Investigation of Dynamic Thiol/Disulfide Homeostasis in Children With Acute Immune Thrombocytopenia

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Summary: Oxidative stress may play a role in the pathogenesis of immune thrombocytopenia (ITP), but the role of dynamic thiol/disulfide homeostasis has not been studied. The objective of this study was to assess whether there is a change in thiol/disulfide homeostasis in children with acute ITP. A total of 40 children with acute ITP and 50 healthy age-matched and sex-matched controls were included in this study. Serum total thiol and native thiol levels have been measured with a novel automatic spectrophotometric method. The amount of dynamic disulfide bonds and related ratios were calculated from these values. The average total thiol and native thiol levels of the patient group were found to be significantly lower than those levels of controls ($P < 0.01$). However, intravenous immunoglobulin (IVIG) treatment with 1 g/kg/d prevented these reductions. disulfide level was slightly, but not significantly, depressed in ITP patients, but it recovered following IVIG treatment. We detected no marked changes in disulfide/total thiol, disulfide/native thiol, and native thiol/total thiol ratios between groups. These results are the first to demonstrate that thiol/disulfide homeostasis plays a role in ITP pathogenesis, and IVIG treatment can prevent the reduced thiol levels in children.

Key Words: immune thrombocytopenia, childhood, thiol, disulfide, oxidative stress

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Immune thrombocytopenia (ITP) is an acquired autoimmune disorder characterized by a low circulating platelet count (ie, platelet count $< 100\times10^9$/L) and the generation of autoantibodies against glycoproteins expressed on the platelet surface.[1] Primary ITP is defined as isolated thrombocytopenia in the absence of any obvious cause or illness, whereas secondary ITP refers to the presence of a concurrent underlying disorder responsible for disturbed immune function leading to thrombocytopenia. Primary ITP is further classified according to its duration since diagnosis: newly diagnosed (acute, <3 mo), persistent (3 to 12 mo), and chronic (>12 mo).[1] Both the destruction of platelets and impaired megakaryopoiesis contribute to thrombocytopenia.[2] The pathophysiology of ITP is complex. Antiplatelet autoantibodies are detectable in only 50% to 60% of patients, and there are no ITP-specific diagnostic markers.[3,4] ITP is considered one of the most common hematologic disorders in children, affecting 4 to 8 per 100,000 children each year, and this number is increasing.[2,4,5] The incidence of ITP is the highest among children aged between 2 and 5 years.[6–8] ITP is an acute disease in most children, as thrombocytopenia resolves within a few weeks to months in the majority of children. Furthermore, the clinical manifestation of ITP is extremely variable. Although patients may be severely thrombocytopenic, they rarely present with severe bleeding.[9] Therefore, ITP is generally recognized as a benign condition, with the majority of children having minor or no bleeding despite extremely low platelet counts.[4,7] Nevertheless, the clinical course is often acute, and severe hemorrhages, such as intractable epistaxis, gastrointestinal bleeding, or intracranial hemorrhages, may occur.[10] Treatments for cases with ITP are aimed at achieving a safe platelet count, preventing serious bleeding, and improving quality of life.[11] A recent study reported increasing trends in hospitalization rates and health care costs.[12]

Plasma thiol pool is mostly formed by albumin and protein thiols and, to a lesser extent, by low–molecular-weight thiols such as cysteine, homocysteine, cysteinylglycine, glutathione, hydrogen sulfide, and γ-glutamylcysteine.[13,14] Although glutathione is the most common low–molecular-weight thiol involved in counteracting oxidative stress,[15] low levels of glutathione in erythrocytes have been reported in ITP patients.[16] The thiol-disulfide couple maintains a homeostatic intracellular and tissue redox status.[17] Thiols can undergo oxidation reaction via oxidants and form disulfide bonds.[18] These disulfide bonds are the earliest sign of protein oxidation, but they are reversible.[19] Disulfide bonds can be reduced back to thiol groups; thus, dynamic thiol-disulfide homeostasis is maintained.[17] Thus, disulfide bridges are thought to be a marker of oxidative stress, whereas thiols are suggested as members of the antioxidant system. There is a growing body of evidence showing that an aberrant thiol/disulfide homeostasis state is involved in the pathogenesis of a variety of diseases.[14,20] To the best of our knowledge, no study has investigated dynamic thiol/disulfide homeostasis in children with ITP. Therefore, the purpose of this study was to evaluate the dynamic thiol/disulfide homeostasis as an oxidative stress parameter in children with acute ITP by using a novel and automated assay.

MATERIALS AND METHODS

Study Population

A total of 40 children (23 boys and 17 girls) with acute ITP who were treated in the Pediatric Hematology and Oncology Department of the Gaziantep University Hospital, between May 2017 and July 2018, were enrolled in this study. In addition, 50 healthy children (26 boys and 24 girls), selected from the hospital's outpatient clinic, were included as age-matched and sex-matched controls. Children from 3 months to

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16 years of age who presented with newly diagnosed ITP, having a platelet count of $<100\times10^9$/L, and those taking no prior immunomodulating (eg, intravenous immunoglobulin [IVIG] or corticosteroids) treatments before diagnosis were included in the study. Children with laboratory and clinical features that were not compatible with a diagnosis of acute ITP, such as the presence of organomegaly, other cytopenias besides thrombocytopenia, other autoimmune phenomena, or features suggestive of infectious disease, for example, the Epstein-Barr virus infection and hepatitis, patients with chronic ITP (thrombocytopenia that persisted >1 year after the onset of illness), those taking immunomodulating treatments before diagnosis, patients with severe or life-threatening bleeding at presentation, children with acute or chronic inflammatory or infectious diseases, with a history of steroid usage, or who were previously treated for ITP with IVIG or other treatment modality were excluded from the study. Infants younger than 3 months were excluded to limit the inclusion of children with inherited or alloimmune thrombocytopenia.

The diagnosis of acute ITP was based on the presence of isolated thrombocytopenia (platelet count $<100\times10^9$/L), without any underlying disease. Compatible history, physical examination, normal hemoglobin concentration, white blood cell count and peripheral blood smear, and absence of underlying conditions/malignancy cases were required for the diagnosis. Bone marrow aspiration was performed only in case of atypical laboratory or clinical presentation. Children with acute ITP were treated with IVIG (1 g/kg/d, intravenous infusion) for 2 days, and followed up for 12 months following initial diagnosis. Treatment efficacy was assessed on the basis of clinical signs and laboratory findings. This study was approved by the local clinical ethics committee, and written informed consent was obtained from the parents or guardians of both the patients and the controls. The present research was conducted according to the principles described in the Declaration of Helsinki.

Blood Samples and Laboratory Measurements

In patients (for ITP-before treatment group) and controls, blood sampling was performed on admission. Blood samples in the ITP-after treatment group were collected when the platelet count exceeded the international consensus level of 100×10^9/L. Biochemical analyses were carried out within 30 minutes after blood collection. For thiol/disulfide measurements, venous blood specimens were stored for 20 minutes for clotting, and their sera were separated by centrifugation at 1500*g* for 10 minutes, placed into plain tubes, and stored at −80°C until analysis.

The serum native thiol (–SH) and total thiol (–SH+–S–S–) levels were measured using commercially available kits (Rel Assay Diagnostics, Mega Tıp, Gaziantep, Turkey). These spectrophotometric methods, developed by Erel and Neselioglu,[17] were assayed in an autoanalyzer. The reducible disulfide bonds were first reduced to free-form functional thiol groups. The unused reductant, sodium borohydride, was consumed and removed with formaldehyde, and all of the thiol groups, including the reduced and native ones, were measured after reacting with 5,5′-dithiobis-(2-nitrobenzoic) acid. Half of the difference between the total and native thiols yielded the dynamic disulfide (–S–S) content.

Statistical Analyses

All values were shown as the mean (SD) or percentage. The distribution of all variables was checked with the Kolmogorov-Smirnov test. Mann-Whitney *U* test was used for data with abnormal distribution. Otherwise, the differences between the mean values of 2 groups were analyzed using the unpaired Student *t* test. Kruskal-Wallis test was applied for comparison of >2 groups, and Dunn's multiple comparisons test was used as post hoc test. Sex of the 2 groups was analyzed using the χ^2 test. The Pearson test was used to assess the correlations, but the Spearman correlation analysis was performed for the data with abnormal distribution. GraphPad Instat (version 3.05; GraphPad Software Inc., San Diego, CA) statistical software was used. A *P*-value <0.05 denoted a statistically significant difference.

TABLE 1. Demographic and Laboratory Data of the Children With Acute Immune Thrombocytopenia and of Healthy Controls

	Control (N = 50)	ITP-Before Treatment (N = 40)	*P*
Age (y)	4.9 ± 3.3	5.3 ± 3.5	0.5795
Sex			0.7584
Male	26 (52)	23 (57.5)	
Female	24 (48)	17 (42.5)	
Weight (kg)	13.9 ± 3.5	14.2 ± 3.8	0.6983
Petechia	0	40 (100)	
Purpura	0	39 (97.5)	
Epistaxis (mild)	0	18 (45)	
Ecchymosis	0	15 (37.5)	
Hemoglobin (g/dL)	14.0 ± 2.9	13.4 ± 1.7	0.2499
Hematocrit (%)	38.8 ± 4.9	35.6 ± 4.2	0.0019
White blood cells ($\times10^3$/mm^3)	9.4 ± 1.9	9.2 ± 3.6	0.0648
Neutrophils ($\times10^3$/mm^3)	3.9 ± 1.5	4.1 ± 1.6	0.5433
Lymphocytes ($\times10^3$/mm^3)	3.0 ± 1.8	3.2 ± 1.7	0.5928
Platelet count ($\times10^3$/mm^3)	324.9 ± 65.4	21.8 ± 26.4	<0.0001
MPV (fL)	8.8 ± 1.2	9.0 ± 1.1	0.4172
CRP (mg/L)	1.0 ± 0.1	11.8 ± 7.1	<0.0001

Data are represented as mean ± SD values and n (%).
CRP indicates C-reactive protein; ITP, immune thrombocytopenia; MPV, mean platelet volume.

RESULTS

The demographic and clinical data of the subjects are presented in Table 1. There were no significant differences in the average age, sex, weight, hemoglobin, white blood cells, neutrophils, lymphocytes, and mean platelet volume between the patient and control groups. However, hematocrit and platelet counts were markedly low in the patient group ($P=0.0019$ and $P<0.0001$, respectively) when compared with controls. After IVIG treatment, hematocrit increased to 36.7% ± 3.0%, but this value was still markedly different from controls (38.8% ± 4.9%, $P=0.0338$). Platelet counts reached to $273.7\pm94.3\times10^3$/mm^3 after IVIG treatment, but this value was significantly low when compared with controls ($324.9\pm65.4\times10^3$/mm^3, $P=0.0044$). We detected an increase in C-reactive protein (CRP) levels (from 1.0 ± 0.1 to 11.8 ± 7.1 mg/L, $P<0.0001$). However, this increase in CRP was suppressed after IVIG treatment (2.6 ± 3.4 mg/L, $P=0.6877$).

All patients had petechia. In addition, purpura, epistaxis (mild), and ecchymosis were observed in 97.5%, 45.0%, and 37.5% of the patients, respectively. However, we have not recorded any hematuria, gastrointestinal bleeding, splenomegaly, or hepatomegaly in our patient group.

TABLE 2. Dynamic Thiol/Disulfide Parameters of the Children With Immune Thrombocytopenia and of Healthy Controls

	Mean ± SD			
	Control (n = 50)	ITP-Before Treatment (n = 40)	ITP-After Treatment (n = 40)	*P*
Native thiol (μmol/L)	384.6 ± 99.3	311.8 ± 106.8	466.2 ± 159.2	< 0.01* < 0.001†
Total thiol (μmol/L)	451.4 ± 111.7	370.0 ± 126.5	545.0 ± 172.9	< 0.01* < 0.001†
Disulfide (μmol/L)	33.4 ± 16.9	29.1 ± 17.7	39.4 ± 14.7	< 0.01†
Disulfide/native thiol (%)	9.1 ± 4.6	9.6 ± 5.6	9.1 ± 3.5	> 0.05
Disulfide/total thiol (%)	7.5 ± 3.3	7.7 ± 3.8	7.5 ± 2.6	> 0.05
Native thiol/total thiol (%)	85.1 ± 6.6	84.5 ± 7.5	84.9 ± 5.2	> 0.05

*ITP-before treatment versus control group.
†ITP-before treatment versus ITP-after treatment.
ITP indicates immune thrombocytopenia.

Table 2 and Figures 1 and 2 show the results of the dynamic thiol/disulfide measurements. Native thiol and total thiol values were significantly low when compared with the controls ($P < 0.01$ for both parameters, Fig. 1). These values were recovered after IVIG treatment, and marked changes were noted when compared with before and after IVIG treatment ($P < 0.001$ for both parameters, Fig. 1). No significant changes were observed in disulfide levels, but marked changes were observed when the before and after treatment values were compared ($P < 0.01$, Fig. 1). However, there were no statistically significant changes in disulfide/native thiol, disulfide/total thiol, and native thiol/total thiol ratios between the groups ($P > 0.05$, Fig. 2).

Although we performed correlation analysis, no significant correlations between thiol/disulfide parameters and white blood cells, neutrophils, lymphocytes, mean platelet volume, CRP, hematocrit, and platelet counts were noted ($P > 0.05$ for all values). In addition, there were no marked correlations between CRP and hematocrit or platelet counts ($P > 0.05$).

DISCUSSION

To the best of our knowledge, the present study is the first investigation on the association between dynamic thiol/disulfide homeostasis in pediatric acute ITP patients. The results of the present study demonstrated that native thiol and total thiol levels were markedly depressed in acute ITP, and IVIG treatment prevented these depressions. Slight changes in disulfide levels were also reversed with IVIG treatment. Thus, our findings suggest that endogenous thiols were depleted, and dynamic thiol/disulfide homeostasis is affected by acute ITP.

There are only a few studies that have addressed the involvement of oxidative stress in the pathogenesis of ITP. Brox et al[21] reported on the successful use of ascorbic acid, a well-known antioxidant, in the treatment of a small group of refractory ITP adult patients. However, several groups

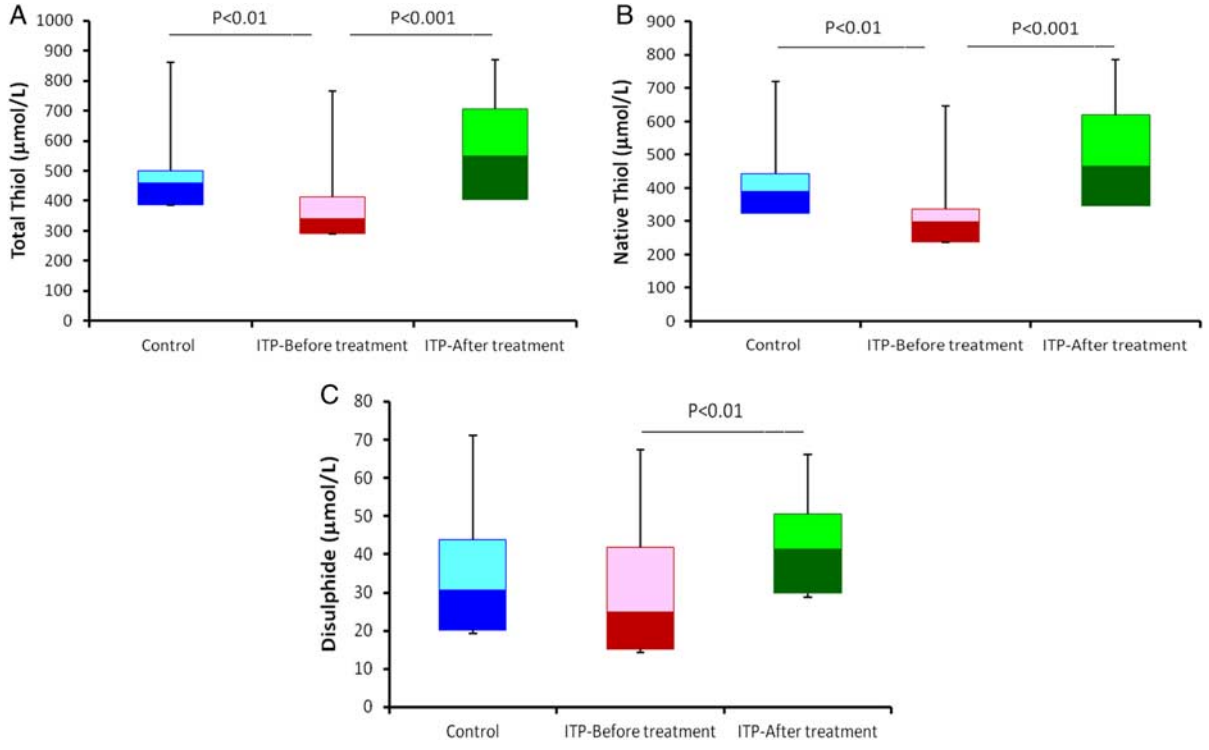

FIGURE 1. Box-plots of native thiol (A), total thiol (B), and disulfide levels (C) between the study groups. Lower and upper margins of each box represent the 25th and 75th percentiles, horizontal lines in the middle of the boxes represent median value, and whiskers represent lowest and highest values. ITP indicates immune thrombocytopenia. full color online

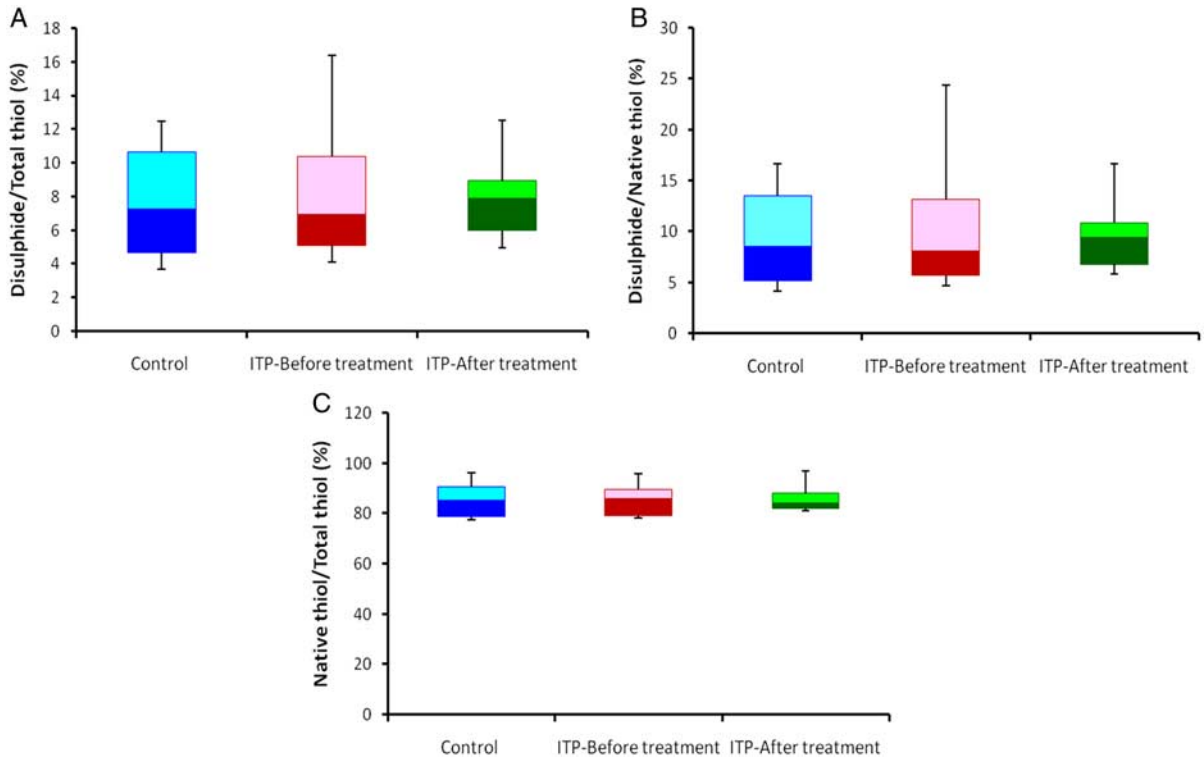

FIGURE 2. Box-plots of disulfide/native thiol (A), disulfide/total thiol ratios (B), and native thiol/total thiol ratio (C) between the study groups. Lower and upper margins of each box represent the 25th and 75th percentiles, horizontal lines in the middle of the boxes represent median value, and whiskers represent lowest and highest values. ITP indicates immune thrombocytopenia. full color online

failed to reproduce these results and suggested that ascorbic acid is not very effective in patients with refractory or chronic ITP.[22,23] In adult ITP patients, elevated levels of plasma and erythrocyte malonyldialdehyde, and reduced erythrocyte glutathione and ascorbic acid levels have also been reported.[16] Akbayram et al[24] investigated antioxidant parameters including serum malonyldialdehyde, total antioxidant capacity, and total oxidant status, which were found to be significantly elevated in acute and chronic ITP. Jin et al[25] showed that serum catalase, superoxide dismutase, glutathione peroxidase, glutathione levels, and total antioxidant status were significantly lower in adult patients with chronic ITP than in controls, whereas serum oxidized glutathione, malondialdehyde levels, and total oxidant status were significantly higher. Overexpression of vanin-1 (*VNN1*) gene, an oxidative stress sensor in epithelial cells, has been shown to be strongly associated with progression to chronic ITP in children.[26] Increased lipid peroxidation has been observed in ITP, with elevation in malondialdehyde levels.[16,24,25,27] Moreover, free radical-mediated protein modifications are highly immunogenic and can generate an antibody response by stimulating the adaptive immune system.[28] These findings suggested that augmented oxidative stress may be a prominent factor in the destruction of the platelet membrane, leading to the loss of cell membrane elasticity, increased cell fragility, and a shortened cellular life span. Thus, scavenging of oxygen radicals may provide a theoretical basis for the treatment of ITP patients. Indeed, Elalfy et al[27] reported that platelet count and bleeding score markedly improved in patients receiving antioxidant compared with placebo; thus, adjuvant antioxidant therapy might be considered as a complementary therapy for patients with ITP.

Reactive nitrogen species may also play a predominant role in ITP. Increased serum nitric oxide (NO) level in ITP patients has been reported.[25] NO has the potential of inhibiting apoptosis in human platelets.[29,30] NO rapidly interacts with superoxide to form peroxynitrite, which can oxidize a wide variety of biomolecules including thiols.[31] Peroxynitrite is able to induce apoptosis in platelets, thus reducing platelet life time and becoming clinically manifested as thrombocytopenia.[32] Collectively, these data imply that NO and peroxynitrite can contribute to thiol/disulfide homeostasis in ITP.

We detected lower hematocrit in patients with acute ITP-before treatment than in those in the control group. In addition, hematocrit remained in the diminished state after IVIG treatment. Our result is in agreement with the data reported by Akbayram et al[24] who found that hematocrit was low in children with ITP.

CRP is being used as a clinical marker for acute inflammation and infections. We observed a significant elevation in CRP levels at diagnosis, and decline of CRP levels after IVIG treatment in acute ITP patients. Our results support the previous study, which showed that slow platelet count recovery is associated with elevated CRP levels at diagnosis, and treatment with IVIG results in dropping of CRP levels accompanied by reduced clinical bleeding severity and normalization of platelet counts.[33] Furthermore, it is suggested that CRP may amplify antibody-mediated platelet destruction.[33] Thus, developing interventions that are aimed at diminishing CRP levels or inhibiting its function may be beneficial to the ITP patients.

In conclusion, the results of this study showed that thiol/disulfide homeostasis was disrupted in children with acute ITP. Our findings imply that reduced thiol levels play

a role in the pathogenesis of ITP, and measuring thiol levels might be useful for evaluating treatment response in ITP patients. Our data may also generate a basis for further studies evaluating the effect of thiol-based therapy to avoid oxidative stress-mediated autoimmunity, and to reduce bleeding risk of patients with ITP.

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