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ORIGINAL PAPER

# Leukocyte DNA damage in children with iron deficiency anemia: effect of iron supplementation

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**Abstract** Iron deficiency is frequently associated with anemia. Iron is a transition-metal ion, and it can induce free radical formation, which leads to formation of various lesions in DNA, proteins, and lipids. The aim of this study was to investigate baseline oxidative DNA damage and to clarify the role of the administration of a therapeutic dose of iron on DNA oxidation in children with iron deficiency anemia (IDA). Twenty-seven children with IDA and 20 healthy children were enrolled in the study. Leukocyte DNA damage (strand breaks and Fpg-sensitive sites) was assessed using comet assay before and after 12 weeks of daily iron administration. Before the iron administration, the frequency of DNA strand breaks in the children with IDA was found to be lower than those in the control group ($P<0.05$), but there was not a significant difference for frequency of Fpg-sensitive sites. After 12 weeks of iron administration, the frequency of both DNA strand breaks and Fpg-sensitive sites were found to be increased ($P<0.01$). No significant association was determined between DNA damage parameters and hemoglobin, hematocrit, serum iron, total iron binding capacity, and ferritin. In conclusion, basal level of DNA strand breaks is at a low level in children with IDA. After iron administration, DNA strand breaks and Fpg-sensitive sites, which represent oxidatively damaged DNA, increased. However, this increase was unrelated to serum level of iron and ferritin.

**Keywords** Iron deficiency anemia · DNA strand breaks · Fpg-sensitive sites · Comet assay · Iron supplementation

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## Introduction

Iron is an essential trace element, and prevalence of iron deficiency among infants and young children living in developing countries is high. Iron deficiency is a pathological condition, in which there is not sufficient iron for synthesis of iron-containing proteins such as hemoglobin (Hb), myoglobin, cytochromes, and iron-containing enzymes such as catalase and various peroxidases [26]. Iron deficiency is frequently associated with anemia.

Oxidative stress has been defined as the disturbance of the balance of oxidant/antioxidant systems in favor of oxidants. Oxygen and nitrogen-derived free radicals are major oxidants. They are formed by normal cellular metabolism and by exogenous factors [8]. Free radicals can react with cellular lipids, proteins, and DNA leading to permanent damage on these molecules, which causes structural and functional alterations. Iron is a transition metal and can catalyze free radical formation by the Fenton reaction [14, 25]. Free radicals lead to formation of various lesions in DNA including oxidized bases, abasic sites, and DNA strand breaks [14, 30]. The major base oxidation product is 8-oxo-7,8 dihydro-guanine (8-oxo-Gua) which is a highly mutagenic lesion [14]. The frequency of DNA strand breaks can be detected by the Comet assay (single cell gel electrophoresis). This simple, rapid, sensitive fluorescence microscopy technique for estimation of damage in individual cell has been increasingly employed [10,

17]. Comet assay can be used for determination of oxidized bases in DNA by using damage-specific repair endonucleases [9].

Oxidative damage derived from the disturbance of the equilibrium status of oxidant/antioxidant systems has been suggested as a contributory factor in various pathological conditions [7, 8, 22]. Associations between iron deficiency anemia (IDA) and oxidative stress have been investigated by various investigators previously [5, 19, 20, 32]. Increased erythrocyte susceptibility to in vitro oxidative stress [32], decreased antioxidant defense, and increased lipid peroxidation in erythrocytes [19] from adults with IDA have been determined. To date, to the best of our knowledge, the effect of therapeutic iron administration on DNA oxidation has not been investigated in children with IDA. The purpose of this study was to examine baseline oxidatively damaged DNA and to clarify the role of the administration of a therapeutic dose of iron on DNA oxidation in children with IDA.

## Materials and methods

### Subjects

A total of 27 children with IDA attending Bakirkoy Dr. Sadi Konuk Education and Research Hospital, Pediatric Clinic, and 20 age-matched healthy children who periodically attended the same clinic for regular check-ups of their development were included in the present study. The Institutional Ethical Committee approval was taken in accordance with the principles of Declaration of Helsinki, and informed consent was obtained from parents. IDA was defined as serum ferritin concentration <10 ng/ml, indicating the depletion of iron stores, Hb concentration <11 g/dl, and a mean corpuscular volume (MCV) <70 fL for children aged between 12 and 24 months and MCV <73 fL for children aged between 24 and 48 months [29]. Routine blood biochemistry and systemic examination were performed in all children. Children with acute infections, celiac or other chronic diseases, hematological diseases other than iron deficiency anemia, and children who had used antibiotic(s) and/or vitamin(s) in the last 3 months were excluded from the study. In order to exclude the presence of thalassemia, children with MCV <70 fL underwent Hb electrophoresis. By the time the study commenced, none of the children had been supplemented with iron. Demographic and clinical data are summarized in Table 1.

Children with IDA were treated with $Fe^{3+}$ preparation, orally (5–6 mg/kg/day) [16]. Complete blood count, serum levels of ferritin and iron, total iron binding capacity (TIBC), and DNA damage in peripheral leukocytes (frequency of strand breaks and Fpg-sensitive sites) were determined before and after a 12-week iron administration. Venous blood samples were collected after a 12-h fasting into different tubes with EDTA, with heparin and without any anticoagulant. Serum obtained by centrifugation of the tube without anticoagulant was used for determination of ferritin, iron, and TIBC measured in a routine analysis laboratory. Heparinized blood was used for DNA damage measurements, and blood sample with EDTA was used for complete blood count.

### Determination of DNA damage by Comet assay

Baseline DNA strand breaks were detected in DNA of leukocytes with the comet assay established by Singh et al. [27] and as described previously [12]. Twenty microliters of heparinized blood was mixed with 60 µl of 1.4% low-melting-point agarose in Dulbecco's buffer at 37°C. Subsequently, 50 µl of this mixture was layered onto slides that had previously been coated with 0.3% normal melting point agarose and covered with a coverslip at 4°C for at least 5 min. After removing the coverslips, the slides were submersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA–$Na_2$, 10 mM Tris–HCl, 200 mM NaOH, 1% Triton X-100, and 10% DMSO) for 1 h. Slides were then immersed in alkaline electrophoresis buffer (0.3 M NaOH and 1 mM $Na_2$EDTA, pH >13) at 4°C for unwinding (40 min) and then electrophoresed (20 V/400 mA, 24 min). The loops of DNA extend towards the anode, giving an appearance of the tail of a comet when stained with ethidium bromide and viewed by fluorescence microscopy. Undamaged DNA remains within the head. In order to evaluate the degree of damage, comet images were scored visually. Slides were duplicated for each case. Fifty cells per slide were examined, and 100 cells were examined per case. Each comet was classified to five categories (0–4) according to the extent of DNA migration. The comets with bright heads and no apparent tails were assigned to category 0, images with very little heads and long diffused tails to category 4. Comets displaying features intermediate between category 0 and 4 were divided and assigned to easily distinguishable categories 1, 2, and 3 (Fig. 1). The number of comets in each category was counted, and average DNA damage in the case of strand breaks was expressed as arbitrary unit (au) which is related to the percentage of DNA in the tail [9].

The oxidative DNA damage (8-oxo-Gua adducts) is converted into and then measured as a strand break by using 8-oxo-Gua specific endonuclease. In the present study, DNA were incubated with formamidopyrimidine DNA glycosylase (Fpg), a bacterial protein which generates additional breaks at sites containing 8-oxo-Gua, after the lysis step. Subtracting the percent DNA in tail without Fpg incubation from the percent DNA in tail with Fpg

**Table 1** Demographic and clinical data of the study groups

| | Children with IDA group ($n$=27) | | | Control group ($n$=20) |
|---|---|---|---|---|
| | | Before iron administration | After iron administration | |
| Mean age (month) | 16±7 | | | 20±6 |
| Gender | | | | |
| Male | 16 | | | 13 |
| Female | 11 | | | 7 |
| Birth weight (kg) | 3.29±0.50 | | | 3.42±0.60 |
| Height (cm) | 74±7 | | | 69±8 |
| Hb (g/dl) | | 9.43±1.30 | 11.36±1.32* | 10.56±1.29 |
| Hct (%) | | 29±4 | 35±4* | 33±3 |
| MCV (fL) | | 69.94±8.30 | 70.99±6.52 | 80.31±7.24 |
| Serum Fe (μg/dl) | | 18.07±5.87 | 54.96±22.74* | 50.18±18.20 |
| TIBC (μg/dl) | | 350±55 | 335±79 | 298±46 |
| Ferritin (μg/L) | | 7.52±2.39 | 25.92±18.22* | 77.07±22.87 |

*$P$<0.001 versus before iron administration

incubation gives the net amount of base damage represented by Fpg-sensitive sites [11].

### Statistical analysis

Data were expressed as mean ± SD with 95% confidence interval. Statistical analysis was performed with SPSS 10 for Windows; $P$<0.05 was considered statistically significant. Comparisons between control and IDA groups (before iron administration) were carried out by Student's $t$ test. Comparisons between before and after iron administration in the IDA group were performed by paired $t$ test. Correlations between variables were assessed by Pearson's test.

## Results

Hb, hematocrit (Hct), serum iron, and ferritin levels were found to be significantly increased after iron administration in the children with IDA (Table 1). DNA damage parameters in the control and IDA group before iron administration are shown in Fig. 2. There was not a significant difference between male (75±4 and 32±3 au, respectively) and female (74±3 and 32±4 au, respectively) children for frequency of neither strand breaks nor Fpg-sensitive sites. Before the iron administration, frequency of DNA strand breaks in the children with IDA was found to be lower than those in the control group ($P$<0.05), but there was not a significant difference for frequency of Fpg-

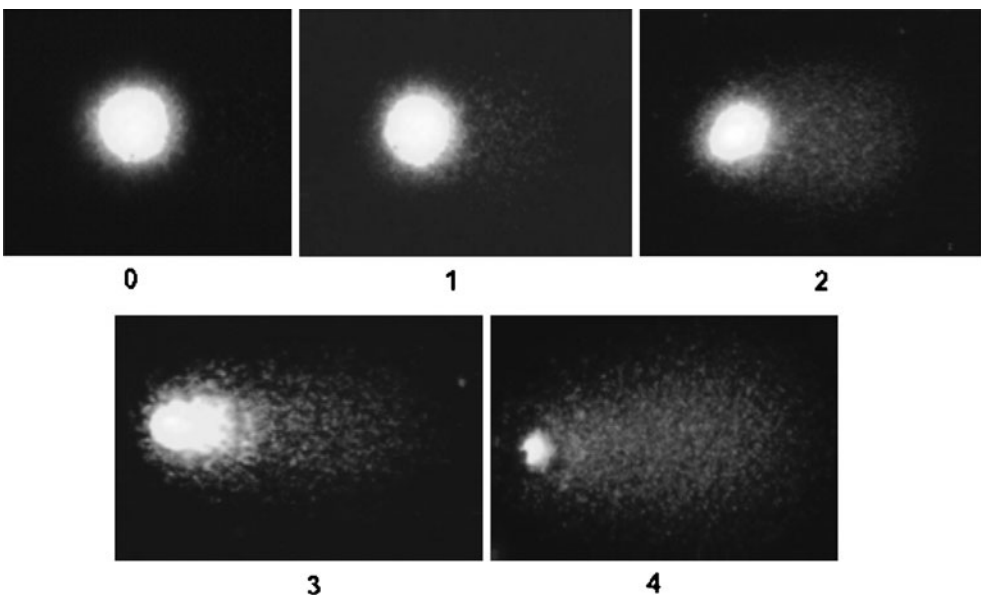



**Fig. 1** Comet classes in visual scoring

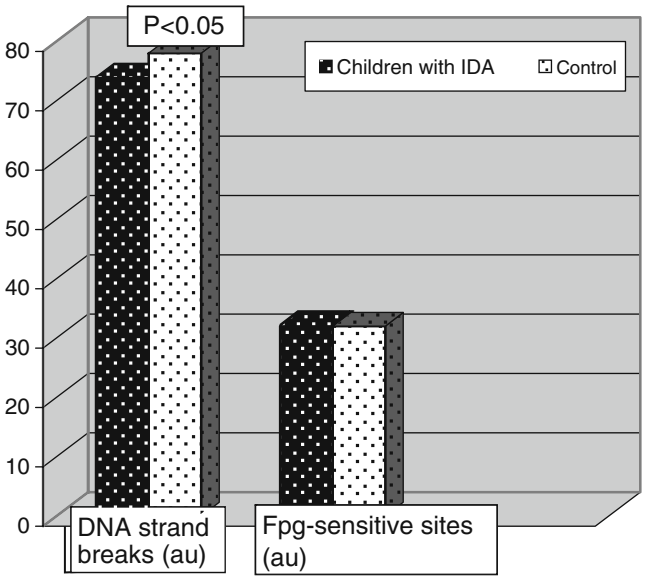



**Fig. 2** DNA damage parameters in the control and iron deficiency anemia group

sensitive sites. After 12 weeks of daily iron administration, frequency of both DNA strand breaks and Fpg-sensitive sites was found to be increased ($P<0.01$). After the iron administration, frequency of DNA strand breaks and frequency of Fpg-sensitive sites in the children with IDA exceeded the levels of the control group (Table 2). However, this increase was significant for frequency of Fpg-sensitive sites, but not significant for frequency of DNA strand breaks. In the children with IDA group, no significant association was determined between DNA damage parameters and age, weight, birth weight, Hb, Hct, serum iron, TIBC, and ferritin.

## Discussion

Non-protein-bound iron increases formation of oxygen free radicals [14, 25]. Iron-induced formation of oxygen free radicals in plasma is inhibited by transferrin, an iron binding protein [20]. The majority of studies on oxidative stress in IDA are based on lipid peroxidation measurements, and their results are contradictory. It has been determined that red blood cell susceptibility to in vitro oxidation is increased in IDA patients [4, 32]; however, some authors have suggested that there is no evidence of increased susceptibility of red blood cells to lipid peroxidation [1, 23]. Biomolecular targets of free radicals extend beyond lipids, and DNA oxidation may have more severe consequences. Oxidative DNA damage having mutagenic potential is a much more dangerous condition than lipid peroxidation. As far as we know, there is not any study examining oxidative DNA damage in children with IDA. Recently, Aslan et al. [2] have found lower plasma total antioxidant capacity and increased DNA damage (strand breaks) in lymphocytes of adult females with IDA. They have suggested that oxidants were increased, and antioxidants were decreased, and as a result, oxidative/antioxidative balance shifted toward oxidative side in patients with IDA. However, conflicting results have been reported [18, 23]. On the other hand, DNA strand breaks do not represent oxidative DNA damage exclusively. They are also produced by repair processes of damaged DNA as an intermediate step [13]. Therefore, in order to determine oxidative DNA damage conclusively by the comet assay, the frequency of Fpg-sensitive sites should be measured in addition to frequency of DNA strand breaks. The Fpg-sensitive sites represent the additional breaks generated by the 8-oxo-Gua-specific repair enzyme, Fpg [11]. In the present study, before, the iron administration frequency of DNA strand breaks in the children with IDA was found to be lower than those in the control group, whereas Fpg-sensitive sites were not statistically significant. In the case of IDA, oxygenation of tissues decreases which in turn leads to a deceleration in general metabolism. Decreased frequency of strand breaks may be derived from decreased formation of free radicals and decreased repair processes due to decelerated metabolism in patients with IDA.

After 12 weeks of daily iron administration, various blood parameters, Hb, Hct, iron, and ferritin were found to be significantly increased as well as the frequency of both DNA strand breaks and Fpg-sensitive sites. However, there was not a reliable correlation between blood parameters and

**Table 2** DNA damage parameters in the control and iron deficiency anemia group before and after iron administration

| | Children with IDA group ($n$=27) | | | | Control group | |
|---|---|---|---|---|---|---|
| | Before iron administration | 95% CI | After iron administration | 95% CI | $n$=20 | 95% CI |
| DNA strand breaks (au) | 74±4* | 73–76 | 81±8** | 77–84 | 78±6 | 75–81 |
| Fpg-sensitive sites (au) | 32±3 | 31–3 | 36±7*, ** | 33–39 | 32±3 | 30–34 |

*CI* confidence interval

*$P<0.05$ versus control

**$P<0.01$ versus before iron administration

DNA damage parameters. These increases seem independent of serum level of iron. Although increased lipid peroxidation after an acute iron loading has been shown [15, 21, 33], Bacon et al. suggested that when iron is administrated at a correct dose, additional oxidative stress is not formed [3]. In the present study, increased DNA strand breaks and Fpg-sensitive sites after 12 weeks of iron administration may be a result of accelerated metabolism in response to iron loading.

Iron is essential for DNA synthesis. Its deficiency may lead to impaired DNA synthesis and subsequent alterations in levels of apoptosis, programmed cell death. Berrak et al. [5] showed that neutrophil- and monocyte-apoptotic responses were significantly less in IDA patients compared to the control group, and low apoptotic responses of IDA patients rose to levels of the control group by day15 of iron therapy. Apoptosis can be initiated by activation of both intrinsic and extrinsic caspase pathways [6, 29]. The intrinsic pathway begins with release of cytochrome c from mitochondria [31]. Iron is an essential component of cytochrome c [24], and in the case of iron deficiency, cytochrome c-mediated apoptosis may be impaired. Oxidative stress and apoptosis are in a close association. Apoptosis can be induced as a response to oxidative stress. Oxygen free radicals lead to an increase in the formation of DNA strand breaks, which in turn activates p53 gene. p53 protein acts to induce cell cycle arrest or apoptosis in response to DNA damage [28]. Data of this apoptosis study in patients with IDA (Berrak) are in accordance with the findings of the present study.

In conclusion, baseline level of DNA strand breaks was at a low level in children with IDA. After 12 weeks of daily iron administration, DNA strand breaks and Fpg-sensitive sites, which represent oxidatively damaged DNA, increased. However, this increase was free from corresponding changes in the serum level of iron and ferritin. We suggest that this increase is a result of increased oxidative stress due to an accelerated metabolic rate by the rehabilitation of oxygenation in organism. Whether antioxidant support should be added to iron administration in children with IDA remains unclear. Due to the limited number of cases included, more studies in larger groups should be performed to document the impact of iron treatment on oxidative DNA damage, subsequently, malignancy in children with IDA.

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