The Turkish Journal of Pediatrics 2022; 64: 876-881
https://doi.org/10.24953/turkjped.2022.73

Original Article

Decreased antioxidant capacity with serum native thiol and total thiol levels in children with hemophilia A: a prospective case-control study

Serçin Taşar[1], Ayşe Gül Güven[1], Asburçe Olgaç[2], Salim Neşelioğlu[3], Özcan Erel[3], Bülent Alioğlu[4]

[1]Department of Pediatrics, Ankara Training and Research Hospital, Ankara; [2]Department of Pediatric Metabolic Diseases, Dr.Sami Ulus Maternity, Child Health and Diseases Training and Research Hospital, Ankara; [3]Department of Clinical Biochemistry, Ankara Yıldırım Beyazıt University Faculty of Medicine, Ankara; [4]Department of Pediatric Hematology, Ankara Training and Research Hospital, Ankara, Türkiye.

ABSTRACT

Background. Experimental studies have addressed the role of oxidant stress in the pathogenesis of Hemophilia A. This study aimed to determine whether dynamic thiol-disulfide exchange, a recently recognized cellular defense system against oxidative stress, is disturbed in children with hemophilia A.

Methods. This prospective case control study included male children with hemophilia A (n=62) and randomly selected healthy age and sex-matched controls (n=62). Serum native thiol, total thiol and disulfide levels were analyzed with a novel spectrophotometric method. Ratios of disulfide/total thiol, disulfide/native thiol, and native/total thiol were calculated. Statistical comparisons were made using the independent samples t-test or the Mann-Whitney U test, according to whether the data were normally distributed or not.

Results. Serum native thiol (385.0 ± 35.9 versus 418.0 ± 44.3, respectively; p<0.001) and total thiol (424.2 ± 38.7 versus 458.0 ± 46.3, respectively; p>0.001) levels were significantly lower in children with Hemophilia A compared to controls. Children with hemophilia A had significantly lower serum native thiol to total thiol ratio than controls (p=0.024). Serum disulfide levels of children with hemophilia A were close to controls (19.2 [17.6- 22.1] versus 19.8 [17.8- 21.2]), respectively; p=0.879) whereas disulfide to native thiol ratio (p=0.024) and disulfide to total thiol ratio (p=0.024) were significantly higher.

Conclusions. Decreased antioxidant capacity with levels of serum native thiol and total thiol in children with hemophilia A might be regarded as evidence for the disturbance of thiol/disulfide balance. Antioxidant treatment can be a future target of therapy in children with hemophilia A.

Key words: Hemophilia A, children, thiol, oxidative stress.

Hemophilia A is a bleeding disorder caused by Factor VIII deficiency due to an X-linked single-point mutation. Although different novel treatment options are emerging in children with hemophilia A, early initiation of bleeding prophylaxis with factor replacement remains the most widely accepted approach.[1] With the prevention of bleeding complications as well as the decrease in fatal viral transmission rates, the life expectancy of the children with hemophilia A has reached to the normal population, and the quality of life of individuals has increased significantly. In the last decade, novel therapeutic products and gene therapy have become popular research topics as novel management strategies in patients with hemophilia A.[2,3]

In-vitro studies and experimental models have suggested an important role of increased oxidative stress in the emergence and

✉ Serçin Taşar
sercin_gozkaya@yahoo.com

Received 17th January 2022, revised 1st June 2022, accepted 26th June 2022.

progression of symptoms in several single-point mutation diseases, including hemophilia A.[4] Misfolding of newly synthesized Factor VIII in the endoplasmic reticulum triggers a pathway cited as unfolded protein respond, which in turn induces apoptosis by increasing oxidative stress. The reaction could be reversed with antioxidant treatment in-vitro and in mice.[5] Immunogenicity and antibody development in patients with Hemophilia A as a complication of Factor VIII replacement were shown to be increased by ex-vivo oxidation of Factor VIII, which was resistant to in vivo treatment with N-acetylcysteine.[6] Moreover, it has been shown that the joint and bone destruction in hemarthrosis, a typical hallmark of Hemophilia A, is caused by the inability of macrophages to adequately eliminate the oxidative stress created by the proteins (but not recombinant products) in the plasma-derived Factor VIII concentrates.[7] However, the exact cellular mechanisms by which oxidative stress contributes to Hemophilia A pathogenesis has not yet been clarified.[8]

Dynamic thiol-disulfide exchange has become a common indicator in recent studies focusing on the role of oxidant stress in the pathophysiology of many diseases. Thiols, which are organic compounds, form a balancing defense against oxidative stress with the sulfhydryl (-SH) group they contain, and reversible disulfide bonds are formed as a result of the oxidation of these sulfhydryl groups.[9] The instability of Factor VIII was suggested to be due to the lack of non-covalent bonds in the A2 subunit and in-vitro and in vivo studies demonstrated that creating genetically engineered disulfide covalent bonds to this domain improved the stability of Factor VIII, indicating a novel target of therapy.[10,11] Thus, we hypothesized that children with hemophilia A may have dysregulated thiol/disulfide homeostasis associated with an increase in oxidative stress as well as a compensatory change in disulfide levels due to Factor VIII instability. The present study investigated whether serum native thiol, total thiol, and disulfide levels, as well as the balance between these parameters as an indicator of antioxidant capacity, were significantly altered in children with hemophilia A compared to healthy children.

Material and Methods

A prospective case-control study was conducted in the Department of Pediatric Hematology, Ankara Training and Research Hospital, between August 2020 and December 2020. The local ethics committee approved the study (No: E-20:378), which was performed according to the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from parents of all participating children.

A total of 62 male children aged between 2 and 18 years, diagnosed with hemophilia A were included (study group). The criterion for inclusion in the study group was that the children had received at least one Factor VIII concentrate prior to the study. One of the physicians in the pediatrics department interviewed and examined the subjects regularly at one-month intervals. The exclusion criteria were active infection, coexisting chronic hepatic, renal, cardiac, autoimmune, and rheumatological diseases. The control group consisted of randomly selected 62 healthy age- and sex-matched children who were examined in the pediatric hematology outpatient clinics. Clinical characteristics, including the duration of disease, Factor VIII levels, the frequency and the latest time of Factor VIII infusion, were recorded.

Venous blood samples were obtained from the participants, and the separated serum samples after centrifugation at 1500 x g for 10 minutes were stored at -80 °C. The measurements of native thiol (–SH) and total thiol (–SH+–S–S–) levels in serum samples of both children in the study group and the controls were performed by commercially available kits (Rel Assay Diagnostics, Turkey), using the spectrophotometric method developed by

Erel and Neselioglu.[12] As described by the developers, reducible disulfide bonds were first reduced to free functional thiol groups with sodium borohydride. The reductant sodium borohydride was removed with formaldehyde and the total thiol content of the sample was measured using Ellman reagent. Half of the value obtained after subtraction of the native thiol content from the total thiol content gave the amount of disulfide bond.

Statistical analysis

Descriptive statistics were given as mean ± standard deviation and median with interquartile range (IQR) of 25% to 75% for continuous variables depending on their distribution. Numbers and percentages were used for categorical variables. The Kolmogorov-Smirnov test was used to analyze the normal distribution of numerical variables and checked by Q-Q plots and histograms. The Levene test was used to check the homogeneity of the variances. The Independent Samples t-test was used to compare independent groups with variables with normal distribution. For variables without a normal distribution, the Mann-Whitney U test was applied. For statistical analyses, "Jamovi project (2020), Jamovi (Version 1.6.9) [Computer Software] (Retrieved from https://www.jamovi.org) and JASP (Version 0.14.1) (Retrieved from https://jasp-stats.org) were used. A p value of less than 0.05 was considered as statistically significant.

Results

Baseline characteristics are given in Table I. The mean age was similar between children with hemophilia A and controls (11.20 ± 5.40 vs. 11.16 ±5.41 years respectively; p=0.967). The median duration of hemophilia A was 11.0 years. The Factor VIII levels of 10 children (16.1%) were <2%, 15 (24.2%) were 2% - <5%, 37 (59.7%) were 5% - 20%.

Children with hemophilia A had similar hemoglobin (13.3 ± 1.8 versus 13.9 ± 1.7 g/dL, respectively; p=0.064) and hematocrit (39.5% [37.7- 44.4] versus 42.0% [37.8- 46.5], respectively; p=0.167) levels as controls (Table II). Serum native thiol (385.0 ± 35.9 versus 418.0 ± 44.3, respectively; p<0.001) and total thiol (424.2 ± 38.7 versus 458.0 ± 46.3, respectively; p>0.001) levels were significantly lower in children with Hemophilia A compared to controls. Serum native thiol to total thiol ratio was significantly lower in children with hemophilia compared to controls (0.911 [89.7 - 91.6] versus 0.914 [90.3 - 92.3], respectively; p=0.024). Serum disulfide levels of children with hemophilia A were close to controls (19.2 [17.6- 22.1] versus 19.8 [17.8- 21.2]), respectively; p=0.879) whereas disulfide to native thiol ratio (4.9 [4.6 - 5.7] versus 4.7 [4.2 - 5.4], respectively; p=0.024) and disulfide to total thiol ratio (4.4 [4.2- 5.1] versus 4.3 [3.9- 4.8], respectively; p=0.024) were significantly higher (Table II).

Discussion

The present study aimed to determine whether serum native thiol, total thiol, and disulfide levels and the ratio between these variables changed in children with hemophilia A compared to normal healthy controls, indicating an increase in oxidative stress. We found that serum native thiol and total thiol levels, as well as native thiol/total thiol ratios, were significantly lower in children with hemophilia A compared to healthy children. Our findings indicate that the

Table I. Clinical characteristics of children with hemophilia A (n=62).

Variable	Value
Duration (year)†	11.0 [6.0- 17.0]
Level of Factor VIII activity‡	
<2%	10 (16.1)
2%-<5%	15 (24.2)
5%-20%	37 (59.7)
Interval for last exposure (days)‡	
≤2	13 (21.0)
2-≤3	35 (56.5)
3-≤4	8 (12.9)
4-≤7	6 (9.7)

†: median [Q1- Q3], ‡: n (%). FVIII: clotting factor VIII.

Table II. Comparison of oxidative markers in children with hemophilia A and healthy control groups.

Variables	Hemophilia A (n=62)	Control (n=62)	p
Hemoglobin§	13.3 ± 1.8	13.9 ± 1.7	0.064
Hematocrit (%)†	39.5 [37.7- 44.4]	42.0 [37.8- 46.5]	0.167
Native thiol (μmol/L)§	385.0 ± 35.9	418.0 ± 44.3	<0.001
Total thiol (μmol/L)§	424.2 ± 38.7	458.0 ± 46.3	<0.001
Native /total thiol (%)†	0.911 [89.7- 91.6]	0.914 [90.3- 92.3]	0.024
Disulfide (μmol/L)†	19.2 [17.6- 22.1]	19.8 [17.8- 21.2]	0.879
Disulfide/native thiol (%)†	4.9 [4.6- 5.7]	4.7 [4.2- 5.4]	0.024
Disulfide/total thiol (%)†	4.4 [4.2- 5.1]	4.3 [3.9- 4.8]	0.024

§: mean ± SD, †: median [Q1- Q3]. IMA: ischemia-modified albumin

thiol/disulfide exchange may be dysregulated with a decrease in thiol levels in response to increased oxidant stress in children with hemophilia A.

We found that serum disulfide levels did not differ significantly in children with Hemophilia A, but both disulfide/total thiol and disulfide/native thiol levels were significantly higher. In case of oxidative stress, disulfide levels would be expected to be higher relative to healthy controls, and whether this is related to Factor VIII instability remains to be clarified. The stability and activation of Factor VIIIa heterotrimer, the cleaved and activated form of Factor VIII, depends on the non-covalent linkage of its A2 subunit with the A1/A3C1C2 dimer.[13,14] Genetically engineered disulfide covalent bridges between the A2 subunit and A3 extended the FVIIIa half-life[10], and the creation of an engineered disulfide interdomain bond between A2-A3 increased factor VIIIa's clotting activity by 90%.[11] It was claimed that disulfide bonds only serve to stabilize protein molecules, but over time research has proven that dynamic thiol-disulfide homeostasis acts as a defense mechanism against oxidative stress by establishing redox responsive covalent disulfide bonds between oxidation-sensitive thiol groups. The increase in oxidative stress causes the electrons transferred by the oxidative products to form reversible disulfide bonds by oxidizing the redox sensitive native thiol compounds. In this dynamic process, disulfide bonds are reversibly formed and then reduced, creating a dynamic switch between the conformational and functional states of redox sensitive molecules. However, when oxidative stress continues, the native thiol capacity decreases while the disulfide level increases linearly.[15] Disulfide levels in patients with hemophilia A did not increase proportionally with the decrease in thiol levels, we think that it may be because the disulfide formation-reduction exchange compensates for Factor VIII protein stabilization.

Inability of thiol-disulfide homeostasis to adequately cope with oxidative stress has been reported to have a role in many chronic inflammatory and hematological diseases.[16-18] Also, although thiol-disulfide homeostasis dysregulation was suggested to be associated with several protein misfolding diseases[19], no study has so far investigated whether thiol-disulfide homeostasis is dysregulated in patients with hemophilia A. There have been controversial results for the levels of native thiol, total thiol, disulfide, and IMA in various hematological diseases.[16-18,20-22] In one study on oxidative biomarkers in sickle cell disease, the authors reported decreased levels of thiol and disulfide.[21] Acute immune thrombocytopenia was another condition, in which decreased native and total thiol levels were detected.[17] Increased IMA and disulfide levels and reduced native and total thiol were reported in adult patients with myelodysplastic syndrome. Moreover, the disulfide/native thiol was found to be an independent risk factor for mortality in

myelodysplastic syndrome.[16] Beta-thalassemia and iron deficiency anemia were associated with increased levels of native thiol, total thiol, disulfide, and disulfide to total ratio.[18,20,22,] The authors proposed several mechanisms for the high levels of thiols, such as a compensatory antioxidant response against excessive oxidative stress, and transfusion-dependent elevation in a proportion of younger red blood cells.[18,20] We suggest that prospective studies are needed to clarify such controversies.

There were several limitations of our study which need to be addressed. First, the lack of power analysis for determining the sample size might cause smaller study group that was insufficient to interpret the meaning of the observed results. Second, the analysis of serum albumin levels to clarify the reciprocal relationship between serum thiol and disulfide levels and serum albumin was lacking. Third, potential complications including hemophilic arthropathy and number of annual bleeding status of the patients were not presented. Associations between theses and oxidative stress might have strengthened the message of the article. Lastly, enzymatic and non-enzymatic investigations of oxidative stress biomarkers were not used to compare thiol and disulfide levels.

Decreased antioxidant capacity with levels of serum native and total thiols in children with hemophilia A might be regarded as some evidence for the disturbance of thiol/disulfide balance. In addition to gene therapy and new therapeutic products, antioxidant therapy can be a future research topic in terms of both inhibitor development and prevention of complications in children with hemophilia A.

Ethical approval

Ethical approval was obtained from the Ethics Committee of Ankara Training and Research Hospital prior to initiation of the research work (No: E-20:378).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: ST, AG; data collection: ST, AG, AO; analysis and interpretation of results: AG, SN; draft manuscript preparation: ST, ÖE, BA. All authors reviewed the results and approved the final version of the manuscript.

Source of funding

The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

REFERENCES

1. Mancuso ME, Male C, Kenet G, et al. Prophylaxis in children with haemophilia in an evolving treatment landscape. Haemophilia 2021; 27: 889-896. https://doi.org/10.1111/hae.14412
2. Mannucci PM. Hemophilia therapy: the future has begun. Haematologica 2020; 105: 545-553. https://doi.org/10.3324/haematol.2019.232132
3. Hassan S, Monahan RC, Mauser-Bunschoten EP, et al. Mortality, life expectancy, and causes of death of persons with hemophilia in the Netherlands 2001-2018. J Thromb Haemost 2021; 19: 645-653. https://doi.org/10.1111/jth.15182
4. Prasad KN, Bondy SC. Can a micronutrient mixture delay the onset and progression of symptoms of single-point mutation diseases?. J Am Nutr Assoc 2022; 41: 489-498. https://doi.org/10.1080/07315724.2021.1910592
5. Malhotra JD, Miao H, Zhang K, et al. Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. Proc Natl Acad Sci U S A 2008; 105: 18525-18530. https://doi.org/10.1073/pnas.0809677105
6. Peyron I, Dimitrov JD, Delignat S, et al. Oxidation of factor VIII increases its immunogenicity in mice with severe hemophilia A. Cell Immunol 2018; 325: 64-68. https://doi.org/10.1016/j.cellimm.2018.01.008

7. Bertling A, Brodde MF, Visser M, et al. Components in plasma-derived factor VIII, but not in recombinant factor VIII downregulate anti-inflammatory surface marker CD163 in human macrophages through release of CXCL4 (Platelet Factor 4). Transfus Med Hemother 2017; 44: 351-357. https://doi.org/10.1159/000472157

8. Poothong J, Pottekat A, Siirin M, et al. Factor VIII exhibits chaperone-dependent and glucose-regulated reversible amyloid formation in the endoplasmic reticulum. Blood 2020; 135: 1899-1911. https://doi.org/10.1182/blood.2019002867

9. Erel Ö, Erdoğan S. Thiol-disulfide homeostasis: an integrated approach with biochemical and clinical aspects. Turk J Med Sci 2020; 50: 1728-1738. https://doi.org/10.3906/sag-2003-64

10. Radtke K-P, Griffin JH, Riceberg J, Gale AJ. Disulfide bond-stabilized factor VIII has prolonged factor VIIIa activity and improved potency in whole blood clotting assays. J Thromb Haemost 2007; 5: 102-108. https://doi.org/10.1111/j.1538-7836.2006.02283.x

11. Gale AJ, Pellequer J-L. An engineered interdomain disulfide bond stabilizes human blood coagulation factor VIIIa. J Thromb Haemost 2003; 1: 1966-1971. https://doi.org/10.1046/j.1538-7836.2003.00348.x

12. Erel O, Neselioglu S. A novel and automated assay for thiol/disulphide homeostasis. Clin Biochem 2014; 47: 326-332. https://doi.org/10.1016/j.clinbiochem.2014.09.026

13. Hakeos WH, Miao H, Sirachainan N, et al. Hemophilia A mutations within the factor VIII A2-A3 subunit interface destabilize factor VIIIa and cause one-stage/two-stage activity discrepancy. Thromb Haemost 2002; 88: 781-787

14. Pipe SW, Saenko EL, Eickhorst AN, Kemball-Cook G, Kaufman RJ. Hemophilia A mutations associated with 1-stage/2-stage activity discrepancy disrupt protein-protein interactions within the triplicated A domains of thrombin-activated factor VIIIa. Blood 2001; 97: 685-691. https://doi.org/10.1182/blood.v97.3.685

15. Messens J, Collet J-F. Thiol-disulfide exchange in signaling: disulfide bonds as a switch. Antioxid Redox Signal 2013; 18: 1594-1596. https://doi.org/10.1089/ars.2012.5156

16. Ali UM, Tombak A, Dagdas S, et al. Is dynamic thiol/disulfide homeostasis associated with the prognosis of myelodysplastic syndrome?. J Med Biochem 2020; 39: 336-345. https://doi.org/10.2478/jomb-2019-0050

17. Beyazit H, Demiryürek AT, Temel MT, Pekpak E, Demiryürek S, Akbayram S. Investigation of dynamic thiol/disulfide homeostasis in children with acute immune thrombocytopenia. J Pediatr Hematol Oncol 2019; 41: 463-467. https://doi.org/10.1097/MPH.0000000000001494

18. Guzelcicek A, Cakirca G, Erel O, Solmaz A. Assessment of thiol/disulfide balance as an oxidative stress marker in children with β-thalassemia major. Pak J Med Sci 2019; 35: 161-165. https://doi.org/10.12669/pjms.35.1.307

19. Yadav K, Yadav A, Vashistha P, Pandey VP, Dwivedi UN. Protein misfolding diseases and therapeutic approaches. Curr Protein Pept Sci 2019; 20: 1226-1245. https://doi.org/10.2174/1389203720666190610092840

20. Odaman Al I, Ayçiçek A, Ersoy G, Bayram C, Neşelioğlu S, Erel Ö. Thiol disulfide homeostasis and ischemia-modified albumin level in children with beta-thalassemia. J Pediatr Hematol Oncol 2019; 41: e463-e466. https://doi.org/10.1097/MPH.0000000000001535

21. Özcan O, Erdal H, İlhan G, et al. Plasma ischemia-modified albumin levels and dynamic thiol/disulfide balance in sickle cell disease: a case-control study. Turk J Haematol 2018; 35: 265-270. https://doi.org/10.4274/tjh.2018.0119

22. Topal I, Mertoglu C, Sürücü Kara I, Gok G, Erel O. Thiol-disulfide homeostasis, serum ferroxidase activity, and serum ischemia modified albumin levels in childhood iron deficiency anemia. Fetal Pediatr Pathol 2019; 38: 484-489. https://doi.org/10.1080/15513815.2019.1627626