RESEARCH LETTER

The role of red blood cells in hemostasis and whole blood clot contraction in adults after the Fontan procedure

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Introduction The incidence of thromboembolic complications in patients undergoing the Fontan procedure is high. It ranges from 8% to 33%, and is associated with a poor prognosis.[1] Although the exact mechanism of such a thrombotic risk is still poorly understood, the following risk factors have been suggested: imbalance of procoagulant and anticoagulant factors, increased platelet (PLT) activity, endothelial injury, and impaired clot susceptibility to lysis.[2]

Similarly to in vivo processes, whole blood clots formed in vitro undergo dynamic changes, including their volume reduction called clot contraction.[3-5] The blood clot contraction is driven by activated PLTs that interact with fibrin via, inter alia, cytoskeletal motility proteins, and generate a contractile force.[3-5] However, it has been suggested that reduced clot contraction is a risk factor for thromboembolic complications, such as acute ischemic stroke and residual vein obstruction.[3,5] Previous studies have linked impaired clot shrinkage to lower PLT count, PLT dysfunction, higher concentration of fibrinogen, elevated hematocrit (HCT), or leukocytosis.[3-8]

During clot contraction, internally packed red blood cells (RBC) change their shape and form geometric polyhedral structures called polyhedrocytes.[6-8] Increased number of polyhedrocytes has been linked with longer ischemia and poor glycemic control.[7,8] The clinical relevance of polyhedrocytes is under investigation. Taking into account high incidence of polyglobulia and a nonpulsatile flow in the Fontan circulation (FC), further research is justified.[1,2,9-11]

The aim of this study was to investigate clot contraction and polyhedrocyte formation in vitro, and their potential associations with clinical and routine laboratory parameters in the Fontan population.

Patients and methods The study was conducted between January 2018 and December 2020, and included patients after the Fontan surgery hospitalized in our center. The control group comprised random members of the hospital personnel and their families aged between 18 and 40 years. Healthy controls with total cholesterol level of 5 mmol/l or higher, increased fibrinogen levels (>4 g/l), prolonged prothrombin time (PT) (>13 s), increased levels of liver enzymes (alanine aminotransaminase >41 U/l, aspartate aminotransferase [AST] >40 U/l, and γ-glutamyltransferase >40 U/l), or pregnancy were excluded. The Local Ethical Committee in Kraków approved the study (130/KBL/OIL/2018), and each participant provided their written informed consent.

Venous blood samples were drawn from all participants from the antecubital vein with minimal stasis after an overnight fast. Fibrinogen concentration and PT (international normalized ratio [INR]) were measured using an automated coagulometer (Behring Coagulation System [BCS], Siemens Healthcare Diagnostics, Marburg, Germany). Coagulation factors V, VII, and X (one-stage PT with the use of factor depleted plasma; Siemens Healthcare Diagnostics) were evaluated using the BCS. Anticoagulants were suspended for at least 24 hours more than the time needed to abolish their effect (INR below 2 for vitamin K antagonist [VKA], 7 days for aspirin, and 48 hours for non-VKA oral anticoagulants [NOACs]).

Clot contraction and polyhedrocytes The clot contraction analysis was conducted by assessing clot

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TABLE 1 Basic characteristics and selected parameters in the study population

Parameter	Fontan (n = 40)[a]	Control (n = 23)	*P* value
Age, y	21.5 (20–26)	29 (21–33)	0.02
Women	17 (42.5)	11 (55)	0.52
BMI, kg/m^2	21.6 (20.4–24.3)	21.56 (18.8–23.9)	0.43
WBC, 10^3/µl	5.4 (4.5–6.6)	6.5 (4.7–7.2)[b]	0.24
RBC, 10^6/µl	5.6 (5.4–5.9)	5.0 (4.7–5.2)[b]	<0.001
Hb, g/dl	16.5 (15.6–17.9)	14.3 (13.2–15.6)[b]	<0.001
HCT, %	49.3 (46.1–52.0)	42.4 (39.8–45.3)[b]	<0.001
MCV, fl	87.0 (84.8–89.8)	85.2 (83.7–87.6)[b]	0.15
MCH, pg	29.0 (28.3–30.4)	29.4 (28.4–30.4)[b]	0.88
MCHC, g/dl	33.5 (32.9–34.4)	34.0 (33.5–34.6)[b]	0.10
RDW, %	13.1 (12.6–14.3)	12.4 (12.0–12.8)[b]	<0.001
PLT, 10^3/µl	150 (121–198)	229 (212–244)[b]	<0.001
Creatinine, µmol/l	74.5 (67.5–84.5)	66.5 (61–78)	0.09
Glucose, mmol/l	4.7 (4.5–5.1)	5.0 (4.9–5.1)	0.04
Iron, µmol/l	17.2 (11.1–22.2)	16.9 (14.5–21.8)[b]	0.79
GGT, U/l	67 (43–102)	14.5 (12.0–19.5)[b]	<0.001
ALT, U/l	25 (20–30.5)	14.5 (12–19)[b]	<0.001
AST, U/l	26 (23–30)	18 (16–22)[b]	<0.001
Bilirubin, µmol/l	17.5 (14.4–29.6)	8.8 (6.6–11.7)[b]	<0.001
Albumin, g/l	42.1 (40.4–43.5)	40.5 (39.7–42.2)	0.12
Fibrinogen, g/l	2.3 (2–2.5)	2.6 (2.4–2.8)[b]	0.01
INR	1.22 (1.15–1.48)	1.02 (0.99–1.1)	<0.001
Factor V, %	49 (39–63)	79 (70–92)	<0.001
Factor VII, %	54 (44–64)	99.0 (84–104)	<0.001
Factor X, %	76.5 (59.5–87.0)	101.0 (97.0–109.0)[b]	<0.001
Clot contraction, µl	478 (434–528)	554 (532–576)	<0.001

Data are presented as median (interquartile range [IQR]) unless indicated otherwise.

a Median time from the surgery 20.5 (IQR, 18.0–22.0) years

b Data for 3 participants were not available.

SI conversion factors: to convert ALT, AST, and GGT to µkat/l, multiply by 0.0167; and hemoglobin to g/l by 10.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, γ-glutamyltransferase; Hb, hemoglobin; HCT, hematocrit; INR, international normalized ratio; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PLT, platelets; RBC, red blood cells; RDW, red cell distribution width; WBC, white blood cells

size differences after contraction using the methods described previously.[12] After appropriate sample preparation, the clot size was measured as a difference between the initial sample volume and the fluid volume around the clot after its contraction. The evaluation of polyhedrocytes within the whole blood clots was made under a scanning electron microscope, according to our previous experience[3,7,8] and literature.[5,6] More methodological details are given in Supplementary material.

Statistical analysis Statistica 13.0 software (StatSoft, Statistica 13.0, Tulsa, Oklahoma, United States) was used for statistical analysis. Values of continuous data were presented as means with SD or median with interquartile range (IQR), and qualitative data as numbers and percentages. The χ^2 test and the Fisher test were used to compare qualitative data, while the *t* test, the Mann–Whitney test, and analysis of variance were used for the comparison of quantitative data. Continuous variables were first checked for normal distribution with the Shapiro–Wilk test. To analyze the variables with non-normal distribution, the Mann–Whitney test was used, and the normally distributed data were assessed with the *t* test. Associations between the continuous variables were obtained by the Spearman rank-order correlation test. The adjustment for the confounding variable (age) involved a 2-way analysis of variance. For the adjustment analysis, 3 age categories were adopted (<20, 21–30, >30 years old). For all tests, a *P* value below 0.05 was considered significant.

Results The study included 40 Fontan patients (17 women [42.5%]; median age, 21.5 [IQR, 20–26] years) and 23 healthy controls. The Fontan patients were younger by about 8 years than the controls (TABLE 1). Most FC participants (33 [82.5%]) matched class II of the New York Heart Association (NYHA) classification of heart failure symptoms, 12.5% were in class III, and 5% in class I. As many as 13 Fontan patients (32.5%) had thromboembolic complications after the Fontan surgery. Pulmonary embolism was found in 7 patients (17.5%), ischemic stroke in 2 (5%), while FC thrombus formed in 4 individuals (10%). Most of the patients received aspirin (22 [55%]), 12 (30%) were treated with VKA, and 5 (12.5%) with NOACs.

RBC count and HCT were higher in the Fontan patients than in the control group (TABLE 1). There were no differences between the groups with regard to mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and mean corpuscular volume (MCV). The Fontan patients had lower PLT count, with thrombocytopenia (PLT <150 × 10^3/µl) in 20 (50%) cases. Moreover, fibrinogen concentration was by 11.5% lower in the Fontan group, along with increased activity of the liver enzymes (TABLE 1).

Clot contraction was by 12.4% lower in the Fontan patients than in the controls (*P* <0.001; TABLE 1), even after adjustment for age (*P* <0.001). The patients on aspirin or oral anticoagulants (VKAs and NOACs) had similar mean contraction values as those without everyday thromboprophylaxis (467.95 [61.5] µl vs 500.5 [65.6] µl vs 464.8 [95.8] µl, respectively; *P* = 0.35). The most relevant correlations for clot contraction (all *P* <0.05) were observed for the PLT / fibrinogen ratio (*R* = 0.53), PLT (*R* = 0.43), MCV (*R* = –0.45), and liver enzymes, such as γ-glutamyltransferase (GGT) (R = –0.23) or AST (*R* = –0.22).

The presence of polyhedrocytes was additionally assessed in 11 cases in the Fontan group and in 11 controls (groups not different in terms of age and sex). The area covered by polyhedrocytes was by 30.6% lower in the Fontan patients than in the controls (mean, 50.2 [22.9]%

vs 72.3 [9.4]%; P <0.001), even after adjustment for age (P = 0.02). The percentage of polyhedrocytes correlated with clot contraction (R = 0.48), HCT (R = 0.45), hemoglobin concentration (R = 0.42), coagulation factor V (R = 0.3), total bilirubin concentration (R = –0.54), white blood cell count (R = –0.42), and with time from surgery (R = –0.55) (all P <0.05).

Discussion This study is the first to show impaired clot contraction in the Fontan patients. This phenomenon may support the concept that reduced clot contraction is an additional prothrombotic factor in the Fontan population, as has been demonstrated in ischemic stroke and residual vein obstruction.[3,5]

The factors influencing the clot contractility are still poorly known. Previous studies suggested that higher HCT, older age, and higher body mass index may have a negative impact on clot contractility in the healthy population.[3,5] Interestingly, positive correlation was found for PT, but only in healthy individuals, not in stroke patients.[5] In turn, in the stroke patients a weak positive correlation was found with monocyte count.[5]

It was showed that PLT count in the clot cross-section defines the contractile force.[4] In our study, we observed a positive correlation between the clot contraction and PLT count, similarly to the results in patients with residual vein obstruction and stroke.[3,5] This may suggest that the high prevalence of thrombocytopenia in the Fontan group may be one of the main factors associated with poorer clot contraction. Moreover, platelet function also changed after the Fontan procedure, similarly to other states where reduced clot contraction was observed.[5,10,11] Our results, revealing a high correlation coefficient for the PLT / fibrinogen, also support the concept that clot contraction depends mainly on the complex interactions between these factors.[5,6]

Contrary to the patients with venous thromboembolism, we found no association between the clot contraction and red cell distribution width (RDW),[3] probably due to the fact that the Fontan participants have higher RDW than controls, resulting from exacerbated apoptosis.[14] Instead, we observed an inverse correlation between the clot contraction and MCV. A significant deviation in the volume of erythrocytes is frequent in the Fontan population, and it is associated, among other things, with liver injury and a high risk of iron deficiency.[14,15]

The results of our study support the concept that liver dysfunction may play a major role in abnormal clot function in the Fontan patients. Following the Fontan procedure, almost all patients develop chronic liver disease that progresses over time.[9,10,13] Hepatic dysfunction in our study was reflected by higher levels of GGT and AST, with subsequent deficiency of coagulation factors synthesized in the liver.[1,2,9,10,13] We showed that hepatic dysfunction contributes to reduced clot contraction and polyhedrocytes formation. Moreover, reduced ability to form polyhedrocytes correlated with the time since surgery, which may additionally support the significance of liver damage progression.

The main limitation of our study is the fact that the analyzed groups of participants differed in age. However, we confirmed a small effect of this difference on the main results in an additional analysis. Moreover, the study groups were relatively small. Still, these are preliminary data and further research in a larger population with prospective observation is needed.

In conclusion, our study is the first to show that the Fontan patients have impaired clot contraction related to their liver dysfunction. In the next steps, clinical significance of our preliminary findings needs to be elucidated.

SUPPLEMENTARY MATERIAL

Supplementary material is available at www.mp.pl/paim.

ARTICLE INFORMATION

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