The Effect of Modified Ultrafiltration on Angiopoietins in Pediatric Cardiothoracic Operations

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Background. Cardiopulmonary bypass subjects patients’ blood to hemodilution and nonphysiologic conditions, resulting in a systemic inflammatory response. Modified ultrafiltration (MUF) counteracts hemodilution and has also been postulated to improve outcomes by proinflammatory cytokine removal. The objective of this study was to investigate whether the benefits of MUF include the removal of proinflammatory mediators, such as angiopoietin-2 (angpt-2). We hypothesize that some of the clinical benefits of MUF are related to the preferential removal of angpt-2.

Methods. We performed a prospective cohort study in children 18 years old or younger undergoing cardiopulmonary bypass. Serum samples were obtained from each patient preoperatively, after cardiopulmonary bypass, and on intensive care unit admission. A fluid sample from the MUF effluent was also analyzed. Angpt-1, angpt-2, interleukin-8, and interleukin-10 levels were determined by enzyme-linked immunosorbent assay.

Results. Thirty-one patients were enrolled. Angpt-1 levels significantly decreased across all time points (p < 0.01). Angpt-2 concentrations were significantly elevated at intensive care unit admission when compared with both preoperative and post–cardiopulmonary bypass levels (p < 0.01). The angpt-2:1 ratio significantly increased after cardiopulmonary bypass to intensive care unit admission (p < 0.01). There was no significant difference between the angpt-2 or angpt-1 percentage of extraction within MUF effluent. Interleukin-8 and interleukin-10 significantly increased from preoperative to intensive care unit admission (both p < 0.01).

Conclusions. The results of this study demonstrate that MUF removes both proinflammatory and antiinflammatory mediators equally. This study suggests that the clinical benefits of MUF cannot be attributed to the removal of larger quantities of proinflammatory mediators such as angpt-2 and interleukin-8.


Cardiopulmonary bypass (CPB) is integral in the surgical intervention of complex congenital heart disease. However, the process also subjects the patient’s blood to hemodilution, hypothermia, and nonendothelialized surfaces. These factors, along with surgical trauma, ischemia-reperfusion injury, and heparin–protamine interactions, act as a potent stimuli for a systemic inflammatory response. As a consequence of the resulting vascular leak, a variety of clinical corollaries ranging from postoperative edema to end-organ dysfunction and death can occur [1]. The effects of vascular leak and increased total body water are especially seen in children because of their relatively lower total blood volumes in relation to the CPB primer volume [2]. Numerous antiinflammatory strategies have been attempted to counteract the effect of CPB [3]. The ability of modified ultrafiltration (MUF) to serve as an antiinflammatory strategy is one that is still currently under investigation [4]. MUF was introduced in 1991 and is the process of ultrafiltration immediately after the completion of CPB [2]. In children, MUF has been shown to have clinical benefit by decreasing total body water, improving myocardial contraction, decreasing transfusion requirements, and decreasing intensive care unit (ICU) lengths of stay [2, 4, 5]. The most accepted beliefs for the clinical improvement are the removal of excess free water and its subsequent hemococoncentrational effects [2, 4]. There remains controversy whether MUF also removes proinflammatory mediators. Most of these studies have been difficult to reproduce because of small patient numbers and varying hemodilutional and hemococoncentrational effects secondary to the processes of CPB and MUF, respectively [4].
Angiopoietins (angpt) are a family of vascular growth factors involved in angiogenesis. Angpt-1 and angpt-2 play opposing roles in vascular permeability through their interaction with the Tie-2 receptor [6-9]. Angpt-1 is constitutively produced by pericytes and maintains vascular quiescence, inhibiting apoptosis and stabilizing intercellular junctions. Additionally, it activates the phosphoinositide-3-kinase/Akt cell survival signaling pathway and inhibits NFκB and Rho kinase [9]. By contrast, angpt-2 typically possesses proinflammatory properties by competitive inhibition of the angpt-1/Tie-2 signaling cascade. The angpt-2 interaction with the Tie-2 receptor causes widened intercellular gaps through the Rho kinase pathway, resulting in vascular leakage and transmigration of leukocytes [9]. Angpt-2 is preformed and is rapidly released during periods of stimulation with interleukins, hypoxia, histamine, or thrombin [7, 10]. Children undergoing CPB have elevated angpt-2:1 ratios for as long as 24 hours postoperatively. In addition, the angpt-2:1 ratio correlates with ICU length of stay [10]. Additionally, angpt-2 has been shown to be preferentially removed with the use of plasma exchange [11].

Our aim was to describe the effects of MUF on plasma angiopoietins. Inasmuch as angpt-2 is preformed and is rapidly released during CPB, we hypothesize that some of the clinical benefits of MUF are related to the preferential removal of angpt-2.

Material and Methods

This study was approved by the Yale University Human Research Protection Program and registered on ClinicalTrials.gov, NCT01489475. All patients under 18 years of age undergoing CPB were offered enrollment from December 2011 to June 2013. Patient demographics, including age at operation, diagnosis, Risk Adjustment in Congenital Heart Surgery (RACHS-1) surgical severity score [12], duration of CPB, aortic cross-clamp time, and ICU length of stay were collected. The ICU length of stay was calculated in hours by calculating the difference between the admission vital signs and the first vital signs obtained in the inpatient department or the last set of vital signs before discharge if the patient was discharged from the ICU.

Preoperative and Operative Technique

Anesthesia for all patients was performed under the supervision of a single attending anesthesiologist (D.G.). Most commonly, induction was performed with inhalation agents. Maintenance anesthesia was performed with a combination of inhalation agents, intermittent narcotics, intermittent benzodiazepines, and muscle relaxants.

The CPB circuit used for all patients was a roller pump with heparin primed in the circuit. The CPB circuit was primed with methylprednisolone 30 mg/kg and Amicar 75 mg/kg. It was also primed with red blood cells and fresh frozen plasma for patients weighing less than 10 kg. The degree of hypothermia for each patient was determined by the cardiothoracic surgeon and perfusionist on a case-by-case basis.

A Hemocor HPH hemoconcentrator (Minntech Corporation, Minneapolis, MN) was used for MUF. Immediately after CPB, MUF was initiated. Blood traveled retrograde from the arterial cannula and was directed across the hemoconcentrator membrane. The filter pore size is rated to allow passage of particles less than 65 kD (Minntech Hemocor HPH Hemoconcentrator, Minntech). The blood was then returned to the patient through the venous cannula. MUF was completed when the bypass circuit was emptied of blood for most cases. Filtration typically occurred for approximately 10 minutes or until additional plasma water was not able to be safely removed.

Sampling

Serum samples for angpt-1, angpt-2, interleukin (IL)-8, and IL-10 were taken at three different time points. The first serum sample was drawn from the arterial line in the operating room before surgical intervention. The second serum sample was drawn from the CPB circuit, after surgical intervention and just before MUF, while the patient was being weaned from CPB. The last serum sample was drawn from the arterial line on ICU admission (ICU). A final fluid sample was taken from the discarded MUF effluent after the completion of MUF. The percentage of MUF extraction was calculated by dividing the angiopoietin or interleukin concentration in the MUF effluent by the concentration after CPB, which was immediately before MUF. Serum samples were collected in tubes containing sodium citrate and were centrifuged at 4,000 × g for 10 minutes to separate the serum from the cellular components. The samples were then stored in 1 mL aliquots at −70 °Celsius until analysis.

Angpt-1 and angpt-2 levels were measured with the use of commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN). Briefly, 96-well microtiter plates were coated with the appropriate capture antibodies (100 μL at 4 μL/mL) in phosphate-buffered saline (PBS) for 2 hours. The plates were washed with PBS containing 0.05% Tween-20 three times, and nonspecific binding sites were blocked by incubation with 300 μL 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature. After washing, 100 μL standard (recombinant human antibodies) or angiopoietin samples were added and incubated for 2 hours at room temperature. The plates were subsequently washed, and the detection antibody was added. After 2 hours at room temperature, the plates were washed and incubated with streptavidin-horseradish peroxidase 1:200 in 1% BSA in PBS for 20 minutes at room temperature. The plates were washed, and 100 μL substrate solution (1:1 mixture of hydrogen peroxide and tetramethylbenzidine) was added. This reaction was stopped after 20 minutes with an acidic stop solution. The optical density was measured at 450 nm with a wavelength correction of 540 nm.

The IL-8 and IL-10 levels were also measured with the use of commercially available sandwich ELISA kits (R&D Systems). This process was similar to that used for
angiopoietins, with incubation times as outlined by the manufacturer’s instructions.

**Statistical Analysis**

All statistical analyses were performed with SAS 9.2 software (Cary, NC). Preoperative, post-CBP, and ICU values were compared with Friedman’s test followed by Wilcoxon signed rank tests for pairwise comparisons between time points. The Bonferroni method was used to correct for multiple comparisons. For MUF percentage extraction, the Wilcoxon signed rank test was used to assess differences comparing angpt-1 with angpt-2 and IL-8 with IL-10. For undetectable concentration levels, the value of 1 pg/mL was given for calculations of ratios.

**Results**

Eighty-four patients under 18 years of age underwent cardiac operations requiring CPB and MUF during this time period. Thirty-one patients were enrolled (37% enrollment). The patients’ demographic data are shown in Table 1. The median CPB times, cross-clamp times and ICU lengths of stay were 98 minutes (range, 28 to 202 minutes), 54 minutes (range, 0 to 114 minutes), and 94 hours (range, 27 to 860 hours), respectively. There were a variety of surgical repairs or palliations performed, with ventricular septal defect repairs predominating (Table 2). The RACHS-1 risk categories are shown in Figure 1A. Some patients underwent more than one intervention, which accounts for the larger number of surgical procedures for the number of patients. The patients included in our study had statistically greater RACHS-1 risk categories compared with the other eligible but not enrolled surgical patients (means 2.61 vs 2.23; p = 0.04) (Fig 1B). All children survived to hospital discharge. All patients had adequate serum samples for analysis of angiopoietin and interleukin levels. However, 2 patients did not have adequate MUF effluent samples available for angpt or IL analysis. Three others had only enough MUF effluent available for angpt analysis.

There was a statistically significant decrease in angpt-1 levels at all sampling time points (medians 1,902 pg/mL, 1,263 pg/mL, and 902 pg/mL, respectively; p < 0.01). Paired comparison also revealed significant decreases within the group (Fig 2A). Conversely, both preoperative and post-CBP angpt-2 levels did not significantly change (median 5,860 pg/mL vs 5,059 pg/mL; p = 0.11). There was a statistically significant increase in angpt-2 at ICU admission in comparison with preoperative and post-CBP levels (median 7,456 pg/mL; p = 0.007 and p = 0.002, respectively) (Fig 2B).

Given that both angiopoietins interact with the Tie-2 receptor with equal affinity, we also analyzed the angpt-2:1 ratio. Pairwise comparisons of angpt-2:1 ratios between preoperative to post-CBP showed no significant difference (median 3.06 vs 3.49; p = 0.13). After the process of MUF, there was a statistically significant increase in the angpt-2:1 ratio at ICU admission compared with the previous two time points (median 8.92; p = 0.007 and p = 0.002) (Fig 2C). No correlation was found between the angpt-2:1 ratio after CPB or ICU admission and ICU lengths of stay, patient age, or patient weight (Table 3).

Angpt-1 reached detectable levels in the MUF effluent in 20 of 29 patient samples (69%). Angpt-2 was detected in 25 of 29 MUF effluent samples (86%). There was no statistical difference between the number of patients with detectable MUF angpt-1 and angpt-2 levels (p = 0.21). The percentage of MUF extraction for angpt-1 and angpt-2 was similar (medians 0.74% interquartile range [IQR] 0.15, 1.28% and 1.02% IQR 0.6, 2.12%, respectively; p = 0.54).

Additionally, IL-8 concentrations were detectable in 21 of 31 patients preoperatively, in 25 of 31 patients after CPB, and in 30 of 31 patients at ICU admission. There was no statistically significant difference from preoperative to post-CBP (median 80 pg/mL vs 260 pg/mL; p = 0.29) or from post-CBP to ICU admission (median 260 pg/mL vs 760 pg/mL; p = 0.06). The increase from ICU admission was statistically significant when compared with preoperative (p = 0.004) (Fig 3A).

IL-10 concentrations were detectable in 10 of 31 patients preoperatively, in 28 of 31 patients after CPB, and in all patients on ICU admission. There was a statistically significant increase from preoperative compared with post-CBP (median 1 pg/mL vs 179 pg/mL; p < 0.01). The

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**Table 1. Baseline Patient Characteristics**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Values</th>
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<tr>
<td>Age, mo</td>
<td>6 (0.07–122)</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>Weight kg</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>14 (45)</td>
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IQR = interquartile range.
increase from post-CPB and ICU admission did not reach statistical significance (179 pg/mL vs 478 pg/mL; \( p = 0.25 \)). The increase from ICU admission reached statistical significance when compared with preoperative (\( p < 0.01 \)) (Fig 3B).

For analysis of MUF effluent, IL-8 reached detectable levels in only 7 of 26 patients (27%). IL-10 reached detectable levels in only 11 of 26 patients (42%). There was no statistical significant difference in the number of patients with detectable MUF levels of IL-8 and IL-10 (\( p = 0.38 \)). Owing to the large number of undetectable values for these two cytokines, we were unable to perform meaningful statistics on the percentage of extraction.

Comment

Although MUF is the standard of care, its clinical benefits are still being elucidated. We describe here that the percentage of MUF extraction of angpt-2 is not statistically different from that of its predominantly anti-inflammatory counterpart, angpt-1. In addition, there is an increasing angpt-2:1 ratio from post-CPB to ICU admission. Given that angpt-2 is preformed, stored in intracellular Weibel-Palade vesicles, and released on endothelial activation [7], we hypothesized that this inflammatory cytokine would be preferentially removed by MUF. Our results refute our hypothesis because the process of MUF appeared to remove angpt-2 and angpt-1 equally without an appreciable decrease in angpt-2 levels after MUF.

The pore size of the filter used for MUF is rated to allow passage of particles less than 65 kD (Minntech Hemocor HPH Hemoconcentrator, Minntech). Angpt-1 and angpt-2 have similar molecular weights of approximately 70 kD [13, 14]. Given the similar molecular weights, it is not surprising that both are filtered at equal percentages. Although angpt-2 has been shown to be removed by plasma exchange preferentially over angpt-1, this did not occur when MUF was used [11]. Given that angpt-2 is a preformed acute phase reactant, we were surprised that its levels remained the same after CPB. We suspect that hemodilution affected both the angpt-2 levels and part of the decrease seen in angpt-1. Secondarily, patient rewarming, not exposure to the CPB circuit, may be the primary inflammatory nidus that activates endothelial cell release of angpt-2. More study is needed to determine whether implementing MUF after rewarming will further improve clinical outcomes. In addition, IL-8 (molecular weight approximately 8 kD) and IL-10 (molecular weight approximately 18 kD) had similar numbers of patients with detectable MUF levels, indicating that they are also removed by the MUF filter equally [15–17]. It is unclear why some patients had undetectable IL-8 and IL-10 preoperative and post-CPB levels, but similar results have been reported [18, 19].

Our study was not powered to provide clinical correlations; however, prior work by others has demonstrated a correlation between vascular integrity and angpt-2 concentration. This appears to be reversible after the introduction of recombinant angpt-1 [6, 8]. In addition, our group has previously shown a correlation between the angpt-2:1 ratio and ICU length of stay [10]. Given that quantitatively more angpt-2 is present after endothelial activation after bypass, it is tempting to speculate that proportionally more angpt-2 is removed after MUF and that the angpt-2:1 ratio on ICU admission is lower than it would have been if MUF were not used. With MUF being the standard of care in most pediatric cardiothoracic programs, this speculation will not be easily tested. An alternative theory is that proinflammatory mediators may increase during the process of MUF because this process exposes blood to additional nonendothelialized tubing. Again, this concept will need further investigation.

There are a few limitations of our study. These are single-center data composed of low-risk to moderate-risk surgical patients. This may have skewed the data because patients with more complex conditions may have had higher circulating angpt-2 levels. Although gross concentrations of proinflammatory mediators would likely differ, given our results, it is unlikely that this would lead to a difference in percentage of proinflammatory or antiinflammatory MUF extraction. Second, our results are based on a single institution’s perfusion and MUF.
protocol, which may limit generalizability. In addition, given that MUF is provided for all patients at our institution, there was no control group. Third, middle serum sample was drawn from the bypass circuit rather than by interruption of arterial line monitoring while patients were coming off bypass. This was done in an effort to optimize patient safety and also after ample time had passed to allow for blood equilibration. We assumed in our study that the sample from the bypass circuit represented the concentration of inflammatory mediators in the patient; however, a better comparison could have been made if all samples had been drawn from the arterial line. Fourth, the post-MUF sample was obtained at ICU admission. The time from MUF completion to ICU admission is variable and depends on many factors, including hemostasis, chest wall closure, rewarming, and ease of patient transport to the ICU. These issues could have had an impact on inflammatory mediator concentrations, confounding the effect of MUF. However, this was done in an effort to minimize blood draws in our surgical patients, and it prompted our decision to assess the discarded MUF effluent for percentage of protein extraction. Last, our sample size was small, with a recruitment rate of only 37%. This low recruitment rate was attributed to a single recruiter and to the reluctance of families to agree to research studies during the surgical process. Despite these limitations, this study is
the first to investigate the percent extraction of both proinflammatory and antiinflammatory angiopoietins in MUF effluent after pediatric cardiothoracic operations.

Conclusions
Angiopoietin removal after MUF is not significantly different between angpt-2 and angpt-1. The ratio of angpt-2:1 continues to increase after the process of MUF. These results call into question whether the clinical benefits of MUF are attributable to the selective removal of angpt-2.

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