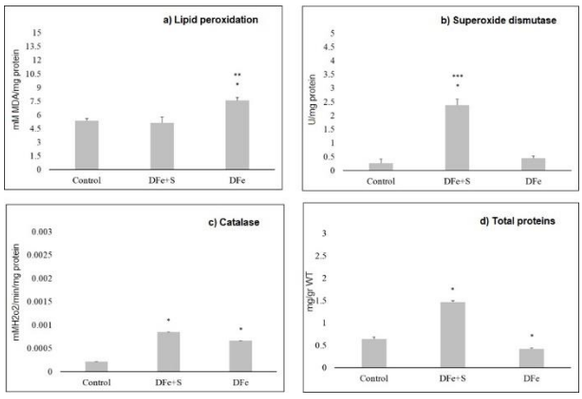
Determination of antioxidant effect

Super oxide dismutase: Figure 1b shows that among the study groups, ID+S subjects reported the highest SOD levels, 253% higher than control females. In ID subjects, SOD levels are 116% higher than in control females and 81.2% lower than in ID+S subjects.

Catalase: CAT levels in the study groups are shown in figure 1c. Catalase levels in DFe+S and DFe females do not show significant differences, however, they are higher than those of control females. 228% higher in DFe+S females and 180% higher in DFe females.

Total protein: As shown in figure 1d, the group with the highest total protein was the ID+S group. 102 % more than the control group and 136 % more than the ID group.

ID females exhibited the lowest total protein levels, 14.6 % lower than control females.



* Female of the control group (p≤0.05)
**Female from Iron Deficient+Supplemented group (p≤0.05)
***Female from the Iron Deficient group (p≤0.05)

Graphic 1 Oxidative stress and antioxidant effect in iron-deficient and supplemented females. Graph "a" shows the levels of lipid peroxidation in the study subjects and is indicative of oxidative stress. Graphs b, c and d; showed the antioxidant effect: b) superoxide dismutase, c) catalase and d) total proteins

Discussion

Iron deficiency (DFe) is a worldwide public health problem, as it causes multiple conditions with major health impacts (DeMaeyer & Adiels-Tegman, 1985; Kassebaum *et al*., 2014; Stoltzfus, 2001). During neurodevelopment, DFe leads to disturbances such as: disturbances in motor function, anxiety, cognitive and behavioural defects, auditory and visual dysfunction, as well as psychiatric conditions. The pathophysiology of these neurological disorders is complex and includes dysfunctional myelination, abnormal dendritogenesis and synaptogenesis, and even impaired neurotransmitter and hormone synthesis (Algarin *et al*., 2003; Bakoyiannis *et al*., 2015).

The neurological alterations of DFe have been attributed to several factors, mainly to a decrease in the incorporation of iron as a molecular structure of proteins. At the antioxidant level, iron is known to be a structural part of catalases (Isler *et al*., 2002), and it is also known that this trace element participates in oxidative stress processes through the Fenton reaction (Lloyd *et al*., 1997). Therefore, high levels of iron can cause oxidative stress, and low levels of iron can affect antioxidant defences. It is worth noting that most iron-oxidative stress studies have been conducted at the blood level (Akarsu *et al*., 2013; Askar *et al*., 2017b; Diaz-Castro *et al*., 2008), but it is necessary to know what happens at the central level to learn more about the involvement of iron in neurological effects. Iron has been shown to play an important role in CNS development as it is essential for myelination and axonal development, in fact, it has been established that late supplementation in iron-deficient infants does not restore neurodevelopmental damage (de Ungria *et al*., 2000). On the other hand, it has been shown at the brain level that iron deficiency tends to increase reactive oxygen species and decrease the in vivo activity of antioxidant enzymes (Thompson *et al*., 2003) but studies are usually performed in experimental models using males as a sample and little is known in females.

In a previous study, we demonstrated that organs increase iron demand under deficiency conditions, indicating altered organ function in the presence of iron deficiency (Vieyra-Reyes *et al*., 2017). In the present study, we found that iron-deficient females show the highest levels of lipid peroxidation, indicating that they are the most damaged at the oxidative level. This indicates that oxidative stress cannot be counteracted by antioxidant enzymes. It can be seen that although the levels of superoxide dismutase in iron-deficient females are similar to those of control females, the high brain lipid peroxidation cannot be counteracted by this antioxidant defence. In the case of catalase, the levels are significantly higher than in control females, but they are not sufficient to reduce the damage. This provides further information for the understanding of the neurological, cognitive and behavioural alterations associated with iron deficiency.

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When analysing the effect of supplementation, it was found that the levels of iron bound to haemoglobin are lower than those of control females, however, they are not significantly different. This helps greatly in the regulation of oxidative stress, since thanks to the supplementation, the levels of superoxide dismutase and catalase increase, preventing the levels of lipid peroxidation from being altered. This is also reflected in the increase of total protein levels in supplemented females. This is a transcendent result that supports and sustains the importance of the use of iron supplementation for the prevention of the development of neurological problems and their respective concomitant pathologies.

Conclusions:

- Chronic iron deficiency in females greatly affects the levels of lipid peroxidation in the brain and this effect cannot be counteracted by the antioxidant defence of superoxide dismutase and catalase.

- Iron supplementation in females that suffered from iron deficiency at perinatal level, during gestation and until weaning, equivalent to 21 days postnatal, show normal levels of lipid peroxidation due to the high antioxidant defence activated by increased levels of superoxide dismutase and catalase.

Perspectives:

To develop comparative studies, between females and males suffering from chronic iron deficiency to evaluate the levels of oxidative stress and to establish strategies in order to reach possible solutions to this major public health problem.

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