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Fibrin network changes in neonates after cardiopulmonary bypass

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Abstract

Background—Quantitative and qualitative differences exist between the hemostatic systems of neonates and adults, among them the presence of ‘fetal’ fibrinogen, a qualitatively dysfunctional form of fibrinogen that exists until one year of age. The consequences of ‘fetal’ fibrinogen on clot structure in neonates, particularly in the context of surgical associated bleeding, have not been well characterized. Here we examine the sequential changes in clotting components and resultant clot structure in a small sample of neonates undergoing cardiac surgery and cardiopulmonary bypass (CPB).

Methods—Blood samples were collected from neonates (n=10) before surgery, immediately after CPB and following the transfusion of cryoprecipitate (i.e. adult fibrinogen component). Clots were

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formed from patient samples or purified neonatal and adult fibrinogen. Clot structure was analyzed using confocal microscopy.

Results—Clots formed from plasma obtained after CPB and after transfusion were more porous than baseline clots. Analysis of clots formed from purified neonatal and adult fibrinogen, demonstrated that at equivalent fibrinogen concentrations, neonatal clots lack three-dimensional structure while adult clots were denser with significant three-dimensional structure. Clots formed from a combination of purified neonatal and adult fibrinogen were less homogenous than those formed from either purified adult or neonatal fibrinogen.

Conclusions—Our results confirm that significant differences exist in clot structure between neonates and adults, and that neonatal and adult fibrinogen may not integrate well. These findings suggest that differential treatment strategies for neonates should be pursued to reduce the demonstrated morbidity of blood product transfusion.

Introduction

Bleeding after cardiopulmonary bypass (CPB) is a serious complication of cardiac surgery and often requires significant transfusion of adult blood products to achieve hemostasis¹⁻⁴. Neonates are particularly susceptible to the coagulopathic effects of CPB. Their immature coagulation system, massive hemodilution from large circuit primes and extensive suture lines that accompany complex congenital cardiac repairs all potentiate the bleeding experienced by neonates following CPB^{5,6}. To restore hemostatic balance, transfusion of packed red blood cells (pRBCs), platelets, cryoprecipitate (fibrinogen component) and coagulation factors derived from adult blood is essential. However, these treatment options pose multiple risks. In addition, little is known about how the immature coagulation system of neonates interacts with adult derived blood products to establish hemostasis.

Hemostasis is a complex physiologic process that regulates the formation and dissolution of clots. Thrombin, a critical enzyme to clot formation, mediates the proteolytic cleavage of fibrinogen into fibrin. Fibrin monomers polymerize into a fibrin network, which is subsequently cross-linked by factor XIII (FXIII)⁷. At birth all of the key components of the hemostatic system are present; however, important differences exist between neonates and adults, including the presence of 'fetal' fibrinogen^{8,9}. Although mean plasma fibrinogen concentrations in neonates are comparable to those in adults, evidence suggests that neonatal fibrinogen is qualitatively dysfunctional and exists in a fetal form until approximately one year of age¹⁰. The original hypothesis that a fetal form of fibrinogen might exist was based upon observations that thrombin and reptilase clotting times are prolonged in neonates and infants compared to adults¹¹. These tests measured the rate of conversion of fibrinogen to fibrin after the addition of either thrombin or reptilase and suggested that the polymerization of neonatal fibrin is slower than that of adult fibrin¹¹. Biochemical studies showed that neonatal fibrinogen has a different electrical charge and higher phosphorus content than adult fibrinogen^{12,13}. Studies utilizing whole blood viscoelastic tests, specifically thromboelastography (TEG), further supported functional differences between fetal and adult fibrinogen¹⁴. Adult fibrinogen values demonstrate excellent correlation with the TEG maximum amplitude after modification with a glycoprotein IIb/IIIa receptor blocker that uncouples platelet-fibrinogen interactions. However, in neonates and infants this correlation

is lost, indicating that an altered state of fibrinogen may exist in these patients. While these previous studies highlight that differences exist between neonatal and adult fibrinogen, the structural and functional consequences of ‘fetal’ fibrinogen to the fibrin network in neonates have not been well characterized.

In this investigation, our primary aim was to assess the structural characteristics of the fibrin network formed from neonates undergoing complex cardiac surgery at baseline, immediately after CPB and following subsequent transfusion of adult fibrinogen. Our overarching hypothesis was that the fibrin network formed from neonatal fibrinogen would be less dense and more susceptible to fibrinolysis than that formed from adult fibrinogen. We additionally examined the influence of thrombin and fibrinogen concentration and the integration of adult fibrinogen on neonatal fibrin networks.

Materials and Methods

In accordance with the Emory University IRB (irb.emory.edu) and following informed written parental consent, we enrolled 16 neonates undergoing cardiac surgery requiring CPB in this prospective observational study. Premature neonates (< 36 weeks gestation), neonates with APGAR scores <7 at 5 minutes, and those with known coagulopathy, coagulation defect, or mother with coagulation defect were excluded.

All neonates underwent nonpulsatile hypothermic CPB with a nonheparin-coated system, a Terumo RX-05 hollow-fiber membrane oxygenator (Terumo Cardiovascular Systems, Ann Arbor, MI, USA) and COBE SMARt neonatal circuit tubing (Sorin Group USA, Inc., Arvada, CO, USA). Our anticoagulation protocol consisted of a heparin bolus of 400 units/kg to the patient with an additional 1000 units in the CPB prime. All circuits contained a 250 ml priming volume with pRBCs added to the circuit as needed to achieve and maintain a hematocrit of 30% throughout the duration of CPB. As per institutional protocol, all neonates received tranexamic acid (100 mg/kg to the patient, 100 mg/kg to the pump and a continuous infusion of 10 mg/kg/hr throughout the duration of the operation). After heparin neutralization with protamine, continued bleeding after CPB was treated with our standard transfusion protocol of a quarter of a platelet apheresis unit and three units of cryoprecipitate. Further blood product administration was at the discretion of the attending anesthesiologist or intensivist.

Collection of neonatal plasma samples

All blood samples were drawn from an indwelling arterial line after the induction of anesthesia. Five mls of blood were aspirated prior to collection to prevent heparin contamination of the sample. All samples were immediately centrifuged to yield platelet poor plasma (PPP), placed in a cryotube and stored at -80°C . The first ten patients formed the basis of our testing. Three blood samples were obtained: baseline (before CPB), immediately after the termination of CPB and heparin reversal, and after transfusion of our standard transfusion protocol of a quarter of a platelet apheresis unit and three units of cryoprecipitate. In the final six patients, we collected a single baseline sample of blood. These six samples were not used for testing, but were utilized to isolate purified neonatal fibrinogen.

Fibrinogen, FXIII and prothrombin ELISAs

We sequentially measured the effect of CPB and the transfusion of adult cryoprecipitate on the concentrations of fibrinogen, FXIII and prothrombin using ELISAs (Abcam, Cambridge, MA, USA). Plasma samples were diluted into 50 µl of diluent buffer to reach a concentration within the working range of each assay. Concentrations were determined through the generation of a standard curve. All samples were analyzed in triplicate.

Isolation of Purified Neonatal Fibrinogen

Neonatal fibrinogen was isolated from PPP samples through affinity chromatography using glycyl-L-prolyl-L-arginyl-L-proline (GPRP)-sepharose beads ¹⁵. Following a one-hour incubation period, fibrinogen was dissociated from the beads using 1M sodium borate, 50mM sodium acetate, pH 5.4. Isolated fibrinogen was subsequently exchanged into 25mM Hepes, 150mM NaCl, pH 7.4 and stored at -80°C until utilized. Purification was verified by gel electrophoresis.

Analysis of clot structure of patient derived clots

Clots formed from samples collected at baseline, after CPB and after transfusion were analyzed with confocal microscopy. Clots (50 µl) were formed from PPP and 0.25 U/mL of human thrombin in a 9:1 volume ratio. To allow for visualization, clots were prepared with 10 µg/ml of labeled fibrinogen (Alexa-Fluor 647 nm, Invitrogen, Carlsbad, CA, USA). Clots were formed in the presence of thrombin between a glass slide and coverglass, allowed to polymerize for 1 hour and then imaged using a Zeiss Laser Scanning Microscope (LSM 510 VIS, Zeiss Inc., Thornwood, NY, USA) at 63x magnification ¹⁶. A minimum of four random 10 µm z-stacks were acquired per gel.

Analysis of structure of clots comprised of purified adult and neonatal fibrinogen

We hypothesized that differences between adult and neonatal fibrinogen may lead to incomplete integration between the two fibrin networks and an altered fibrin network structure following the transfusion of adult fibrinogen. To explore this hypothesis, we examined the structural differences in clots formed from purified adult fibrinogen, purified neonatal fibrinogen or a mixture of the two at specific concentrations of fibrinogen and thrombin observed during the course of CPB and after transfusion. Clots (30 µl) were formed from purified adult (FIB 3, Enzyme Research Laboratories, South Bend, IN, USA) or neonatal fibrinogen at a concentration of 2.5 or 3.5 mg/ml fibrinogen in 25 mM Hepes, 150 mM NaCl, 5 mM CaCl₂, pH 7.4. Clots were formed from a range of thrombin (human α-thrombin, Enzyme Research Laboratories, South Bend, IN, USA) concentrations (5, 10 or 20 µg/ml) and then analyzed via confocal microscopy. These ranges of fibrinogen and thrombin concentrations were chosen to mimic ranges observed in samples collected at baseline, after CPB and after transfusion. Mixed fibrinogen clots were also formed by combining neonatal and adult fibrinogen in a 2.5:1 or 1:2.5 ratio to mimic concentrations observed after transfusion with a total fibrinogen concentration of 3.5 mg/ml in the presence of 10 µg/ml thrombin. To allow for visualization, clots were prepared with the addition of 10 µg/ml Alexa-Fluor 555 (adult) and/or Alexa-Fluor 488 (neonatal) labeled fibrinogen.

Polymerization and imaging was performed as described above in the previous section entitled “*Analysis of clot structure of patient derived clots*”.

Quantification of fibrin fiber alignment

Quantification of clot fiber alignment was achieved through application of an algorithm based on a fast Fourier transform (FFT) that we have previously employed¹⁷. Briefly, each confocal image was padded with redundant data, a Gaussian decay (standard deviation = 0.25) and two-dimensional Hann window function were applied to minimize edge effects, and the images were transformed into the frequency domain using a two-dimensional FFT (Supplemental Figure 1A–E). Fiber alignment was determined by polar coordinate analysis of the resulting power spectrum and quantification of the relative intensity (RT) of pixels in angular bins (Supplemental Figure 1F). The predominant fiber angle was defined as the angle with the maximum RT, and the alignment index (AI) was quantified as the fraction of fibers aligned within $\pm 20^\circ$ of the preferred fiber alignment (PFA) normalized to a random distribution of oriented fibers. Note that the higher the AI, the greater alignment of the fibers. Analysis was performed on each image for all stacks.

Fibrin degradation assays

Because one potential consequence of the structural differences between neonatal and adult clots could manifest as differences in the rate of clot degradation, we assessed differences in fibrin degradation between neonatal clots, adult clots and clots formed from a mixture of the two using a series of dynamic fibrinolysis assays. A custom microfluidics-based microscopy approach was utilized to measure clot degradation rates. Similar methods using microscopy to quantify fibrin degradation have been previously described by other investigators^{18–21}. Results obtained from analysis of fibrinolysis using microscopy correlate with fibrinolytic activity measured using quantification of D-dimer²⁰. A device consisting of a single major and minor channel, which lies perpendicular to the main channel, was utilized for fibrinolysis assays. Devices were constructed by casting polydimethylsiloxane into an aluminum mold. Sylgard 184 was used in a 9:1 weight ratio of elastomer base to crosslinker. Following curing, the device was plasma-treated and bonded to a slide to create a sealed channel. Clots were produced in 25mM HEPES, 150mM NaCl, 5mM CaCl₂ buffer with fibrinogen concentrations of 2.5 mg/ml. 10% Alexa-Fluor 645 labeled adult fibrinogen was added for visualization. Polymerization was initiated with the addition of 0.5 U/ml of thrombin and 40 μ l of the clot solution was immediately injected into the device. After polymerizing for an hour, the device was mounted on a Nikon TE widefield microscope for imaging. Plasmin (0.01 mg/ml) was added to a primary channel inlet and images of the clot were taken every five minutes for twelve hours. ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to derive degradation rates by comparing the first and final frames and measuring distance along a line perpendicular to the edge of the clot. Clot degradation is described as the distance of clot degraded divided by twelve hours.

Statistical analysis and blinding methods

All patient samples were de-identified prior to sample transfer to researchers performing plasma and clot analysis. Researchers performing plasma and clot analysis were therefore blinded to the patient clinical outcomes. For confocal studies, clots were given a numeric

identifier so the researcher performing imaging was blind to the condition at the time of imaging. For image processing, an automated algorithm was used to calculate fiber alignment. Due to the large number of images being processed, blinding was not feasible. However, because the image analysis used an automated algorithm, minimal input was required from the researcher.

All statistical analyses were performed by a repeated measures one-way ANOVA with a Tukey's posthoc test using Prism (Graphpad Software Inc., La Jolla, CA, USA). Due to the exploratory intent of this investigation, a power analysis was not performed. Statistical significance was achieved for $p < 0.05$.

Results

Ten neonates were enrolled for testing in this prospective, observational study. The median (range) age was 5 (1–10) days and the median (range) weight was 3.9 (2.7–4.6) kgs. The median (range) CPB time was 136 (55–376) minutes with an ischemic time of 73 (23–167) minutes and a lowest temperature of 25 (18–34) °C. One patient underwent deep hypothermic circulatory arrest for a total time of 2 minutes.

Plasma Composition

We sequentially measured the effect of CPB and transfusion of cryoprecipitate (i.e. adult fibrinogen) on the concentrations of fibrinogen, FXIII and prothrombin in neonatal plasma samples (Figure 1A). Mean data summarized from all samples are presented in Figure 1B and concentrations for each individual neonate are presented in Figure 1C. Fibrinogen concentration decreased significantly after CPB from baseline values and subsequently increased again after transfusion ($p < 0.001$). Mean fibrinogen concentrations were similar at baseline and after transfusion (3.2 ± 0.7 mg/ml and 3.5 ± 1.3 mg/ml, respectively), but decreased in the samples obtained immediately after CPB (2.1 ± 0.7 mg/ml; $p < 0.001$). FXIII concentration also decreased significantly after CPB from 17 ± 6.5 µg/ml to 10 ± 6.9 µg/ml ($p < 0.001$); however, after transfusion, its concentration was significantly higher than baseline (20 ± 8.9 µg/ml; $p < 0.001$). When combined, there were no significant changes in prothrombin concentration over the three time points (baseline: 25 ± 6.7 µg/ml; post-CPB: 26 ± 14 µg/ml; post-transfusion (PTF): 27 ± 9 µg/ml). Conversely, when examining prothrombin concentration for each neonate separately, a significant degree of inter-patient variability was observed.

Clot Structure in Patient Samples

The effects of CPB and transfusion on clot structure were analyzed using confocal microscopy. Representative images of clots formed from three neonatal patient plasma samples collected at baseline, immediately after CPB and after the transfusion of adult cryoprecipitate are shown in Figure 2. Clot structure varied widely between individual patients. However, in general, clots were denser following CPB compared to baseline. Clot structure was further altered after the transfusion of adult cryoprecipitate with the resultant clots appearing highly heterogeneous with numerous regions of dense fibrin.

Next, we investigated the effect of thrombin concentration, as determined through the previous ELISAs, on clot structure. Representative images of clots formed from neonatal plasma samples collected at baseline, after CPB and after transfusion organized by low (<10 $\mu\text{g/ml}$), medium (10–19 $\mu\text{g/ml}$) or high (>20 $\mu\text{g/ml}$) thrombin concentrations are shown in Figure 3. No baseline samples were available for the low thrombin concentration. Baseline clots formed in the presence of medium thrombin concentrations were more porous and comprised of thicker fibers than those formed at higher concentrations. Interestingly, an opposite trend was observed in clots formed from plasma collected after CPB. In clots formed after transfusion, at low thrombin concentrations, highly heterogeneous fibrin strands with large amounts of open space were observed. Clots became denser with increasing thrombin concentrations.

To eliminate the role of fibrinogen dilution following CPB in the observed alterations in clot structure, we normalized fibrinogen levels in samples collected after CPB to baseline levels through the addition of exogenous purified neonatal fibrinogen. The addition of exogenous purified neonatal fibrinogen to samples collected after CPB (to match baseline fibrinogen concentration) resulted in predominately homogenous clots (Figure 3). To understand the effect of adding adult fibrinogen, again replicating post-transfusion fibrinogen concentrations, we performed identical experiments with exogenous purified adult fibrinogen. The addition of identical levels of adult fibrinogen resulted in heterogeneous clots that were more porous and comprised of thicker fibers than those formed in the presence of purified neonatal fibrinogen (Figure 3).

Structure of clots formed from purified neonatal and adult fibrinogen

At equivalent fibrinogen concentrations, neonatal clot structure differs from adult clot structure (Figure 4). Clots formed with purified adult fibrinogen were dense, highly branched and had significant three-dimensional structure while clots formed with neonatal fibrinogen were comprised of mostly aligned fibers and lacked significant three-dimensional structure.

We then examined the structure of clots formed from purified neonatal fibrinogen alone, adult fibrinogen alone or a mixture of the two (Figure 5). Samples formed from neonatal only fibrinogen at a concentration of 2.5 mg/ml formed clots that appeared sheet-like without significant three-dimensional structure. Increasing neonatal fibrinogen concentration to 3.5 mg/ml resulted in clots with thicker fibers but, again, these clots lacked significant three-dimensional structure. As anticipated, adult clots had more three-dimensional structure, were more homogenous and increased in density with increasing fibrinogen concentrations. Clots formed from a mixture of neonatal and adult fibrinogen with a higher ratio of neonatal fibrinogen, 2.5:1 mg/ml respectively, displayed distinct architectures between the adult and neonatal networks. The neonatal network was similar to that seen in the neonatal only sample, while the adult network formed a highly aligned network lacking its normal three-dimensional structure. Clots formed from a mixture of the two proteins, but with a higher ratio of adult to neonatal fibrinogen, 2.5:1 mg/ml respectively, were less aligned and demonstrated more network overlap than observed in the samples with a higher ratio of neonatal fibrinogen.

Analysis of clot fiber alignment

Fiber alignment was quantified for clots derived from patient samples or purified neonatal and adult fibrinogen (Table 1). In general, clots comprised of neonatal fibrinogen were found to display a significantly higher degree of alignment compared to those comprised of adult fibrinogen. Clots formed from patient samples obtained at baseline and after CPB were found to be significantly more aligned than samples obtained after transfusion with alignment index (AI) values of 1.17 ± 0.1 , 1.19 ± 0.1 and 1.15 ± 0.1 at baseline, post-CPB and PTF, respectively ($p < 0.01$, baseline vs. PTF; $p < 0.001$, post-CPB vs. PTF). AI was also quantified for clots formed from purified adult or neonatal fibrinogen, or a mixture of the two, at various thrombin and fibrinogen concentrations. At all thrombin concentrations analyzed ($5\text{--}20 \mu\text{g/ml}$), clots comprised purely of neonatal fibrinogen were more aligned than those comprised purely of adult fibrinogen. Mixed clots, except for the 1:2.5 neonatal to adult fibrinogen $10 \mu\text{g/ml}$ thrombin samples, were more significantly aligned than adult only clots. Additionally, alignment of adult versus neonatal fibers in these mixed clots was only found to be significantly different in the 2.5:1 neonatal to adult fibrinogen $20 \mu\text{g/ml}$ thrombin samples ($p < 0.001$), potentially due to differing polymerization kinetics of the two fibrinogen species.

Fibrin degradation

Clots formed from adult fibrinogen had a significantly slower degradation rate ($17.78 \pm 10.7 \mu\text{m/hr}$) than those formed from neonatal fibrinogen ($38.88 \pm 9.0 \mu\text{m/hr}$, $p < 0.01$; Figure 6). Mixed clots formed at a 2.5:1 or 1:2.5 ratio of adult:neonatal fibrinogen had degradation rates similar to those formed from adult fibrinogen (12.47 ± 5.9 and $10.62 \pm 6.5 \mu\text{m/hr}$, respectively), indicating that the features of the adult network are likely dominating in these experiments.

Discussion

Our results demonstrate that the presence of ‘fetal’ fibrinogen in neonates creates an altered fibrin network structure that is significantly different from that seen in adults. Baseline neonatal clots lack significant three-dimensional structure, are more porous than adult clots and are composed of highly aligned fibers. Even after the transfusion of adult cryoprecipitate (fibrinogen), the resultant clot more closely resembles neonatal clots, suggesting that neonatal and adult fibrinogen may not completely integrate during fibrin formation. Indeed, clots formed from a mixture of neonatal and adult fibrinogen, but with a higher ratio of neonatal fibrinogen, displayed an architecture similar to that seen in the neonatal only sample. In contrast, clots formed with a higher ratio of adult to neonatal fibrinogen more closely resembled the structural characteristics of adult clot. Furthermore, we demonstrate that clots formed with neonatal fibrinogen display an increased rate of degradation suggesting that at least one functional consequence, an increased susceptibility to fibrinolysis, exists as a result of the altered neonatal fibrin structure. However, mixed clots formed at a 2.5:1 or 1:2.5 ratio of adult to neonatal fibrinogen had degradation rates similar to those formed from adult fibrinogen indicating that the adult network is likely dominant.

In our initial experiments, we sequentially measured the effect of CPB on concentrations of fibrinogen, FXIII and prothrombin. As expected we observed significant decreases in both fibrinogen and FXIII concentrations following CPB that are likely the result of the substantial hemodilution that occurs in neonates following the institution of CPB. The subsequent increase observed after transfusion is almost certainly due to the delivery of adult cryoprecipitate. In contrast, we observed a large degree of inter-patient variability in prothrombin concentration, and there was no overarching trend to these changes. Prothrombin is modulated by a number of factors, including inflammatory responses, thus differences in prothrombin concentration for individual patients may be due a variety of environmental influences. Further investigation into the basis of these differences may provide insight into the crosstalk between blood coagulation and systemic inflammation.

In our experiments, we also show that neonatal clots are more highly aligned than adult clots. The functional significance of fiber alignment in the context of fibrin clots has only been characterized in a limited number of studies, with these studies focusing on the effect of fluid flow in generating clots with aligned fibers. It has been observed in polarized light microscopy images of *ex vivo* coronary artery and aortic thrombi that these thrombi are comprised of highly aligned fibers²². Clots formed from adult fibrinogen *in vitro* under static conditions are not aligned and, yet, we observed highly aligned fibers under static conditions when clots were formed from neonatal fibrinogen. This could be due to differing polymerization dynamics in neonatal versus adult fibrinogen. In particular, the highly aligned networks coupled with the lack of three-dimensional structure suggest that polymerization of neonatal fibrinogen favors elongation rather than branching. Future studies characterizing the molecular differences in neonatal fibrin polymerization dynamics may shed further light on these discrepancies.

In addition to structural characteristics, we also examined one potential functional consequence of neonatal fibrinogen, namely its susceptibility to fibrinolysis. Clots formed from adult fibrinogen and all ‘mixed’ clots regardless of their ratio of adult to neonatal fibrinogen had a significantly slower degradation rate than clots formed from neonatal fibrinogen alone. It has been clinically shown that pediatric patients following cardiac surgery, especially neonates, have an increased risk of thrombosis compared to other hospitalized pediatric patients and that this increased risk is associated with suboptimal outcomes²³. Based on our results, it is possible that the fibrin network resulting from the ‘mixing’ of neonatal fibrinogen and transfused adult fibrinogen is poorly degraded by the neonatal fibrinolytic system, and thus associated with an increased risk of thrombosis. This difference between the neonatal and adult clot is worthy of our attention and could potentially impact clinical outcomes.

The results of our study have important implications in the future development of treatment options for bleeding in neonates. Differential treatment strategies for neonates should be pursued to reduce the morbidity associated with blood product transfusion. In particular, understanding the molecular mechanisms between adult and neonatal fibrin polymerization may provide insight into the hemostatic dysfunction experienced by neonates after CPB and following the transfusion of adult cryoprecipitate. If indeed adult and neonatal fibrin polymers integrate poorly, other therapeutic options to increase the effectiveness of the

neonatal fibrin network should be explored. Procoagulant agents, such as prothrombin complex concentrates, capable of augmenting neonatal plasma thrombin concentrations,²⁴ may enhance the properties of the neonatal fibrin network more efficiently than the transfusion of adult fibrinogen. Further research aimed at investigating such options is warranted.

Our study has several limitations. First, it is unclear if our *ex vivo* results can be accurately applied to the *in vivo* state since they do not account for the contribution of platelets or the endothelial system to the coagulation process. Also, they were performed under static conditions and do not take into account the rheological elements of coagulation encountered under physiologic conditions. In addition, we assayed neonatal fibrinogen under conditions that have been the standard for adult fibrinogen. With all the noted differences between neonatal and adult clotting, it is highly probable that other components or conditions of the neonatal clotting cascade differ from their adult counterparts. It is possible that the clotting conditions for neonatal fibrinogen (ion concentrations, blood factors, pH, etc.) are different enough to account for some of the discrepancies observed between neonatal and adult fibrin clots. Analysis of these factors would further clarify the contribution of neonatal fibrinogen to bleeding complications following surgery. Another limitation of our study is a lack of quantitative analysis of fiber number, length, and density and number of branch points in fibrin clots. Although we attempted to perform these analyses, the lack of significant three-dimensional structure observed in neonatal clots prevented reliable extraction of the data. The image-processing steps performed to extract the data include normalizing and thresholding the raw image data to allow identification of the individual fibers. The resulting binary image can be skeletonized and individual fibers identified to extract fiber number and length, as well as the number of branch points. Further, the ratio of fiber to total pixels can be determined to quantify fiber density. In an adult clot, these processing steps yield quantitative data that aid in characterizing the organized clot structure (Supplemental Figure 2). However, performing the same processing steps on the neonate imaging data did not provide the same quantitative data (Supplemental Figure 2). The inability to extract these data is due to the nearly uniform pixel value across the image plane. As a result, thresholding the images, regardless of the defined threshold value, did not yield a binary image that allowed for identification of individual fibers. While not quantitative, the ability to extract morphological data from adult clots and not from neonatal clots provides further indication of the differences in their respective clot structures. Future studies will utilize other methods, such as scanning electron microscopy and atomic force microscopy, to obtain more quantitative data from neonatal clots.

In summary, we conclude that there are significant structural differences between neonatal and adult fibrin networks that result in at least one verified functional consequence – clot degradation rates. Our studies confirm that neonatal fibrinogen, endogenous or purified, forms a fibrin network that lacks significant three-dimensional structure, is more porous than that of adults and is composed of highly aligned fibers. After CPB, neonatal clots further deteriorate and are not fully restored despite the transfusion of adult fibrinogen. We also demonstrate that, similar to adults, increasing thrombin concentration promotes the formation of a more robust fibrin network. Thus it is possible that procoagulant therapies designed to augment thrombin generation could potentially assist clinicians in achieving

hemostasis in neonates after major surgery. Further translational studies into the molecular mechanisms of neonatal coagulation may inform more educated treatment decisions for neonates experiencing bleeding and result in improved patient outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box Summary**What we already know about this topic**

- Bleeding after cardiac surgery is particularly common and problematic in neonates
- Fetal fibrinogen, present until 1 year of age, is dysfunctional compared to adult fibrinogen and may contribute to bleeding after cardiopulmonary bypass

What this article tells us that is new

- In blood samples from 10 neonates after cardiopulmonary bypass, clots formed were more porous than before surgery
- Clots formed from purified fibrinogen from neonates alone or mixed with adult fibrinogen were less dense than adult clots, suggesting that transfusion of adult fibrinogen may be less effective in neonates than adults

Summary Statement

The structural consequences of ‘fetal’ fibrinogen on the fibrin network in neonates have not been well characterized. Here we examine the fibrin network of neonates undergoing complex cardiac surgery at baseline, after cardiopulmonary bypass and after transfusion of adult fibrinogen. We focus our studies on the structural differences between the neonatal and adult fibrin network.

Figure 1. Patient sample collection scheme and analysis of clotting component concentrations via ELISA

The sample collection scheme is shown in A. Plasma samples were collected from 10 patients at baseline, after CPB (post-CPB) and after transfusion (PTF) and then fibrinogen, factor XIII and prothrombin concentrations were analyzed through ELISA. Data pooled from all patient samples are shown in B and concentrations for each patient sample are shown in C. *** represents $p < 0.001$

ELISA = enzyme-linked immunosorbent assay, CPB = cardiopulmonary bypass, FXIII = factor XIII, Prothmb = prothrombin

Figure 2. Confocal microscopy analysis of clots constructed from patient plasma samples collected at baseline, after CPB and after transfusion of cryoprecipitate

To allow for visualization, clots were prepared with the addition of Alexa-647 labeled fibrinogen. Representative images of clots formed from plasma collected at baseline (A, D, G), after CPB (post-CPB; B, E, H) and after transfusion (PTF; C, F, I) for three patients, Patient 1 (A–C), Patient 5 (D–F), and Patient 8 (G–I) are shown. Scale bar = 20 μ m
CPB = cardiopulmonary bypass

Figure 3. Influence of thrombin concentration on patient derived clot structure

To allow for visualization, clots were prepared with the addition of Alexa-647 labeled fibrinogen. Representative images of clots formed from neonatal PPP collected at baseline (A–B), after CPB (post-CPB; C–E) and after transfusion (PTF; F–H) organized by low ($<10 \mu\text{g/mL}$; C, F, I, L), medium ($10\text{--}19 \mu\text{g/mL}$; A, D, G, J, M) or high ($>20 \mu\text{g/mL}$; B, E, H, K, N) thrombin concentrations (as determined by ELISAs) are shown. No baseline samples were available for the low thrombin concentration. To analyze the effect of adult fibrinogen on clot structure after transfusion, purified neonatal (I–K) or adult fibrinogen (L–N) was added to samples collected after CPB (post-CPB) to bring the fibrinogen level to those commensurate with baseline levels. Scale bar = $20 \mu\text{m}$

PPP = platelet poor plasma, CPB = cardiopulmonary bypass, ELISAs = enzyme-linked immunosorbent assays

Figure 4. Confocal microscopy analysis of clots constructed from purified neonatal or adult fibrinogen

Clots were formed from purified adult (A, B, E, F, I, J) or neonatal (C, D, G, H, K, L) fibrinogen at a concentration of 2.5 (A, C, E, G, I, K) or 3.5 (B, D, F, H, J, L) mg/ml and a thrombin concentration of 5 (A–D), 10 (E–H) or 20 (I–L) $\mu\text{g}/\text{mL}$ and then analyzed via confocal microscopy. These ranges of fibrinogen and thrombin concentrations were chosen to mimic ranges observed in samples collected at baseline, after CPB, and after transfusion. To allow for visualization, clots were prepared with the addition of Alexa-555 (adult) or Alexa-488 (neonatal) labeled fibrinogen. Representative images are presented. Scale bar = 20 μm

CPB = cardiopulmonary bypass

Figure 5. Confocal microscopy analysis of clots constructed from both purified neonatal and adult fibrinogen

Clots were formed from purified adult (A–B) or neonatal (C–D) fibrinogen at a concentration of 2.5 (A, C) or 3.5 mg/ml (B, D) fibrinogen in the presence of 10 $\mu\text{g/ml}$ thrombin. Clots were also formed by combining neonatal and adult fibrinogen in a 2.5:1 (E, G, H) or 1:2.5 (F, I, J) ratio to create clots simulating conditions observed after transfusion with a total fibrinogen concentration of 3.5 mg/ml in the presence of 10 $\mu\text{g/ml}$ thrombin. Neonatal fibrinogen was labeled with Alexa-Fluor 488 (green) to allow for separate visualization of adult and neonatal fibrinogen labeled with Alexa-Fluor 555 (red). Representative images are presented. For combined fibrinogen samples, overlaid neonatal and adult fibrinogen channels (E, F) and corresponding individual neonatal (G, I) and adult fibrinogen (H, J) channels are presented. Scale bar = 20 μm

Figure 6. Analysis of fibrinolysis

Clots were formed from purified adult, neonatal, and mixed fibrinogen clots and degradation rates were measured using a custom-made fluidics device. An overlay of the initial and final frames with false color added to aid visualization are shown in A. Initial frame: red; final frame: pink. Scale bar = 100 μ m

An image of the fluidics setup on the microscope (B), a close-up image of the device with channels labeled (C) and degradation data (D) are presented. *** represents $p < 0.001$

Table 1

Analysis of clot fiber alignment

Patient Samples		Samples							
		Baseline			Post-CPB			PTF	
		1.17 +/- 0.1			1.19 +/- 0.1			1.15 +/- 0.1	
Purified Fibrinogen Samples	<i>Thrombin Concentration</i>	A2.5	A3.5	N2.5	N3.5	GN2.5A1	RN2.5A1	GN1A2.5	RN1A2.5
	5 ug/mL	1.07 +/- 0.1	1.14 +/- 0.2	1.67 +/- 0.2	1.22 +/- 0.1	1.31 +/- 0.2	1.29 +/- 0.2	1.49 +/- 0.3	1.52 +/- 0.3
	10 ug/mL	1.05 +/- 0.1	1.13 +/- 0.1	1.21 +/- 0.1	1.21 +/- 0.1	1.27 +/- 0.2	1.23 +/- 0.1	1.11 +/- 0.1	1.08 +/- 0.1
	20 ug/mL	1.09 +/- 0.1	1.11 +/- 0.1	1.21 +/- 0.1	1.24 +/- 0.1	1.21 +/- 0.1	1.12 +/- 0.1	1.20 +/- 0.1	1.23 +/- 0.2

Fiber alignment index was determined for clots derived from patient samples or purified neonatal and adult fibrinogen. Patient samples were collected at baseline, after CPB (post-CPB) or after transfusion (PTF). Clots formed from purified fibrinogen were produced by polymerization of adult (denoted A in Table), neonatal (denoted N in Table) or a combination of the two in a 2.5:1 or 1:2.5 ratio to create clots with a total fibrinogen concentration of 3.5 mg/mL. Clots were formed in the presence of 5, 10, or 20 µg/mL thrombin, therefore simulating concentrations observed following transfusion. For mixed fibrinogen samples, neonatal and adult channels were analyzed separately to determine if alignment differed between the two populations within the same sample; data obtained from neonatal channels in these mixed samples are denoted G and data obtained from adult channels in these mixed samples are denoted R.

CPB = cardiopulmonary bypass. PTF = post-transfusion