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Evaluation of hypercoagulability with rotational thromboelastometry in children with iron deficiency anemia

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ABSTRACT

Objective: Iron deficiency anemia (IDA) has been demonstrated to be a risk factor for thromboembolic events, although the pathogenesis of the development of thromboembolism in IDA is as yet unclear. The likelihood of children with IDA contracting hypercoagulability was evaluated in this *cross-sectional* study using rotational thromboelastometry (ROTEM).

Material and Method: A total of 57 children with IDA (median age 11 years; 37 female, 20 male) and 48 healthy children (median age 9.9 years; 23 female, 25 male) were enrolled in the study. Whole blood count, serum iron, transferrin saturation, ferritin level, prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen levels were ascertained, while ROTEM assays [intrinsic TEM (INTEM) and extrinsic TEM (EXTEM)] were used to measure and analyze coagulation time (CT), clot formation time (CFT), maximum clot firmness (MCF) and rate of maximum lysis (ML60%). *This study conforms to ethical standards, has been approved by the appropriate Institutional Review Board.*

Results: Hemoglobin, serum iron, transferrin saturation and ferritin levels were lower in the IDA group than in the control group ($p < 0.001$, for all), while the EXTEM and INTEM CT in the two groups were similar ($p > 0.05$). The EXTEM and INTEM MCF in the IDA group was higher than in the control group, while the INTEM CFT and rate of ML60% were lower than in the control group ($p < 0.001$, $p < 0.001$, $p < 0.05$, $p < 0.001$, respectively).

Conclusion: The ROTEM results suggest that although the platelet count and coagulation tests were within normal ranges in IDA, the tendency to coagulate may have been increased.

KEYWORDS

Iron deficiency; anemia; child; rotational thromboelastometry

Introduction

Iron deficiency anemia (IDA) is the most common type of anemia around the world [1–4]. Anemia is known to be an important risk factor in the development of many cardiovascular diseases [1–4]. The rate of development of ischemic stroke was found to be higher in those with IDA than in healthy individuals in a recent study [5,6], while IDA was diagnosed in more than half of the children who had suffered a stroke with no underlying disease. Furthermore, iron deficiency was identified as an important risk factor leading to strokes in small healthy children [7].

The association between IDA and venous thromboembolism has yet to be completely explained, having remained under-researched. It has been indicated that the most probable cause is secondary thrombocytosis [8]. Changes in the oxidative balance (i.e. decreased antioxidative defense and increased oxidative stress), accompanied by increased thrombotic risk, are associated with a high platelet count, increasing the risk of platelet aggregation [9]. In this regard, it has been suggested that the abnormal platelet count

and function seen in IDA can possibly act synergistically to increase the development of thrombosis [10]. Other factors that are considered to contribute to the formation of thrombosis include changes in erythrocyte morphology, increased blood flow due to hypoxia and endothelial damage [11–13]. *However, the procoagulant state in IDA was not supported by laboratory tests.*

Hypercoagulability refers to a situation in which blood demonstrates increased coagulative properties before the development of thrombosis, although not all the steps of coagulation can be evaluated through routine coagulation tests. The advantage of rotational thromboelastometry (ROTEM) is that it shows the dynamic changes during the coagulation process, including the coagulation factors, platelets, fibrin and every process of fibrinolysis, and that it can be used to make an efficient evaluation of the coagulation status of the patient [14]. Successful results have been achieved in establishing procoagulant conditions in many diseases through thromboelastometry [15–18].

The present study aims to measure the coagulation time (CT), clot formation time (CFT), maximum clot

firmness (MCF) and maximum lysis (ML60%) rate, and to identify any associations that may exist between these parameters and hemoglobin and iron levels and platelet count.

Material and method

Participating in this cross-sectional study were 57 children diagnosed with IDA between October 2016 and October 2017, as well as 48 healthy children. Children with acute or chronic infections, malabsorption syndrome or parasitosis in their medical history, and who had used iron supplements in the last three months were excluded from the study. Blood samples were taken from the patient group before the initiation of iron therapy. Anemia was defined as a hemoglobin level lower than the normal range, taking into account the age of the individual [19]. The WHO 2001 diagnostic criteria were used for the diagnosis of iron deficiency, with a ferritin level $<12 \mu\text{g/l}$ and transferrin saturation $<16\%$ accepted as iron deficiency [19]. The control group was composed of 48 healthy children of similar age and gender with no anemia or iron deficiency.

This study was approved by the College of Medicine Institutional Review Board (ethical approval no: 80558721/25). *Informed consent was obtained from all parents of the participating children*, and the study was conducted according to the Declaration of Helsinki.

Collection of blood samples

All blood samples were collected after eight hours of fasting and were studied immediately. Specimens for the ROTEM and coagulation tests were drawn into tubes containing a 3.2% buffered sodium citrate (0.129 mol/L) as the anticoagulant (9:1). Complete blood counts were measured using a Beckman Coulter LH750 machine (Kraemer Blut. Brea, CA, US). Prothrombin time (PT, s), activated partial thromboplastin time (aPTT, s) and fibrinogen (mg/dL) tests were performed immediately on a Siemens BCS XP machine (Tem International, Marburg, Germany). Serum iron levels were measured using a photometric method (Hitachicobas c 502, Roche Diagnostics, Germany), while serum ferritin levels were determined through a chemiluminescent immunoassay method (Cobas E-602, Roche Diagnostics, Germany). Transferrin saturation was calculated as $\text{serum iron} \times 100 / \text{total iron-binding capacity}$.

ROTEM analysis

A Rotation TEM was performed according to the manufacturer's guidelines using a Thromboelastometry Coagulation Analyzer model Gamma 2500 (Tem International, Munich, Germany). The two standard ROTEM

assays, termed intrinsic TEM (INTEM) and extrinsic TEM (EXTEM), were performed, in which the intrinsic and the extrinsic coagulation pathways are triggered, respectively [20,21]. In INTEM, coagulation is activated with 20 μL of contact activator (partial thromboplastin-phospholipid from rabbit brain extract and ellagic acid, in-TEM[®]; Tem International, Munich, Germany). In EXTEM, coagulation is activated by a 20 μL of tissue factor (TF, tissue thromboplastin from rabbit brain extract, ex-TEM[®]; Tem International, Munich, Germany). The mean parameters obtained were CT (s), CFT (s), MCF (mm) and ML (LY60%). CT is the time from the beginning of the coagulation analysis until an increase in amplitude of 2 mm, which reflects the initiation phase of the clotting process, while CFT is the time taken for the amplitude of the thromboelastogram to increase from 2 to 20 mm, and reflects the propagation phase of the entire blood clot formation. MCF is the maximal amplitude reached during TEM [20], and correlates with platelet count and function as well as with the concentration of fibrinogen [22]. ML60% is the percentage decrease in amplitude 60 minutes post MCF. A 'hypercoagulable profile' was defined as a shorter CFT and a higher MCF than the corresponding values in the healthy controls [23].

Statistical analysis

Statistical analyses were performed using SPSS for Windows version 15.0 software (SPSS, Inc., Chicago, IL, US). Categorical data were compared using a Chi-square test, while the normality of distributions was evaluated using a Kolmogorov–Smirnov test. Descriptive statistics were reported as mean and standard deviation (mean $\pm$ SD) for the normally distributed variables, and the median and interquartile distribution (Q1–Q3) range was used for other variables. Group comparisons with non-normal distribution were analyzed using the non-parametric Mann–Whitney U-test, and otherwise, an independent sample t-test. In order to evaluate the correlation between the variables, a Spearman correlation test was applied. A value of $P < 0.05$ was considered statistically significant.

Results

Among the IDA group, 37 (65%) children were female and 20 (35%) were male, while among the control group, 23 (48%) were female and 25 (52%) were male. The median age was 11 (2–14) years and 9.9 (8–13) years in the IDA and control groups, respectively. The age and gender distribution was similar in the two groups ($p > 0.05$). The median hemoglobin (Hb)

value, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and red cell distribution width (RDW) were lower, while platelet count was higher, in the IDA group than in the control group (p

<0.001, $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.01$, respectively). The median serum iron, transferrin saturation and ferritin levels were significantly lower in the IDA group than in the control group ($p < 0.001$, for all). No significant differences were identified in the PT, aPTT or fibrinogen levels of the two groups ($p > 0.05$, for all). The clinical characteristics, blood count, iron parameters and coagulation test results of the IDA and control groups are presented in Table 1.

The ROTEM values of both the study and control groups were within normal reference ranges. The median INTEM CFT value of the IDA group was lower, and the mean EXTEM MCF and INTEM MCF values were statistically significantly higher in the IDA group when compared to the control group ($p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively) (Table 2). The median EXTEM ML and INTEM ML values were statistically significantly lower in the patient group than in the control group ($p < 0.001$, for both) (Table 2).

Positive correlations were found between EXTEM and INTEM MCF and the platelet count, and negative correlations were found between EXTEM and INTEM MCF and hemoglobin, MCV, MCH and serum iron ($p < 0.001$, for all). Negative correlations were found between EXTEM CFT and INTEM CFT and platelet count ($p < 0.001$), while positive correlations were

found between INTEM CFT and hemoglobin, MCV and MCH ($p < 0.05$, for all). Positive correlations were found between EXTEM ML and INTEM ML and MCV, MCH and serum iron ($p < 0.001$, for all), and a positive correlation was found between INTEM ML and hemoglobin ($p < 0.001$). No correlations were found between the ROTEM parameters and PT, aPTT and fibrinogen levels.

Discussion

There are various studies in literature analyzing thrombotic complications in adults and children with IDA. IDA has been shown to represent a significant risk in children who have suffered an ischemic stroke [7]. Azab et al. reported that the possibility of children with a prior history of stroke to have IDA was 3.8 times higher than in children without a history of stroke [24]. Benedict et al. reported that IDA was a preventable cause of sinovenous thrombosis, and that a diagnosis of IDA should be considered in small children when a cerebral sinovenous thrombosis is diagnosed [25].

Thrombotic complications have been considered to develop secondary to the changes in platelet count and/or function in IDA [26] and that the mechanism of the etiology is as possible reactive thrombocytosis and erythropoietin-mediated platelet hypersensitivity [25,27]. Anemic patients require more blood flow to overcome oxygen deficiencies, and the increased blood flow can result in endothelial damage and platelet aggregation, and hence a thrombus formation cascade [11].

In this study, we observed that CFT decreased while MCF increased in children with IDA. The findings suggest that the tendency to coagulation increased, that clot stability was impaired and the clot strength increased [23]. MCF is associated with platelet count and platelet functions [14,28], and is a direct marker of the maximum dynamic properties of GPIIb / IIIa mediated-fibrin and platelet aggregation [29]. In our study, a positive correlation was identified between platelet count and MCF, and a negative correlation between platelet count and CFT. This finding suggests that platelet count is an important parameter in the formation of a tendency for coagulability in IDA. However, observations of thrombosis in patients without thrombocytosis has led some to believe that other pathological changes may be present in the pathogenesis of thrombosis, in addition to the platelet count [6]. Abnormal platelet activation and function are possibly more important than the absolute platelet count [10]. It has been suggested that increased platelet activity in iron deficient patients is associated with oxidative stress [9]. Tekin et al. reported that the collagen and adenosine diphosphate (ADP) aggregation responses, in addition to high glutathione levels in children with IDA, were significantly higher than in healthy controls,

Table 1. Clinical characteristics, blood count, iron parameters and coagulation test results of the IDA and control groups.

	IDA group (n = 57)	Contol group (n = 48)	p
Age	11.0 (2–14)	9.9 (8–13)	>0.05
Female/Male	37/20	23/25	>0.05
Hb (g/dl)	8.4 (6.8–9.4)	12.80 (12.0–13.2)	<0.001
MCV (fl)	58.20 (54.35–62.75)	79 (77–81)	<0.001
MCH (pg)	17.90 (16.05–19.45)	26 (24.7–27.55)	<0.001
RDW (%)	20.20 (18.75–22.15)	13.40 (12.97–15.80)	<0.001
Platelet ($\times 10^9/L$)	350.50 (280–436.25)	285.50 (242.25–370.25)	0.003
Serum iron($\mu g/dl$)	20 (15–27.5)	37.50 (29–61)	<0.001
Saturation (%)	4.66 (3.48–6.30)	16.6 (11.19–21.22)	<0.001
Ferritin(ng/ml)	5.50 (3.65–9.70)	28.24 (22.25–36.90)	<0.001
PT (s)	12.60 (12–13)	12.8 (12–13.5)	>0.05
aPTT (s)	28.60 (26.80–31.10)	30.10 (32.70–35)	>0.05
Fibrinogen (mg/dl)	251 (227–301)	275 (250–299)	>0.05

Note: Hb: Hemoglobin, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, TIBC: Total iron binding capacity, PT: Partial thromboplastin time, aPTT: activated partial thromboplastin time.

Table 2. Thromboelastometry values of the IDA and control groups.

	IDA group (n = 57)	Control group (n = 48)	p
EXTEM			
CT(s)	84(74–93)	84.5(73.25–102.75)	>0.05
CFT(s)	87(73–107)	90.5(78.25–101.0)	>0.05
MCF (mm)	69.64 $\pm$ 0.73	63.93 $\pm$ 0.6	<0.001
ML %60	5(3–8)	10(7–12)	<0.001
INTEM			
CT(s)	192.07 $\pm$ 4.41	189.51 $\pm$ 5.76	>0.05
CFT(s)	69(59–80.50)	77.50(67.25–86)	0.025
MCF(mm)	67.05 $\pm$ 0.58	62.14 $\pm$ 0.60	<0.001
ML %60	4(3–7)	7(4–9.75)	<0.001

Note: CT: clotting time, CFT: clot formation time, MCF: maximum clot firmness, ML60%: maximum lysis.

and concluded that increased oxidative stress may result in increased thrombocyte aggregation tendencies [9]. Accordingly, the cause of the increased MCF in IDA may be the changes in the platelet count, in addition to increased platelet activity.

Platelets play an important role in coagulation physiology by maintaining hemostatic balance, and are associated with such pathologies as atherosclerosis and thrombosis. Recent studies have revealed the possibility of using micro RNAs released from platelets as markers for atherosclerosis and ischemic events (micro RNA, platelet, thrombocytosis) [30]. The level of oxidative stress, changes in the coagulation system, and platelet activation and the micro RNA profile may play a role in the pathophysiology of the tendency to thrombosis.

This present study has demonstrated that although CT was similar between the two groups, CFT MCF together result in a clot formation with increased stability, in addition to the active platelets and fibrin interactions during the coagulation cascade. In a study in which the safety of ROTEM was evaluated in thromboembolic events among patients who had undergone non-cardiac surgery, no difference in the CTs of patients with and without thromboembolic events was demonstrated, while INTEM MCF was found to have the best predictive value for thromboembolic events [23].

Changes in erythrocytes are one of the mechanisms highlighting the association between IDA and thrombosis. Microcytic erythrocytes are known to affect blood flow and to increase the tendency to clotting by increasing blood viscosity [12]. In the present study, erythrocyte parameters such as MCV and MCH were found to be associated with all other steps of coagulation other than CT, and this result demonstrates that the degree of anemia, and changes in the erythrocyte volume and hemoglobin concentration play a role in the tendency to coagulate.

One of the findings of this study related to the decreased rate of ML in children with IDA, when compared to healthy children. It is possible to explain this result with two possible mechanisms. The first of these relates to the changes in the level of plasminogen activator inhibitor-1 in patients with IDA, which was demonstrated to be markedly high in an earlier study [31]. An increased plasmin activator inhibitor prevents plasminogen from converting into plasmin, and thus decreases fibrinolysis [32]. Another possible mechanism may be the changes in the structure of the fibrin clot in IDA. Increased clot strength and impaired stability are resistant to fibrinolysis, and may stimulate thrombosis [33–35]. The factors defining clot stiffness are number of platelets, local calcium concentration and the diameter and geometry of fibrin fibers [36]. A positive correlation has been found in the present study between MCF and platelet count, and a strong

negative correlation between the MCF and iron was demonstrated. The presence of a negative correlation between iron levels and MCF may mean, theoretically, that the clot firmness would decrease as iron levels increase, the reason for which may be hidden in the *in vitro* studies evaluating changes in the coagulation system due to the presence of iron ions. Jankun et al. demonstrated that the *in vitro* addition of Fe^{+3} into the plasma resulted in prolonged coagulation and a relatively unstable fibrin formation, as a result of its interaction with the proteins of the coagulation cascade in the early period [37]. Platelet aggregation and thrombin levels were demonstrated to be decreased, and the clotting capacity of fibrinogen was found to be impaired when ferrous sulphate was added to plasma [38]. As can be seen from the findings of these studies, increased iron concentrations in *in vitro* environments results in a weakened coagulation mechanism.

The limitations of this study include the small size of the study sample, and the fact that the ROTEM parameters were not studied following treatment.

In conclusion, iron plays an important role in ensuring the coagulant and anticoagulant systems work in harmony. CFT and ML rates are decreased in children with IDA, without change to the CT. These findings point out that the tendency to coagulate is increased in children with IDA. Changes in the erythrocyte morphology and iron levels, as well as changes in the platelet count and activity, may play a role in the development of a tendency to coagulate. *In our study, platelet counts were within the normal range in 79% of the patient group, suggesting that coagulability may be present without thrombocytosis in IDA. Based on these results, we believe that there may be other factors that contribute to coagulability in IDA, other than thrombocytosis, and that there is a need for studies to be carried out in this regard.* Although ROTEM demonstrated an increased tendency for coagulability, the nominal values within normal ranges may explain the rare incidence of thrombotic events in this group of children.

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Disclosure statement

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