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Original Article

Altered endothelial function following the Fontan procedure

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Abstract *Objective:* Thrombosis has been widely described after the Fontan procedure. The vascular endothelium plays a central role in the control of coagulation and fibrinolysis. The aim of this study was to investigate if patients undergoing a modified Fontan procedure have impaired endothelial function and fibrinolysis in the late postoperative course. *Patients and methods:* We compared 23 patients aged from 7 to 26 years with age-matched healthy volunteers, collecting blood samples prior to and following standardized venous occlusion testing. Plasma levels of von Willebrand factor antigen, tissue-type plasminogen activator antigen, plasminogen activator inhibitor-1, and D-dimer were measured with enzyme-linked immunosorbent assay. *Results:* We found increased plasma levels of von Willebrand factor antigen in patients when compared to controls ($p = 0.003$). At the basal condition, concentrations of tissue-type plasminogen activator antigen and plasminogen activator inhibitor-1 antigen in the plasma, as well as their activity, were not significantly different between patients and controls. Following venous occlusion, concentrations of tissue-type plasminogen activator antigen in the plasma were significantly increased both in patients and controls, compared to pre-occlusion values. D-dimer was within the reference range. Multivariate discriminant analysis differentiated patients and their controls on the basis of differences for plasminogen activator inhibitor-1 and von Willebrand factor antigen ($p = 0.0016$). *Conclusions:* Our data suggest that patients with the Fontan circulation may have endothelial dysfunction, as indicated by raised levels of von Willebrand factor. Fibrinolysis seems to be relatively preserved, as suggested by appropriate response to venous occlusion.

Keywords: Functionally univentricular heart; fibrinolysis; thrombosis

Thromboembolism is a well recognized long-term complication of the Fontan circulation, and may decrease life expectancy and functional state of patients with functionally univentricular hearts. Several studies have looked for factors associated with the late occurrence of thromboembolism in this setting, implicating local and haemodynamic conditions,[1–4] as well as haematological abnormalities, the most frequent being deficiency of protein C.[3,5–10]

The vascular endothelium is known to play a vital role in the local regulation of pulmonary vascular tone, and in the function of vascular smooth muscle cells, as well as in the control of coagulation, fibrinolysis, and inflammation. A number of techniques are currently available for assessment of endothelial function. Most of them examine the ability of the endothelium to cause vasodilation in response to the pharmacological and physiological stimuluses that increase the release of nitric oxide. Endothelial activation, and/or injury, may also result in the release of various factors in the plasma, which can be used as markers of endothelial dysfunction. Selectins, von Willebrand factor, tissue-type plasminogen activator, thrombomodulin, and endothelins are among the endothelial markers frequently measured in the plasma.[11]

Endothelial-mediated vasodilation has been studied in patients with the Fontan circulation.[12,13] Endothelium-dependent vasomotion, however, may not be representative of other important aspects of endothelial function, such as modulation of thrombosis and fibrinolysis. Currently, there is no

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consensus concerning the postoperative type and duration of prophylaxis for anticoagulation in patients with the Fontan circulation. Altered endothelial function and fibrinolysis, if present, could emphasize the need for more aggressive strategies. Thus, the aim of our study was to look for evidence of impaired endothelial function and fibrinolysis in the late postoperative course of patients undergoing a modified Fontan procedure.

Methods

Population studied

We included in the study 23 patients who had previously undergone a modified Fontan procedure, and who were being followed-up in the outpatient clinic of the Department of Pediatric Cardiology, Heart Institute, São Paulo, Brazil. We used 15 gender and aged-matched, healthy volunteers as controls. The patients, and their parents, were informed about the research purpose of the collection of data and gave their informed consent. The study was approved by the Scientific Committee of the Heart Institute.

Collection of blood

All collections of blood were performed between 08:00 and 10:00 hours. After a period of 15 minutes resting supine, we collected peripheral venous blood via a single venepuncture in the antecubital fossa. We either avoided the use of a tourniquet, or limited it to less than one minute. Blood was collected in 1 to 10 volumes of 3.8% sodium citrate.

We then performed the venous occlusion stress test in patients and control subjects, inflating a cuff on the upper arm to pressures between systolic and diastolic values for 5 minutes. Samples were obtained from the occluded arm before deflation of the cuff.[14–16]

All samples were centrifuged at 3,000 revolutions per minute for 20 minutes. Plasma was separated and stored at minus 80 degrees Celsius until analysis. Aliquots were thawed only once for use. All post-stasis values were corrected for the haematocrit, using the following correction factor (F):

$$F = H_1(1 - 0.9 \times H_2)/H_2(1 - 0.9 \times H_1)$$

where H_1 represents the haematocrit before, and H_2 the haematocrit after occlusion.[17]

General laboratory determinations

A further sample was collected into ethylenediaminetetraacetic acid for determinations of the haematocrit, platelet count, and factor V. Saturations of oxygen were measured in room air using finger pulse oximetry.

Biochemical determinations

Levels of D-dimer (Asserachrom D-DI Diagnostica Stago, France), tissue-type plasminogen activator antigen (Imubind total tissue-type plasminogen activator, American Diagnostica, USA), plasminogen activator inhibitor-1 antigen (Imubind PAI-1, American Diagnostica, USA) and von Willebrand factor antigen (Imubind vWF, American Diagnostica, USA) were measured in the plasma using enzyme-linked immunosorbent assays. In addition, we used a chromogenic assay (Spectrolyse PAI, American Diagnostica, USA), for the quantitative determination of plasminogen activator inhibitor-1 activity. Samples were processed in duplicate. Results were obtained by comparison with a standard curve with reagents provided by the manufacturer. Results were expressed as nanograms per millilitre for D-dimer, plasminogen activator inhibitor-1 and tissue-type plasminogen activator antigen, and as units per decilitre for von Willebrand factor antigen.

Statistical analysis

Results are expressed as mean plus or minus standard deviations, or median and range, as appropriate. Differences between patients and controls were tested using Student's t test or the Mann-Whitney test according to the distribution of data. Differences between values obtained before and after occlusion were tested using the paired Student's t-test or the Wilcoxon test. Discriminant analysis was used to identify biochemical variables able to differentiate patients from controls. p values less than 0.05 were considered statistically significant.

Results

Patient characteristics

We enrolled 23 patients aged from 7 to 26 years, with a median of 14 years, 6 months to 19 years, with a median of 3.5 years following a modified Fontan procedure. Of the patients, 14 were female. Cardiac diagnoses were tricuspid atresia in 14, double inlet left ventricle in 1, pulmonary atresia with intact ventricular septum in 1, and miscellaneous lesions in the other 7. The Fontan circulation had been created by a direct atriopulmonary connection in 1 patient, a total cavopulmonary connection using a lateral tunnel technique in 9, and with an extracardiac conduit in 12 patients. The median age of the control group was 16 years.

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General clinical and laboratory evaluation

Clinical assessment showed 18 patients to be in the first class of the grading system of the New York Heart Association, and 5 to be in the second class. Systemic ventricular ejection fraction assessed by gated radionuclide angiography ranged from 40 to 73%, with a median of 55%. The peripheral oxygen saturation at rest was 92.3 plus or minus 5.6%. The haematocrit was 40.0 plus or minus 9.5%. The platelet count was within the reference range for all patients. Activity of factor V in the plasma was 62.1 plus or minus 14.95, with a median of 64%. Mean plasma levels of D-dimer did not differ from those in the control group.

Endothelial markers

Biochemical determinations are shown in Table 1. We found increased levels of von Willebrand factor ($p = 0.0003$) when compared to controls. At the basal condition, concentrations of tissue-type plasminogen activator antigen and plasminogen activator inhibitor-1 in the plasma, as well as their activity, were not significantly different between patients and controls. Despite that, 5 patients had levels of plasminogen activator inhibitor-1 in the plasma, at 55.38, 63.04, 72.59, 72.92 and 76.31 ng/mL respectively, above the maximum value found in the control group, which was 50.57 ng/mL. In 2 of these, the ratios of plasminogen activator inhibitor-1 to tissue-type plasminogen activator antigen, at 11.8 and 12.1, were above the maximum value observed in controls, which was 9.2. The same observation was found regarding tissue-type plasminogen activator antigen levels, with six values, of 11.24, 12.89, 14.91, 15.66, 28.18 and 36.92 ng/mL, above the upper limit found in the control group, specifically 9.86 ng/mL.

Multivariate discriminant analysis showed that differences existed between the patients and their control for plasminogen activator inhibitor-1 and von Willebrand factor antigen ($p = 0.00164$), but not tissue-type plasminogen activator antigen and D-dimer. The discriminant model correctly classified 79% of all individuals as patients or controls.

Following venous occlusion, concentrations of tissue-type plasminogen activator antigen in the plasma were significantly increased both in patients and controls, compared to pre-occlusion values. Plasminogen activator inhibitor-1 antigen did not differ from basal values (Table 2). No differences in levels of biochemical markers in the plasma were

Table 1. Basal biochemical markers of endothelial function of patients and controls (median and range).

Biochemical markers	Patients (n = 23)	Controls (n = 15)	p value
D-dimer (ng/mL)	140.01 46.06–1288.79	158.67 68.87–526.68	0.30
vWF:Ag (U/dL)	123.2 79.14–571.16	95.38 67.34–122.11	0.0003
t-PA Ag (ng/mL)	6.87 4.36–36.92	6.16 2.55–9.86	0.06
PAI-1 Ag (ng/mL)	33.87 10.91–76.31	31.62 1.5–50.57	0.08
PAI activity (U/mL)	15.15 1.48–36.72	18.57 8.68–32.29	0.43

vWF:Ag: von Willebrand factor antigen; t-PA Ag: tissue-type plasminogen activator antigen; PAI Ag: plasminogen activator inhibitor antigen.

Table 2. Response to venous occlusion in patients and controls (median and range).

	Patients		Controls	
Biochemical marker	Before VO	Following VO	Before VO	Following VO
t-PA Ag (ng/mL)	6.87 4.36–36.92	10.22* 6.25–60.04	6.16 2.55–9.86	7.72** 3.82–14.68
PAI-1 Ag (ng/mL)	33.87 10.91–76.31	36.42 4.25–88.51	31.62 1.5–50.57	31.38 1.5–52.94
PAI-1/t-PA	4.3 0.43–12.08	2.92 0.25–9.36	4.17 0.59–9.2	3.09 0.39–7.33
PAI activity (U/mL)	15.15 1.48–36.72	13.9 4.81–35.65	18.57 8.68–32.29	22.14 5.46–32.7

VO: venous occlusion; t-PA Ag: tissue-type plasminogen activator antigen; PAI-1 Ag: plasminogen activator inhibitor; *$p = 0.0004$ vs. pre-VO, **$p = 0.0007$ vs. pre-VO.

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found according to whether or not patients were receiving warfarin.

Discussion

One of the major functions of the endothelium is to maintain a nonthrombogenic and anticoagulant blood-tissue interface. This is mainly mediated by anticoagulant proteins, such as proteins C and S, tissue factor pathway inhibitor, as well as heparan sulfate-rich (antithrombin III) and condroitin sulfate-rich (thrombomodulin) proteoglycans. Prostacyclin and nitric oxide play a pivotal role as inhibitors of platelet aggregation. Under pathological conditions, the endothelium assumes a procoagulant phenotype, by down regulating its anticoagulant functions and increasing the secretion of adhesive molecules, such as fibronectin, von Willebrand factor and selectins.[11] Endothelial cells also secrete both tissue-type plasminogen activator and its inhibitor, plasminogen activator inhibitor-1, and both may be altered in pathological conditions, thereby impairing fibrinolysis.[18]

Our findings suggest that patients with the Fontan circulation have altered endothelial function as demonstrated by increased levels of von Willebrand factor in the plasma. Since this protein is synthesized only in endothelial cells and megakaryocytes, elevated levels in the plasma are a relatively specific indicator of endothelial dysfunction.[19,20] Furthermore, in contrast to thrombomodulin, which is increased in plasma only following proteolytic damage to the endothelial cell membrane, von Willebrand factor is secreted by "activated", or dysfunctional, but not necessarily "damaged", endothelial cells.[20] This is the reason why we decided to determine the levels of von Willebrand factor in our patients. Our results indicated, however, that not all endothelial functions were impaired. Thus, there was an appropriate release of tissue-type plasminogen activator in response to venous occlusion, resulting in a significant increase of levels in the plasma, as was observed in controls. High levels of plasminogen activator inhibitor-1, and increased ratios of plasminogen activator inhibitor-1 to tissue-type plasminogen activator, nonetheless, were observed in individual subjects at baseline.

It is well known that conversion to the Fontan circulation leads to loss or great reduction of pulsatility in the pulmonary circulation. It has been suggested that abnormal shear stress on the wall of the pulmonary vasculature may alter endothelial function, with vasoconstriction and an increased risk for formation of thrombus.[12,21,22] The role of endothelial dysfunction in patients with functionally univentricular physiology has been addressed recently, suggesting impaired endothelial-dependent vasodilation.[13,23]

Our results in patients with the Fontan circulation may be analyzed in parallel to our previous findings in a group of adolescents with a superior cavopulmonary connection, or bidirectional Glenn shunt, in whom varying degrees of hypoxaemia were present.[24] That subset of cyanotic patients had increased levels of von Willebrand factor in the plasma, similar to what we found in the present study. They also had increased levels of tissue-type plasminogen activator and reduced levels of thrombomodulin, an endothelial cell membrane protein that accelerates the activation of protein C. It seems reasonable to suppose that, at least in part, these findings were the result of hypoxaemia, as it is known that hypoxia shifts the endothelial phenotype towards a prothrombotic state.[25–27] The results of the present study indicate that the absence of hypoxaemia did not prevent patients with the Fontan circulation from endothelial activation or dysfunction, suggesting that other factors may be involved, possibly persisting since before the completion of the functionally univentricular circulatory pattern. It is possible that a compound influence of low and non-pulsatile flow, chronic venous congestion and hepatic dysfunction may play a role.

Recent studies have shown that increased levels of von Willebrand factor may be predictive of cardiovascular events in patients with known cardiovascular disease.[19,28] It has also been demonstrated that levels of von Willebrand factor in the plasma correlated inversely with measured flow-mediated vasodilation assessed by high-resolution ultrasound.[28] The clinical implications of altered levels of von Willebrand factor in the plasma on the development of thrombotic lesions, as well as on the long-term prognosis of this subset of patients, remains to be demonstrated.

Abnormal fibrinolysis is found in around two-fifths of adults with thromboembolic disorders, occurring with considerably greater frequency than deficiencies of protein C, protein S and antithrombin III.[29] Fibrinolysis is mediated primarily by endothelial cells with the release of the two major components, tissue-type plasminogen activator and plasminogen activator inhibitor-1. Basal levels of these proteins, however, do not reflect the overall functioning of the fibrinolytic process. Measurements of the endothelial responses to various stimuluses have been used in vascular function tests. Using standardized methods, venous occlusion physiologically induces release of tissue-type plasminogen activator from the endothelium, providing a reliable assessment of fibrinolytic reserve. Plasminogen activator inhibitor-1 behaviour is not uniform,

although reduced levels in the plasma are reported by some authors.[14,30]

In our study, the fibrinolytic function appeared to be relatively preserved, as suggested by the appropriate release of tissue-type plasminogen activator in response to venous occlusion. Fibrinolysis might be impaired in some instances, as demonstrated by elevated levels of plasminogen activator inhibitor-1 in the plasma, and increased rations of plasminogen activator inhibitor-1 to tissue-type plasminogen activator on an individual basis.

Our data suggest, therefore, that patients with the Fontan circulation may have endothelial dysfunction, as indicated by raised levels of von Willebrand factor. Further studies are necessary, involving a larger population of patients, better to understand the risk factors for thromboembolism in this setting.

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