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Postnatal longitudinal analysis of serum nitric oxide and eosinophil counts in extremely preterm infants

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ABSTRACT

Background: Nitric oxide (NO) may be related to the pathogenesis of several morbidities in extremely preterm infants, including late-onset adrenal insufficiency. However, eosinophilia is observed under pathological conditions with adrenal insufficiency. Therefore, this study explored postnatal changes in NO levels and eosinophil counts in extremely preterm infants with and without morbidities.

Methods: Nineteen extremely preterm infants with a median gestational age of 27.0 weeks and median birth weight of 888 g were enrolled in this study. Serum levels of nitrogen oxides (NOx) and peripheral blood eosinophil counts were measured at birth and every 2 weeks thereafter. Morbidities of the study group were diagnosed using a single criterion.

Results: Serum NOx levels (mean ± standard deviation) were 22.5 ± 14.9 μmol/L, 51.2 ± 23.7 μmol/L, 42.4 ± 15.2 μmol/L, and 33.8 ± 9.4 μmol/L at birth and 2, 4, and 6 weeks of age, respectively. The serum NOx level at 2 weeks of age was significantly higher than that at birth and 6 weeks of age. Eosinophil counts, which increase with adrenal insufficiency, were measured simultaneously and were 145 ± 199/μL, 613 ± 625/μL, 466 ± 375/μL, and 292 ± 228/μL at birth and 2, 4, and 6 weeks of age, respectively. These values showed that the eosinophil count was significantly higher at 2 weeks of age than at birth and 6 weeks of age. The serum NOx level of infants without chorioamnionitis was significantly increased at 4 weeks of age, and the eosinophil count of infants with necrotizing enterocolitis was significantly increased at 2 weeks of age. No correlation with the NOx level or eosinophil count was observed in infants with late-onset circulatory collapse.

Conclusion: The postnatal serum NOx level and eosinophil count were significantly correlated with each other and peaked at 2 weeks of age.

1. Introduction

Nitric oxide (NO) is an endothelium-derived relaxing factor produced by vascular endothelial cells. NO is generated *in vivo* by converting L-arginine to L-citrulline by NO synthase (NOS). NOS is classified into neuronal NOS, inducible NOS, and endothelial NOS (eNOS) based on its calcium dependence and main expression sites.[1–3] In addition to its vasodilative effect, NO is involved in various *in vivo* reactions, such as atherosclerosis and neuronal apoptosis. Several studies have indicated that NO production is induced during pathological conditions, including systemic inflammation, infection, and respiratory failure. NO is also involved in circulation adaptation during the early postnatal period.[3,4] Furthermore, previous studies[5,6] have reported that adrenal insufficiency induces eNOS expression, which increases NO levels.

When preterm infants are separated from the placenta at birth, their adrenal glands are immature; as a result, they are in a state of adrenal insufficiency until they are older than 36 weeks of age.[7–9] Adrenal insufficiency in preterm infants contributes to the pathogenesis of late-onset circulatory collapse (LCC) and chronic lung disease.[10–12] Therefore, we speculated that NO production might be increased in preterm infants with latent adrenal insufficiency.

In contrast, eosinophils are controlled by cytokines, such as

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interleukin (IL)-5 or granulocyte-macrophage colony-stimulating factor. Increased eosinophil counts in peripheral blood have been observed under pathological conditions with adrenal insufficiency.[13] The responsible mechanism is the induction of eosinophil apoptosis by glucocorticoids.[14] Transient hypereosinophilia occurs in 14 %–76 % of preterm infants during hospitalization in the neonatal intensive care unit.[15] Previously,[16] we demonstrated that hypereosinophilia in preterm infants was associated with LCC caused by relative adrenal insufficiency.

NO production *in vivo* was measured based on nitrogen oxides (NOx), which are the sum of the metabolites. Currently, there is no literature regarding the longitudinal evaluation of serum NOx in blood samples from preterm infants or the relationship between serum NOx and eosinophils in preterm infants. During this study, we tested the hypothesis that the postnatal trends of serum NOx levels and eosinophil counts would reflect relative adrenal insufficiency in extremely preterm infants.[5,6]

2. Methods

This multicenter, prospective, observational study was planned at Okayama University Hospital and conducted with the cooperation of Tokyo Women's Medical University Hospital and Okayama Medical Center. This study was approved by the ethics committees of each institution (ethics committee approval numbers: K1510-016, 4103 and H27-RINKEN-JINSOKU53, respectively). This study included infants who were born before 28 weeks of gestation and admitted to either institution between April 2016 and March 2018. Written informed consent was obtained from the parents of the patients. Infants with chromosomal abnormalities, complex cardiac malformations, central nervous system abnormalities, inborn errors of metabolism, and immunodeficiency syndromes were excluded. They were also excluded if the attending physicians considered their participation inappropriate.

LCC was diagnosed based on the clinical criteria (Table S1 in the Supplementary Appendix). Chronic lung disease was diagnosed based on the need for respiratory support to maintain arterial O_2 saturation at 90%–95 % for ≥3 days at the postmenstrual age of 36 weeks.[17] Symptomatic patent ductus arteriosus and persistent pulmonary hypertension of the newborn were diagnosed using ultrasonic echocardiography.[18,19] Histological chorioamnionitis (hCAM) was diagnosed based on the results of the histological examination of the placenta. Intraventricular hemorrhage was diagnosed using intracranial echography and categorized as grades I to IV.[20] Furthermore, necrotizing enterocolitis (NEC) was diagnosed based on the blood test results and abdominal radiography findings.[21] Respiratory distress syndrome was confirmed using a stable microbubble rating and chest radiography before surfactant administration.[22] Sepsis was diagnosed based on positive blood culture results.

Specimens were collected from umbilical cord blood at birth and peripheral vein blood every 2 weeks. They were also collected when needed until the infant was 8 weeks of age. Depending on the infant's condition, sample collection could be delayed for up to 1 week. After the specimens were collected, the serum was centrifuged and stored at −40 °C. NO is unstable *in vivo*, converted to nitrite (NO_2^-) as the stable compound, and oxidized to nitrate (NO_3^-)[23,24]; therefore, serum NO_2^- and NO_3^- levels were measured using the Greiss method.[25] Simultaneously, blood cells were analyzed using automated blood cell analyzers (XE5000 and XN350; Sysmex Corporation, Kobe, Japan) to measure white blood cell counts. A trained laboratory technician measured eosinophil counts. We excluded data regarding NOx levels and eosinophil counts obtained within 24 h after steroid administration to avoid steroid effects.

We used EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria), for statistical analyses.[26] The Wilcoxon signed-rank sum test was performed to evaluate the trends of NOx and eosinophil counts. Student's *t* test was performed to evaluate the association with each disease. Pearson's product–moment correlation coefficient was used to determine the correlation between NOx and eosinophil counts. Statistical significance was set at $p < 0.05$.

3. Results

Eighty-five infants born before 28 weeks of gestation were admitted to the centers during the study period. Of the 15 infants who were excluded, 7 experienced early neonatal death. Of those seven infants, severe neonatal asphyxia was the cause of death for four and intraventricular hemorrhage (grades 3–4) was the cause of death for three. Nineteen extremely preterm infants were included in the study with parental consent (Fig. 1). Table 1 shows the patients' characteristics. The median gestational age was 27.0 weeks, the median birth weight was 888 g, and all infants were appropriate for gestational age. Serum NOx levels were 22.5 ± 14.9 μmol/L, 51.2 ± 23.7 μmol/L, 42.4 ± 15.2 μmol/L, and 33.8 ± 9.4 μmol/L at birth and 2, 4, and 6 weeks of age, respectively (Fig. 2). The NOx level at 2 weeks of age was significantly elevated compared to that at birth ($p < 0.01$). A comparison of the levels at 2 and 6 weeks of age showed that they significantly decreased ($p < 0.05$). Eosinophil counts were 145 ± 199/μL, 613 ± 625/μL, 466 ± 375/μL, and 292 ± 228/μL at birth and 2, 4, and 6 weeks of age, respectively (Fig. 3). Similar to the serum NOx levels, the eosinophil count at 2 weeks of age was significantly elevated compared to that at birth ($p < 0.05$). In parallel with the NOx levels, eosinophil counts decreased significantly between 2 and 6 weeks of age ($p < 0.05$). As shown in Figs. 2 and 3, the postnatal longitudinal transition of NOx was significantly correlated with the changes in eosinophil counts ($p < 0.01$; $r = 0.996$).

We also investigated the relationship between serum NOx levels and eosinophil counts and the following complications that affect the prognosis of preterm infants: LCC; chronic lung disease; symptomatic patent ductus arteriosus; hCAM; intraventricular hemorrhage; NEC; respiratory distress syndrome; persistent pulmonary hypertension of the newborn; and sepsis. No significant correlation with any of those diseases at birth was observed. In contrast, the serum NOx level was significantly higher ($p < 0.05$) in the group without hCAM at 4 weeks of age, and the eosinophil count was significantly higher ($p < 0.01$) in the group with NEC at 2 weeks of age (Table 2).

4. Discussion

To the best of our knowledge, this is the first study to evaluate the longitudinal transition of serum NOx levels in extremely preterm infants. We found that serum NOx levels in extremely preterm infants born before 28 weeks of gestation were transiently and significantly elevated at 2 weeks of age. Furthermore, postnatal changes in serum NOx levels were significantly correlated with changes in eosinophil counts, which were measured simultaneously.

The findings of a previous study[3] indicated that postnatal trends of urinary NOx in newborns significantly increased from day 1 to day 4 in both term and preterm infants. Endo et al.[4] showed that serum NOx levels in full-term infants increased significantly from day 0 to day 5 and decreased significantly at day 30. A comparison with the results of previous studies showed that NOx levels in extremely preterm infants in this study were transiently elevated after birth but peaked later. However, their NOx levels did not appear different. The difference in peaks between term and preterm infants may arise because preterm infants are exposed to more oxidative stress, such as intensive care and oxygen administration, and have less antioxidative activity. Prolonged periods of high NOx concentrations are expected to have various effects on the pathology in preterm infants. This pilot study focused on the longitudinal changes of NOx; therefore, further research is needed to prove the relationship between NOx and the pathology of preterm infants.

Similar to the findings of previous studies,[16,27,28] this study showed

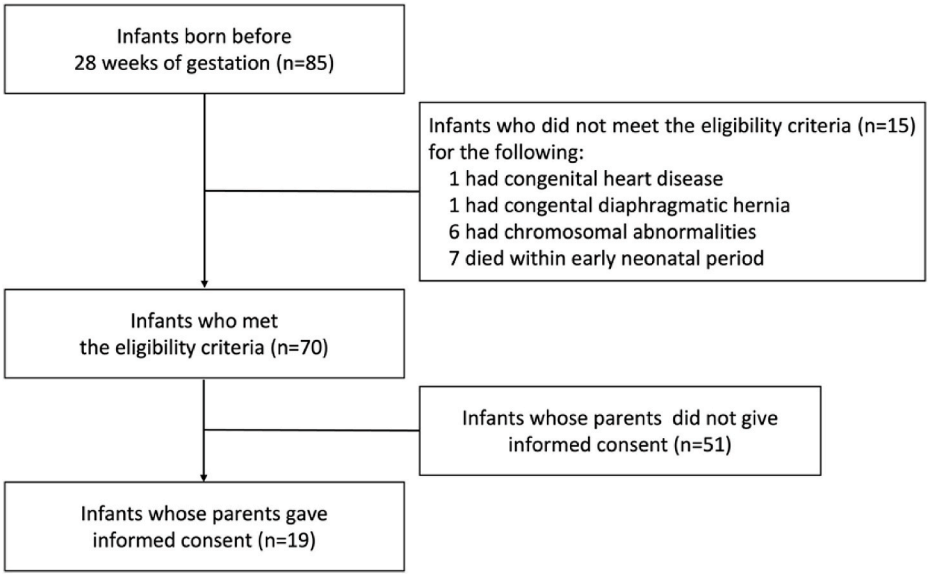

Fig. 1. Patient disposition flow chart.

Table 1
Baseline characteristics of the study participants.

	Total (n = 19)
Gestational age (wk)	27.0 (23.3–27.6)
Birth weight (g)	888 (558–1212)
Appropriate for gestational age	19 (100 %)
Sex (male/female)	11/8
Apgar score	
1 min	4 (1–8)
5 min	7 (3–9)
Antenatal steroids	13 (68 %)
Histological chorioamnionitis	16 (84 %)
Nonreassuring fetal status	1 (5 %)
Cesarean delivery	10 (53 %)

Values are presented as the number (percentage) or median (range).

that eosinophil counts increased transiently after birth and then declined. Juul et al.[15] also reported that term and preterm infants experienced a transient increase in eosinophil counts after birth; however, the peak occurred later in preterm infants than in term infants. Several studies have indicated that preterm infants are in a state of relative adrenal insufficiency that persists after birth.[7–9] We previously reported[16] that steroid administration significantly reduced the eosinophil count. Steroids inhibit eosinophil production and shorten the eosinophil lifespan by inhibiting granulocyte-macrophage colony-stimulating factor, IL-3, and IL-5,[29] which may be related to relative adrenal insufficiency in preterm infants and eosinophil trends. Because NO production also may be elevated in the pathogenesis of relative adrenal insufficiency,[5,6] we expected that serum NOx and eosinophil trends would be correlated. Therefore, we examined that relationship and clarified that a correlation existed between serum NOx levels and eosinophils. During a study of rodents, Wallerath et al.[5] found that glucocorticoids reduced the activity of the eNOS promoter by up to 70 % by decreasing the stability of the eNOS messenger ribonucleic acid and reducing the binding activity of the essential transcriptase GATA. Liu et al.[6] also reported that a three-fold increase in physiological glucocorticoid levels significantly reduced the expression level of eNOS. These results suggest that glucocorticoids have an inhibitory effect on NO and eosinophils. During this study, our results showed a correlation between postnatal trends of serum NOx and eosinophils in extremely preterm infants. These results may reflect the relative adrenal insufficiency that potentially emerges in all extremely preterm infants.

LCC is a condition in which hypotension and oliguria suddenly occur without any apparent cause after respiratory and circulatory conditions have been stabilized in preterm infants.[7,10] Relative adrenal insufficiency has been demonstrated as the underlying cause.[7,10] During our previous study on the pathogenesis of LCC, we found that dilatation of systemic blood vessels occurred at the onset of LCC.[11] Additionally, we reported eosinophilia at the onset of LCC because eosinophil counts increased during adrenal insufficiency.[16] During this study, trends of serum NOx were correlated with those of eosinophil counts, although no significant correlation existed at each point. Therefore, we could not prove that NOx was affected by relative adrenal insufficiency. We found statistically significant differences between hCAM and NOx and between NEC and eosinophils, but the number of cases in each of these diseases was small; therefore, further studies are needed for clinical interpretation.

This study had some limitations. First, obtaining parental consent was difficult because of the severity in extremely preterm infants. Therefore, the number of cases was small, and some complications had a very low incidence. As a result, the relationship between each complication and NOx or eosinophils was not been clarified during this study. Second, we could not evaluate steroid profiles or cytokines that affected NO and eosinophil counts during this study. The limited number of specimens that were collected from extremely preterm infants during this study made frequent testing or examining multiple items simultaneously difficult. Third, the relationship between the NOx levels and eosinophil counts was correlated according to the average trend, but they were not correlated according to individual data. This is because of the limitation of complete blood cell count, which is affected by various conditions, not only adrenal insufficiency, as well as various pathological conditions, such as infection, bone marrow status, and postnatal stress.

During this study, we found that serum NOx levels in preterm infants born before 28 weeks of gestation were transiently and significantly elevated at 2 weeks of age and considerably decreased at 6 weeks of age. Postnatal changes in serum NOx levels were significantly correlated with changes in eosinophil counts, which were measured simultaneously. We intend to conduct further large-scale and long-term studies to clarify the relationship between NOx and pathophysiological conditions of preterm infants.

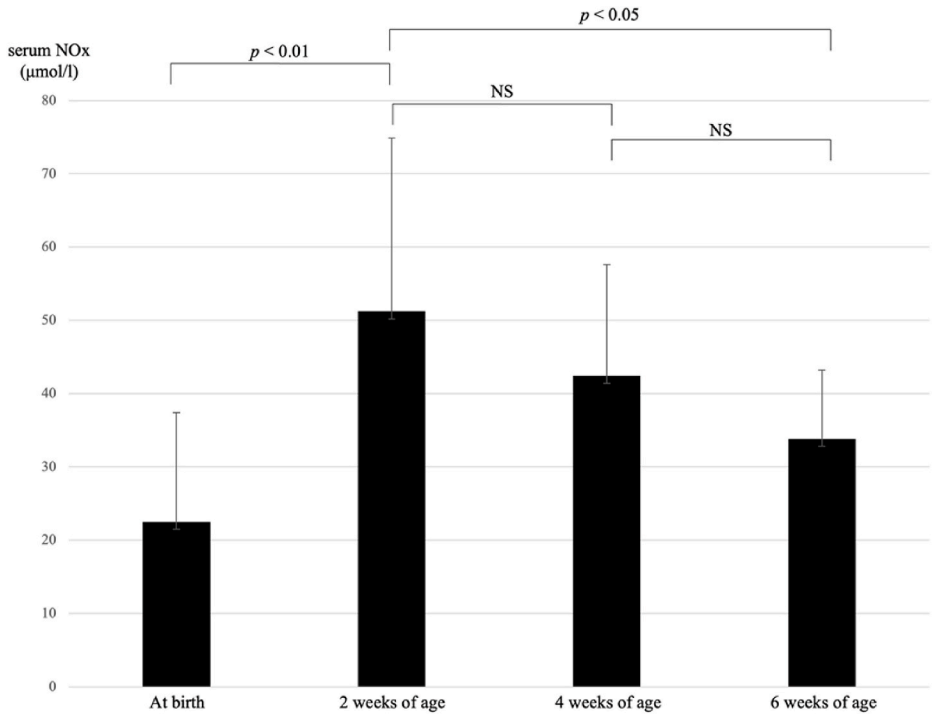

Fig. 2. Serum NOx levels (i.e., the sum of serum nitrite and nitrate) of preterm infants. NOx: nitrogen oxides; NS: not significant.

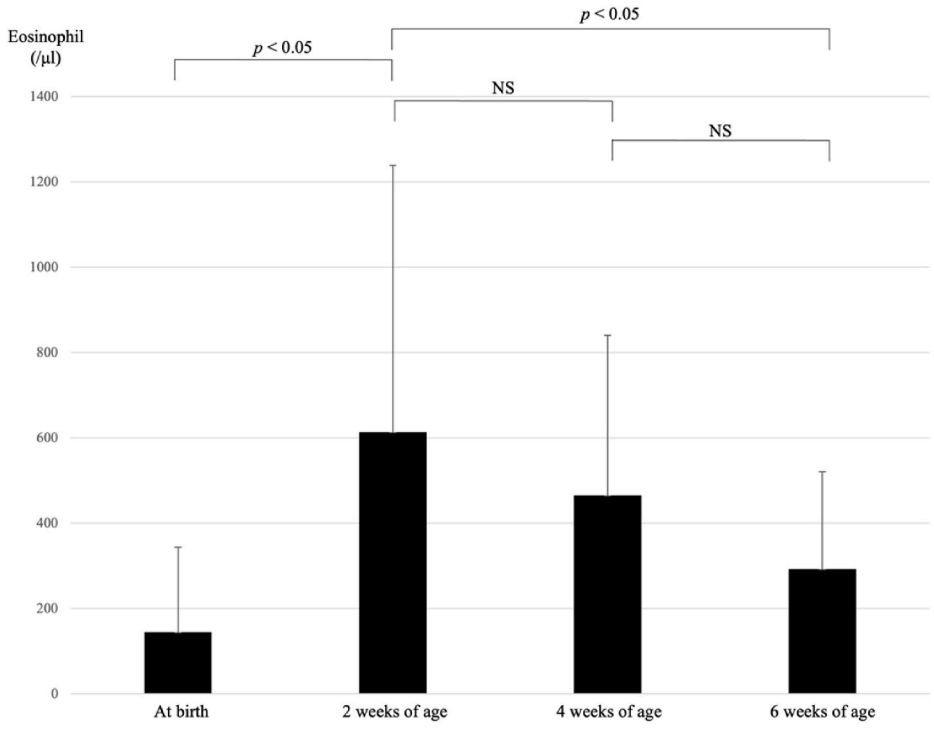

Fig. 3. Eosinophil counts of preterm infants. NS: not significant.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Statement of financial support

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Table 2
Correlation of serum NOx and eosinophil counts with postnatal diseases.

Disease		Serum NOx, μmol/L (n)		*p* value	Eosinophil count,/μL (n)		*p* value
		With disease	Without disease		With disease	Without disease	
hCAM	At birth	21.7 ± 15.2 (13)	33.3 (1)	0.48	158.4 ± 216.3 (16)	71.3 ± 123.6 (3)	0.52
	2 weeks of age	47.1 ± 22.5 (15)	82.2 ± 15.1 (2)	0.052	628.5 ± 685.8 (15)	537.7 ± 464.6 (3)	0.83
	4 weeks of age	38.6 ± 13.5 (15)	61.2 ± 12.5 (3)	0.017	445.4 ± 333.6 (15)	566.3 ± 682.5 (3)	0.63
	6 weeks of age	32.3 ± 9.7 (13)	43.2 ± 0.1 (2)	0.15	273.7 ± 219.3 (15)	386.7 ± 339.8 (3)	0.46
RDS	At birth	22.5 ± 17.9 (9)	22.4 ± 9.1 (5)	0.99	164 ± 224.8 (14)	90.6 ± 136.8 (5)	0.51
	2 weeks of age	50.0 ± 23.6 (14)	57.6 ± 32.7 (3)	0.66	663.5 ± 711.4 (14)	438.0 ± 315.8 (4)	0.55
	4 weeks of age	43.0 ± 16.5 (13)	40.9 ± 14.8 (5)	0.81	522.2 ± 393.0 (13)	318.2 ± 361.5 (5)	0.33
	6 weeks of age	33.1 ± 10.2 (10)	35.0 ± 9.8 (5)	0.74	347.9 ± 242.0 (13)	148.6 ± 152.1 (5)	0.11
PPHN	At birth	17.8 ± 3.4 (2)	23.3 ± 16.1 (12)	0.65	385 ± 289.9 (2)	116.4 ± 183.5 (17)	0.078
	2 weeks of age	71.0 ± 41.1 (2)	48.6 ± 22.3 (15)	0.24	792.0 ± 691.6 (2)	591.0 ± 657.6 (16)	0.69
	4 weeks of age	41.0 ± 3.0 (2)	42.6 ± 16.6 (16)	0.9	143.0 ± 38.2 (2)	505.9 ± 390.8 (16)	0.22
	6 weeks of age	30.7 (1)	34.0 ± 10.1 (14)	0.76	483.5 ± 398.1 (2)	268.7 ± 215.7 (16)	0.23
IVH	At birth	20.7 ± 10.1 (7)	24.3 ± 19.3 (7)	0.67	187.3 ± 271.8 (9)	106.3 ± 119.9 (10)	0.4
	2 weeks of age	53.3 ± 27.2 (9)	48.9 ± 22.5 (8)	0.73	373.4 ± 278.9 (9)	853.3 ± 819.9 (9)	0.12
	4 weeks of age	39.0 ± 8.0 (7)	45.1 ± 19.8 (11)	0.43	436.3 ± 394.8 (8)	489.0 ± 397.6 (10)	0.78
	6 weeks of age	33.1 ± 11.7 (7)	34.4 ± 8.4 (8)	0.8	224.8 ± 166.9 (8)	346.8 ± 274.3 (10)	0.29
sPDA	At birth	16.8 ± 3.7 (6)	26.8 ± 18.9 (8)	0.87	131.7 ± 197.6 (14)	181 ± 242.3 (5)	0.66
	2 weeks of age	46.6 ± 19.6 (13)	66.3 ± 35.0 (4)	0.16	568.5 ± 704.1 (13)	730.2 ± 498 (5)	0.65
	4 weeks of age	42.2 ± 18.3 (13)	42.8 ± 6.0 (5)	0.94	529.2 ± 414.7 (13)	300.2 ± 261.5 (5)	0.27
	6 weeks of age	33.4 ± 11.0 (12)	34.7 ± 5.8 (3)	0.83	296.1 ± 219.1 (13)	283.4 ± 300.9 (5)	0.92
NEC	At birth	NA	22.5 ± 14.9 (14)		0 (1)	152.7 ± 207.2 (18)	0.48
	2 weeks of age	38.4 (1)	52 ± 24.9 (16)	0.6	2706 (1)	490.3 ± 387.3 (17)	0.000043
	4 weeks of age	19.9 (1)	43.7 ± 15.0 (17)	0.14	648 (1)	454.8 ± 394.6 (17)	0.64
	6 weeks of age	25.5 (1)	34.4 ± 9.8 (14)	0.74	602 (1)	274.4 ± 228.8 (17)	0.18
Sepsis	At birth	12.5 (1)	23.3 ± 15.3 (13)	0.51	0 (1)	152.7 ± 207.2 (18)	0.48
	2 weeks of age	43.2 (1)	51.7 ± 25.1 (16)	0.75	52 (1)	646.4 ± 647.2 (17)	0.39
	4 weeks of age	NA	43.4 ± 15.6 (18)		NA	465.6 ± 385.5 (18)	
	6 weeks of age	NA	33.8 ± 9.7 (15)		NA	292.6 ± 235.0 (18)	
LCC	At birth	21.2 (1)	22.6 ± 15.5 (13)	0.93	0 (1)	152.7 ± 207.2 (18)	0.48
	2 weeks of age	NA	51.2 ± 24.3 (17)		NA	613.4 ± 643.3 (18)	
	4 weeks of age	17.8 (1)	43.8 ± 14.8 (17)	0.11	736 (1)	449.6 ± 391.2 (17)	0.49
	6 weeks of age	29.6 (1)	34.1 ± 10.0 (14)	0.68	195 (1)	298.3 ± 240.9 (17)	0.68
CLD	At birth	16.8 ± 3.7 (6)	26.8 ± 18.9 (8)	0.23	184 ± 197.3 (8)	116.1 ± 213.9 (11)	0.49
	2 weeks of age	60.3 ± 26.4 (7)	44.9 ± 22.0 (10)	0.21	507.3 ± 481.0 (7)	680.9 ± 742.9 (11)	0.59
	4 weeks of age	40.7 ± 13.0 (8)	43.7 ± 18.0 (10)	0.7	476.0 ± 546.7 (8)	457.2 ± 219.2 (10)	0.92
	6 weeks of age	31.7 ± 9.8 (7)	35.6 ± 10.0 (8)	0.47	269.4 ± 248.6 (8)	311.1 ± 235.3 (10)	0.72

CLD: chronic lung disease; hCAM: histological chorioamnionitis; IVH: intraventricular hemorrhage; LCC: late-onset circulatory collapse; NA: not available; NEC: necrotizing enterocolitis; NOx: nitrogen oxides; PPHN: persistent pulmonary hypertension of the newborn; RDS: respiratory distress syndrome; sPDA: symptomatic patent ductus arteriosus. Values are presented as the mean number of samples ± standard deviation.

Authors' contributions

Hirokazu Watanabe contributed to the study design, data interpretation, and writing the first draft and revision of the manuscript. Yosuke Washio contributed to the study design, acquisition of the data, data analysis and interpretation, and revision of the manuscript. Kei Tamai, Daisaku Morimoto, Tomoka Okamura, Junko Yoshimoto, Misao Kageyama, Hidehiko Nakanishi, Atsushi Uchiyama, Hirokazu Tsukahara, and Satoshi Kusuda contributed to the study design, providing important intellectual content, and revision of the manuscript. All the authors approved the final manuscript.

Consent statement

The authors obtained written informed consent from the parents of the patients.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pedneo.2023.08.006.

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