Oxidative stress and antioxidant status in beta-thalassemia heterozygotes

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Background: *Several studies have evaluated the oxidant and antioxidant status of thalassemia patients but most focused mainly on the severe and intermediate states of the disease. Moreover, the oxidative status has not been evaluated for the different beta-thalassemia mutations.*

Objective: *To evaluate lipid peroxidation and Trolox equivalent antioxidant capacity in relation to serum iron and ferritin in beta thalassemia resulting from two different mutations (CD39 and IVS-I-110) compared to individuals without beta-thalassemia.*

Methods: *One hundred and thirty subjects were studied, including 49 who were heterozygous for beta-thalassemia and 81 controls. Blood samples were subjected to screening tests for hemoglobin. Allele-specific polymerase chain reaction was used to confirm mutations for beta-thalassemia, an analysis of thiobarbituric acid reactive species was used to determine lipid peroxidation, and Trolox equivalent antioxidant capacity evaluations were performed. The heterozygous beta-thalassemia group was also evaluated for serum iron and ferritin status.*

Results: *Thiobarbituric acid reactive species (486.24 ± 119.64 ng/mL) and Trolox equivalent antioxidant capacity values (2.23 ± 0.11 mM/L) were higher in beta-thalassemia heterozygotes compared to controls (260.86 ± 92.40 ng/mL and 2.12 ± 0.10 mM/L, respectively; p-value < 0.01). Increased thiobarbituric acid reactive species values were observed in subjects with the CD39 mutation compared with those with the IVS-I-110 mutation (529.94 ± 115.60 ng/mL and 453.39 ± 121.10 ng/mL, respectively; p-value = 0.04). However, average Trolox equivalent antioxidant capacity values were similar for both mutations (2.20 ± 0.08 mM/L and 2.23 ± 0.12 mM/L, respectively; p-value = 0.39). There was no influence of serum iron and ferritin levels on thiobarbituric acid reactive species and Trolox equivalent antioxidant capacity values.*

Conclusion: *This study shows an increase of oxidative stress and antioxidant capacity in beta-thalassemia heterozygotes, mainly in carriers of the CD39 mutation.*

Keywords: *Oxidative stress; Beta-thalassemia; Lipid peroxidation; Beta-globins; Thiobarbituric acid reactive substances; Mutation*

Introduction

Beta-thalassemia is a heterogeneous group of blood disorders characterized by decreased or absent synthesis of the beta-globin chain. The disorder may be caused by several molecular defects, most of them mutations, that affect the expression of the ß-globin gene. The gene is located on the short arm of chromosome 11p15.5. Modifications in the ß-globin gene that result in a partially active gene are called ß+ thalassemia, while defects related with the absence of expression are termed ß0[1].

In individuals with beta-thalassemia, unpaired globin chains and high cellular iron concentrations may promote oxidative damage to red blood cells with corresponding decreased survival in the bloodstream. Although iron is essential for metabolic processes, an iron excess can lead to the production of free radicals[2,3]. In subjects submitted to blood transfusions, iron overload is closely related to increased oxidative damage of tissues; chelating therapy is used to prevent the harmful effects of excess iron such as cardiomyopathy[4].

To protect themselves from pro-oxidant agents, which are generated both during cellular metabolism and as a result of pathologic processes, cells have both enzymatic and non-enzymatic defense systems. The oxidative state of the cell is related to the balance between the formation of oxide-reducing agents and the antioxidant defense system. Oxidative stress aggravates the symptoms of many diseases, such as hemolytic anemia[5]. According to Bogdanska et al.[6], antioxidant enzymes can be used as biological markers of some changes, and enzymatic determination is important to assess the response of the organism to stress agents.

Several studies have evaluated the oxidant and antioxidant statuses of thalassemia patients[3]. However, most studies have focused mainly on the severe and intermediate states and thus few data are available for the heterozygote profile[7]. Moreover, the oxidative status has not been evaluated for the different beta-thalassemia mutations, although such an assessment is important due to the large genotypic and phenotypic heterogeneity of different populations[8]. Thus, the objective of this study was to evaluate lipid peroxidation and Trolox equivalent antioxidant capacity (TEAC) in relation to serum iron and ferritin

in subjects who are heterozygous for beta thalassemia resulting from two different mutations (CD39 and IVS-I-110) compared to those in individuals without beta-thalassemia.

Methods

This study was approved by the Ethics Committee *of Instituto de Biociências, Letras e Ciências Exatas/Universidade Estadual Paulista* in São José do Rio Preto (IBILCE/UNESP). All subjects who participated in the study gave informed consent. One hundred and thirty subjects were evaluated including 49 who were heterozygous for beta-thalassemia (all Caucasians, 34 women and 15 men with ages varying between 18 to 79 years) and 81 healthy subjects who were not beta-thalassemia heterozygotes and thus comprised the control group (74% Caucasians, 36 women and 45 men with ages from 18 to 62 years). All individuals are from the northwestern region of Sao Paulo state.

All subjects underwent venipuncture for the drawing of blood samples which were submitted to the following procedures: hemoglobin screening tests, allele-specific PCR, used to confirm the mutations for beta-thalassemia, an analysis of thiobarbituric acid reactive species (TBARS) used to determine lipid peroxidation and a test for TEAC. The beta-thalassemia subjects were also evaluated for serum iron and ferritin status.

Beta-thalassemia screening tests

The samples were evaluated by hemoglobin electrophoresis at pH 8.6 and pH 6.2 and by high-performance liquid chromatography (HPLC) VARIANT (Bio-Rad Laboratories Brazil) using a Beta-thalassemia Short Program Kit[9]. The presence of hemoglobin A2 values above the normal range (2.5 to 3.5%) along with a clinical evaluation was used for the laboratory diagnosis of beta-thalassemia carriers. All of the individuals in the control group were tested for hemoglobinopathies. None of the control group members had signs or symptoms of anemia.

Molecular analysis of beta-thalassemia

Genomic DNA was extracted using the phenol/chloroform method[10]. Confirmation of mutations was by gene amplification using allele-specific sense and antisense primers. The 5'-GGC TGT B5A CAT CAC TTA GAC CTC-3' (sense) and 5'-AGA AGG B5B GGA AAG AAA ACA TCA-3' (antisense) primers, which produce a 659 base pair (bp) fragment were used as an internal control reaction[11].

The following pairs of primers were used to identify the CD39, IVS-I-110 and IVS-I-6 mutations: PS39W 5'-GAC TCA AAG AAC CTC TG-3' (sense) and PS39M 5'-GAC TCA AAG CTC AAC TA-3' (antisense); TB110W 5'-GGG TGG GAA AAT AGA CC-3' (sense) and TB110M 5'-GGG TGG GAA AAT AGA CT-3' (antisense); and IVSI6W 5'-GTC TTG TAA CCT TGA TA- 3' (sense) and IVSI6M 5'-GTC TTG TAA CCT TGA TG-3' (antisense). These primer pairs produce 439 bp, 377 bp and 273 bp fragments[11].

The amplification process involved denaturing at 95°C for seven minutes (CD39) or 94°C for seven minutes (IVS-I-110

and IVS-I-6), followed by 32 cycles of 94°C for 50 seconds (CD39 and IVS-I-110) or 35 cycles at 94°C for 50 seconds (IVS-I-6), an annealing step of 54°C for 50 seconds (CD39 and IVS-I-6) or 58°C for 50 seconds (IVS-I-110), and an extension step of 72°C for 50 seconds (CD39 and IVS-I-110) or 72°C for one minute (IVS-I-6), with a final extension at 72°C for seven minutes[11].

Evaluation of lipid peroxidation and total antioxidant capacity

The plasma level of TBARS was analyzed to evaluate lipid peroxidation. The measurement of TBARS is based on the reaction of malondialdehyde and other aldehydes with thiobarbituric acid (TBA) at low pH and high temperature forming a complex with an absorption peak at 535 nm. Values of up to 440 ng/mL are considered normal[12].

TEAC was determined according to its equivalence to Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid), an antioxidant that is a synthetic water-soluble analog of vitamin E. TEAC was measured using the colorimetric method. This method is based on the reaction of 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) with potassium persulfate ($K_2S_2O_8$) that produces the radical cation ABTS•+, which is a blue-green chromophore. The addition of a sample containing antioxidants to the preformed radical cation reduces the ABTS again, and the discoloration is analyzed at 734 nm[13]. The normal values established in this study were 1.85 to 2.31 mM/L, which correspond to the mean value ± three times the standard deviation. This interval corresponds, theoretically, to 99.73% of a data set with a Gaussian distribution.

Serum iron and serum ferritin

Serum iron was obtained by a two-point enzymatic test, using the Fe VITROS Chemistry Slides Kit (Ortho-Clinical Diagnostics Products; Johnson & Johnson), and ferritin was assessed by direct chemiluminometric technology using the ADVIA Centaur® system. Transferrin saturation was calculated by dividing the serum iron level by the total binding capacity of iron and multiplying the result by 100. Reference values were provided by the manufacturer as follows: 37 to 170 µg/dL serum iron for men and women, 22 to 322 ng/mL ferritin for men, 10 to 291 ng/mL ferritin for women, and 20 to 50% transferrin saturation for men and women.

Statistical analysis

Data were tested for a normal distribution by graphic inspection of the residues and for homogeneity of variances using the Levene test. This test confirmed that the use of a parametric test was appropriate for subsequent analyses. Therefore, the t test was used to compare TBARS and TEAC values of the beta-thalassemia and control groups, and between beta-thalassemia heterozygotes carrying the CD39 or IVS-I-110 mutation. The influence of serum iron, ferritin and transferrin saturation on TBARS values, independent of the mutation carried and gender

was evaluated by simple regression analysis. Statistical analyses were carried out using the Statistica 7 software (Statsoft Inc.) and the level of significance was set at 5% (p-value < 0.05).

Results

Analyses of lipid peroxidation and antioxidant status showed significantly higher mean values for TBARS and TEAC in beta-thalassemia carriers compared with the control group (independent t-test; p-value < 0.01: Table 1). It was noted that 65% (n = 32) of individuals who were heterozygous for beta-thalassemia had TBARS values above the reference limit. For the TEAC analysis, eight samples (16%) had values outside of the reference range, including one with a lower value and seven with higher values than the normal range established in this study.

To evaluate the possible effect of thalassemia mutations on the TBARS and TEAC values, samples were subdivided according to mutation. Of the 49 subjects who were heterozygous for beta-thalassemia, 18 (37%) carried the CD39 mutation, 23 (47%) carried the IVS-I-110 mutation, one (2%) carried the IVS-I-6 mutation, and seven (14%) had negative results for these three mutations. The individual carrying the IVS-I-6 mutation and those without an identified mutation were not included in the statistical analyses due to the small sample size.

The values for TBARS and TEAC for the CD39 and IVS-I110 mutations are presented in Table 2. Higher TBARS values were detected in individuals carrying the CD39 mutation compared to those with the IVS-I-110 mutation (independent t-test: p-value = 0.04), but no significant difference in mean TEAC values was observed between the groups (independent t-test: p-value = 0.39). In the TBARS evaluation, 14 (78%) of the individuals carrying the CD39 mutation and 12 (52%) individuals carrying the IVS-I-110 mutation had values above the reference limit.

Serum iron, ferritin and transferrin saturation values for the group with beta-thalassemia are shown in Table 3. There was no influence of iron levels ($r^2 < 0.09$: p-value > 0.05), transferrin saturation ($r^2 < 0.06$: p-value > 0.09) or ferritin ($r^2 < 0.10$: p-value > 0.05) on TBARS and TEAC values.

Discussion

As expected, an increase in TBARS levels, a lipid peroxidation indicator was observed in beta-thalassemia carriers compared to the control group thus confirming the increased exposure of beta-thalassemia carriers to oxidative stress. Additionally, in this disease, a relationship has been observed between increased oxidative stress and decreased levels of the paraoxonase 1 (PON1) protein expressed in the liver and found in blood associated with high-density lipoprotein (HDL)[7]; this relationship suggests an antioxidant capacity of PON1[14]. Thus, the decrease in PON1 also reflects a greater susceptibility to the oxidation of low-density lipoproteins (LDL)[2,7], a known risk factor for cardiovascular disease[15,16].

In beta-thalassemia, there is an excess of free alpha chains and a release of intra-erythrocytic heme which triggers a

Table 1 - TBARS and TEAC values for the control group and beta-thalassemia heterozygotes

		Control n = 81	Beta-thalassemia heterozygotes n = 49	Independent t-test p-value
TBARS (ng/mL)	mean ± SD	260.86 ± 92.40	486.24 ± 119.64	< 0.01
	Range	74-428	204-848	
TEAC (mM/L)	mean ± SD	2.12 ± 0.10	2.23 ± 0.11	< 0.01
	Range	1.85-2.31	1.78-2.55	

Reference range: TBARS = 0-440 ng/mL; TEAC = 1.85-2.31 mM/L

Table 2 - TBARS and TEAC values for the CD39 and IVS-1-110 mutations

Parameters		CD39 n = 18	IVS-I-110 n = 23	Independent t-test p-value
TBARS (ng/mL)	mean ± SD	529.94 ± 115.60	453.39 ±121.10	0.04
	Range	408-848	204-769	
TEAC (mM/L)	mean ± SD	2.20 ± 0.08	2.23 ±0.12	0.39
	Range	2.03-2.35	1.78-2.37	

Reference range: TBARS = 0-440 ng/mL; TEAC = 1.85-2.31 mM/L

Table 3 - Serum iron, ferritin and transferrin saturation values for heterozygous beta-thalassemia heterozygotes

Parameters		Beta-thalassemia n = 49
Serum iron (μg/dL)	mean ± SD	100.15 ± 26.66
	Range	(43-151)
Ferritin (ng/mL)	mean ± SD	165.04 ± 158.32
	Range	(10-709)
Transferrin saturation (%)	mean ± SD	31.53 ± 9.83
	Range	(13.3-51.3)

Reference range: TBARS = 0-440 ng/mL; TEAC = 1.85-2.31 mM/L

hemoglobin oxidation pathway, thus causing damage to the red blood cells[17]. With beta-thalassemia, auto-oxidation reflects the formation of superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2) resulting in oxidative stress. This study evaluated the effect of beta-thalassemia mutations on lipid peroxidation and antioxidant capacity, represented by TBARS serum levels and TEAC. The relationships between variations in TBARS and TEAC levels and the mutation type were also tested. The increased lipid peroxidation in subjects with the ß0 CD39 mutation compared to those with the ß+ IVS-I-110 mutation was expected because of the greater number of free alpha chains in ß0 thalassemia[1]. Some studies have found decreased catalase in beta thalassemia trait patients when compared to the reference range. However the decreased blood catalase activity is not related to specific beta-thalassemia mutations[18].

The TBARS measurement is a good indicator of pro-oxidant stimuli[2,3]. Although heterozygous beta-thalassemia is not related to increased iron as observed in the severe and intermediate forms of thalassemia, heme/free iron can inhibit the action of various cytoplasmatic enzymes thereby altering cellular homeostasis[19].

Thus, the cell experiences oxidative stress and there may be significant protein degradation and lipid peroxidation in the cell membrane[20]. In this study, there was no relationship between TBARS and TEAC values and serum iron, ferritin or transferrin saturation values, three parameters commonly used to assess iron levels[21]. However, in beta-thalassemia, excess iron can lead to organ damage, especially of the liver and heart, and to endocrine dysfunction[22]. Recent studies have shown the importance of other markers such as non-transferrin bound iron (NTBI) and labile plasma iron (LPI) to detect iron excess in thalassemia patients due to the direct correlation of these markers with the formation of free radicals[23,24].

The oxidizing capacity of beta-thalassemia heterozygotes identified in this study by increased TEAC values could be a response to increased oxidative stress. In fact, increased levels of detoxification enzymes, including superoxide dismutase, catalase and glutathione peroxidase, have been detected in thalassemia patients, indicating a possible mechanism for protecting against oxidative stress[25,26]. There are several hypotheses to explain the enhanced antioxidant status in patients with ß-thalassemia major. Two probable mechanisms are the compensating antioxidant response arising from excessive oxidative stress, and the transfusion-dependent elevation in the proportion of younger red blood cells. Another mechanism explaining the increase in antioxidant capacity may be related to chelation therapy by deferoxamine[27,28]. However, some studies have shown that individuals with homozygous thalassemia have a reduced antioxidant capacity, with depletion of vitamin E, decreased superoxide dismutase and glutathione peroxidase activity and an increase in ferritin, indicating that thalassemic individuals exhibit enhanced oxidative stress. Thus, decreased antioxidant enzyme activity may reflect the role of antioxidants in modulating the severity of the disease[29,30].

Conclusions

This study shows an increase of oxidative stress and antioxidant capacity in beta-thalassemia heterozygotes, mainly in CD39 mutation carriers, most likely due to the larger number of free alpha chains in ß0 thalassemia. It is possible that oxidative stress influences the increase in oxidative capacity that was detected in this study, regardless of the type of mutation (CD39 or IVS-I-110), indicating a possible mechanism for mitigating oxidative stress in this disease.

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