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Derangement of Arginine and Related Amino Acids in Children Undergoing Surgery for Congenital Heart Disease With Cardiopulmonary Bypass

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Objectives: Arginine is a conditionally essential amino acid, the precursor for nitric oxide and a key factor in cell proliferation, protein synthesis, and energy metabolism. When there is increased demand in the setting of inflammation, ischemia-reperfusion injury, and organ dysfunction, endogenous arginine production falls short, and external supplementation may be necessary. The goal of this study was to assess changes in concentrations of plasma arginine, citrulline, ornithine, glutamine, and plasma arginase in infants and children undergoing surgery for congenital heart disease with cardiopulmonary bypass.

Design: Prospective observational study.

Setting: The study was conducted in the Heart Center at Texas Children's Hospital.

Subjects: Children undergoing surgery for congenital heart disease with cardiopulmonary bypass.

Interventions: None.

Measurements and Main Results: Serial perioperative blood samples were collected for quantification of amino acids, arginase, nitric oxide metabolites, and markers of organ function (lactate, Pa_{O_2}/F_{IO_2} ratio, and creatinine clearance). Thirty children (18 males) were included in the study; median (interquartile range) age 0.5 years (0.3–0.9 yr). The mean ± SD for plasma amino acid concentrations before cardiopulmonary bypass: arginine 62 ± 20 μmol/L, citrulline 24 ± 6 μmol/L, ornithine 53 ± 32 μmol/L, and glutamine 591 ± 126 μmol/L. Arginine concentration was decreased within the first 24 hours (43 ± 15 μmol/L; $p = 0.004$), citrulline and glutamine concentrations decreased over the first 48 hours (11 ± 4 μmol/L; $p < 0.001$ and 493 ± 131 μmol/L; $p = 0.019$, respectively) and were associated with an increase in arginase (3.8 ± 3 μg/mL; $p < 0.05$). There was an increase in Vasoactive-Inotropic Score (5.9 ± 19 vs 0.5 ± 2; $p < 0.001$), decrease in creatinine clearance (76 ± 24 vs 93 ± 31; $p = 0.002$), and Pa_{O_2}/F_{IO_2} ratio (243 ± 138 vs 374 ± 200; $p = 0.007$) comparing to baseline.

Conclusions: A widely variable degree of arginine, citrulline, and glutamine depletion occurs in children after surgery for congenital heart disease. These findings were associated with increased arginase and coincide with some of the markers of organ perfusion.

Key Words: arginase; arginine; cardiopulmonary bypass; citrulline; congenital heart disease

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Children undergoing surgery for congenital heart disease (CHD) requiring cardiopulmonary bypass (CPB) are at risk for developing a low output state leading to reduced systemic oxygen delivery. This can be associated with additional end-organ dysfunction, increased duration of mechanical ventilation, ICU length of stay, as well as higher mortality (1–3).

Major factors playing a role in developing organ injury after CPB include neutrophil activation secondary to exposure to foreign surfaces, particularly the CPB circuit, systemic inflammatory response syndrome, hemolysis, and ischemia-reperfusion injury and associated endothelial dysfunction (1, 4). There is increasing interest in the role of arginine and citrulline in the pathophysiology

of organ dysfunction in the presence of systemic inflammation and critical illness. Arginine is a conditionally indispensable amino acid which in situations of increased demand (rapid growth, infection, inflammation, or organ dysfunction) becomes essential due to the inability to increase its de novo synthesis (5, 6). Citrulline, a nonessential amino acid, is the precursor for the endogenous synthesis of arginine. Nitric oxide, a signal molecule resulting from the oxidation of the imino group of arginine by nitric oxide synthase, is required for the maintenance of endothelial function, vascular tone, and organ perfusion (7, 8). In addition, arginine plays a central role in cell proliferation, protein synthesis, energy metabolism, and ammonia detoxification (6).

Arginine depletion has been described in various disease states, especially when there is a significant systemic inflammatory response such as in sepsis (9, 10). In the pediatric population, arginine depletion has also been demonstrated after CPB (11, 12). However, there is limited evidence regarding the mechanism underlying this depletion. Increased expression of arginase I, which is the predominant isozyme of the divalent cation-dependent arginase enzyme, has been observed in disease conditions such as sickle cell, leishmaniasis, sepsis, ischemia-reperfusion injury, and myocyte injury (13–17). It is possible that the combination of increased arginase expression and activity, together with organ dysfunction after CPB, may contribute to the net arginine depletion that has been observed in this patient population.

In this prospective observational study, we report the plasma profile of arginine, citrulline, ornithine, and glutamine, as well as plasma arginase concentration, nitric oxide metabolites (NOx), and hematological markers of inflammation in pediatric patients undergoing surgery for CHD with the use of CPB, to explore associations between the changes in plasma concentrations of arginine and related amino acids with markers of organ dysfunction.

MATERIALS AND METHODS

Patients

This prospective observational study was conducted in the Heart Center at Texas Children's Hospital between December 1, 2016, and February 28, 2017. The study protocol was approved by the Institutional Review Board for Investigations in Human Subjects of the Baylor College of Medicine. Preoperative written consent was obtained from parents. The inclusion criteria were any patient younger than 18 years old undergoing surgery for CHD requiring CPB. Patients were excluded if they weighed less than 2 kg, had prior history of hemolytic anemia, or a mechanical prosthetic valve. A sample size of 30 subjects was calculated to provide a 95% CI of the mean of 7% (i.e., mean ± 7%), assuming a coefficient of variation of 20% (Primer of Biostatistics, McGraw-Hill, 2002). Clinical data were collected from the electronic medical records (EPIC hyperspace, v2015, Verona, WI) from December 2016 to February 2017, 30 patients were enrolled in the study.

Study Protocol

A baseline (pre-CPB) blood sample was drawn from an indwelling venous or arterial catheter immediately before CPB cannulation. In addition, a blood sample was obtained from the primed CPB circuit to assess the possible dilution effect on circulating amino acids. Subsequent patient samples were obtained at 90 minutes, 6 hours, 12 hours, 24 hours, and 48 hours after CPB, and a final sample was obtained on day 5. EDTA coated tubes were used for sample collection; samples were immediately centrifuged at 3,000 g for 15 minutes and plasma separated and frozen at –80°C. Variables such as creatinine level, vasoactive medication dosage, and Pao_2 and Fio_2 were also recorded at each time-point.

Amino Acids, Arginase, and NOx Analysis. Plasma amino acid concentrations were determined by heated electrospray ionization liquid chromatography-mass spectrometry following derivatization to their corresponding dansylated derivatives and separation using a Kinetex C18 column (Phenomenex, Torrace, CA) (18). A cell-free U-[$^{13}C^{15}N$] amino acid mix (Cambridge Isotope Laboratories, Andover, MA) was used as internal standard. Arginase I was measured by biolayer interferometry (Octet RED384 System; Pall ForteBio, Fremont, CA) using anti-arginase I monoclonal antibody (Abcam, Cambridge, MA). Monoclonal antibody was biotinylated 1:1 using a standard protocol (Thermofisher, Waltham, MA). High precision streptavidin biosensors (Pall ForteBio) were loaded with the respective biotinylated antibody. Binding kinetics were measured and a standard curve obtained from a serial dilution of the corresponding antigen. Serum samples were analyzed with loaded biosensors at 37°C. Plasma samples were diluted to achieve optimal binding. Plasma samples were prepared for measurement of NOx levels by the reduction of nitrate to nitrite using cadmium and ammonium chloride. Total plasma NOx was subsequently measured using negative chemical ionization-gas chromatography-mass spectrometry using a SP 2230 column (Supelco, Bellefonte, PA) after conversion to pentafluorobenzyl derivative using pentafluorobenzyl bromide (Sigma Aldrich, St. Louis, MO) (19). Sodium [$^{15}N^{18}O_3$] nitrate (ISOTEC, Miamisburg, OH) was used as internal standard.

WBC and Subclass Counts. Complete blood cell count was measured by Sysmex XN-9000 (Sysmex America, Lincolnshire, IL). WBC subclasses were reported for neutrophils, bands, metamyelocytes, lymphocytes, basophils, and eosinophils. Immature to total neutrophil ratio was calculated by dividing the immature count by the sum of neutrophils, band cells, and metamyelocytes.

Clinical Data and Markers of Organ Function. The Vasoactive-Inotropic Score (VIS), as a surrogate for hemodynamic status, was calculated according to a standard formula (20). Creatinine clearance, as a marker of adequacy of renal function, was calculated using a modified Schwartz formula (21). Pao_2 to Fio_2 (P/F) ratio, a marker of pulmonary function, was measured by routine arterial blood gas analysis. Clinical outcome measures such as hospital length of stay, ICU length of stay, duration of mechanical ventilation, and mortality rate were recorded.

Nutrition and Perioperative Fluid Management

As per institutional protocol, patients remained—nil per os for at least 8 hours prior to surgery and typically started enteral or parenteral feeds on the first postoperative day. During the early postoperative period, all patients were routinely fluid restricted. Intake was increased daily with the goal to be at full maintenance fluid, primarily composed of nutrition, at around 72 hours after

CPB. Breast milk or standard infant formulae were used according to patient's preference or availability, and total parenteral nutrition (TPN) was used if there was a clinical indication for withholding enteral feeds. TPN formulations used in our institution contain amino acid mixtures (trophamine 10%, premasol 10%, or travasol 10%) that have similar concentrations of arginine (1.2 g/100 mL).

Statistical Analysis

Standard descriptive statistics was used to describe baseline data. Values reported are means ± SD for normally distributed variables or medians (interquartile range [IQR]) for variables not normally distributed. Repeated measure analysis of variance was used to determine changes in variables over time. Fisher least significant difference was used for post hoc analysis. Bivariate analysis was performed using Pearson correlations of coefficient test with two-tailed analysis and alpha of 0.05. Additionally, to assess the effect of CPB duration and body temperature on arginine metabolism, subjects were divided into two groups using median CPB duration and median intraoperative temperature as the cutoff value; unpaired *t* test was performed to compare the two groups. Statistical analysis was performed using SPSS v25 statistics software (IBM, Armonk, NY).

RESULTS

Patient Characteristics

The median age of the patients was 0.5 years (IQR, 0.3–0.9 yr), male (18/30), and Caucasian (16/30) (**Table 1**). There was no hospital mortality. Patient characteristics, intraoperative data, and surgical procedures are given in Table 1. All patients received a single dose of methylprednisolone (20 mg/kg) intraoperatively prior to CPB cannulation, as per institutional protocol. A wide range of procedures were included in the study (Table 1). The median CPB duration was 149 minutes (IQR, 112–201 min), median ICU length of stay was 3 days (IQR, 2–6 d), and median hospital length of stay was 7.5 days (IQR, 4–11 d) (Table 1). The median duration of mechanical ventilation was 12 hours (IQR, 0–24 hr) and 43% of the patients (13/30) were extubated in the operating room or immediately after arrival to ICU. The majority of patients (26/30) received nutrition 24 hours after surgery, of which 21 were fed enterally, and the remainder parenterally. No patient received inhaled nitric oxide during the study period.

WBC and Subclass Count

Total WBC count at baseline (pre-CPB) was $9.5 \pm 4.6 \times 10^3$ with a neutrophil count of $4.3 \pm 4.0 \times 10^3$, lymphocyte count of $4 \pm 2.3 \times 10^3$, and monocyte count of $0.8 \pm 0.6 \times 10^3$ (**Fig. 1**). WBC count progressively increased during the first 48 hours after CPB ($p < 0.001$) and remained elevated until day 5 ($p = 0.028$; Fig. 1). Subclass analysis showed that this increase in WBC was mainly related to the neutrophil count ($p < 0.001$; Fig. 1). Monocyte count increased significantly by 24 hours after CPB ($p = 0.012$; Fig. 1), whereas the lymphocyte count progressively declined during the first 48 hours ($p < 0.001$; Fig. 1) with a subsequent rise to baseline values. The neutrophil to lymphocyte ratio (N/L) showed a significant peak at 24 hours ($p < 0.001$; Fig. 1), and the immature white cell percentage (band count) increased at 6 hours after cannulation ($p = 0.03$; Fig. 1).

TABLE 1. Patient Characteristics, Surgical Interventions, and Clinical Outcome

Patient Characteristics	Values (IQR) or *n* (%)
Age, yr	0.5 (0.3–0.9)
0–12 mo	22 (73)
12 mo to 5 yr	2 (7)
> 5 yr	6 (20)
Weight, kg	5.9 (4.6–9.2)
Race	
White	16 (54)
Hispanic	10 (34)
Black	1 (3)
Middle east	2 (6)
Asian	1 (3)
Sex	
Male	18 (60)
Female	12 (40)
Surgical interventions	
Biventricular repair	18 (60)
Single ventricle palliations	6 (26)
Others	
Arch repair with CPB	1 (3)
Mitral valve repair	1 (6)
Total anomalous pulmonary venous return repair	1 (3)
Septal myomectomy for left ventricular outflow tract obstruction	3 (10)
CPB duration, min	149 (112–201)
ICU length of stay, d	3 (2–6)
Hospital length of stay, d	7.5 (4–11)
Initiation of nutrition after surgery, hr	24 (12–36)
Duration of mechanical ventilation, hr	12 (0–24)

CPB = cardiopulmonary bypass, IQR = interquartile range.

Clinical Data

The VIS at baseline was 0.5 ± 2 and increased in the first 12 hours after CPB to 5.9 ± 19 ($p < 0.001$; **Fig. 2**) and then declined. The baseline plasma lactate was 0.8 ± 0.3 mmol/L and subsequently remained elevated 2.5 ± 1 ($p < 0.001$) during the first 24 hours after CPB (Fig. 2). Creatinine clearance at baseline was 93 ± 31 mL/(min × 1.73 m^2) and significantly decreased 76 ± 24 ($p = 0.002$; Fig. 2) during the first 24 hours after CPB. Likewise,

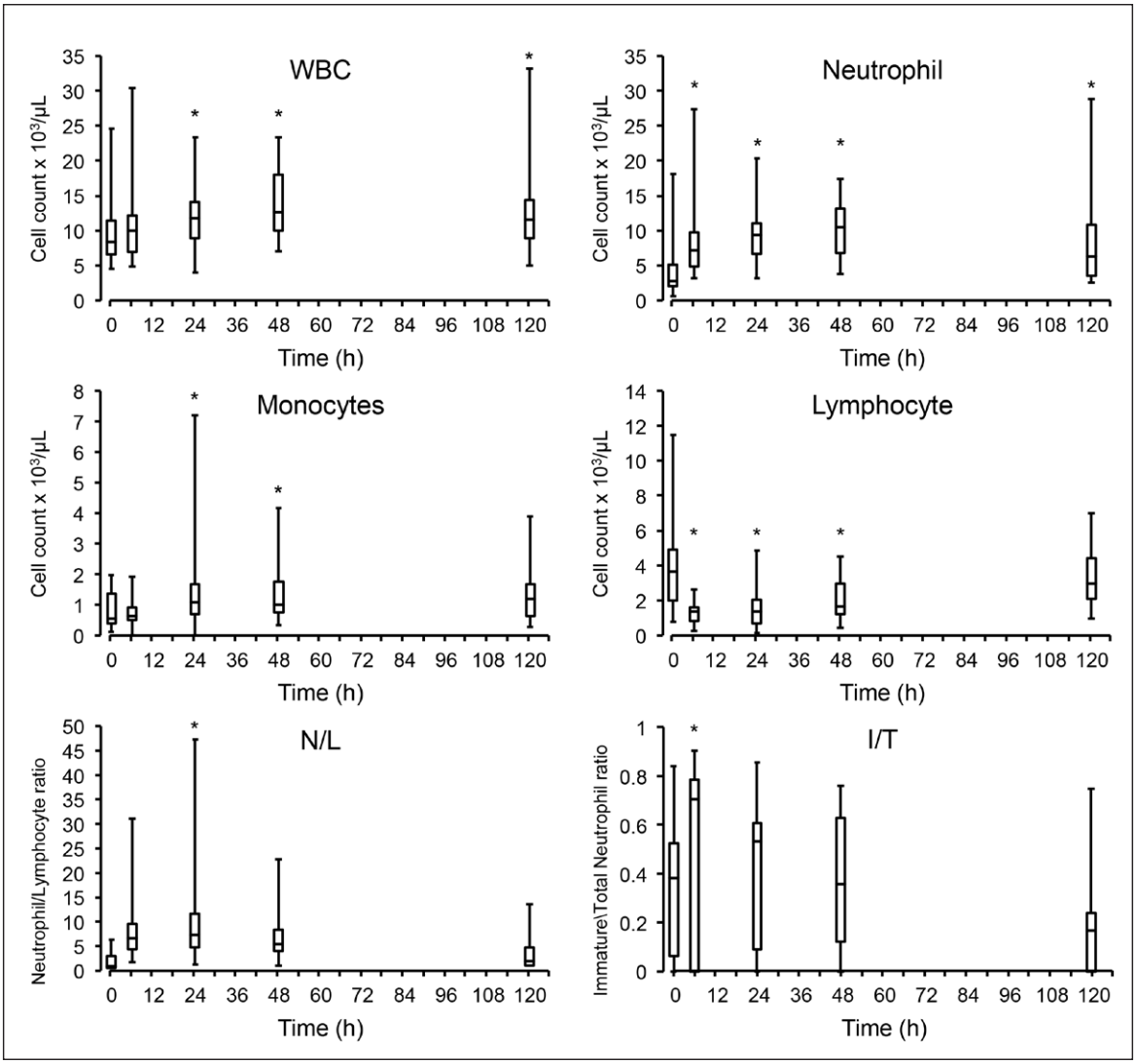

Figure 1. WBC and subclass cell counts (neutrophil, monocyte, and lymphocyte), neutrophil to lymphocyte ratio (N/L), and immature to total neutrophil ratio (I/T) after cardiopulmonary bypass (CPB) in a pediatric population. Symbols are means ± SD, $n = 30$. Asterisk denotes difference ($p < 0.05$) from baseline (pre-CPB) (*comparing to time zero, $p < 0.05$, symbols are means ± SD).

P/F ratio at baseline was 374 ± 200 and significantly decreased during the first 12 hours 243 ± 138 ($p = 0.007$; Fig. 2).

Amino Acids, Arginase, and NOx Analysis

The patients' baseline plasma amino acid levels prior and after CPB, including CPB blood prime are given in **Supplemental Table 1** (Supplemental Digital Content 1, http://links.lww.com/CCX/A226). All amino acid concentrations showed an immediate decrease 90 minutes after cannulation (**Fig. 3**). Arginine concentration was significantly reduced 6 hours after CPB ($p = 0.034$) and continued to decrease until 24 hours after CPB ($p = 0.004$; Fig. 3). Plasma arginine concentration returned to baseline levels once patients were started on enteral and/or parenteral nutrition. Plasma ornithine concentrations showed a similar pattern of depletion in the first 24 hours after CPB ($p = 0.057$; Fig. 3) and returned toward baseline after feeding. Plasma citrulline concentration progressively declined immediately after cannulation which remained low throughout the rest of the study duration ($p < 0.001$; Fig. 3). The fall in arginine concentration during first 12 hours was associated with decline in plasma citrulline concentration (r = 0.6; $p < 0.001$). Plasma glutamine concentrations declined during the first 48 hours after CPB ($p = 0.019$; Fig. 3) and remained low for the remainder of the study.

The plasma arginase concentration at baseline was 2.2 ± 2 μg/mL, increased in the first 6 to 12 hours ($p = 0.004$; Fig. 3) with a subsequent decline. The increase in plasma arginase concentration was correlated with plasma arginine depletion at 12 hours after CPB ($r = 0.5$; $p = 0.002$; Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/CCX/A226). Also, increase in arginase was weakly correlated with plasma citrulline depletion at 24 hours ($r = 0.4$; $p = 0.04$) and glutamine depletion at 48 hours ($r = 0.6$; $p = 0.03$). Plasma NOx concentration at baseline (pre-CPB) was 70 ± 52 μmol/L and there were no significant changes during the observation period (Fig. 3).

Clinical Findings and Laboratory Data

CPB duration was inversely correlated with increase in plasma arginase ($r = 0.5$; $p = 0.02$). During the postoperative period, the

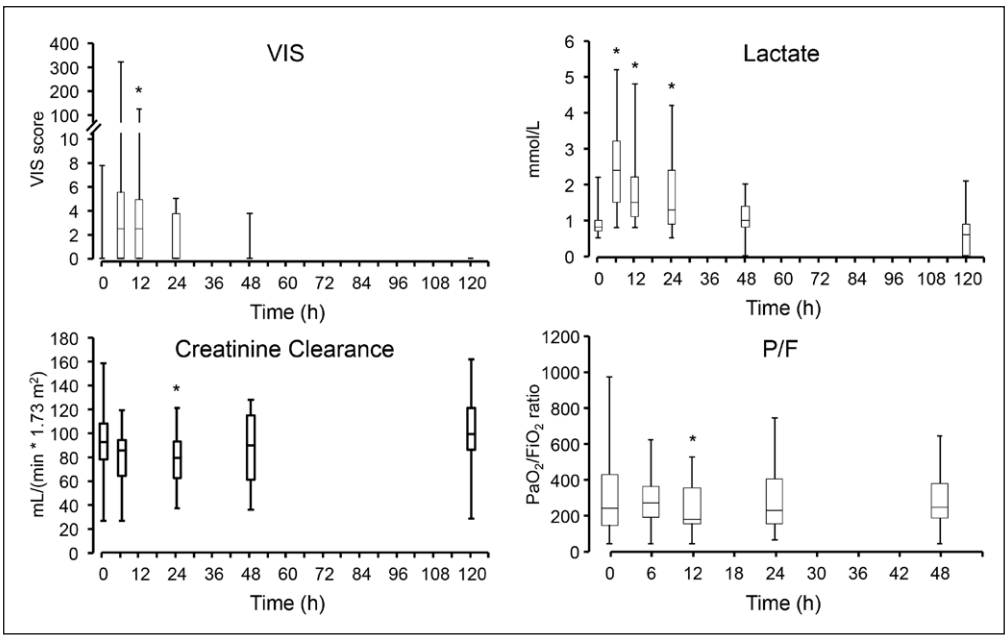

Figure 2. Vasoactive-Inotropic Score (VIS), plasma lactate concentration, creatinine clearance, and Pao_2 to Fio_2 ratio (P/F) after cardiopulmonary bypass (CPB) in a pediatric population. Symbols are means ± SD, $n = 30$. Asterisk denotes difference ($p < 0.05$) from baseline (pre-CPB) (*comparing to time zero, $p < 0.05$, symbols are means ± SD).

VIS at 6 hours was associated with increase in serum lactate concentrations ($r = 0.5$; $p = 0.02$), as well as, prolonged duration of ventilation ($p = 0.007$). There were no other significant associations between clinical findings and arginine (**Supplemental Table 2**, Supplemental Digital Content 2, http://links.lww.com/CCX/A227; and **Supplemental Table 3**, Supplemental Digital http://links.lww.com/CCX/A228). The duration of CPB was inversely associated with the monocyte count at 24 hours ($r = 0.5$; $p = 0.02$). Also, the increase N/L ratio ($r = 0.4$; $p = 0.04$) was weakly associated with a decrease in creatinine clearance at 6 hours after CPB (**Supplemental Table 4**, Supplemental Digital Content 4, http://links.lww.com/CCX/A229).

DISCUSSION

Our study has demonstrated that children who undergo surgery for CHD with CPB have an early depletion in plasma arginine levels with a simultaneous increase in plasma arginase levels and that these both returned to baseline by day 5 after surgery. In addition, we observed a fall in citrulline and glutamine levels without recovery by day 5. These changes were not associated with NOx.

Patients requiring CPB experience complications secondary to low systemic oxygen delivery and endothelial injury-related organ dysfunction (2). Arginine is the precursor for nitric oxide and has been shown to be depleted in plasma after CPB in congenital heart surgery (11, 12). Our study also confirmed that there is a reduction in plasma arginine concentration within the first 24 hours after CPB. Plasma arginine concentration reflects the balance between the supply and utilization of this amino acid (22, 23). In our study population, the recovery of arginine was observed after 48 hours once parenteral or enteral nutrition was provided. Early perioperative nutrition has been shown to improve plasma arginine concentration (24, 25). Approximately 30% reduction in arginine concentration identified in our study is similar to reported arginine depletion in various diseases states such as sepsis, malaria, sickle cell, and coronary artery disease with cardiogenic shock (26–29). Clinical consequences of arginine depletion in these conditions were mainly associated with endothelial dysfunction and nitric oxide imbalance; however, pathophysiologic consequences of the arginine deficiency are yet to be elucidated. In this study, we did not find any statistical correlation between clinical markers of organ perfusion as well as NOx.

Citrulline is not only the precursor for arginine synthesis but also a marker for gut mass and function (30, 31). During congenital cardiac surgery with CPB, multiple episodes of ischemia-reperfusion injury take place and the splanchnic circulation is the first to be affected (11, 32, 33). The depletion of citrulline coincides with enterocyte damage, relaxation of gap junctions, and increased intestinal permeability (33). We also demonstrated a depletion of citrulline that failed to recover to baseline concentrations even 5 days after exposure to CPB. A reduction in citrulline production will result in a decrease in endogenous arginine synthesis which may also contribute to the depletion of arginine (34). Despite lack of recovery in postoperative citrulline concentration, arginine concentration recovered after 48 hours. It is known that de novo arginine synthesis only contributes 10–15% of the arginine flux (35). Also, lack of recovery of citrulline may be due to its slow production rate secondary to ischemia-reperfusion injury of the gut.

After CPB, there is a well-known inflammatory response due to exposure of inflammatory cells to foreign surfaces of the bypass circuit, mechanical shear stress, tissue ischemia and reperfusion, hemodynamic changes, and exposure to blood products (1, 4). Upon exposure to foreign surfaces, neutrophils are activated and initiate an inflammatory cascade which results in macrophage activation and lymphocyte depletion (1, 36). In our study, we also demonstrated a similar pattern of inflammation with a peak of leukocytosis at 24 hours comprised of neutrophilia followed by monocytosis and a reduction in the number of lymphocytes. This inflammatory response took place despite the large dose of steroids provided preoperatively. The depletion of lymphocytes during early postoperative period after CPB has been well described and is thought to be secondary to apoptosis (37). As a result, N/L ratio showed a significant increase 24 hours after CPB in this pediatric population. In adults undergoing bypass, it has been reported that increased N/L ratio is associated with adverse outcomes such as renal impairment (38); our data support this observation because increased N/L ratio and monocytosis was associated with decreased creatinine clearance during the early postoperative

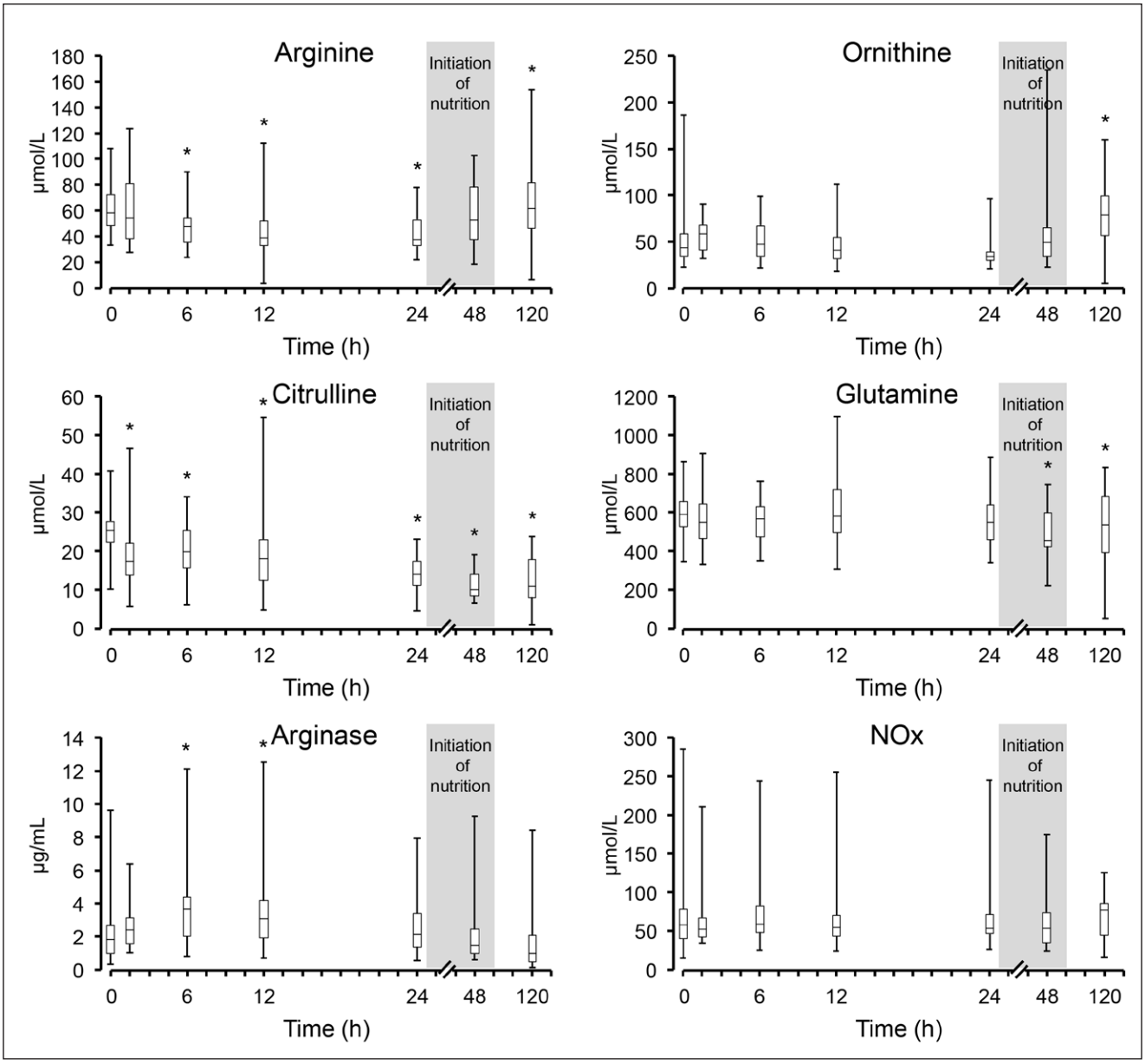

Figure 3. Plasma arginase, nitric oxide metabolites (NOx), and amino acid concentrations after cardiopulmonary bypass (CPB) in a pediatric population. *Shaded area* denotes the commencement of nutrition. Symbols are means ± SD, $n = 30$. Asterisk denotes difference ($p < 0.05$) from baseline (pre-CPB) (*comparing to time zero, $p < 0.05$, symbols are means ± SD).

period. Additionally, the association between the severity of inflammation and depletion of arginine and citrulline in critically ill children has been described in the literature (39). It is known that T-cells are exquisitely sensitive to nutritional status and the proliferation of T-cells is blunted in the arginine-depleted state (40). The mechanisms involved in the association of arginine and inflammation remains under investigation.

In this study, we observed an increase in plasma arginase I concentration after the CPB. Arginase is a divalent cation-dependent enzyme that catalyzes the hydrolysis of L-arginine to produce L-ornithine and urea. Mammals express two different isozymes: cytosolic arginase I and mitochondrial arginase II. Arginase I is the predominant isoform that is expressed in liver, RBCs, and activated macrophages (13). In addition to increased expression during inflammation (14), injury to arginase I expressing cells potentially leads to the release of this enzyme into the circulation. An increase in plasma arginase activity has been observed in certain disease states such as sickle cell disease, leishmaniasis, and sepsis (15). Furthermore, increased activity has also been observed in cardiac myocytes after ischemia-reperfusion injury (16, 17).

In our study, most patients initiated on parenteral or enteral nutrition 24 hours after surgery. In postoperative patients, due to concerns for fluid overload, feeding or parenteral nutrition has been advanced slowly. Of note, most parenteral nutrition formulations do not contain citrulline or glutamine. Therefore, suboptimal nutrition could potentially play a role in diminished citrulline and glutamine concentrations postoperatively.

Overall, there were multiple risk factors that could contribute to the arginine depletion in our study population. First, patients remained undernourished during early postoperative period. Second, there was a depletion of citrulline, which is the precursor for arginine synthesis. Third, there was an increase in plasma arginase concentration. And finally, arginine requirements were likely increased due to inflammation, surgical tissue damage, and ischemia-reperfusion injury (41).

This study had some limitations. Inherently, different age groups may show variations in amino acid content; however, the subgroup analysis was limited due to size and the diversity of the study population. We performed an observation on the potential effect of nutrition on arginine replenishment; however, direct measurement of the effect can only be observed via a tracer infusion study. In this study, we showed an increased concentration of arginase in plasma after CPB; however, due to limitation of resources and sample volume, activity level was not measured. Further analysis of the arginase activity level may better demonstrate the impact of this change on arginine depletion.

CONCLUSIONS

We conclude that in our study population, after pediatric congenital cardiac surgery with CPB, inflammation along with multiple organ damage developed as well as depletion of conditionally essential amino acids (arginine, citrulline, and glutamine). Depletion of arginine, citrulline, and glutamine is possibly multifactorial and can be associated with increase in arginase and low nutritional status. These findings have potential implications in our understanding of pathophysiology of systemic inflammatory response syndrome, hemolysis, and ischemia-reperfusion injury and associated endothelial dysfunction resulting in low systemic oxygen delivery and multiple organ dysfunction in this population

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