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# Effect of γ-irradiation on Fe content in developing neural precursor cells

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## ABSTRACT

The hypothesis of this work is that the effects of γ-irradiation on Fe allocation in developing brain requires the interaction of mother-fetus, and that Fe content changes mostly reflect the γ-irradiation effects over neural precursor cells. Two irradiation protocols of cultures were performed: i) direct γ-irradiation of cells after isolation, and ii) γ-irradiation *in utero* followed by isolation and culture of the cells. In cells irradiated *in vitro*, no changes neither in the total Fe content nor in the labile Fe pool (LIP) were observed from 1 to 4 h pi, as compared to these parameters in non-irradiated cells. In cells irradiated *in utero*, an increase (3.3-fold) in the total Fe content of the neural precursor cells was observed at 4 h pi, as compared to the content in non-irradiated cells. Also, the LIP content was decreased after 1 h pi. Thiobarbituric reactive substances (TBARS) content showed an increase at 4 h pi, as compared to control values, and nitric oxide (NO) generation was increased by 2.6-fold at 2 and 4 h pi. The data reported here suggested that the increase in Fe content was associated to γ-irradiation in the developing brain, requiring the mother-fetus interaction, and that NO seems to play a key role.

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**KEYWORDS:** γ-irradiation, Fe, brain cell culture, nitric oxide, oxidative stress

## INTRODUCTION

Mammalian brain exposure to ionizing radiation might happen as a consequence of military or industrial accidents, or after antineoplasic therapeutic treatments [1]. A broad spectrum of congenital abnormalities, growth retardations, developmental delays and functional defects are associated with irradiation of the mammalian fetus [2] since developing central nervous system (CNS) is especially sensitive to radiation damage [3]. Developmental radiation-induced abnormalities of the brain cortex of fetuses are expressed in different ways, depending on the dose and on the gestational day of exposure [4]. Ionizing radiation induces tissue damage on developing CNS through different simultaneous mechanisms, including apoptosis and reactive oxygen species (ROS)-dependent effects [5]. Radiation induced white matter necrosis in the rat spinal cord is preceded by changes in permeability of the blood brain-barrier, reduced blood flow, and infarction so that the necrosis is an ischemic necrosis [6]. Moreover, tissue damage in cerebral ischemia may be produced by acidosis-induced delocalization of intracellular Fe which acts as a catalyst in oxidative reactions [7, 8] and these effects were significantly decreased by Fe depletion [9]. Fe is essential for many brain cell functions [10], but must be stringently regulated, as it is one of the most abundant redox-active metals, capable of catalyzing the production of ROS [11]. Neurons and glial cells require Fe for electron transport,

NADPH reductase activity, myelination of axons and as a cofactor for enzymes involved in the synthesis of neurotransmitters [12]. An imbalance in brain Fe has been associated with normal aging and neurodegenerative disorders [13] and perturbation of Fe homeostasis proteins can lead to neuropathology in mouse models [14]. The labile iron pool (LIP) is defined as a low-molecular-weight pool of weakly chelated Fe that rapidly passes through the cell. It likely consists of both forms of ionic Fe ($Fe^{2+}$ + $Fe^{3+}$) associated with low affinity ligands for Fe ions [15]. It has been proposed that Fe is complexed by several chelators, such as citrate and other organic ions, phosphate, carbohydrates, etc [16]. The LIP can catalyze the conversion of normal by-products of cell respiration, like superoxide anion and hydrogen peroxide, into highly damaging hydroxyl radical through the Fenton reaction, or by the $Fe^{2+}$ catalyzed Haber-Weiss reaction, or into equally aggressive ferryl ions or oxygen-bridged $Fe^{2+}$/$Fe^{3+}$ complexes. Moreover, $Fe^{3+}$ can be reduced either by superoxide anion or by ascorbate ($AH^-$) leading to further radical production [17] that could be responsible for lipid peroxidation, DNA strand breaks and degradation of biomolecules [18]. The more immature antioxidant defenses and the higher abundance of labile Fe found in the developing CNS, together with the high proportion of dividing neuroblasts, might be some of the reasons for the high radiosensitivity of developing brain [5]. In a model of homogenates from developing rat brain exposed *in utero* to γ-irradiation total Fe, ferritin and LIP content were assessed by Robello *et al.* [19]. The summary of the data showed in Table 1 suggested that the induced damage in the developing rat brain could be auto-limited by affecting the cellular availability of catalytically active Fe, even though Fe content in maternal plasma was significantly increased. Moreover, using the same model, Gisone *et al.* [2] proposed that ionizing radiation induces an early increase in NOS activity that correlates with a further increase in NO steady state concentration, which could play an antioxidant role in the developing rat brain irradiated *in utero* (Table 1). To further characterized γ-irradiation effects, specifically on neural precursor cells, an *in vitro* model was used [20]. Gisone *et al.* [21] reported in this *in vitro* model an NO generation burst, and cellular apoptosis through the activation of caspases. However, up to now there is no experimental evidence that indicate that this *in vitro* exposure

**Table 1.** General features of the model for studying effects of γ-irradiation *in uterus* on rat brain fetus homogenates.

| | Control | γ-irradiation | Time pi (h) | Ref. |
|---|---|---|---|---|
| Total Fe (pmol/mg FW) | 60 ± 5 | 140 ± 6* | 1 | [19] |
| LIP brain (pmol/mg FW) | 4.2 ± 0.6 | 1.0 ± 0.2* | 2 | [19] |
| LIP maternal plasma (μM) | 1.9 ± 0.3 | 3.7 ± 0.7* | 1 | [19] |
| Ft content (%) | 100 ± 15 | 23 ± 15* | 2 | [19] |
| NO content (pmol/g FW) | 37 ± 4 | 216 ± 6* | 2 | [2] |
| Total NOS (pmol/g 30 min) | 39 ± 5 | 59 ± 5* | 1 | [2] |
| iNOS (pmol/g 30 min) | 13 ± 1 | 14 ± 4 | 1 | [2] |
| TBARS (nmol/mg prot) | 0.96 ± 0.02 | 1.45 ± 0.12 | 4 | [2] |

*significantly different from control values $p<0.01$, ANOVA.

could reproduce adequately the effects on Fe distribution by γ-irradiation *in utero* on developing brain.

Since previous data from Robello *et al.* [19] suggested that a complex interaction between Fe and NO could be triggered in fetal brain homogenates, as part of the network of the cellular response to limit irradiation-dependent injury, the hypothesis of the present work is that the effects of γ-irradiation on Fe allocation in developing brain requires the interaction of mother-fetus, and that the Fe content changes mostly reflect the γ-irradiation effects over neural precursor cells. To test this hypothesis upon the consequences on Fe availability, two irradiation protocols of cultures of neural precursor cells were performed: i) direct γ-irradiation of cells after isolation (no mother-fetus interaction after exposure), and ii) γ-irradiation *in utero* followed by isolation and culture of the neural precursor cells (mother-fetus interaction after exposure). In both models, total Fe and LIP content were evaluated during 1 to 4 h post-irradiation (pi) period.

## MATERIALS AND METHODS

### Breeding and culture preparation

Female Wistar rats, from the School of Pharmacy and Biochemistry, University of Buenos Aires, were mated overnight and the following morning sperm-positive vaginal smear was checked to assess day 0 of pregnancy. Fetal brains were removed at 17$^{th}$ gestational day (17 GD) under general anesthesia (sodium pentobarbital 120 mg/kg ip). Primary cell cultures from fetal brain were obtained and prepared as previously described by Michelin *et al.* [20] with modifications, where the precursor nature of cells was confirmed by nestin and GFAP immunoreactivity and by the capacity of differentiation in neuronal and glial cells after 5 days in culture. Housing, handling and experimental procedures followed the rules of the School of Pharmacy and Biochemistry (UBA), according to the Scientific Procedures of the United Kingdom animals (Act 1986). RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) and 20 mM L-glutamine, 1000 U/l penicillin, and 5 mg/l streptomycin was employed at a final density of $10^6$ cells ml$^{-1}$ into Petri dishes. Cultures were kept at 37ºC in a humidified atmosphere of 95% air and 5% $CO_2$.

### Irradiation conditions

For *in utero* irradiation studies, the pregnant rats (17 GD) were placed in plastic restraining cages and exposed to 2 Gy γ-irradiation using a teletherapy γ unit ($^{60}$Co) at a rate of 0.7 Gy/min. Control rats were sham-irradiated under similar conditions. Fetal brains were removed under general anesthesia (sodium pentobarbital 120 mg/kg ip to the pregnant rat).

For *in vitro* irradiation studies, isolated cells (17 GD) were irradiated with a dose of 2 Gy using a teletherapy γ unit ($^{60}$Co) at a rate of 0.7 Gy/min. The exposure period started 2 h after plating in order to allow cell attachment. Control samples were sham irradiated. The cells were then incubated and harvested at the time-points indicated at each assay.

### Total Fe content

Fetal neural precursor cells (5 x $10^6$ cells) were dried until constant weight in an oven at 70ºC. Then, the samples were mineralized in $HNO_3$/ $HClO_4$ (1:1) according to Laurie *et al.* [22]. Fe content was determined spectrophotometrically after reduction with thioglycolic acid, measuring the $Fe^{2+}$-batophenanthroline complex absorbance at 535 nm [23].

### Labile iron pool (LIP)

The LIP was determined both, by Electron Paramagnetic Resonance (EPR) at 77K, according to Woodmansee and Imlay [24], and by a fluorescence technique employing the Fe sensor calcein (CA) according to Darbari *et al.* [25] with modifications by Robello *et al.* [26]. For $Fe^{3+}$ + $Fe^{2+}$ determinations by EPR the cells were homogenized in 10 mM Tris-HCl buffer, 120 mM KCl, 1 mM deferoxamine mesylate (DF), pH 7.4, at a final concentration of 20 x $10^6$ cells ml$^{-1}$. For the measurements at 77 K the homogenate was transferred to a Dewar finger. The measurements were performed in a Brucker (Karlsruhe, Germany) espectrometer ECS 106 with a cavity ER 4102ST using the following instrument settings: modulation frequency, 50 kHz; microwave power, 20 mW;

microwave frequency, 9.42 GHz; center field, 1,600 G; time constant, 81.92 ms; modulation amplitude, 4.759 G; and sweep width, 800 G. For the fluorescence measurements the cells were homogenized in 40 mM potassium phosphate buffer, 120 mM KCl, pH 7.4, at a final concentration of 20 x $10^6$ cells $ml^{-1}$. The homogenate was centrifuged at 10,000 × *g* for 15 min at 4ºC. The supernatant was passed through filters with 30 kDa nominal molecular weight limit. The filtered solution was then reduced with thioglycolic acid to a final concentration of 4% (v/v). Fe content in the reduced samples of fetal brain cells was measured in the presence of 1 µM CA solution in 40 mM potassium phosphate buffer, 120 mM KCl, pH 7.4. The fluorescence ($\lambda_{exc}$ = 497 nm, $\lambda_{em}$ = 518 nm) was monitored until stabilization of the signal, and then DF was added to a final concentration of 800 µM. The fluorescence was monitored until a new stabilization of the signal. Then $Fe^{2+}$ + $Fe^{3+}$ concentration was assessed according to Robello *et al*. [26].

**Fe reduction rate**

Fe reduction rate by fetal neural precursor cells was determined spectrophotometrically measuring the absorbance at 520 nm ($\varepsilon$ = 13.6 $mM^{-1}$ $cm^{-1}$) in the presence of 5 mM 2,2´-bipyridyl, 0.1 mM NADPH and 50 µM Fe:EDTA in 100 mM potassium phosphate buffer, pH 7.4 [27].

**Detection of ascorbyl radical ($A^{\bullet}$) by EPR**

Measurements were performed at room temperature in a Brucker (Karlsruhe, Germany) espectrometer ECS 106 with a cavity ER 4102ST. Fetal neural precursor cells (120 x $10^6$ cells) were homogenized in 1 mM DF in DMSO and immediately transferred to a Pasteur pipette for $A^{\bullet}$ detection. Instrument settings were as follows: modulation frequency, 50 kHz; microwave power, 10 mW; microwave frequency, 9.75 GHz; centered field, 3487 G; time constant, 327.68 ms; modulation amplitude, 1 G; and sweep width, 15 G [28]. Quantification of the $A^{\bullet}$ was performed using an aqueous solution of 2,2,5,5-tetramethyl piperidine 1-oxyl (TEMPO) introduced into the same sample cell used for the measurements. EPR spectra for both sample and TEMPO solutions were recorded at exactly the same spectrometer settings and the first derivative EPR spectra were double integrated to obtain the area intensity, then the concentration of radical was calculated according to Kotake *et al.* [29].

**Ascorbate ($AH^-$) content**

The content of $AH^-$ was measured by reverse phase HPLC with electrochemical detection. Fetal neural precursor cells (40 x $10^6$ cells $ml^{-1}$) were homogenized in metaphosphoric acid 10% (w/v) according to Kutnink *et al.* [30].

**Thiobarbituric reactive substances (TBARS) content**

The content of TBARS in the cells was determined as previously reported by Malanga *et al.* [31]. An aliquot of 5 x $10^6$ cells in 100 µl 100 mM potassium phosphate buffer, pH 7.4 were mixed with 50 µl 3% (w/v) SDS and 12.5 µl of 4% (w/v) butyl hydro-xytoluene (BHT) in ethanol and vortexed before adding 500 µl of 0.1 N HCl, 75 µl of phosphotungstenic acid 10% (w/v) and 250 µl of thiobarbituric acid (TBA) 0.7% (w/v). After this, the reaction mixture was incubated at 100ºC for 45 min in a water bath and, after cooling, TBARS were extracted once with 1.25 ml n-butanol. TBARS concentration was measured by a fluorescence technique in a spectrofluorometer Hitachi F-3010 ($\lambda_{exc}$ = 515 nm, $\lambda_{em}$ = 555 nm).

**NO generation in neural precursor cells**

Fetal neural precursor cells (40 x $10^6$ cells $ml^{-1}$) were homogenized at 4ºC in 30 mM Tris-HCl containing 0.5 mM DTT, 1 mM EDTA, 1% SDS, pH 7.4, and protease inhibitors: 0.5 µg/ml leucopeptin, 0.7 µg/ml pepstatin A, 40 µg/ml phenylmethylsulfonyl fluoride, 0.5 µg/ml aprotinin and 0.6 mM $MgSO_4$. For NO generation determinations 1.6 x $10^6$ cells were supplemented with the adequated substrates or cofactors: 1 mM $CaCl_2$, 1 mM NADPH and 500 µM L-arginine in presence of 10 µM DAF-2. NO reaction with DAF-2 produces the highly fluorescent product Triazolofluorescein (DAF-2T) ($\lambda_{exc}$ = 494 nm y $\lambda_{em}$ = 515 nm) [32]. Samples were incubated at 37ºC during 30 min in order to allow NO generation and its reaction with DAF-2 giving place to DAF-2T formation. Samples were centrifuged at 10,000 *g* for 5 min, and then DAF-2T fluorescence was measured in the supernatant (32).

**Statistical analyses**

Data in the text and tables are expressed as mean ± S.E. of four independent experiments, with two replicates in each experiment. For each experiment, the fetal neural precursor cells from one litter culture were pooled and two aliquots of the pool were analyzed. Statistical tests were carried out using ANOVA followed by a Bonferroni post test. All statistical analysis was performed using the computer program GraphPad InStat® Version 3.01, 32 bit for Win 95/NT.

**RESULTS**

Previous data from Michelin *et al*. [20] and Gisone *et al*. [21] have shown that cells cultured on 17 GD are neural precursors that undergo *in vitro* differentiation. Viability of the cells was not significantly affected over the initial 4 h of culture (98 ± 1%). $A^{\bullet}$ content in cells in culture over the 1 to 4 h period, was assessed by EPR. The cells exhibited an EPR signal with the spectral features ($a_H$ = 1.88 G, g = 2.0054) of $A^{\bullet}$ (Fig. 1A), that was significantly increased after 2 h of

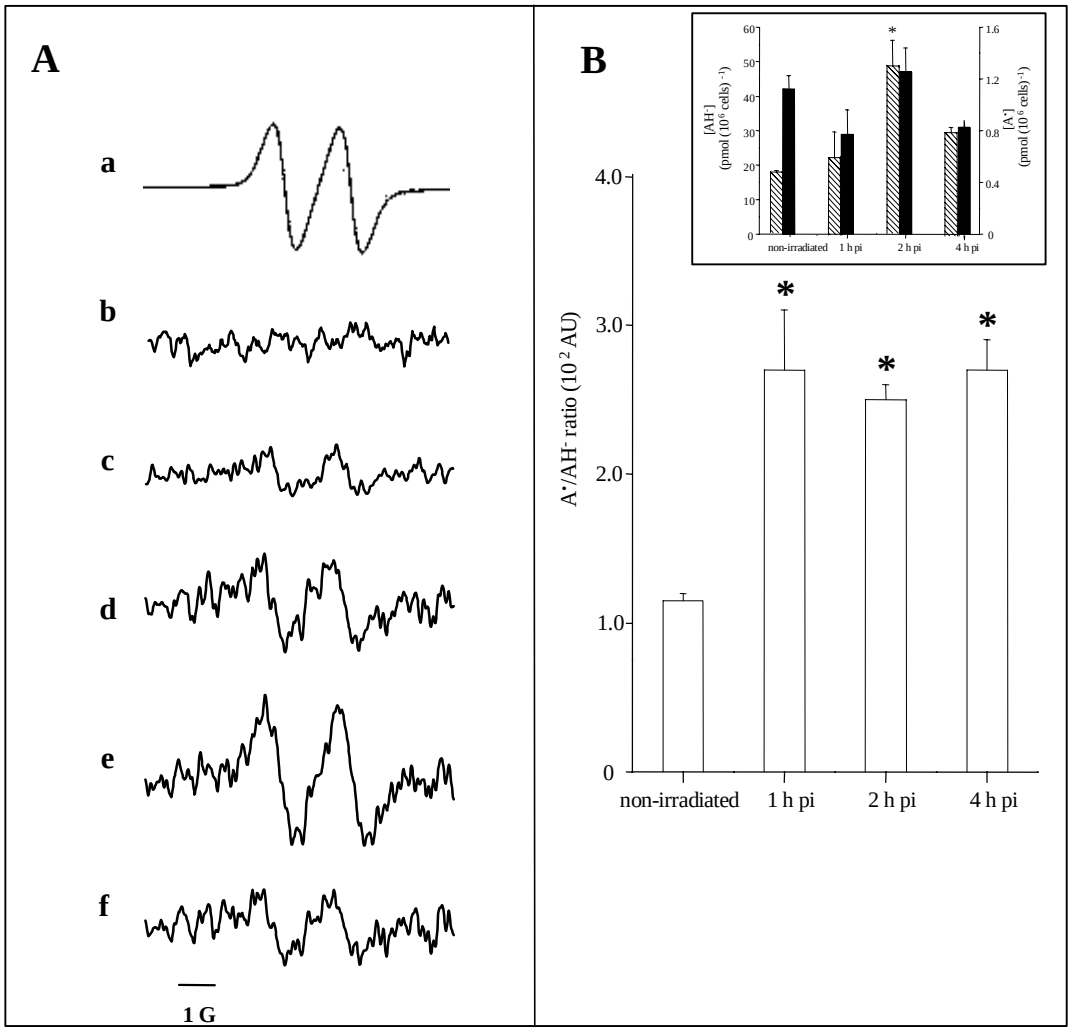


**Figure 1.** A) Typical EPR spectra of $A^{\bullet}$ in neural precursor cells irradiated *in vitro.* EPR signal from: (a) computer simulated spectrum employing the $A^{\bullet}$ parameters ($a_H$ = 1,88 G, g = 2,0054); (b) 1 mM DF in DMSO; (c) non-irradiated neural precursor cells; (d) neural precursor cells at 1 h pi; (e) at 2 h pi; (f) at 4 h pi. B) Effect of *in vitro* irradiation on the $A^{\bullet}/AH^{-}$ ratio in neural precursor cells. Inset: $A^{\bullet}$ (▨) and AH-(■) content in neural precursor cells irradiated *in vitro* at 1, 2 o 4 h pi. *significantly different from value in non-irradiated cells ($p < 0.05$).

exposure to γ-irradiation (Fig. 1B inset). $A^{\bullet}$ may be a terminal free radical product of free radical transformation in the biological antioxidant defense system but the $A^{\bullet}/AH^{-}$ is considered as a more appropriate index of oxidative stress [33]. Total content of $AH^{-}$ in precursor cells in culture was not significantly affected, as compared to the measured value in non-irradiated cells (Fig. 1B inset). The $A^{\bullet}/AH^{-}$ ratio was significantly increased in cells exposed to γ-irradiation, as compared to control cells in culture (Fig. 1B), suggesting that γ-irradiation of the cells in culture resulted in the triggering of oxidative stress in the cells within the first hour pi.

Since Fe is involved in the catalysis of reactions leading to reactive species generation, total Fe content was studied on the early stages after γ-irradiation. Non-significant changes in the total Fe content of the neural precursor cells irradiated *in vitro* were observed from 1 to 4 h pi, as compared to the content in non-irradiated neural precursor cells (Fig. 2B). Moreover, the LIP is the fraction of the total Fe directly responsible for catalyzing radical reactions. EPR detection of LIP by the signal of the DF-$Fe^{3+}$ complex is understood as a reliable method for detection, and the fluorescence method employing CA is seen as a very sensitive approach [34]. The LIP was measured by both methods to confirm the accuracy of the data. The typical EPR signal of DF-$Fe^{3+}$ complex is shown in Fig. 2A. Quantification of the spectra showed non-significant differences among the time period pi ($0.6 \pm 0.1$, $0.7 \pm 0.2$, $0.7 \pm 0.3$ and $0.6 \pm 0.2$ nmol $(Fe^{2+} + Fe^{3+})$ $(10^{6}cell)^{-1}$, in control and cells 1, 2 and 4 h pi, respectively). The effect of γ-irradiation on the LIP in the fetal neural precursor cells was studied by the fluorescence technique employing CA. As it is shown in Fig. 2B, LIP in

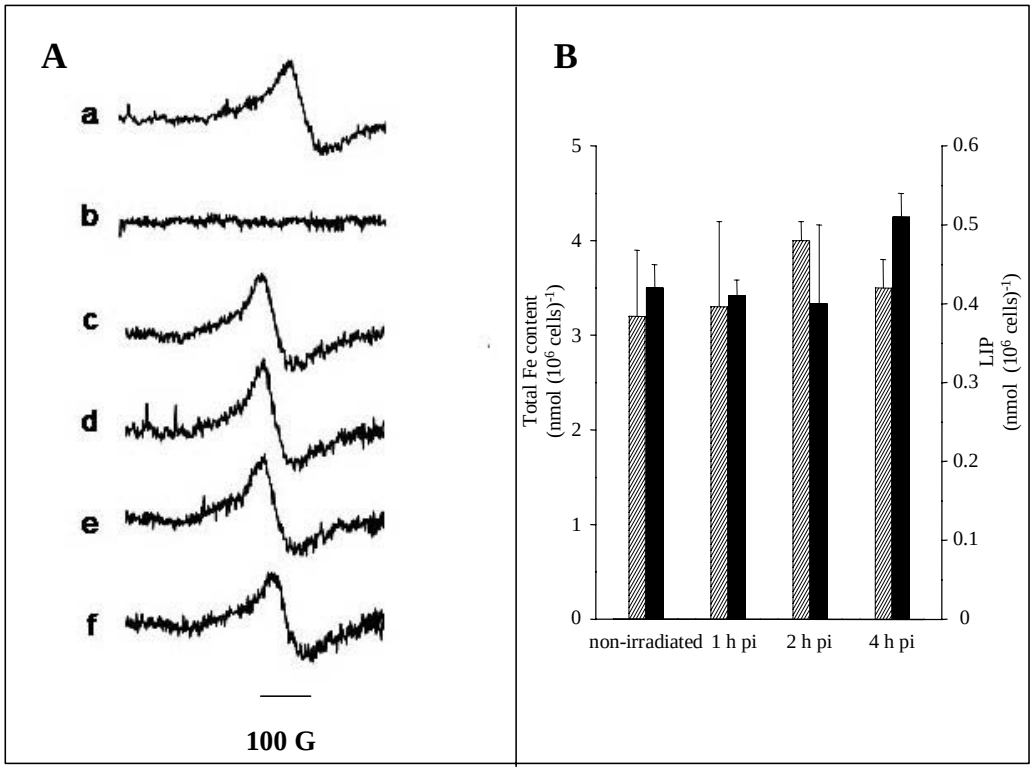


**Figure 2.** Total Fe and LIP content in neural precursor cells irradiated *in vitro*. A) LIP determination by EPR detection of DF-$Fe^{3+}$ complexes. EPR signal from: (a) 30 μM $Fe^{3+}$-100 mM DF (b) 100 mM DF alone; (c) non-irradiated neural precursor cells; (d) neural precursor cells at 1 h pi; (e) at 2 h pi; (f) at 4 h pi. B) Total Fe (▨) and LIP (■) content quantified by a fluorescence technique with the Fe sensor CA in non-irradiated neural precursor cells and in cells at 1, 2, and 4 h pi.

the cells was non-significantly affected upon the studied period, as compared to values in non-irradiated cells. The data shown here indicate that the LIP was unchanged by irradiation, and that the Fe pool available for detection seems to be the same, independent of the methodology employed in the cells irradiated *in vitro*.

In agreement with previous data from Gisone *et al*. [2] where TBARS content was shown to be significantly increased 4 h pi, TBARS content of the neural precursor cells irradiated *in utero* was 1.1 ± 0.3, 0.7 ± 0.3, 1.0 ± 0.3 and 2.1 ± 0.3 nmol $(10^6 \text{ cell})^{-1}$, in control and cells 1, 2 and 4 h pi, respectively, showing a significant increase 4 h pi as compared to control values. These data strongly suggested that the effect seen, employing brain homogenates, mostly reflected the response in the neural precursor cells. Accordingly, a significant increase (3.3-fold) in the total Fe content of the neural precursor cells irradiated *in utero* was observed 4 h pi, as compared to the content in non-irradiated cells (Fig. 3). Even though the capability to reduce ferric iron and transport it into the cytosol of neurons depends on the presence of molecules with ferric reductase activity, and it is not clear whether neurons contain a ferric reductase [35], the maximum capacity for reduction of Fe was not affected over the studied period pi (Fig. 3). Data in Fig 3 showed that the LIP, assessed by CA-fluorescence methodology significantly decreased (-31%) in cells 1 h pi, as compared to the non-irradiated cells in culture.

Ionizing radiation has been shown to induce an early increase in NO steady state concentration in homogenates from developing rat brain irradiated *in utero* with 1 Gy of γ-radiation on the 17 GD (Table 1). Data in Fig. 4 show that NO generation in the neural precursor cells from fetuses irradiated *in utero*, are significantly increased by 2.6-fold at 2 and 4 h pi.

## DISCUSSION

The experiments described here were performed to test if the effects on Fe increase observed in developing brain homogenates after exposure to γ-irradiation *in utero* (Table 1) were either due to (i) γ-irradiation direct effects through free radical generation that severely damaged cellular membranes allowing Fe to get freely in the cells, or (ii) γ-irradiation indirect effects through the mother-fetus interaction. The neural precursor

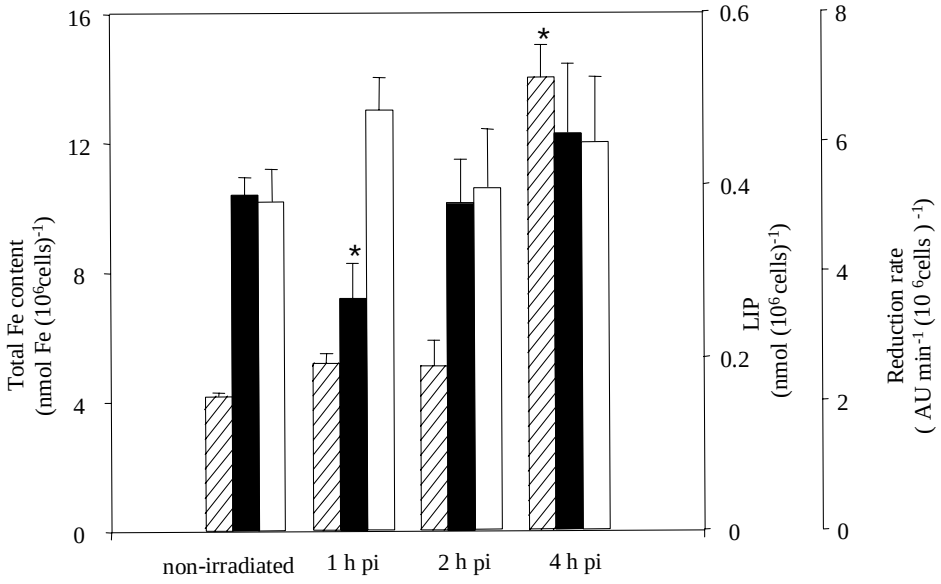


**Figure 3.** Total Fe and LIP content in neural precursor cells irradiated *in utero*. Total Fe (▨), LIP (■) content quantified by a fluorescence technique with the Fe sensor CA, and Fe reduction rate (□) in non-irradiated cells, and cells at 1, 2, and 4 h pi. *significantly different from value in non-irradiated cells ($p < 0.05$).

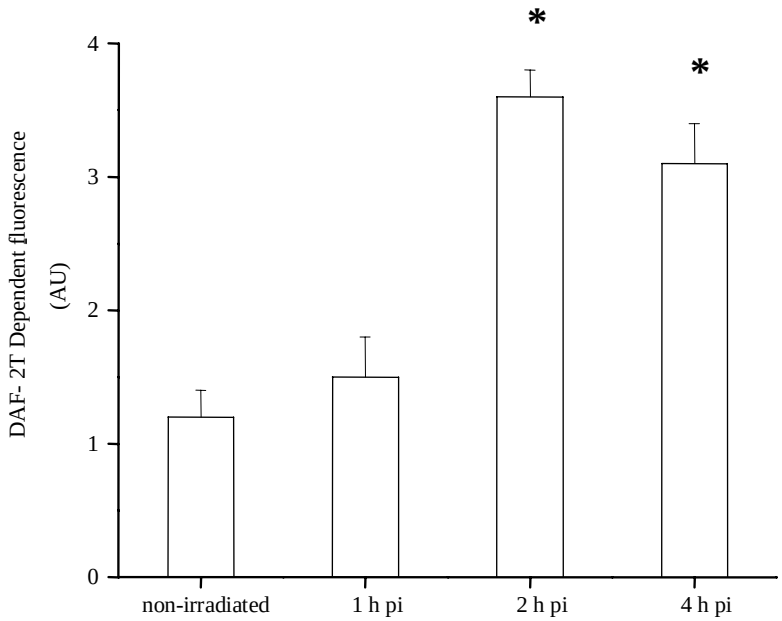


**Figure 4.** NO generation in neural precursor cells irradiated *in utero*. NO production was assessed employing the DAF-2T fluorescence technique. *significantly different from value in non-irradiated cells ($p < 0.05$).

cells irradiated *in vitro* showed a significant increase in the $A^{\bullet}/AH^{-}$ ratio indicating that oxidative stress was triggered in the hydrophilic cellular medium by exposure to γ-irradiation. Thus, the treatment could have been responsible for the establishment of radical-dependent damage that would lead to an intracellular Fe increase, by allowing the non-restricted Fe uptake from the incubation medium due to membrane deterioration. However, Gisone *et al*. [2] reported that both the $A^{\bullet}/AH^{-}$ ratio and oxidative damage were not affected in rat brain homogenates exposed to γ-irradiation *in utero* at the initial stages after irradiation as compared to non-irradiated brain homogenates. Thus, the *in vitro* irradiation of neural precursor cells did not seem to adequately reproduce the effect observed after *in utero* exposure to γ-irradiation. Moreover, as it is shown here, employing the model of irradiated neural precursor cells *in vitro*, neither total Fe nor the LIP content were increased under these experimental conditions. The results obtained in this study, strongly suggested that Fe increase observed in fetus brain homogenates after irradiation *in utero*, could not be interpreted as a direct effect of irradiation, even though the Fe content in the incubation media RPMI 1640 ($107 \pm 22$ μM) was high enough to allow Fe to get inside the cell where the Fe concentration is significantly lower, if the membranes had lost the permeability restrictions.

A whole different picture was seen when γ-irradiation was performed *in utero,* and a mother-fetus interaction was allowed over the studied period. Increases in both, Fe content and lipid peroxidation, were detected after 4 h of the exposure to γ-irradiation, following the profile previously described in brain homogenates from fetus irradiated *in utero* (Table 1). However, it should be pointed out that a different kinetic profile of the response was shown in neural precursor cells suggesting the contribution to the measured parameters of other cellular populations in the brain in the case of the whole homogenate as compared to the neural precursor cells culture. As fast as 1 h pi, the Fe content in the maternal plasma was increased by approximately 2-fold (Table 1), and a significant increase (2.3-fold) in the total Fe content of the fetal rat brain irradiated *in utero* was observed from 1 to 4 h pi, as compared to the content in non-irradiated fetal rat brain homogenates (Table 1). The data reported here showed that Fe content in the neural precursor cells was increased by approximately 3-fold 4 h pi,

suggesting that other cell types present in the brain (e.g. reticulo-endothelial cells) could be getting the Fe at an initial step to be later on transfer to the neural precursor cells. This assumption is consistent with the observation that TBARS generation, assumed as to strongly dependent on Fe content, was significantly increased by approximately 2-fold in neural precursor cells 4 h pi. However, the scenario seems to be more complex since the kinetic behavior of the LIP content did not follow this profile. One hour pi the LIP content in the neural precursor cells was decreased by 31%, and as previously postulated by Robello *et al*. [19] γ-irradiation of the developing rat brain could have triggered a network of signals that affected Fe distribution, and among the possible candidates to exert this action, NO is a multi-faced molecule with dichotomous regulatory roles. The rate of NO production and the interaction with biological molecules such as metal ions, thiols, protein tyrosines, and ROS, seems to determine the effect of NO [36]. Previous data from our laboratory [2] showed an early increase in nNOS activity detected 1 h after irradiation, and suggested that NO could play a protective role in the developing rat brain. The observed significant decrease in the LIP in neural precursor cells by γ-irradiation could strike as a paradox that could be interpreted assuming that Fe was chelated to NO generated as a response to γ-irradiation, leading to an increase in the concentration of dinitrosyl-Fe, dinitrosyl-diglutathionyl-Fe or dinitrosyl-glutathionyl-Fe complexes, among others. These complexes would be unable to induce oxidative stress, as it was suggested by Sergent *et al*. [37], in hepatocytes. Also, Lu and Koppenol [38] demonstrated, employing a chemical system, that NO can inhibit the Fenton reaction by reacting with $Fe^{2+}$ to form a nitrosylferrate$^{2+}$ complex. These authors suggested that in complex biological systems an excess of NO would bind to $Fe^{2+}$ and slow the Fenton reaction. This hypothesis may explain the beneficial effects of NO since the nitrosyl Fe complexes are stable and they are not detected as components of the catalytically active Fe pool identified by CA [19]. Even though the data reported here in isolated neural precursor cells, showed a significant increase in NO 2 h pi, NO is a diffusible molecule that, if it was being generated by other cells in the fetus brain as a consequence of the increase in the activity of nNOS, could get access to neural precursor cells to chelate catalytically active Fe *in vivo*. Moreover, in agreement with this hypothesis, at 4 h pi of neural precursor cells total Fe content was significantly increased, but the LIP was unchanged as compared to non-irradiated cells, probably due to the fact that NO generation was increased by 2.6-fold allowing the content of nitrosyl Fe complexes to be increased, but further experiments are needed to test this hypothesis.

Cells of the CNS, like other cells, have natural resistance mechanisms to nitro-oxidative stress exerted by the free radicals, and NO seems to be involved in several pathways, e.g. Bishop and Anderson [39] found that when motor neurons are pretreated with a subtoxic flux of NO (25 nM/s) they gain resistance to a normal cytotoxic flux (250 nM/s) of NO. As it was postulated by Robello *et al*. [19], the role of NO production could be understood (a) as a triggering factor for damaging cellular compounds, (b) as a cellular attempt to minimize cellular injury, or (c) a combination of the above. For NO both actions could be possible since it has a dual nature (induction of adaptive resistance and toxicity). The final effect will depend on its concentration, rate of release, the cellular environment into which it is released and the alteration of the cellular LIP. On this scenario, it could be postulated that the radiation-dependent damage, evidenced as cellular deterioration that lead to severe pathologies, could be related to the lack of success of maintaining over time the control of the activity of the potentially damaging species generated by γ-irradiation. Nevertheless, the data presented in this work showed that, as part of the network of the cellular responses to limit irradiation-dependent injury, a complex interaction between Fe and NO could be triggered not only in the whole brain homogenates, but also in isolated neural precursor cells as well.

## CONCLUSION

Taken as a whole, the data reported here, is the first evidence that the increase in Fe content associated to γ-irradiation in the developing brain requires the interaction of mother-fetus and it is

not a direct effect of irradiation. γ Irradiation *in uterus*, seems to be responsible for affecting Fe uptake by brain fetus, not only for increasing Fe availability in the maternal plasma but also for transporting Fe appropriately bound (e.g. to transferrin) that could easily be taken by the brain cells, as a substantial difference with Fe supplementation in the control medium of the culture. However, the nature of these interactions of mother-fetus is not clear yet. Further analyses are required to establish if alterations on the content in the mother, by oxidative situations or pathologies, could modify oxidative Fe metabolism in the developing brain by a common mechanism.

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