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Oxidative Stress and Genomic Damage Induced In Vitro in Human Peripheral Blood by Two Preventive Treatments of Iron Deficiency Anemia

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Abstract

Iron deficiency is the most prevalent nutritional deficiency and the main cause of anemia worldwide. Since children aged 6–24 months are among the most vulnerable groups at risk, daily supplementation with ferrous sulfate is recommended by the Argentine Society of Pediatrics as preventive treatment of anemia. However, a single weekly dose would have fewer adverse side effects and has been therefore proposed as an alternative treatment. Ferrous sulfate is known by its pro-oxidative properties, which may lead to increased oxidative stress as well as lipid, protein, and DNA damage. We analyzed the effect of daily and weekly preventive treatment of iron deficiency anemia (IDA) on cell viability, oxidative stress, chromosome, and cytomolecular damage in peripheral blood cultured in vitro. The study protocol included the following: untreated negative control; bleomycin, hydrogen peroxide, or ethanol-treated positive control; daily 0.14 mg ferrous sulfate–supplemented group; and weekly 0.55 mg ferrous sulfate–supplemented group. We assessed cell viability (methyl-thiazolyl-tetrazolium and neutral red assays), lipid peroxidation (thiobarbituric acid reactive substances assay), antioxidant response (superoxide dismutase and catalase enzyme analysis), chromosome damage (cytokinesis-blocked micronucleus cytome assay), and cytomolecular damage (comet assay). Lipid peroxidation, antioxidant response, and chromosome and cytomolecular damage decreased after weekly ferrous sulfate supplementation ($p < 0.05$), suggesting less oxygen free radical production and decreased oxidative stress and genomic damage. Such a decrease in oxidative stress and genomic damage in vitro positions weekly supplementation as a better alternative for IDA treatment. Further studies in vivo would be necessary to corroborate whether weekly supplementation could improve IDA preventive treatment compliance in children.

Keywords Ferrous sulfate · Anemia · Iron deficiency · Oxidative stress · DNA damage · Pediatrics

Introduction

Micronutrients play a key role in DNA stability since most of them act as substrates and/or cofactors for metabolic regulation. Thus, inadequate micronutrient levels would impair the activity of enzymes required for genomic stability [1–4].

Iron, zinc, vitamin A, some B-group vitamins such as riboflavin, folic acid, and niacin, and essential fatty acids are among the main micronutrients required for infant growth. Iron is essential for a variety of biological functions, from oxygen transport and mitochondrial oxidation to dopamine synthesis [5] and also required for the development of neuronal connections and neurotransmitter function [6].

Iron deficiency is the leading cause of anemia, a pathological condition characterized by insufficient iron for the synthesis of iron-containing proteins and enzymes, such as hemoglobin, myoglobin, and catalases [7, 8]. Infants aged 6–24 months and pregnant women are among the populations at higher risk of developing iron deficiency anemia (IDA). In infants, the main cause of IDA is concerned with increased growth rates. At early life stages, IDA affects growth and development [6, 9, 10] and influences the physical, mental, and social aspects of infant health.

Anemia is an important global public health problem [9, 11–14]. The prevention of IDA can be achieved by consumption of an adequate diet, fortified food, and iron supplementation,

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either separately or as combined measures. Pharmacological supplementation is particularly relevant in vulnerable populations, where adequate intakes cannot be ensured. In this sense, the World Health Organization (WHO) recommends pharmacological supplementation of breastfeeding infants and pregnant women when IDA prevalence is above 40% [15]. In Argentina, the National Nutrition and Health Survey [12] reported an overall 34.5% prevalence of anemia in infants < 2 years and 50.8% in breastfeeding infants aged 6–9 months. In this context, the Argentine Society of Pediatrics (SAP) recommended the supplementation of infants with daily ferrous sulfate [16]. However, since the 1990s, different studies have shown the effectiveness of weekly over daily iron supplementation. According to a previously published study, compliance was better and adverse side effects were lower in weekly supplemented school children [17–19]. Other authors found better nutritional effectiveness with weekly iron supplementation in pregnant and non-pregnant women [20, 21].

Iron absorption would be markedly reduced a few days after daily oral iron supplementation, whereas absorption levels would remain high after iron intermittent supplementation [22]. In the case of iron transport mechanisms and the associated symptoms of intolerance, they could be saturated by daily supplementation. On the other hand, weekly supplementation would enhance iron absorption through transport mechanisms, thus decreasing such symptoms [23].

Ferrous sulfate ($FeSO_4$) is known by its pro-oxidative properties, which would lead to increased oxidative stress, i.e., increased concentration of reactive oxygen species (ROS) and reactive nitrogen species (RNS). The toxicity of superoxide anion and hydrogen peroxide is the result of their iron-dependent conversion into the highly reactive hydroxyl radical (OH), which may severely damage lipids, proteins, and DNA. Thus, oxidative stress may induce damage on tissues and favor the development of diseases such as cancer, neurodegenerative disease, and cardiovascular disease [24–26].

Studies dealing with the effect of iron treatment on DNA oxidation in IDA infants showed significantly increased DNA strand breaks and oxidized bases as compared with healthy infants [7]. A recent study demonstrated that iron highly induced the formation of 8-oxo-7, 8-dihydroguanine (8-OxoG) and caused more double- than single-strand DNA breaks [27]. Mice supplemented with ferrous sulfate showed an increased frequency of micronuclei in bone marrow [24]. A research conducted in women with IDA found increased oxidative stress and DNA damage [28]. Thus far, however, there are no studies combining both types of preventive treatments (daily and weekly supplementation) and evaluating oxidative stress, antioxidant response, and DNA damage together.

In view of the abovementioned, the aim of the present study was to analyze the effect of daily and weekly ferrous sulfate preventive treatment of IDA on cell viability, oxidative stress, and chromosome and cytomolecular damage in vitro in human peripheral blood cultures.

Methods

Whole Blood Culture

Blood samples were obtained from healthy female donors aged 20–40 years. They were collected by venous puncture with pre-heparinized syringes and cultured in Falcon T25 flasks (Nunc, Denmark) containing HAM F12 medium (Sigma-Aldrich, St. Louis, MO, USA) with antibiotics (60 UI penicillin, 50 μg/ml streptomycin) (Bagó, Buenos Aires, Argentina) and without fetal bovine serum. Lymphocytes were stimulated with 100 μg/ml phytohemagglutinin (Gibco Thermo Fisher Scientific, Buenos Aires, Argentina). All cultures were performed in a pool of gene samples at 37 °C in 5% CO_2 for 7 days. Cultures were performed for 7 days without changing the medium to evaluate the chronic and cumulative effect of daily ferrous sulfate supplementation. Changing the medium would mean to replenish the ferrous sulfate supplemented daily, causing a new metabolic peak which may skew results. Oxidative stress was assessed in samples cultured in HAM F12 medium without phenol red (EMEVE media) (Lobov Buenos Aires, Argentina).

Experimental Design

Peripheral blood cultures were used to assess cell viability in vitro, oxidative stress, and genomic damage. Peripheral blood cultures were chosen because this system best represents in vivo iron metabolism. At the same time, this experimental design is considered an efficient model to determine the effect of micronutrients on genomic damage and the potential cytotoxicity of exposure to physical and chemical agents [29]. The study protocol included four treatments: (1) negative untreated control (NC); (2) positive control (bleomycin, hydrogen peroxide, or ethanol-treated) (PC); (3) daily supplementation with 0.14 mg ferrous sulfate (DT) (pediatric Ferdrómaco, Laboratorios Andrómaco, Buenos Aires, Argentina); and (4) weekly supplementation with 0.55 mg ferrous sulfate (WT) (Laboratorios Andrómaco). The ferrous sulfate concentration recommended by the Argentine Society of Pediatrics was used to simulate the in vitro effects of DT (1 mg/kg/day per 10 ml culture medium). The proposed WT dose (4 mg/kg/day per 10 ml culture medium) was determined by the concentration used in in vivo studies [30].

The study protocol was approved by the National University of La Plata Ethics Committee. Informed consent was obtained from all individual participants included in the study.

Assays

Quantification of iron in culture supernatants was performed by flame atomic absorption spectroscopy (FAAS) after 7 days of treatment. Then, the following assessments were performed: cell viability by methyl-thiazolyl-tetrazolium (MTT) and neutral red (NR) assays, lipid peroxidation by the thiobarbituric acid reactive substances (TBARS) assay, and antioxidant response with the superoxide dismutase (SOD) and catalase enzyme analysis. Finally, the effect of ferrous sulfate on chromosomes was determined with the cytokinesis-blocked micronucleus cytome assay (CBMNcyt), and cytomolecular damage was assessed with the alkaline version of the comet assay. Experiments were performed in triplicate for each treatment condition.

Flame Atomic Absorption Spectrometry

Flame atomic absorption spectrometry (FAAS) was used to determine iron levels in extracellular media after supplementation. Samples were diluted in trichloroacetic acid and analyzed at IDIP (Instituto de Desarrollo e Investigaciones Pediátricas "Prof. Fernando Viteri"), Hospital de Niños "Sor María Ludovica," La Plata, Buenos Aires, Argentina.

MTT (3-(4,5-Dimethyl-Thiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) Assay

The MTT assay is a colorimetric reaction used to evaluate cell viability (mitochondrial activity). Ten percent ethanol (Merck KGaA, Darmstadt, Germany) was used as positive control. After whole blood culture, red blood cells were discarded using ammonium-chloride-potassium lysing buffer (ACK) (Sigma Aldrich). The remaining cells (including lymphocytes) were washed twice with PBS. Then, 240 μl MTT stock solution (5 mg/ml in PBS, protected from light at 4 °C) was added. After a 3-h incubation, DMSO (100 μl/well) was added, plates were seeded, and readings were performed at 550 nm in a plate spectrophotometer (Multiskan™go, Thermo Fisher Scientific).

Neutral Red Assay

The neutral red (NR) assay is based on the ability of viable cells to incorporate NR in endosomes/lysosomes. Experiments were carried out following the protocol of Borenfreund and Puerner [31]. The same as in the MTT assay, 10% ethanol was used as positive control (Merck KGaA). After culture, red blood cells were discarded using ACK, and NR (0.33% final concentration) was added to the remaining lymphocytes. Cells were incubated for 3 h at 37 °C. After incubation, samples were washed with PBS and 100 μl extraction solution (1% acetic glacial acid, 50% ethanol, and 49% distilled water) per well were added. Finally, supernatant readings were performed in a plate spectrophotometer at 550 nm absorbance (Multiskan™go). Results were expressed as percentage of lysosomal activity.

Thiobarbituric Acid Reactive Substances Assay

The basis of this technique is to react the malondialdehyde (MDA) present in the sample with thiobarbituric acid (TBA). Hydrogen peroxide (Cicarelli Laboratorios, Santa Fe, Argentina) was used as positive control. Heparinized blood was centrifuged for 10 min to separate plasma from red blood cells. Plasma, 8.1% sodium dodecylsufate, 20% acetic acid, 8% TBA dissolved in sodium hydroxide, and distilled water were placed into screw-cap tubes. Each tube was vortexed and then incubated in a thermostatic bath for 1 h at 95 °C. After cooling, distilled water and a butanol-pyridine mixture were added to the tubes, which were centrifuged at 4000 rpm for 10 min, followed by absorbance reading at 532 nm in plate spectrophotometer (Multiskan™go). Data were compared with our own calibration curve, which was built by replacing the sample with 1,1,3,3-tetramethoxypropane (TMP) stock solution at different concentrations. Results were expressed as nanomole MDA per milliliter.

Catalase Assay

This is a colorimetric direct method for oxidative stress measurement. Heparinized blood was centrifuged for 10 min and plasma was separated. Red blood cells were washed with physiological solution and centrifuged. This procedure was repeated twice. Red blood cells were placed in 1/20 distilled water for hemolysis, vigorously shaken and centrifuged for 10 min. The supernatant was collected and diluted (1/100) with 50 mM sodium phosphate buffer (Anedra S.A. San Fernando, Argentina), pH 7.0. Sample and buffer were placed in the quartz cell and homogenized by pipetting. Hydroxide peroxide was added and the sample was homogenized. Absorbance was read at 240 nm in a spectrophotometer ((Multiskan™go) at time 0 and after 5 min. The sodium phosphate buffer was used for the blank sample. One unit of catalase was defined as 1 μmol hydrogen peroxide consumed per minute.

Superoxide Dismutase Assay

The superoxide dismutase (SOD) assay is used for indirect measurement of oxidative stress. The assay was performed with the commercial kit Ransod (catalog no. SD125, Randox Laboratories Ltd., UK), following the manufacturer's instructions.

Cytokinesis-Blocked Micronucleus Cytome Assay

The cytokinesis-blocked micronucleus cytome (CBMNcyt) is the most adequate method to assess damage resulting from micronutrient deficiency and excess [29]. Approximately 28 h before the end of culture, 3 mg/ml cytochalasin B (Sigma) was added to the medium. The positive control was treated with 1 μg/ml bleomycin (Gador S.A., Buenos Aires, Argentina). At the end of culture, the content of the tube was placed into a Falcon tube and centrifuged at 1200 rpm for 10 min. The supernatant was removed and cold hypotonic solution (0.075 M potassium chloride [KCl]) was gently added. The sample was centrifuged again at 1200 rpm for 6 min. The supernatant was removed and fixation solution I (sodium chloride-methanol-acetic acid, 6:5:1) was gently added. Two more changes were performed with fixation solution II (methanol-acetic acid, 5:1) at 1200 rpm for 10 min. Fenech's scoring criteria for micronuclei (MNi), nucleoplasmic bridges (NPBs), and buds (NBuds) determinations were used [32]. Cytostatic effects were analyzed through the nuclear division index (NDI) [32]. Standard procedures were applied for the analysis of 1000 cells per experimental point [32].

Alkaline Version of the Comet Assay

The comet assay, also called single cell gel electrophoresis, is used to quantify DNA damage in individual cells. In this study, the alkaline version of the assay was used according to Singh et al. [33], with slight modifications. Cells were embedded in 0.5% low melting point agarose and placed in slides previously covered with 150 μl normal agarose (0.5%). Then, slides were immersed in lysis solution (2.5 MNaCl, 100 mM Na_2EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10) for 24 h. The positive control was treated with 1 μg/ml bleomycin (Gador S.A.). Afterwards, slides were immersed in an alkaline solution (300 mMNaOH, 1 mM Na2EDTA) for 20 min before electrophoresis at 4 °C, 20 V, and 250 mA for 20 min. After electrophoresis, slides were washed with neutral solution and cells were stained with Sybrgreen. A total of 200 images per experimental point were analyzed in an Olympus BX40 fluorescence microscope equipped with a 515–560-ηm excitation filter. The degree of DNA damage was determined by the length of the comet tail. Accordingly, cells were classified into five categories, from 0 (no detectable tail) to 4 (detectable head of the tail, but more DNA in the tail) [34, 35]. With these data, damage index (DI) was obtained according to Collins [35]. The total score for the sample gel was between 0 and 400 "arbitrary units." Visual scoring (arbitrary units) is rapid as well as simple, and there is a very close agreement between this method and computer image analysis (percentage of DNA in the tail) [35].

Statistical Analysis

One-way ANOVA and multiple range test were used to analyze normally distributed variables. Kruskal-Wallis test was used to analyze non-normally distributed variables. The program Statgraphics® 5.1 (Manugistics Inc., Rockville, MD) was used for data analysis, considering $p < 0.05$. Results were expressed as mean ± SD.

Results

FAAS Assay

Flame atomic absorption spectrometry (FAAS) was used to determine iron levels in extracellular media after supplementation. Iron quantification after treatment is shown in Fig. 1. Both NC and PC showed similar blank values (0.5–2 mg/l). In DT and WT, iron was 12.4 mg/l and 7.3 mg/l, respectively. Since quantification was performed in the culture without cells, these results would suggest that iron absorption was more efficient with WT.

MTT Assay

The MTT assay is a colorimetric reaction used to evaluate cell viability (mitochondrial activity). Ten percent ethanol (Merck KGaA) was used as positive control. The Kruskal-Wallis analysis showed significant differences among all groups ($p < 0.01$; Fig. 2a). Cell viability was higher in NC and WT. Differences were highly significant in (a) NC vs. DT, (b) NC vs. WT, and (c) NC vs. PC ($p < 0.01$), and significant in (d) DT vs. WT ($p < 0.05$). These results demonstrate that WT improved cell viability better than DT.

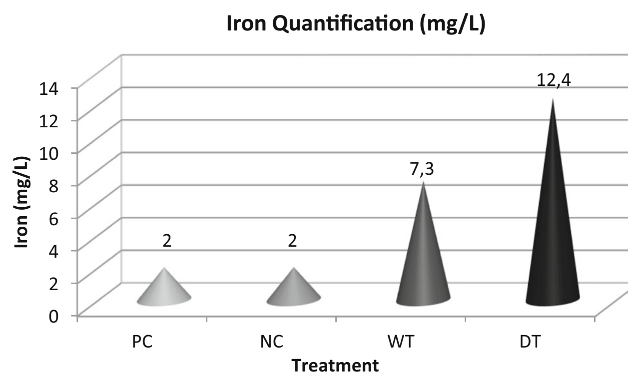

Fig. 1 Iron levels in extracellular media after supplementation measured by AAS. Positive control (PC), negative control (NC), weekly treatment (WT) (0.55 mg ferrous sulfate), daily treatment (DT) (0.14 mg ferrous sulfate)

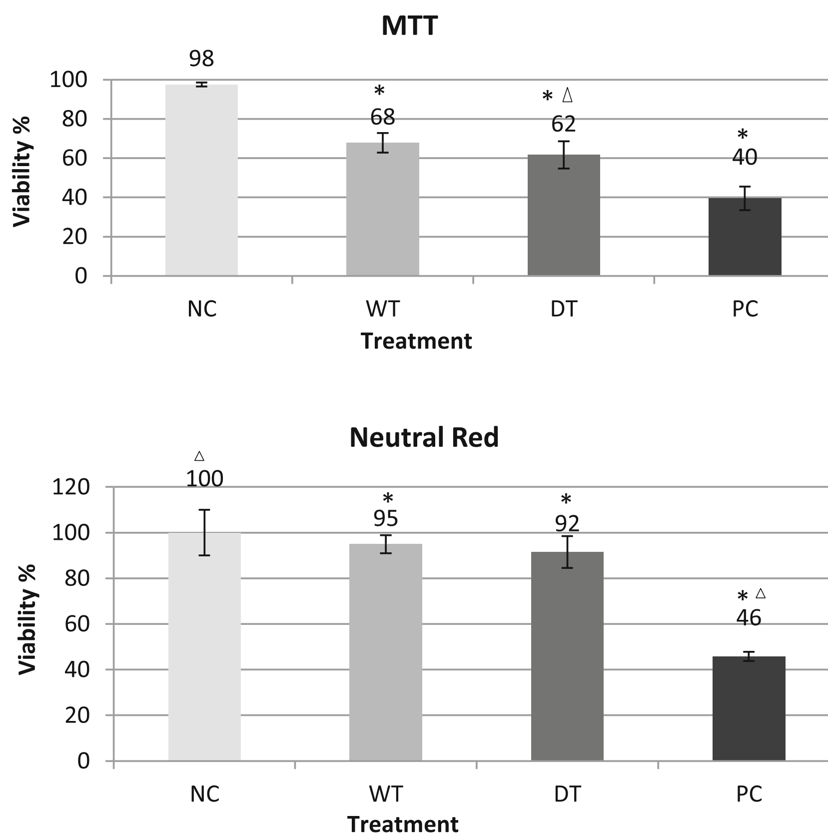

Fig. 2 Cell viability values (%). Negative control (NC), weekly treatment (WT) (0.55 mg ferrous sulfate), daily treatment (DT) (0.14 mg ferrous sulfate), positive control (PC). All assays were performed in triplicate. The asterisk indicates significant differences between the negative control and all the experimental points, and triangles those between WT and all the experimental points. **a** MTT in peripheral human lymphocytes. Data are means ± SD. Kruskal-Wallis ($p < 0.01$) contrast test results were analyzed with Statgraphics® 5.1 software. **b** Neutral red in peripheral human lymphocytes. Data are means ± SD. One-way ANOVA ($p < 0.01$) were analyzed with the Statgraphics® 5.1 software

NR Assay

The NR assay is based on the ability of viable cells to incorporate NR in endosomes/lysosomes. Ten percent ethanol (Merck KGaA) was used as positive control. One-way ANOVA results showed significant differences among treatments ($p < 0.01$; Fig. 2b). Again, cell viability was higher in NC and WT. Multiple range test results showed three well-defined groups: (a) PC, (b) NC, and (c) DT-WT. Differences between DT and WT were not significant.

T-BARS Assay

The basis of this technique is to react the malondialdehyde (MDA) present in the sample with thiobarbituric acid (TBA). Hydrogen peroxide (Cicarelli Laboratorios) was used as positive control. Analysis of lipid peroxidation by thiobarbituric acid reactive substances (T-BARS) is shown in Fig. 3a. ANOVA results showed significant differences among groups ($p < 0.001$). Lipid peroxidation was higher in PC, followed by DT. Multiple range test results showed three clearly differentiated groups: (a) NC-WT, (b) WT-DT, and (c) PC. Thus, WT evidenced higher but not significantly different lipid peroxidation results as compared with DT.

Catalase and SOD Assays

Catalase assay is a colorimetric direct method for oxidative stress measurement. Hydrogen peroxide (Cicarelli Laboratorios) was used as positive control. In both assays, WT had the lowest antioxidant index. In the case of catalase (Fig. 3b), differences among groups were significant ($p < 0.001$). Multiple range test showed three different groups: (a) NC-WT, (b) NC-DT, and (c) PC.

The SOD assay is used for indirect measurement of oxidative stress. In the SOD assay (Fig. 3c), ANOVA showed significant differences ($p < 0.05$), whereas multiple range test showed three homogeneous groups: (a) NC-WT, (b) NC-DT, and (c) PC. In both assays, WT induced the lowest antioxidant response.

CBMNcyt Assay

The CBMNcyt allows analyzing cytostatic effects, cytotoxicity, and chromosomal damage. Bleomycin (Gador S.A.) was used as positive control. The highest NDI values corresponded to NC. Differences among groups were significant ($p < 0.0001$). Multiple range test showed three homogeneous groups: (a) NC, (b) DT-PC, and (c) DT-WT. The NDI was slightly higher in WT than that in DT.

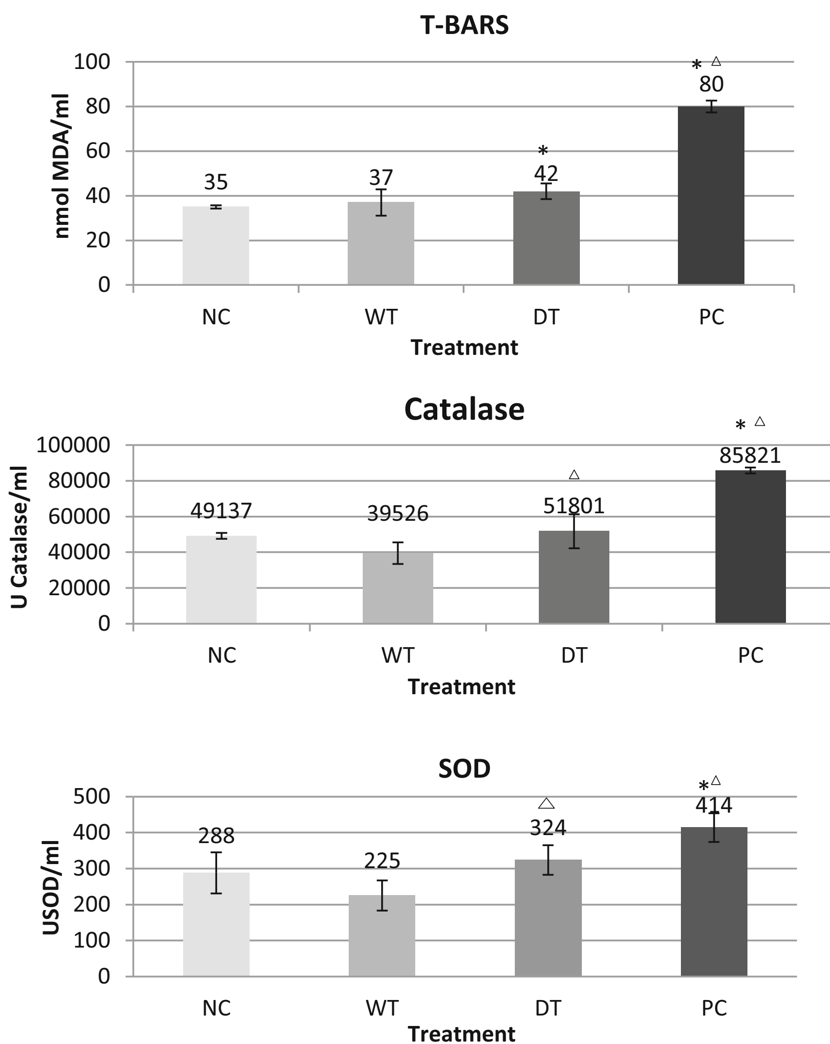

Fig. 3 Oxidative stress average values. Negative control (NC), weekly treatment (WT) (0.55 mg ferrous sulfate), daily treatment (DT) (0.14 mg ferrous sulfate), positive control (PC). All assays were performed in triplicate. Data are means ± SD. Data were analyzed with the Statgraphics® 5.1 software. Asterisks show significant differences between the negative control and all the experimental points, and triangles those between WT and all the experimental points. **a** T-BARS measured in plasma (one-way ANOVA; $p < 0.001$). **b** Catalase measured in erythrocytes (one-way ANOVA; $p < 0.001$). **c** SOD measured in erythrocytes (one way ANOVA; $p < 0.05$)

The frequency of MNi, NPB, and NBuds was determined in 1000 binucleated cells (Table 1). The highest MNi frequency was found in PC followed by DT. ANOVA results showed significant differences ($p < 0.001$), whereas multiple range test showed three groups: (a) NC-WT, (b) DT, and (c) PC. We observed a significant increase of MNi frequency in DT as compared with WT.

Table 1 Average values for nuclear division index (NDI), micronuclei (Mni), nucleoplasmic bridges (NPB), and nuclear buds (NBuds)

Treatment	NDI	MNi ‰	NPB ‰	NBuds ‰
NC	1.21 (0.1)a	0.98 (3.7)a	0.05 (0.6)a	0 (0.0)a
WT	1.18 (0.1)b	1.15 (3.41)a	0.13 (0.9)a	0 (0.0)a
DT	1.17 (0.1)bc	1.95 (3.10)b	0.2 (0.8)ab	0.03 (0.5)ab
PC	1.16 (0.1)c	3.03 (4.0)c	0.3 (1.6)b	0.15 (1)b

NC, negative control; *WT*, weekly treatment; *DT*, daily treatment; *PC*, positive control

Standard deviations are indicated in parentheses. Groups not sharing the same letter are significantly different from each other (*p* values refer to the ANOVA analysis: $p < 0.05$)

The increase of NPB and NBuds frequency was lower in all study groups. ANOVA results for NPB were significant ($p < 0.05$). Data of NBuds did not have a normal distribution and were then analyzed with Kruskal-Wallis. Results of paired comparisons were significant ($p < 0.01$) in PC vs. NC and PC vs. WT. Comparisons between the other groups did not result in significant differences.

This assay revealed higher damage in cells exposed to WT as compared with DT in all the assessed parameters.

Comet Assay

The comet assay is used to quantify DNA damage in individual cells. Bleomycin (Gador S.A.) was used as positive control. Results of DI are shown in Fig. 4 ($p < 0.0001$). The highest DI corresponded to PC, followed by DT. Multiple range test differentiated three groups: (a) NC-WT, (b) DT,

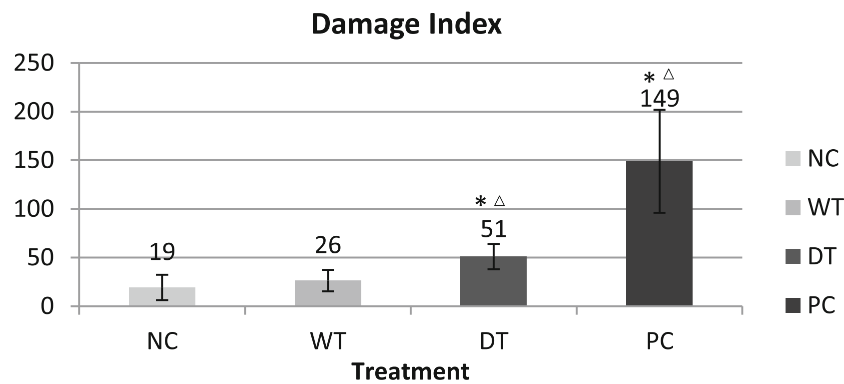

Fig. 4 Damage index assessed with the alkaline version of the comet assay. Negative control (NC), weekly treatment (WT) (0.55 mg ferrous sulfate), daily treatment (DT) (0.14 mg ferrous sulfate), positive control (PC). Data are means ± SD of experiments performed in triplicate. Statistical analysis was performed with Statgraphics® 5.1 software. Asterisks indicate significant differences between the negative control and all the experimental points, and triangles those between WT and all the experimental points (one-way ANOVA; $p < 0.0001$)

and (c) PC. Similar to that described for the CBMNcyt assay, the cytomolecular damage was lower in WT than that in DT.

Discussion

In this study, we compared the effect of two preventive IDA treatments (daily and weekly supplementation of ferrous sulfate) on cell viability, oxidative stress, and chromosome and cytomolecular damage in human blood cultured in vitro.

Our results showed that DT induced a significant decrease in cell viability and an increase in cytotoxicity with respect to NC. In this sense, WT had certain advantages over DT. The MTT assay showed decreased cell viability in DT, which was 10% smaller than in WT. Mean average cell viability was slightly higher in WT than that in DT (NS) when analyzed with the NR assay. Other authors demonstrated the toxicity of ferrous sulfate administered in vitro, but at higher doses than the ones currently used [36, 37]. On the other hand, NDI analysis with the CBMNcyt assay did not show statistically significant differences between DT and WT. All NDI were lower than expected for human lymphocytes under optimal culture conditions [32]. Since these values depend on culture conditions, the decrease observed in the present study could be explained by the time of culture (7 days vs. 72 h in the mentioned report).

Whereas lipid peroxidation was higher in DT compared with NC and WT, the antioxidant response was lower in WT, suggesting that the latter induced less oxidative stress than DT. Altogether, these data suggest that WT caused less oxidative stress than DT. However, in vivo reports are controversial. Hacihamdioglu et al. [38] showed that preventive treatment of anemia with 10 mg/dl ferrous sulfate caused neither lipid peroxidation nor increased antioxidant response in healthy children. Zaka-ur-Rab et al. [39] and Kurtoglu et al. [40] reported that 6 mg/kg/day ferrous sulfate administered to IDA patients decreased lipid peroxidation after 6–8 weeks of treatment. On the other hand, and in agreement with the results presented here, Altun et al. [41] observed that 4 mg/kg/day iron administration during 8–12 weeks increased oxidative stress.

Results of the CBMNcyt assay showed that the frequency of MNi was significantly higher in DT than that in NC and WT, despite the prevalence of MNi in both supplementations was in the normal range established by Fenech [32] for peripheral blood lymphocyte cultures. This was expected considering that both ferrous sulfate treatments were preventive, without exceeding the recommended maximum dose [42]. The same applied to NPB and NBuds since no significant differences were found between WT and DT. Our results suggest that WT would cause less genomic damage than DT. Further, they are in agreement with evidence in the literature reporting a significant increase in MNi frequency in mice supplemented in vivo with daily ferrous sulfate [24]. These authors also reported a negative correlation between MNi frequency and intake of 15 mg/dl iron in children [27]. Premkumar and Bowlus [43] concluded that high dietary ferrous sulfate increased MNi in bone marrow cells of mice treated in vivo. Similarly, Alcântara et al. [37] showed that 640 μg/ml ferrous sulfate increased MNi frequency in an astrocyte cell line and suggested that genotoxic effects could probably be due to iron pro-oxidant properties.

Cytomolecular damage and MNi results were similar, showing a significantly higher DI in DT than in NC and WT. These data agree with those of Aksu et al. [7], who observed that daily iron administration (5–6 mg/kg/day) increased DNA damage and formamidopyrimidine glycosylase sensitivity in children with iron deficiency, suggesting that such increase was the result of higher oxidative stress. Likewise, Ferro et al. [44], using CBMNcyt and comet assay, found increased genotoxic markers induced by an iron overload in patients with thalassemia. Mollet et al. [45] reported that the vascular endothelium can be modified and DNA damage response may be induced with low iron doses. On the

other hand, Franke et al. [26] showed that vitamin C increased DNA damage induced by ferrous sulfate in mice treated in vivo.

Interestingly, the iron final concentration in the supernatant was markedly higher in DT than that in WT and could probably explain the increased oxidative stress observed, with the consequent chromosome and cytomolecular damage recorded in DT.

In general, evidence in the literature shows that ferrous sulfate treatments cause higher DNA damage [24, 37, 43]. Particularly, weekly treatments were more effective than daily treatments in intervention trials [17–19]. However, oxidative stress and antioxidant response to ferrous sulfate supplementation did not present significant differences with controls [38–40].

One of the limitations of the present work was the fact that it was an in vitro study. In this sense, further studies in vivo could corroborate whether weekly supplementation would help reduce adverse effects and achieve better patient compliance with IDA preventive treatment, thereby decreasing IDA prevalence in children < 24 months. On the other hand, the strength of this work is, as far as the authors are concerned, this is the first study combining both types of preventive treatment (daily and weekly supplementation) and evaluating oxidative stress, antioxidant response, and DNA damage together.

Conclusion

In the present in vitro study, daily supplementation of ferrous sulfate moderately increased oxidative stress and therefore increased genomic damage. The observed increased antioxidant response was concomitant with increased lipid peroxidation, indicating an imbalance in the redox system. On the other hand, weekly supplementation did not involve enzymatic antioxidant response or increased lipid peroxidation, and these could probably correlate with the lower genetic damage observed with the CBMNcyt and comet assays. It should be noted that none of the treatments involved toxic doses, which were equivalent to those used in preventive treatments for infants. In any case, the decrease observed in oxidative stress and genomic damage in vitro positions weekly rather than daily ferrous sulfate supplementation as a better alternative for IDA treatment.

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Compliance with Ethical Standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Declarations of Interest The authors declare that they have no conflict of interest.

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