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# Free radicals act as effectors in the growth inhibition and apoptosis of iron-treated Burkitt's lymphoma cells

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**Abstract**
The addition of ferric citrate to Burkitt's lymphoma (BL) cell lines inhibits growth, leads to the accumulation of cells in the phase $G_2$/M of the cell cycle and to the modulation of translocated *c-myc* expression. The increase in the labile iron pool (LIP) of iron-treated BL cells leads to cytotoxicity. Indeed, intracellular free iron catalyzes the formation of highly reactive compounds such as hydroxyl radicals and nitric oxide (NO) that damages macromolecular components of cells, eventually resulting in apoptosis. In this report, we have investigated the possible involvement of free radicals in the response of Ramos cells to iron. When added to Ramos cells, iron increased the intracellular levels of peroxide/peroxynitrite and NO. Moreover, the addition of free radicals scavengers (TROLOX® and Carboxy-PTIO) neutralized the effects of iron on Ramos cells while addition of an NO donor or hydrogen peroxide ($H_2O_2$) to cells generated effects which partially mimicked those induced by iron addition. Collectively, our results suggest the involvement of free radicals as effectors in the iron specific growth inhibition of BL cells observed *in vitro*.

**Keywords:** *Free radicals, iron, oxidative stress, Burkitt's lymphoma*

## Introduction

Iron is an essential nutrient that participates in several biological reactions, ranging from oxygen transport to DNA synthesis. Uptake and storage of iron are highly regulated through post-transcriptional regulatory mechanisms. Cytoplasmic RNA-binding proteins, designated as iron-regulatory proteins (IRPs), respond to changes in cellular iron availability and coordinate the expression of mRNAs bearing IRP binding sites called iron-responsive elements (IRE) [1,2]. IRE motifs are located on a number of mRNAs encoding proteins involved in iron homeostasis such as ferritin and the transferrin receptor TfR [3–5]. These mechanisms operated so as to control intracellular iron levels [6].

Whenever intracellular iron concentration exceeds the metabolic needs of the cell, it may form a low molecular weight pool referred to as LIP, which becomes available for catalyzing the conversion of reactive oxygen species (ROS) into highly toxic free radicals. In conditions of iron excess, LIP can convert normal by-product of cell respiration, like super oxide anion ($O_2^{\cdot -}$) and hydrogen peroxide ($H_2O_2$) into highly damaging hydroxyl radicals or equally aggressive ferryl ions or oxygen-bridged Fe(II)/Fe(III) complexes [7]. Moreover, there is evidence suggesting that iron has a necessary role in supporting transcription of certain genes, such as NO synthase [8] and that accumulation of iron leads to increased steady-state NO levels [9].

These toxic compounds are the main causative agents of oxidative damage to proteins, nucleic acids and lipids [10]. The mechanisms of iron cytotoxicity also involves oxidative destabilization of lysosomes,

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leading to the leakage of digestive enzymes into the cell cytoplasm and ultimately to apoptosis [11].

Diseases related to iron overload, such as hereditary hemochromatosis or transfusional siderosis, are characterized by excessive iron accumulation, resulting in tissue damage and organ failure [12]. Genetic predisposition to iron overload can also manifest itself at the cellular level. Indeed, cells with a high level of expression of TfR and a reduced iron storage capacity owing to genetic predisposition are more susceptible to iron overload [13].

Therefore, the propensity of certain cells to accumulate free iron could favor the production of highly reactive free radicals damaging cellular components. Ramos cells, a Burkitt's lymphoma (BL) cell line, accumulate high levels of free cytoplasmic iron when incubated in the presence of ferric citrate. This treatment results in growth inhibition, blockade in $G_2$/M and down-modulation of translocated *c-myc* expression, which eventually lead to apoptosis [14]. The oxidative stress resulting from iron overload could enhance the formation of free radicals, particularly hydroxyl radicals, which could act as effectors in iron-induced apoptosis of BL cells. In the work described herein, we have evaluated the possible involvement of free radicals in the response of Ramos cells to iron treatment.



## Experimental procedures

### *Materials and cell culture*

Ferric citrate (Sigma, Oakville, Ont.) was dissolved in deionised water (100 mM) and pH adjusted to 7.2 with NaOH. Ferric citrate was used at a final concentration of 1 mM in cell cultures. Diethylenetriaamine NONOate is a NO donor that spontaneously dissociates in a pH-dependent, first-order kinetics with a half-life of 20 and 56 h at 37 and 22–25°C, pH 7.4, respectively, generating two moles of NO per mole of initial reagent. DETA NONOate (Cayman Chemical, Ann Harbor, MI) was dissolved in an alkaline solution of NaOH 0.1 M and used at 50 μM. $H_2O_2$ (Sigma) was used at 100 μM.

Human BL cell line Ramos (ATCC, Manassas, VA) was grown in Iscove's modified Dulbecco's medium (GIBCO/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Cell density and viability were monitored daily by trypan blue dye exclusion using a haemacytometer. The cell line used in this work was mycoplasma-free.

### *Free radicals scavengers*

*Peroxide and peroxynitrite scavenger*. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX®) is a cell-permeable, water-soluble derivative of vitamin E with potent antioxidant properties that prevents peroxide and peroxynitrite-mediated oxidative stress and apoptosis. TROLOX® (CEDARLANE Laboratories Ltd. Hornby, Ont.) was dissolved at 200 mM in methanol and further used in culture at 250 μM. Methanol in culture represent less than 0.1% and do not affect cells (data not shown).

*Nitric oxide (NO) scavenger*. 2-(4-Carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazol-1-yloxy-3-oxide (Carboxy-PTIO) potassium salt reacts with NO to form carboxy-PTI derivatives which in turn inhibits NO synthase. Carboxy-PTIO potassium salt (Sigma) was dissolved in $H_2O$ and used in culture at 20 μM.

### *Detection of intracellular $H_2O_2$*

Dihydrorhodamine 123 (Invitrogen Canada, Inc., Burlington, Ont.) is the uncharged and nonfluorescent reduction product of the membrane-permeable, mitochondrion-selective dye rhodamine 123 which, once inside the cell is oxidized to cationic rhodamine 123. Dihydrorhodamine 123 reacts with intracellular $H_2O_2$ and peroxynitrite. Cells were incubated in the absence or presence of 1 mM ferric citrate and free radicals scavengers (TROLOX and/or Carboxy-PTIO) or NONOate alone for 24 h. Cells were then washed with ice-cold phosphate buffer saline (PBS) and resuspended in 500 μl dihydrorhodamine 123 (10 μg/ml in 1 × PBS). Cells were incubated at 37°C for 30 min and washed with 1 × PBS. Samples were analyzed by flow cytometry on a FACSCalibur flow cytometer using CELLQUEST software (Becton Dickinson, Palo Alto, CA). Statistical comparisons between $Fe^+$ and other conditions were obtained using a two-tailed paired Student's *t*-test.

### *Detection of intracellular nitric oxide*

4,5-Diaminofluorescein diacetate (DAF-2DA) is a cell-permeable, highly sensitive and specific fluorescent indicator for the direct detection of NO. The relatively non-fluorescent DAF-2DA reacts rapidly with NO in the presence of oxygen to yield the highly fluorescent triazolofluorescein compound (DAF-2T). DAF-2DA can detect NO under neutral conditions and can be used in fluorescence microscopy or flow cytometry to measure real-time changes in NO levels in live cells. Cells were incubated in the absence or presence of 1 mM ferric citrate and free radicals scavengers (TROLOX and/or Carboxy-PTIO) or NONOate alone for 72 h. Cells were then washed in ice-cold PBS and resuspended in 500 μl DAF-2DA (EMD Biosciences, San Diego, CA) (1 μM in 1DA PBS). Cells were incubated at 37°C for 30 min and washed with 1 × PBS. Samples were analyzed by flow cytometry on a FACSCalibur flow cytometer for NO detection using CELLQUEST software. Statistical comparisons between $Fe^+$ and other conditions were obtained using a two-tailed paired Student's *t*-test.

*Cell cycle analysis*

Cells were harvested, washed in 1 × PBS and stained with propidium iodide (PI) for DNA content determination, as previously described [15]. Samples were analyzed by flow cytometry on a FACSCalibur flow cytometer using CELLQUEST software. Cytometry results were processed using ModFit LT version 2.0 software (Verity Software House Inc., Topsham, ME). Statistical comparisons between $Fe^{+}$ and other conditions were obtained using a two-tailed paired Student's *t*-test.

*Detection of abasic sites in genomic DNA*

Cells were incubated in the absence of presence of 1 M ferric citrate and free radicals scavengers (TROLOX and/or Carboxy-PTIO), or NONOate alone for 72 h. Afterwards, $6 \times 10^6$ cells were harvested and washed in ice-cold PBS and resuspended in 200 µl 1 × PBS containing 3 mM aldehyde reactive probe (ARP) reagent (N′-aminooxymethylcarbonylhydrazino-D-biotin) (Invitrogen Canada, Burlington, Ont.), which reacts specifically with aldehyde groups exposed by the open ring forms of apurinic/apyrimidinic sites in DNA. Cells were incubated at 37°C for 60 min, washed twice with 1 × PBS and lysed at 50°C for 3 h in 300 µl lysis buffer (100 mM NaCl, 10 mM Tris–HCl pH 8.0, 25 mM EDTA, pH 8.0, 0.5% (w/v) SDS, 0.1 mg/ml proteinase K). Genomic DNA purification was performed by two standard phenol/chloroform/iso-amyl alcohol extractions, followed by precipitation in 2 volumes of 100% ethanol and 1/2 volume of 7.5 M ammonium acetate. DNA was washed with 70% ethanol, resuspended in deionised water, and heated at 65°C for 15 min. Aliquots of genomic DNA (1 and 0.5 µg in final volumes of 5 µl) were heated at 94°C for 5 min and loaded in triplicate on a positively charged nylon membrane (Roche, Laval, QC). DNA was UV cross-linked (UV Stratalinker® 1800, Stratagen, La Jolla, CA) and stained with methylene blue as loading control. The blot was washed with washing buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% (v/v) Tween 20) for 5 min and incubated at room temperature for 30 min in a blocking solution [16]. Streptavidin–alkaline phosphatase conjugate diluted 1/5000 (Avidix-AP, Tropix, Applied Biosystems, Foster City, CA) was then added to the membrane for an additional 30 min. The membrane was washed once with blocking solution for 10 min and subsequently thrice with washing solution for 10 min. Revelation was performed using CDP-Star™ substrate as recommended by the manufacturer (New England Biolabs, Beverly, MA).

*Northern blot analysis*

Total RNA was extracted from $3 \times 10^6$ cells using TRIZOL reagent (Invitrogen, Carlsbad, CA), as specified by the manufacturer. Five microgram of RNA from Ramos cells was analyzed by northern blot using a non-radioactive method described previously [16]. A DIG-labelled *c-myc* probe was prepared by RT-PCR from Jurkat cell RNA using DIG-labelled nucleotides (Roche) and the following primers: forward 5′-GAA-GATCTATGCCCCTCAACGTT-AGCTT-3′ and reverse 5′-GCCTTGAGAACAC-GCAT-TCCTCTAGAAG-3′. A *G3PDH* probe was prepared by PCR on reverse transcription product from Ramos cell RNA, using DIG-labelled nucleotides and the following primers: forward 5′-TGAAGGTC-GGAGTCAACGGATTGG-3′ and reverse 5′-CAC-CACCTGGAGTACCGGGTGTAC-3′. *G3PDH* and *c-myc* were simultaneously detected on the same blot. Assayed mRNAs were normalized using *G3PDH* and AlphaImager densitometry software.

## Results

*The effect of iron growth of Ramos cells is counteracted by free radicals scavengers*

In order to determine whether the iron-induced growth inhibition of Ramos BL cells involves free radicals, we have evaluated the effect of iron in the absence or presence of free radicals scavengers on Ramos cells maintained in culture for up to 72 h. We have previously demonstrated that treatment of Ramos cells with 1 mM ferric citrate substantially decreases cells proliferation and viability [14]. As shown in Figure 1, the addition of TROLOX®, a peroxide and peroxynitrite scavenger, to iron-treated cells does not restore proliferation but slightly reduces cell death. However, the addition of Carboxy-PTIO, a NO scavenger permitted substantial growth of iron-treated cells whereas combining TROLOX® and Carboxy-PTIO completely neutralized the effect of iron, allowing the culture to reach a cellular density comparable to sham-treated cells and to maintain a viability that exceeded 94%. To determine the implication of NO on growth inhibition, NONOate, a NO donor, was added to the cell culture. As shown in Figure 1, we observed that 50 mM of NONOate reproduce the effects of iron; lower concentration had no effect while higher concentrations were toxic for the cells (data not shown). At 24 and 48 h, growth inhibition of Ramos cells induced by NONOate or iron was similar. Thereafter, a slight increase in cell density was observed for NONOate treated cells vs iron treated cells, probably the results from NONOate exhaustion in the culture medium. Moreover, in contrast to the effect of iron, cell viability was barely affected at 72 h. Collectively, these results suggest that the cell growth appears to be mediated by a production of free radicals, since addition of free radicals scavengers to iron-treated cells allows cell proliferation while simultaneously limiting cell death. Moreover, the addition of NONOate to Ramos cells inhibits cell growth, as observed for iron treatment.

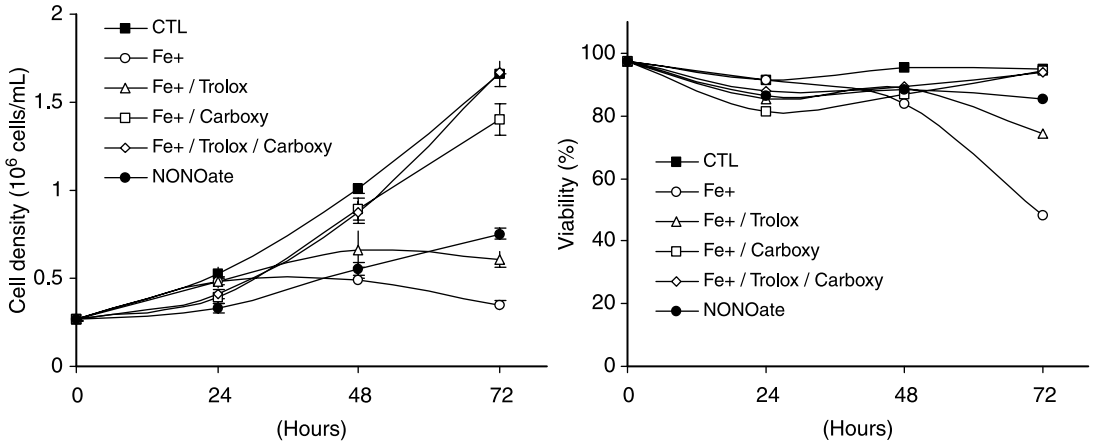


Figure 1. Effect of ferric citrate on Ramos cells in the absence or presence of free radicals scavengers. Ramos cells were grown with or without (CTL) 1 mM ferric citrate, free radicals scavengers (TROLOX® and/or Carboxy-PTIO), or with NONOate alone for up to 72 h. Cell proliferation and viability were evaluated at the indicated times by trypan blue dye exclusion using a standard haemocytometer. Results represent the mean of three independent experiments, with error bars corresponding to standard deviations.



*Iron addition increases intracellular free radicals in Ramos cells*

To confirm that addition of iron to Ramos cells lead to oxidative stress and increased production of free radicals, we have determined the levels of free radicals following iron treatment at different time of the culture. As shown in Figure 2A, iron-treated cells display the higher levels of peroxide and peroxynitrite, after 24 h, which exceed by more than two-fold the basal level detected in sham-treated cells. However, the presence of TROLOX® significantly inhibited peroxide/peroxynitrite accumulation in iron-treated cells ($p < 0.01$). Scavenging intracellular NO through Carboxy-PTIO addition also reduced iron-induced ROS generation, mainly by inhibiting the formation of peroxynitrite. Addition of both scavengers completely blocked the increase of free radicals levels induced by iron treatment ($p < 0.01$). Finally, addition of NONOate alone had no effect on peroxide/peroxynitrite levels.

Since iron could increase the activity of NO synthase [8], we have analyzed the level of NO 72 h following iron addition (optimal response, data not shown). As shown in Figure 2B, iron addition to Ramos cells augments intracellular NO levels. This increase can be partially inhibited by Carboxy-PTIO, whereas inhibition is virtually complete when both TROLOX® or Carboxy-PTIO are added ($p < 0.05$). Results obtained with TROLOX alone are not significant if Ramos cells treated with NONOate alone show a higher level of NO than sham-treated cells; however, the detected level is lower than for iron treated cells which could be explained by the exhaustion of NONOate in culture medium after 72 h of culture.

*ROS mediates $G_2$/M cell cycle arrest in iron-treated Ramos cells*

We have previously reported that Ramos cells treated with iron accumulate in the $G_2$/M cell cycle phase [14]. To gain further insights into the mechanisms leading to this specific effect, we have investigated the relationship between $G_2$/M accumulation and free radical production. As previously described [14] and shown in Figure 3, incubation of Ramos cells in the presence of ferric citrate for 72 h considerably increased the percentage of cells in $G_2$/M, from a steady-state level of 12% for sham-treated cells to 34% for iron-treated cells ($p < 0.01$). However, in the presence of peroxide and peroxynitrite scavengers, iron-treated cells did not accumulate in $G_2$/M, yielding a percentage of cells in $G_2$/M identical to that of untreated cells, thereby strongly suggesting that ROS might play a major role in the cell cycle deregulation observed for iron-treated cells ($p < 0.05$). Interestingly, the use of NO scavenger only slightly decreased the percentage of cells in $G_2$/M from 34 to 27%, which is not significant ($p > 0.5$), whereas addition of NONOate had no effect on $G_2$/M accumulation. These results seem to indicate that NO is not centrally involved in the mechanism by which iron-treated Ramos cells accumulate in $G_2$/M. The minor reduction of $G_2$/M cells observed in Carboxy-PTIO treated cells could be caused by the single inhibition of peroxynitrite formation resulting from the absence of NO. Finally, addition of both scavengers inhibited the accumulation of cell in $G_2$/M, with a percentage of cells in $G_2$/M lower than 15%, which is relatively similar to control cells. Exogenous addition of $H_2O_2$ appears to have no effect on $G_2$/M accumulation, which may rely on the instability of this product.

*Iron treatment of Ramos cells leads to DNA damage through free radicals production*

Oxidative damage to DNA results from the interaction of DNA with ROS, in particular the hydroxyl radical,

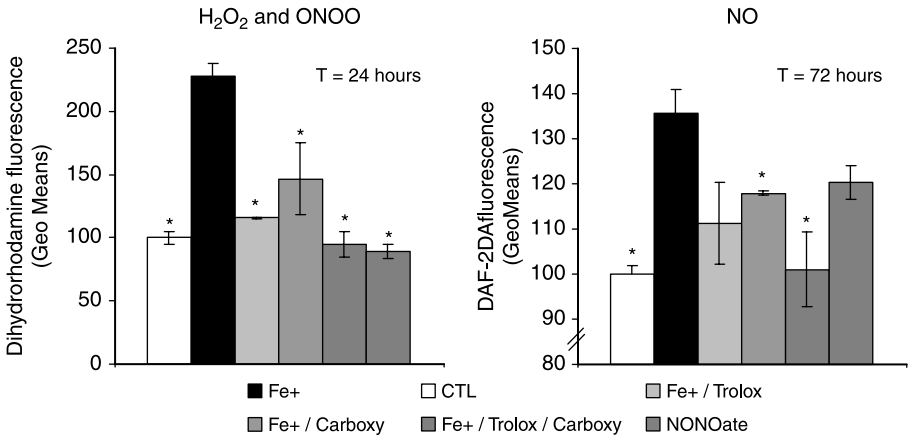


Figure 2. Peroxide, peroxynitrite and NO detection in Ramos cells. Detection of free radicals by flow cytometry in Ramos cells either sham-treated or incubated in the presence of 1 mM ferric citrate and free radicals scavengers (TROLOX® and/or Carboxy-PTIO), or NONOate alone. Detection of peroxide and peroxynitrite was performed using (A) Dihydrorhodamine 123 after 24 h of incubation while (B) NO detection was performed using DAF-2DA after 72 h of incubation. Results represent the mean of three independent experiments, with error bars corresponding to standard deviations. *p* values determined by a two-tailed Student's *t*-test comparing by pairs the level of free radicals of $Fe^+$ treated cells to other conditions were statistically significant ($\star p < 0.05$).

which is converted from superoxide and $H_2O_2$ by Fenton reaction and involves REDOX reactions with iron. Hydroxyl radicals have the potential to generate a number of modifications on the DNA, such as cleavage of the deoxyribose-nitrogenous base bond to release free bases and leaving abasic sites (AP sites), deoxyribose modifications and strand breaks [17]. In fact, AP sites are one of the major types of damages generated by ROS [18]. DNA damage ultimately steer cells to apoptosis. We have previously reported that a 72 h treatment with iron induces apoptosis of Ramos cells [14]. To confirm that the formation of free radicals resulting from iron-induced oxidative stress contributes to apoptosis induction, we have analyzed the effect of iron on DNA damage, in particular, the generation of AP sites. We therefore treated genomic DNA from 72 h iron-treated Ramos cells with ARP reagent, which allows tagging AP sites with biotin. AP sites were then detected using alkaline phosphatase-conjugated streptavidin. Methylene blue staining was used to confirm to equal loading of DNA for each conditions. As shown in Figure 4, genomic DNA extracted from Ramos cells treated with iron gives a clear ARP signal, indicating multiple apurinic/apyrimidinic sites. In contrast, signal produced with DNA from sham-treated cells were close to the limit of detection, likely as a result of basal level of apurinic and apyrimidinic sites. Although, AP sites likely result from the presence of excess ROS in iron-treated cells, TROLOX®, a ROS scavenger, failed to protect the DNA from such damage. One possible explanation for the discrepancy is that a concentration of 250 μM

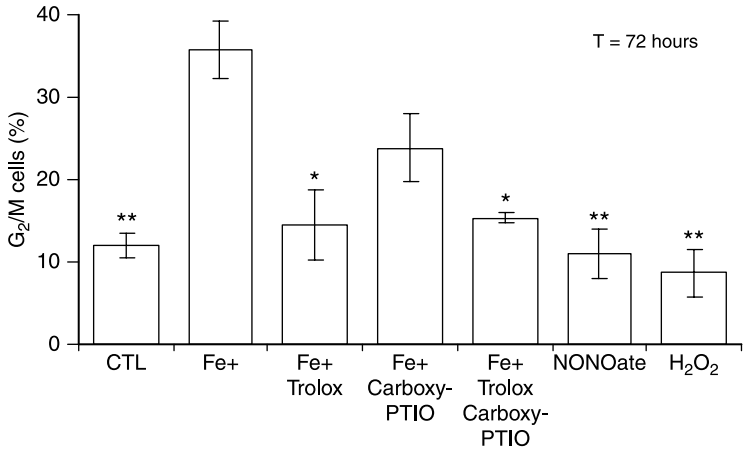


Figure 3. Accumulation of cells in $G_2$/M. Cells were grown for 72 h in the absence (control) or presence of 1 mM ferric citrate and free radicals scavengers (TROLOX® and/or Carboxy-PTIO), or NONOate alone. Cells were processed for cell cycle analysis by flow cytometry after PI staining at 72 h. The percentages of cells in $G_2$/M are displayed. Results represent the mean of three independent experiments, with error bars corresponding to standard deviations. *p* values determined by a two-tailed Student's *t*-test comparing by pairs the percentage of cell in $G_2$/M of $Fe^+$ treated cells to other conditions were statistically significant ($\star p < 0.05$ and $\star\star p < 0.01$).

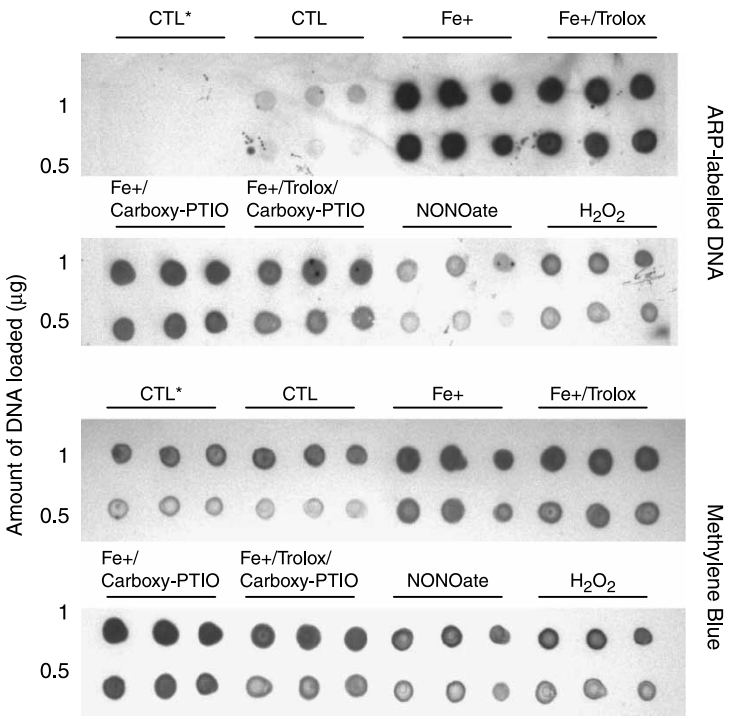


Figure 4. Detection of AP sites in genomic DNA from treated Ramos cells. Ramos cells were sham-treated or grown in the presence of 1 mM ferric citrate and free radicals scavengers (TROLOX® and/or Carboxy-PTIO), NONOate or $H_2O_2$ for 72 h. With the exception of samples labelled "CTL*", cells were treated with ARP$^{\text{-Biotin}}$. Detection of ARP$^{\text{-Biotin}}$ labelled genomic DNA was performed as described in "Experimental procedures" section. Membranes were first stained with methylene blue as DNA loading control (lower panel). Each condition was performed in triplicates for 1 µg and 500 ng of genomic DNA. Data are representative of at least three independent experiments.



TROLOX® might have been insufficient to prevent damage and growth inhibition (Figure 1). In contrast, blocking iron-induced NO formation by Carboxy-PTIO appears to limit DNA damage, reducing the ARP signal by at least two-fold. Likewise, simultaneous scavenging of NO and peroxide/peroxynitrite significantly reduced DNA damage. Finally, exogenous addition of NONOate and $H_2O_2$ could not generate a number of AP sites as high as expected, which could be explained by the degradation of both compounds after 72 h in culture medium and by the difficulty of generating endogenous concentrations equivalent to those induced by iron. Still, for both free radicals donors the detected ARP signals appear to be stronger than those detected from sham-treated cells.

### *c-myc expression is modulated by NO*

Proliferation of BL cells is tightly linked to *c-myc* expression. Moreover, inhibition of cell growth after iron treatment is associated with a decrease in *c-myc* expression [14]. Since iron treatment induces an increase in free radicals levels and that growth inhibition caused by this treatment can be counteracted by the addition of a NO scavenger, we hypothesized that NO could modulate the decrease in *c-myc* expression observed when Ramos cells are treated with iron. We therefore, analyzed the expression of *c-myc* in iron-treated Ramos cells in the presence of free radicals scavengers.

As shown in Figure 5A, the level of *c-myc* mRNA significantly decreased as a function of time in Ramos cells treated with ferric citrate. In general, scavenging peroxide/peroxynitrite by TROLOX® treatment in the presence of iron does not exert any effect on iron-induced *c-myc* decline, suggesting that ROS are not involved in *c-myc* modulation. However, it appears that at 48 h *c-myc* expression is slightly increased in $Fe^{+}$/Trolox vs iron treatment alone. Further studies are required to characterize this phenomenon. In contrast, addition of Carboxy-PTIO, alone or in conjunction with TROLOX®, completely blocked the effect of iron on *c-myc* mRNA, so that *c-myc* profiles were remarkably similar to those found in untreated cells even slightly increased at 48 h. The ability of Carboxy-PTIO to rescue *c-myc* expression in iron-treated Ramos cells strongly suggests that NO is involved in *c-myc* modulation by iron.

To confirm this hypothesis, we have analyzed the effect of an NO donor on *c-myc* expression in iron-treated Ramos cells. *c-myc* mRNA was analyzed by Northern and levels were normalized with *G3PDH* levels (Figure 5B). We observed that subsequent to NONOate addition, *c-myc* levels decreased during the first 24 h of incubation, reaching a level more than two-fold lower than untreated cells and slightly below

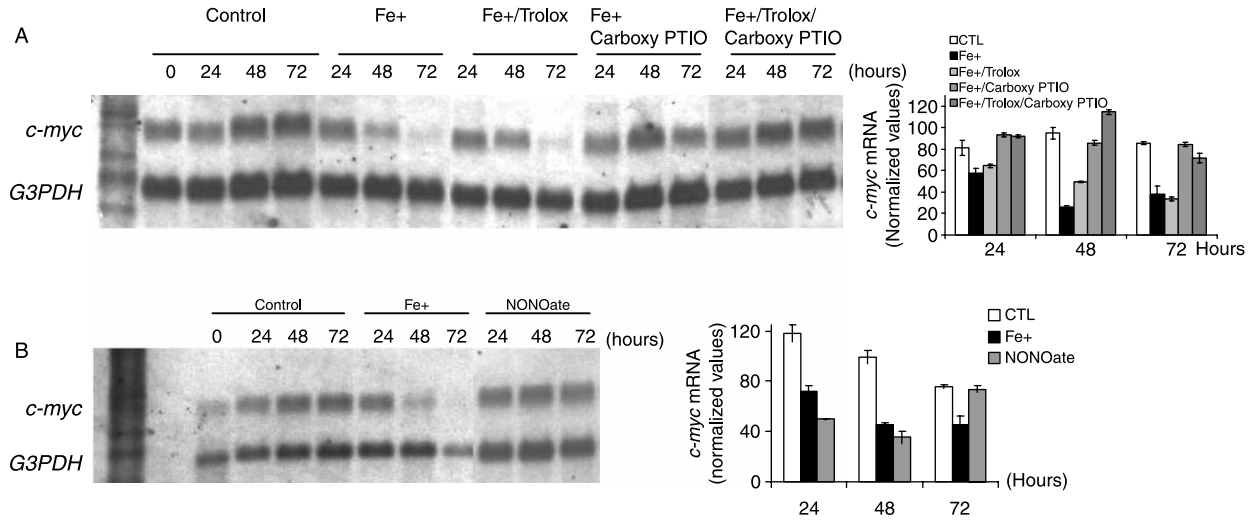


Figure 5. *c-myc* expression in iron-treated Ramos cells in the absence or presence of free radicals scavengers or NO donor. Total RNA from Ramos cells was subjected to Northern blot analysis after 0, 24, 48 or 72 h of culture in the presence of (A) no additive (CTL) or 1 mM ferric citrate ($Fe^{+}$) and free radicals scavengers (TROLOX® and/or Carboxy-PTIO) or (B) no additive (CTL) or 1 mM ferric citrate ($Fe^{+}$) or NONOate. Both blots were simultaneously hybridized with a *c-myc* and a *G3PDH* probes, the latter serving as loading control. Bands corresponding to detected mRNA species are labelled accordingly. Blots are representative of three independent experiments and graphs represent the mean of three independent experiments, with error bars corresponding to standard deviations.

iron-treated cells. This down modulation of *c-myc* expression appears to have the same kinetic that iron-treated cells. However, after 72 h, *c-myc* levels seemed to be restored at the normal that could be explained by the exhaustion of NONOate in the culture media. These results clearly point out the contribution of NO in the regulation of *c-myc* expression and suggest that the effect of iron on *c-myc* implicate the increase of NO originating from the oxidative stress provoked by iron.

## Discussion

BL cells are characterized by an excessive proliferation attributable to the chromosomal translocation and consequent over-expression of the *c-myc* gene. This genomic alteration is also responsible for the disrupted iron homeostasis in cell lines derived from BL, such as Ramos. The over-expression of *c-myc* contributes to the increase of free intracellular iron concentration, mainly by intensifying uptake and reducing storage of iron. BL cells are then more likely to accumulate free iron, which can, in excess, lead to oxidative stress. Indeed, exogenous addition of iron to Ramos has been shown to induce growth inhibition, $G_2$/M accumulation, *c-myc* modulation and apoptosis [14].

The oxidative stress generated by iron overload in Ramos cells leads to an augmentation of free radicals such as NO and ROS. According to our results, addition of 1 mM ferric citrate to Ramos cells increases steady-state levels of peroxide/peroxynitrite by more than two-fold and NO by almost 150%. This strongly suggests that iron contributes to increased mitochondrial metabolism. Indeed, ROS arise chiefly from normal metabolism, primarily from the mitochondrial respiratory chain wherein excess electrons are donated to molecular oxygen and protons to form water molecules. It seems that cells with highly active mitochondria generate more ROS, whose toxicity synergizes with that intracellular iron. This suggests that iron may be preferentially toxic to cells with high mitochondrial activity [11]. Proliferating cells, like cancer cells, possess highly active mitochondria, which might further sensitize cells to iron. Moreover, mitochondrial iron accumulation leads to localized enzymatic damage and reduced mitochondrial functions, which favors cell engagement in apoptosis pathway.

The toxicity resulting from iron overload can be attributed to insults caused by ROS and NO. Our results indicate that growth inhibition resulting from iron treatment can be counteracted by the addition of free radicals scavengers. However, NO scavenging alone is sufficient to prevent growth inhibition and cell death.

According to the present study, the modulation of *c-myc* expression observed upon iron treatment appears to be related to NO levels. In fact, iron-treated cells displayed a significantly reduced *c-myc* mRNA levels, while addition of NO scavengers clearly prevented the down-modulation of *c-myc* expression. Moreover, addition of NO-donor induced a decay in *c-myc* level similar to iron treatment, reinforcing the participation of NO in *c-myc* regulation.

NO plays a critical role as a molecular mediator in a variety of physiological processes. It has been reported that NO donors can directly inhibit the DNA binding activity of NF-κB family proteins and thereby modulate the expression of NF-κB responsive genes [19]. Regulation of *c-myc* is one of the many process

influenced by the transcriptional activity of NF-κB. In the case of BL cells, a study has reported that NF-κB plays an important role in the dysregulation of the translocated *c-myc* gene [20]. Moreover, further evidence suggests that NO modulates the transcriptional activity of the *c-myc* gene by dissociating the active form of the NF-κB complex [21]. On the basis of our results, as well as previous studies, we believe that addition of iron to Ramos cells contributes to an increased NO production through the enhancement of NO synthase activity [8]. Although, in this report we can not conclude on the origin of NO generated following iron treatment, this increase in intracellular NO levels would inhibit NF-κB binding to the *c-myc* promoter, leading to a decreased transcriptional activity [22].

The decline in *c-myc* attributable to NO appears to be insufficient by itself to provoke cell death, since the decrease in *c-myc* expression following NONOate treatment was not accompanied by a notable reduction in culture viability. However, apoptosis initiates in mitochondria, which is the primary site of oxidative stress. Free radicals produced after iron exposure induce DNA damage (apurinic/apyrimidic sites), may contribute to apoptosis induction.

As a result of the aberrant *c-myc* over-expression, iron homeostasis is disrupted. Owing to its transcriptional activities, *c-myc* directly represses transcription of the gene encoding ferritin, a protein that sequesters free iron. *c-myc* also induces the synthesis of IRP-2, a iron regulatory protein that is normally degraded in high iron conditions. IRP-2 post-transcriptionally regulates the stability of *TfR* mRNA, increasing iron uptake capacity of the cell. Simultaneously, IRP-2 blocks the translation of ferritin, thereby mitigating any increases in free intracellular iron. Thus, iron overload can result in oxidative stress through aberrant mitochondrial metabolism. Superoxide and $H_2O_2$ thus produced can be converted into highly toxic free radicals such as hydroxyl radical through a reaction catalyzed by free iron. Moreover, iron directly contributes to increase the activity of NO synthase, which up-regulates intracellular NO levels. Consequently, ROS promote the accumulation of cells in $G_2$/M, whereas NO modulates expression of the *c-myc* gene with consequent growth inhibition. Free radicals also damage DNA, which are contributing factors in apoptosis initiation.

Free radicals produced by iron overload also contribute by themselves to homeostasis disruption. Indeed, many studies have underscored the involvement of $H_2O_2$ and NO in the modulation of cellular iron metabolism [23]. According to these studies, $H_2O_2$ regulates cellular iron acquisition and intracellular iron distribution by blocking both IRP-1-dependent and -independent mechanisms [24]. Moreover $NO^+$, a redox species of NO that interacts primarily with iron, can activate IRP-1 binding activity, resulting in a increased *TfR* mRNA levels and consequently to elevated iron uptake [25,26]. However, in the same study, the authors suggested that NO mediates IRP-2 degradation, while a recent study mentioned that NO promotes IRP-2 stabilization [27]. Nevertheless, these studies suggest that NO and $H_2O_2$ participate in the modulation of iron metabolism by promoting intracellular iron accumulation and consequent increased iron labile pool.

Collectively, it appears that iron exerts pleiotropic effects on Ramos cells by a mechanism involving increased intracellular ROS and NO acting as effectors in the accumulation of cells in $G_2$/M, growth inhibition, decreased *c-myc* expression and ultimately apoptosis. The design of therapeutic strategies based on the mechanisms described herein could improve the treatment of BL, and might also contribute to our knowledge and understanding of iron metabolism.

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