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OBSERVATIONAL STUDY

Oxidative stress and labile plasmatic iron in anemic patients following blood therapy

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Abstract

AIM: To determine the plasmatic iron content and evaluate the oxidative stress (OS) markers in subjects receiving blood therapy.

METHODS: Thirty-nine individuals with unspecified anemia receiving blood transfusions and 15 healthy subjects were included in the study. Anemic subjects were divided into three subgrouP: (1) those that received up to five blood transfusions (n = 14); (2) those that received from five to ten transfusions (n = 11); and (3) those that received more than ten transfusions (n = 14). Blood samples were collected by venous arm puncture and stored in tubes containing heparin. The plasma and cells were separated by centrifugation and subsequently used for analyses. Statistical analyses were performed using Kruskal-Wallis analysis of variance followed by Dunn's multiple comparison tests when appropriate.

RESULTS: The eletrophoretic hemoglobin profiles of the subjects included in this study indicated that no patients presented with hemoglobinopathy. Labile plasmatic iron, ferritin, protein carbonyl, thiobarbituric acid-reactive substances (TBARS) and dichlorofluorescein diacetate oxidation were significantly higher ($P < 0.05$), whereas total thiol levels were significantly lower ($P < 0.05$) in transfused subjects compared to controls. Additionally, the activity of catalase, superoxide dismutase and glutathione peroxidase were significantly lower in the transfused subjects ($P < 0.05$). Antioxidant enzyme activities and total thiol levels were positively correlated ($P < 0.05$), and negatively correlated with the levels of protein carbonyl and TBARS ($P < 0.05$). In contrast, protein carbonyl and TBARS were positively correlated ($P < 0.05$). Altogether, these data confirm the involvement of OS in patients following therapy with repeated blood transfusions.

CONCLUSION: Our data reveal that changes in OS markers are correlated with levels of labile plasmatic iron and ferritin and the number of transfusions.

Key words: Antioxidant enzymes; Labile iron content;

Oxidative stress; Polytransfused subjects

Core tip: Here, the readers will find important information regarding iron accumulation and its correlation with oxidative damage markers in anemic subjects following blood therapy. This research, regarding iron accumulation and its associated toxicology is remarkable because the mechanism(s) involved in its mode of action are not fully understood. Thus, our data are extremely important for research concerning the involvement of iron overload on the development of human diseases.

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INTRODUCTION

Iron is an essential element of cells that participates in various cellular processes due to its ability to accept and donate electrons, interconverting between Fe^{3+} and Fe^{2+} forms[1]. However, this redox property renders iron potentially toxic in biologic systems. The labile plasmatic iron (LPI) component of non-transferrin-bound iron is redox-active, chelatable and capable of permeating into organs to induce tissue iron overload[2]. Thus, LPI is an accessible diagnostic marker of iron overload and cell toxicity[2]. Moreover, LPI can participate in the Fenton reaction and generate a large amount of reactive oxygen species (ROS)[3]. To prevent ROS overproduction, circulating and intracellular free iron are tightly regulated by binding to transferrin, ferritin and other proteins[4,5]. However, the iron balance can be disrupted in some situations, such as with chronic anemia, repeated blood transfusions, and following increased gastrointestinal absorption, which lead to iron overload[6]. Therefore, subjects undergoing repeated blood transfusions are at risk of iron-associated toxicity[7].

Elevated tissue iron can overwhelm protective mechanisms and lead to an increase in iron complexes with small molecules, such as nucleotides and citrate, in the serum of transfusion patients and also within cytoplasm and organelles[8,9]. Furthermore, repeated blood transfusions increase the levels of iron available to generate catalytically active complexes, free radicals and oxidative damage[9]. Thus, LPI promotes free radical formation that culminates in the oxidation of biomolecules. Accordingly, iron overload in humans and in experimental animals is associated with oxidative stress (OS)[10]. Indeed, it is known that an imbalance in the oxidant/antioxidant status of the cell is associated with OS, leading to important cellular macromolecule modifications and cell damage[11]. The cell injury observed in patients with iron overload is attributed to OS[12]. Hence, the oxidation reactions result in the formation of lipid peroxides and protein carbonyls, damaged deoxyribonucleic acid bases, and mitochondrial dysfunction[13]. Additionally, individuals with an iron overload demonstrate impaired antioxidant defenses[6]. Accordingly, the long-term consequence of chronic iron overload is organ injury, which could contribute to the initiation and development of several metabolic disorders, such as endocrinopathies, diabetes mellitus, cirrhosis, hypogonadism and heart failure[14].

In general, oxidative damage of biomolecules can be counteracted by enzymatic as well as non-enzymatic defenses. Indeed, humans have several biologic mechanisms to defend against intracellular OS. One of the most important mechanisms involves the actions of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)[15]. In spite of a well-developed antioxidant defense system, cells can still be oxidatively damaged under some pathologic conditions[11].

Data concerning labile iron accumulation in anemic subjects receiving repeated blood transfusions and the association with oxidative damage markers are scarce in the literature. We hypothesize that OS correlates with LPI in anemic patients following therapy with repeated blood transfusions. In this study, we evaluated OS markers and the activity of enzymatic antioxidant defenses in the blood of patients receiving repeated transfusions and in control subjects (not transfused). Additionally, we determined the LPI and ferritin levels in these subjects and correlated both parameters with other evaluated markers.

MATERIALS AND METHODS

Chemicals

1,1,3,3-tetramethoxypropane, 2-thiobarbituric acid, sodium dodecyl sulfate, 5,5'-dithiobis-(2-nitrobenzoic acid), trichloroacetic acid, 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA), and 2,4-dinitrophenylhydrazine, were purchased from Sigma (St. Louis, MO, United States). The kit for iron determination was obtained from Bio-Systems Corp. (Beloit, WI, United States), kits for measuring SOD (RANSOD) and GPx (RANSEL) were purchased from Randox Laboratories Ltf. (Crumlin, United Kingdom), and the Total Protein kit for protein determination was obtained from BioClin (Delft, Netherlands). All the other chemicals were commercial products of the highest purity grade available.

Subjects

This study was approved by the Ethics Committee in Research of Universidade Federal do Pampa. Altogether, 39 individuals with unspecified anemia receiving blood transfusions and 15 healthy subjects (blood donors) from the Banco de Sangue do Municipio de Uruguaiana were included in the study. Since most of our patients were male, the female patients were excluded from this study. Thus, both anemic and control healthy individuals were male. Anemic individuals were included in the study if they were diagnosed according to the International

Classification of Diseases (anemia unspecified, ICD 10: D64.9), and were not diagnosed with other diseases, such as cancer, renal failure, hepatic disease, blood loss or others. Additionally, anemic patients had received blood therapy during the year prior to collection (*i.e.*, no more than 12 mo from the first transfusion until sample collection). Additionally, it is important to mention here that the sample collection was done before a new transfusion, namely clinical screening. The anemic subjects were divided into three subgrouP: (1) those that received less than five blood transfusions (n = 14); (2) those that received from five to ten blood transfusions (n = 11); and (3) those that received more than ten blood transfusions (n = 14).

Sample collection

Blood from controls and anemic subjects was collected by venous arm puncture and stored in tubes containing heparin. The plasma and cells were separated by centrifugation at 1500 r/min for 10 min and were subsequently used for biochemical analyses. All biochemical assays were done in duplicate or triplicate, depending on availability of samples.

Analysis of hemoglobin

The electrophoretic analysis of hemoglobin was performed using a Minicap system (Sebia, Norcross, France) according to the manufacturer's instructions, and controls were run with each test. The Minicap system uses the principle of capillary electrophoresis in free solution. Charged molecules are separated by their electrophoretic mobility in an alkaline buffer with a specific pH. Separation also occurs according to the electrolyte pH and electro-osmotic flow. Electropherograms were expressed with zones divided from Z1 to Z15 based on standardizing the location of hemoglobin as previously described[16].

Measurement of LPI

LPI refers to non-heme bound, non-ferritin bound and non-transferrin-bound iron (*i.e.*, free iron) according to the previously validated convention[17]. The LPI content was determined by its reactivity with ferrozine, in the presence of the denaturant sodium dodecyl sulfate and the reducing agents ascorbate and sodium metabisulphite, as previously described[18,19]. The results are expressed as μg/dL.

Ferritin

Ferritin content was determined as described by Bernard and Lauwerys[20]. Serum ferritin causes agglutination of latex particles coated with anti-human ferritin that is proportional to the concentration of ferritin and can be measured by turbidimetry. The results are expressed as μg/L ferritin.

Protein carbonyl determination

Protein carbonyl content, which is indicative of oxidation, was determined as described by Levine *et al*[21]. Plasma samples were added to 0.2 mL of 10% trichloroacetic acid and placed on ice for 5 min. After centrifugation (5 min), samples were incubated for 90 min at 37 ℃ with 1 mL of 10 mmol/L 2,4-dinitrophenylhydrazine in 2 mol/L HCl. Finally, proteins were dissolved in 6 mol/L guanidine and interference was removed after washing with ethanol-ethyl acetate 1:1 (v/v). The extent of the damage was estimated by reading absorbance at 370 nm. The results are expressed as nmol carbonyl/mg protein.

Determination of thiobarbituric acid reactive substances levels

Levels of thiobarbituric acid reactive substances (TBARS) in plasma were determined using the method described by Ohkawa *et al*[22]. In brief, samples were incubated in acidic medium containing 0.45% sodium dodecyl sulfate and 0.6% thiobarbituric acid at 100°C for 60 min. After centrifugation, the reaction product was determined at 532 nm using a 1,1,3,3-tetramethoxypropaneas standard and the results are expressed as nmol malondialdehyde/mg protein.

Total thiol determination

Plasmatic total thiol was determined as described by Ellman *et al*[23]. The colorimetric assay was carried out in 1 mol/L phosphate buffer (pH 7.4) and calculated against a standard curve constructed with glutathione. Total thiol content is expressed as nmol total thiol/mg protein.

Determination of DCHF-DA oxidation

The determination of intracellular oxidant production was based on the cleavage of DCHF-DA to DCHF, which fluoresces when oxidized by ROS according to previously described methods[24]. The plasma sample was diluted (1:10) in 10 mmol/L Tris-HCl buffer. Then, 50 μL of diluted plasma was incubated with 10 μmol/L DCHF-DA at 37 ℃ for 20 min. The fluorescence emission at 520 nm was measured using a Perkin-Elmer spectrofluorometer with an excitation wavelength of 488 nm and an emission wavelength of 520 nm. The results are expressed as arbitrary fluorescence units.

CAT activity

CAT activity was measured by the method previously described[25]. Packed erythrocytes were hemolyzed by adding 100 volumes of distilled water, then 20 μL of this hemolyzed sample was added to a cuvette and the reaction was started by the addition of 100 μL of freshly prepared 300 mmol/L H_2O_2 in phosphate buffer (50 mmol/L, pH 7.0) to give a final volume of 1 mL. The rate of H_2O_2 decomposition was measured by a spectrophotometer at 240 nm for a duration of 2 min. The CAT activity is expressed as UI/mg protein.

SOD activity

SOD activity was measured in erythrocytes using a RANSOD kit, which uses xanthine and xanthine

Table 1 Subject characteristics

Characteristics	Controls (n = 15)	Transfusions		
		< 5 (n = 14)	5-10 (n = 11)	> 10 (n = 14)
Age (yr)	40.1 (20–50)	62.8 (24–92)	64.8 (49–84)	57.5 (24–74)
Number of transfusions	0 (0)	3.20 (2–4)	7.17 (5–9)	18.78 (14–26)
Hemoglobin (g/dL)	13.8 ± 0.5	7.5 ± 2.1	6.75 ± 0.5	4.9 ± 0.9
Labile iron content (μg/dL)	108.9 ± 13.8	149.2 ± 45.1	216.2 ± 68.3[a]	366.9 ± 68.5[a]
Ferritin (μg/L)	219.6 ± 18.2	190.3 ± 11.7	221.2 ± 16.1	277.5 ± 27.5[a]

Values are presented as median (range), or mean ± standard deviation; [a]P < 0.05 *vs* control.

oxidase to produce superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazol chloride to form formazan red. The SOD activity was measured by the degree of inhibition of this reaction at 505 nm and is expressed as UI/mg protein.

GPx activity

GPx activity was determined in erythrocytes using the RANSEL kit according to the method previously described[26]. The GPx activity is expressed as UI/mg protein.

Protein determination

The protein content was determined by the biuret method using the Total Protein kit with bovine serum albumin as a standard. The copper ions in an alkaline medium (biuret reagent) react with peptide, producing a purple color, whose intensity is proportional to the concentration of proteins in the samples being measured in a spectrophotometer at 545 nm.

Statistical analysis

All results are reported as median (range) and presented as box-plot graphics for the different grouP of patients. Hemoglobin, LPI and ferritin levels are presented as mean ± SD deviation. A Shapiro-Wilk test was performed to assess the normality of data distributions, and Kruskal-Wallis analysis of variance followed by Dunn's multiple comparison tests were used when appropriate. Spearman's correlational analyses were also performed between variables. For all analyses, we used a GraphPad Prism 5.0 software, and a $P < 0.05$ was considered significant.

RESULTS

Patient characteristics are presented in Table 1. Electrophoretic analyses indicated that all subjects had normal hemoglobin profiles (data not shown). As expected, LPI and ferritin levels were higher in the transfused subjects. Specifically, subjects receiving five or more transfusions had significantly higher LPI levels ($P < 0.05$), and patients receiving more than ten transfusions had significantly higher ferritin levels ($P < 0.05$) compared to controls. Additionally, we found that the number of transfusions was significantly correlated with LPI and ferritin levels ($P < 0.05$) (Table 2).

The OS markers TBARS (Figure 1A), protein carbonyl (Figure 1B) and DCFH-DA oxidation (Figure 1C) were all significantly higher in transfused subjects compared to the control group ($P < 0.05$). However, total thiol levels were significantly lower in subjects receiving more than ten transfusions compared to controls ($P < 0.05$) (Figure 1D).

The activity CAT and GPx were significantly lower in subjects receiving five or more transfusions compared to controls ($P < 0.05$) (Figure 2A and C). SOD activity was significantly lower in subjects receiving more than ten transfusions compared with controls ($P < 0.05$) (Figure 2B). Furthermore, significant negative correlations were observed between the number of transfusions and the activity of these antioxidant enzymes ($P < 0.05$) (Table 2).

Additional correlations were found between LPI levels and OS markers ($P < 0.05$), with the exception of DCDH-DA oxidation (Table 2). LPI and ferritin were negatively correlated with antioxidant enzyme activities and total thiol, and positively correlated with carbonyl and TBARS levels (all $P < 0.05$). Indeed, it was found that antioxidant enzyme activities were positively correlated with total thiol levels, and negatively correlated with the levels of protein carbonyl and TBARS ($P < 0.05$). In contrast, protein carbonyl and TBARS levels were positively correlated ($P < 0.05$).

DISCUSSION

Our data are in accordance with a previous study showing that the increase in LPI content could lead to an increase in ROS generation, and consequently an increase in oxidative damage[12]. Additionally, based on data concerning hemoglobin profile, we discarded hemoglobin disorders in these individuals. These data are extremely important to avoid misinterpretations, as it was previously shown that any imbalance between α and β chains of hemoglobin (α or β-thalassemia, respectively) plays a crucial role in OS[27]. Besides, there are data linking the observed levels of the various biomarkers evaluated in this study to health outcomes, such as in renal failure[28] and breast cancer[29].

Taking into account our results and those previously found, it is plausible to assume that under blood transfusion therapy, the excess of labile (catalytically active) iron must generate free radicals *via* Fenton chemistry, resulting in oxidative damage to biomolecules *in vivo*[30]. Our assumption is further supported by a previous report showing that iron-catalyzed ROS generation leads to an increase in the genomic instability in hematopoietic progenitor cells[31]. Moreover, it was shown in animal models that iron overload causes liver damage *via* both oxidative and nitrosative mechanisms[32]. Indeed, we assume that under repeated blood transfusions, the iron content increases to values that overwhelm the protective mechanisms, leading to an increase in the amount of iron available to form complexes with small molecules, the

Table 2 Spearman's correlations between biochemical and oxidative markers in polytransfused subjects

	LPI	Ferritin	GPx	SOD	CAT	TBARS	DCHF-DA oxidation	Carbonyl	Total thiol
Transfusion number	0.8569[b]	0.7991[b]	-0.8796[b]	-0.7103[b]	-0.8143[b]	0.5114[b]	0.0111	0.5793[b]	-0.5555[b]
Total thiol	-0.4151[b]	-0.3354[b]	0.4830[b]	0.4849[b]	0.5401[b]	-0.2790[b]	0.0106	-0.1164	-
Carbonyl	0.5583[b]	0.5122[b]	-0.5613[b]	-0.3713[b]	-0.4862[b]	0.5208[b]	-0.0627	-	-
DCHF-DA oxidation	-0.1291	-0.0049	0.0370	-0.0446	-0.0401	-0.2293[a]	-	-	-
TBARS	0.4984[b]	0.4144[b]	-0.4638[b]	-0.3113[b]	-0.3457[b]	-	-	-	-
CAT	-0.7266[b]	-0.5944[b]	0.8945[b]	0.7251[b]	-	-	-	-	-
SOD	-0.6085[b]	-0.5744[b]	0.7443[b]	-	-	-	-	-	-
GPx	-0.7973[b]	-0.7144[b]	-	-	-	-	-	-	-
Ferritin	0.9112[b]	-	-	-	-	-	-	-	-

[a]$P < 0.05$, [b]$P < 0.001$ *vs* DCHF-DA oxidation. CAT: Catalase; DCHF-DA: 2',7'-dichlorodihydrofluorescein diacetate; GPx: Glutathione peroxidase; LPI: Labile plasmatic iron; SOD: Superoxide dismutase; TBARS: Thiobarbituric acid-reactive substances.

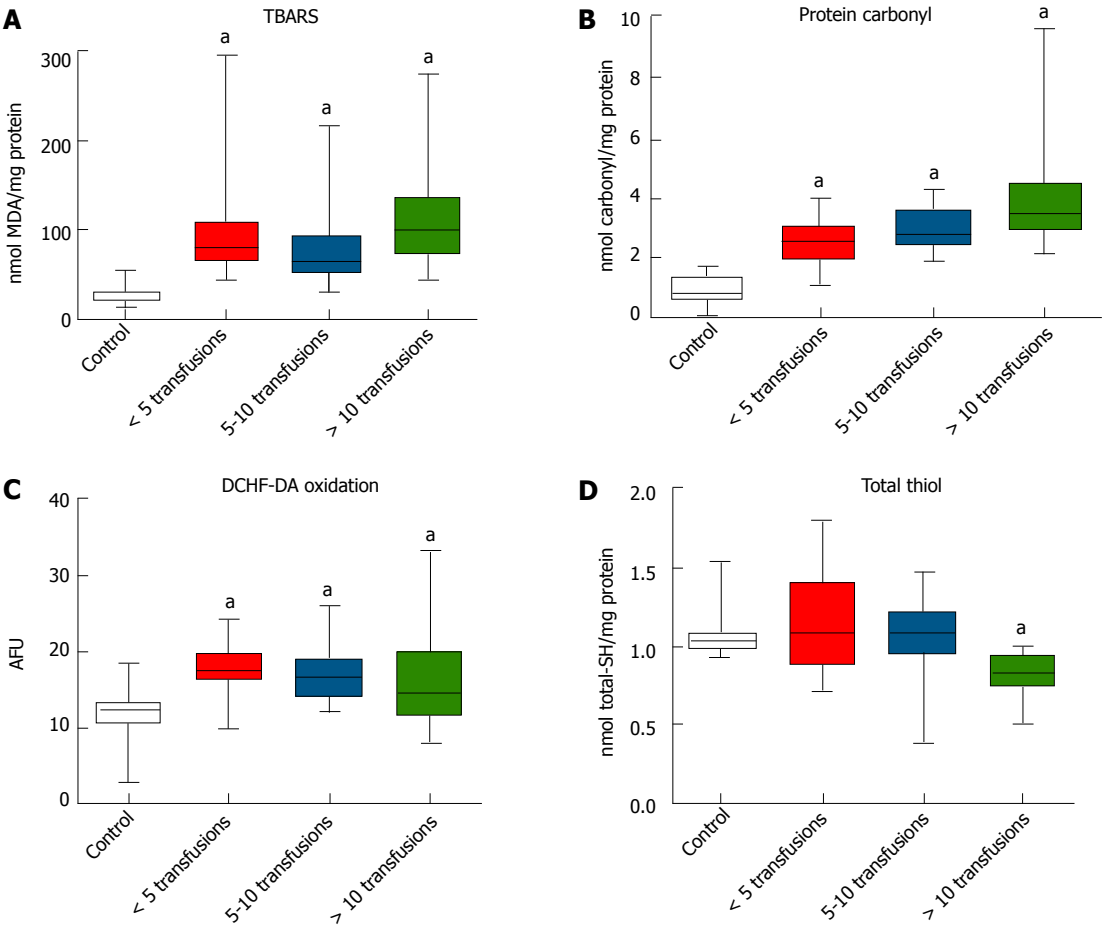

Figure 1 Oxidative stress markers in transfusion patients. A: Thiobarbituric acid-reactive substances (TBARS); B: Protein carbonyl; C: 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA) oxidation; and D: Total thiol levels in controls (n = 15), those receiving < 5 transfusions (n = 14), 5-10 transfusions (n = 11), and > 10 transfusions (n = 14); [a]$P < 0.05$ *vs* controls.

"catalytically active iron complexes". Thus, we assume that the ROS generated are responsible for the oxidation of DCHF-DA found in the transfused subjects, which is supported by a previous report showing that overload with iron (ferric nitrilotriacetate) leads to an increase in DCHF-DA oxidation in cultured rat hepatocytes[33].

Interestingly, we found some changes in the OS parameters even in the absence of significant iron accumulation, suggesting that alterations in OS markers could precede iron accumulation in patients following blood therapy. Accordingly, it seems logical that the differences in other parameters, such as hemoglobin and ferritin levels, could potentially contribute to the different oxidative state among patients. Thus, it is difficult to affirm that iron alone is the primary factor responsible for these differences. This point is extremely relevant and deserves further attention in future investigations.

The results of this study also show that levels of

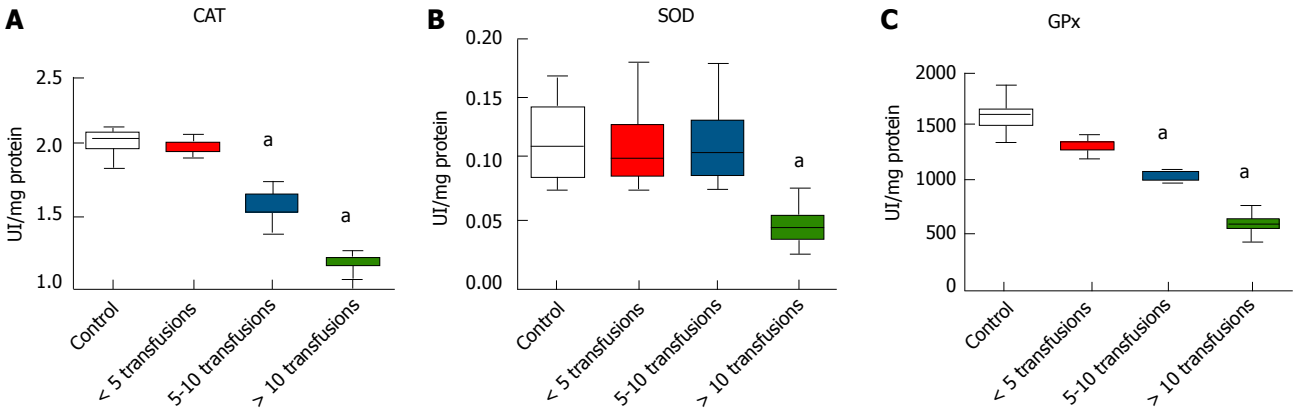

Figure 2 Antioxidant enzymes in transfusion patients. A: Catalase (CAT) activity; B: Superoxide dismutase (SOD) activity; and C: Glutathione peroxidase (GPx) activity in controls (n = 15), those receiving < 5 transfusions (n = 14), 5-10 transfusions (n = 11), and > 10 transfusions (n = 14); $^{a}P < 0.05$ *vs* controls.

TBARS significantly increased in subjects receiving blood transfusions, which was positively correlated to LPI content, ferritin content and the number of transfusions. These findings are in accordance to previous reports showing that the levels of lipid peroxidation products were increased in β-thalassaemic patients receiving blood transfusions[19] and in subjects with hepatic iron overload[6]. Moreover, we found a significant increase in the protein carbonyl in the subjects receiving repeated blood transfusions, which was correlated with LPI content. Additionally, our data are in accordance to a previous paper showing a significant increase in the protein carbonyl content associated with iron overload[33].

A significant reduction in total thiol levels was found in the subjects receiving repeated blood transfusions, which is consistent with a report showing a decrease in thiol content in the liver of rats treated with iron[34]. Albeit not completely understood, we believe that thiols are oxidized (consumed/used) in these subjects due to OS status following iron overload. Another possibility is that the iron could react non-enzymatically with thiols in plasma to generate ROS, which directly leads to reduction of antioxidant capacity in plasma and the increased susceptibility of blood components to oxidation[35]. Thus, this thiol-dependent free radical generation by iron overload might be a potential contributing factor for the changes in the oxidative markers reported here. Our assumptions are supported by a study showing that oxygen radicals can be produced by iron-catalyzed auto-oxidation of cysteine or glutathione[36]. Therefore, the generated ROS (either by Fenton chemistry or by iron-catalyzed auto-oxidation of thiols) may be responsible for the oxidation of other biomolecules reported here, such as lipids and proteins.

The results of the present study demonstrate a marked decrease in antioxidant enzyme activity in the subjects with iron overload, which is consistent with previous reports[30,33]. Moreover, enzyme activities were negatively correlated with LPI, TBARS and protein carbonyl levels. In line with this, we presume that the decrease in the enzymatic activity of antioxidants further contributed to the OS condition. Indeed, Chakraborty *et al*[19] showed that the decrease in antioxidant enzymes strongly contributes to an increase in OS markers (TBARS, protein carbonyl and ROS). Although our data do not support this supposition, we hypothesize that a decrease in the antioxidant enzymes reported here could, at least in part, be due to a decrease in their expression. Indeed, it was previously shown that both CAT and GPx were downregulated under OS conditions in human cells[37]. However, the mechanisms regulating the expression of antioxidant enzymes under iron overload remain to be explored in more detail.

Our data confirms the involvement of OS in patients following therapy with repeated blood transfusions. Additionally, we found that the changes in the OS markers are correlated with iron content, ferritin and the number of transfusions. Thus, iron chelators that efficiently decrease the levels of labile iron are candidates to counteract the iron-induced ROS generation[38]. However, more studies are necessary to better understand the mechanism(s) associated with iron-induced oxidative changes, to minimize the side effects associated to blood transfusion therapy, and to provide some clinical benefits. As antioxidant supplementation is not entirely safe and may cause unfavorable effects to different patients, more discussion on its potential benefits is warranted[39].

In conclusion, our data confirm the involvement of OS and its correlation with LPI and ferritin in unspecified anemic patients following therapy with repeated blood transfusions. However, we found some alterations of OS markers even in the absence of significant iron accumulation, which encourages us to further explore the changes in the OS parameters that occur before iron overload in subjects receiving blood therapy.

COMMENTS

Background

Iron is an essential element that participates in several metabolic activities of cells. However, in excess, iron can be a cause of oxidative stress (OS) in subjects undergoing blood transfusion therapy. Despite this, the relationship between plasmatic iron content, OS markers and the activity of antioxidant enzymes in anemic subjects receiving repeated blood transfusions remains to be better characterized.

Research frontiers

Blood therapy has been used in medical practice to treat anemic patients. However, the increase in the iron level in patients following blood therapy must be considered. Thus, the purpose of this research was to better understand the changes associated with OS markers in patients undergoing blood therapy in order to prevent iron-supported oxidative damage in anemic subjects.

Innovations and breakthroughs

Previous data have shown that blood therapy is associated with iron overload, and consequently, with oxidative changes in various tissues. However, efficient therapies to prevent the side effects associated with repeated blood transfusions are not known. Thus, elucidative studies regarding the plasmatic oxidative changes associated with iron overload are necessary. Here, the authors found that anemic subjects undergoing transfusions show increased levels of plasmatic labile iron, protein carbonyl, thiobarbituric acid reactive substances, and 2',7'-dichlorodihydrofluorescein diacetate oxidation, as well as decreased total thiol levels. Additionally, the activities of superoxide dismutase, catalase, and glutathione peroxidase were significantly lower in the transfused subjects. Significant correlations were found between the number of transfusions, plasmatic iron content, OS markers and the activity of the antioxidant enzymes.

Applications

The results of this study suggest that antioxidants could be associated with blood therapy. Additionally, iron chelators that efficiently decrease the levels of labile iron could be used to counteract the iron-induced generation of reactive oxygen species. However, more studies are necessary to better understand the mechanism(s) associated with iron-induced oxidative changes in order to minimize the side effects associated with blood transfusion therapy and to provide clinical benefits.

Peer review

This is a study that contains important information regarding iron accumulation in anemic subjects receiving repeated blood transfusions and its correlation with the plasmatic oxidative damage markers in these subjects.

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