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NO/ET-1 Imbalance in Preterm Infants at Risk for Necrotizing Enterocolitis

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ABSTRACT

We aimed to evaluate the role of vascular tone regulators in the pathogenesis of necrotizing enterocolitis (NEC) in low birth weight (LBW) neonates with moderate/severe hypoxic-ischemic encephalopathy (HIE). We comparatively analyzed the plasma concentrations of mucin 2 (MUC2), nitric oxide (NO) and endothelin-1 (ET-1), and fecal *human β-defensin* 2 (HBD2) levels of in early postnatal life in 12 infants diagnosed with NEC (group 1), 25 neonates without NEC (group 2), and 20 control infants. The ET-1 and NO concentrations of peripheral blood were elevated in both study groups compared with control infants ($p<0.05$). Increased fecal HBD2 levels were observed in-group 2 compared with control group infants ($p<0.05$), whereas mean plasma MUC2 concentrations were lower in both groups with moderate/severe HIE compared with a control group ($p<0.05$). Spearman's rank-order correlation analysis showed the negative correlation of ET-1 with MUC2 ($r=-0.597$; $p=0.015$) and HBD2 ($r=-0.499$; $p=0.002$), while there were no relationships between NO and intestinal defense markers. It was also found a reliable positive relationship of NO/ET-1 balance with MUC2 ($r=0.643$; $p=0.006$) and HBD2 ($r=0.611$; $p=0.005$). In conclusion, decreased plasma NO/ET-1 ratio in the background of impaired intestinal mucosa defense can serve as a predictive marker for NEC in LBW infants with HIE.

INTRODUCTION

Hypoxia in the antenatal period is a primary cause of numerous disabilities in newborns, particularly in infants born with low birth weight (LBW) [1, 2]. Considerable efforts have been devoted to evaluating alterations in the neurological, cardiopulmonary and renal functions of these infants; however, the intestinal function with regard to hypoxia in the neonate has not been completely evaluated[3,4]. Approximately 55–98% of infants with perinatal hypoxic brain injury suffer from different deviations in the gastrointestinal tract [5]. Disorders of the digestive tract are caused by hemodynamic disturbances because of centralization of the bloodstream and poor circulation in the internal organs, leading to hypoxic damage to the mucosa layer of the gastrointestinal tract, and necrotising enterocolitis (NEC) [6-8]. These disorders may be apparent in the first days of life as in the case of early complications in the form of paresis, motoric defects, and later complications in the form of the vegeto-visceral syndrome, persistent vomiting, and regurgitation [9-10]. Intestinal ischemia or hypoxia initially results in mucosal damage of a part of the intestinal wall that is the most vulnerable to hypoxia. The protective function of the intestinal mucosal barrier depends on the expression and properties of antimicrobial defensive factors such as mucins, which are involved in the regulatory system and generate the main chain of host defense against pathogens [11-12]. The aforementioned factors are actively expressed in the epithelium of the gastrointestinal tract and form the high-molecular-weight viscoelastic layer, which is the protective barrier for the mucosal surface of the gastrointestinal tract, representing a nourishing environment for vital activity of commensal bacteria of the intestine [13-14]. In addition to mucins, defensins play important roles in the formation of the gastrointestinal barrier. Defensins have broad-spectrum antimicrobial activity owing to their ability to disrupt the structure, function of bacteria and viruses, and participate in the stabilization of the mucosal protective layer through interactions with mucins [15].

Immature autoregulation of intestinal blood flow demonstrates that preterm infants are susceptible to intestinal ischemia. The changes of the intestinal microcirculation due to hypoxia-ischemia were reported as an important risk factor for NEC in newborn infants [16]. Ischemia disturbs the balance in the microvascular tone related to the production of vascular regulators such as endothelin-1 (ET-1) and nitric oxide (NO), leads to restriction in blood supply and expansion of ischemic intestinal lesions, which likely play an important role in the pathogenic cascade of NEC [8,17]. However, in the context of broad investigations

confirming the significant effects of NO and ET-1 on the physiology and pathology of vascular homeostasis, the implication of ischemia in the pathogenesis of epithelial injury of the intestinal tract has not been investigated completely. In this study, we aimed to evaluate the role of vascular tone regulators in the formation of the intestinal mucus barrier in LBW neonates with hypoxic-ischemic encephalopathy (HIE). We comparatively analyzed the expression of specific intestinal protective markers, i.e., plasma mucin 2 (MUC2) and fecal human β-defensin 2 (HBD2), and blood markers of endothelial activation, i.e., NO and ET-1, in early of postnatal life.

MATERIALS AND METHODS

Patients and study design

This study was conducted in accordance with the approved guidelines of the Azerbaijan National Committee on Bioethics and Ethics of Science and Technology, and the study was carried out in accordance with the Helsinki Declaration guidelines and regulations. The parents of all of the infants provided written consent for participation in this study after receiving complete information on the study's scope and purpose.

Thirty-seven LBW preterm infants who had moderate/severe perinatal HIE were enrolled in this study and were classified in 2 groups: group 1, infants who presented NEC in the first 7 days of early postnatal life (n=12); group 2, newborn infants without NEC (n=25). The control group (group 3) consisted of 20 LBW neonates who fulfilled all of the following criteria: an uncomplicated maternal history, a 5 min Apgar score of 7 or more, capillary or arterial cord blood pH of 7.00 or higher, a normal delivery after an uncomplicated pregnancy, no neurologic manifestations, normal cranial ultrasound, and no medication during the neonatal period.

We collected intrapartum and neonatal data prospectively and obtained obstetric data from hospital records. Data on maternal preeclampsia, sex, type of delivery, resuscitation measures in the delivery room, type of diet, medication, amount of enteral feeding, and anthropometric measurements (e.g., weight, body length, head, and chest circumference) were included on an individual research card for each infant. Gestational age was determined by the first day of the last menstrual cycle, and when possible, was confirmed or corrected by the first ultrasonography examination with growth measurements. Growth restriction was defined as estimated fetal anthropometric parameters, confirmed at birth, below the 10th percentile for

gestational age and sex. Medical management of all infants in NICU included cardio-respiratory support, followed up by serial detection of blood gases, control of blood parameters. All neonates with NEC underwent total parenteral nutrition and intravenous broad-spectrum antibiotic therapy, and serial abdominal X-rays study.

The study exclusion criteria included the death of the neonate within the first 7 days of life, transfer to other units, clinical or laboratory evidence of TORCH infections (toxoplasmosis, other [syphilis, varicella-zoster, parvovirus B19], rubella, cytomegalovirus, and herpes infections), or congenital malformation.

Assessment of brain pathology.

The diagnosis of asphyxia was determined according to Apgar scores (≤ 5 at 5 min of life), initial capillary or arterial pH of less than 7.00, and initial capillary or arterial lactate of greater than 7.00 mmol/l, according to the American Academy of Paediatrics guidelines [18]. Blood gases were detected within 30 min after delivery. The severity of neonatal encephalopathy was estimated by Sarnat scoring based on abnormal neurologic signs, such as increased irritability and jitteriness, abnormal tone, abnormal primitive reflexes, altered consciousness, or convulsions, within the first 24 h of life [19].

NEC diagnosis

All infants were closely monitored for apnoea and bradycardia, oxygen desaturation, gastric residuals, bilious aspirates, abdominal distension, and grossly bloody stools. The diagnosis of necrotising enterocolitis (NEC) was based on clinical findings modified Bell's staging criteria [20]. All newborns underwent X-ray films for assessment of presence intestinal pneumatosis, portal venous air, dilated bowel, or abnormal gas pattern. NEC stage 1 had been determined in 5, stage 2 in 6 infants, and stage 3 only in 1 newborn.

Blood and stool collection

Venous blood was collected into ethylenediaminetetraacetic acid-containing tubes on days 1-3 and centrifuged for 15 min. No venous punctures were performed for the sole purpose of study-related analysis. Samples were stored in aliquots at -70°C until analysis. Stool samples were prospectively collected and analyzed for HBD2 immediately after sampling on days 3 and 5.

Measurement of serum MUC2 and ET-1 concentrations in peripheral blood

The plasma concentrations of MUC2 (Life Science, Wuhan, China), and ET-1 (Cayman Chemical Company, Ann Arbor, MI, USA) were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits, based on standard enzyme immunoassay procedures. The specimens were diluted according to the manufacturer's instructions for the ELISA kits to obtain the optimal density. The expression levels of MUC2 is reported in ng/ml, whereas that of ET-1 is reported in pg/ml.

Measurement of HBD2 concentrations in feces

HBD2 levels were determined using a standard enzyme immunoassay kit (Immundiagnostik, Bensheim, Germany). Fecal samples were diluted in extraction buffer at a ratio of 1:50. Before standard ELISA, homogenates were centrifuged and dissolved in solution buffer. The concentrations of HBD2 in feces are expressed in ng/g.

Measurement of NO concentrations in peripheral blood

The concentration of NO was measured using the Griess reaction from a commercial kit (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA). This test is based on the conversion of nitrate to nitrite through the action of nitrate reductase enzyme. Before direct assay, samples were ultra-filtered through a 10 000 molecular weight cut-off filter. NO levels were expressed in mmol/l.

Statistical analysis

We performed fecal analyses in triplicate, blood samples in duplicate. Intra and interassay coefficients of variation were <5% and <15%, respectively. Data were tested for normal distributions and found to be nonparametric. Significant differences were determined using the Kruskal-Wallis test, to assess differences in the production of protective markers of the intestinal barrier and endothelial function. Qualitative variables, such as sex, maternal preeclampsia, cesarean section, resuscitation measures in the delivery room, and the degree of HIE were compared using Fisher's exact test. Spearman rank-order correlation coefficients were used to determine the associations between vascular tone regulator markers and intestinal defense proteins. In all instances, significance was established at $p < 0.05$.

RESULTS

As shown in Table 1, there were no significant differences in the mode of delivery or in maternal characteristics between groups 1 and 2, including factors that may have contributed to uteroplacental insufficiency (anemia, preeclampsia). The groups were also similar in baseline neonatal characteristics, including gestational age, Apgar scores, pH and C reactive protein at birth, breast milk feeding, and antibiotic therapy during the 1st week of life.

The concentrations of vascular regulatory markers during the early days of the neonatal period were presented in Figure 1. The NO levels of peripheral blood were significantly elevated in both HIE groups (mean 53.4 mmol/l, range 34-66 mmol/l; mean 49.9 mmol/l, range 23-63 mmol/l; in groups 1 and 2 respectively) compared with control infants (mean 28.2 mmol/l, range 23-35). The ET-1 levels were also considerably higher in-group 1 (mean 4.9 pg/ml, range 2.73-8.50 pg/ml) and group 2 infants (mean 3.81 pg/ml, range 1.3-8.8 pg/ml), and the differences with control group parameters (mean 2.8 pg/ml, range 1.9-3.9 pg/ml) were significant. Figure 2 shows comparisons of the mean total plasma concentrations of MUC2 and fecal concentrations of HBD2 in the study groups. Increased fecal HBD2 levels were observed in infants of both HIE groups compared to control group parameters (mean 98.8 ng/g, range 67-198 ng/g; mean 123.7 ng/g, range 45-243 ng/g; mean 63.3 ng/g, range 28-120 ng/g; respectively in groups 1, 2 and control infants), whereas only the mean total HBD2 concentrations were significantly different between group 2 and control infants. Plasma MUC2 concentrations were considerably lower in infants of group 1 (mean 14.1 ng/ml, range 9-26.4 ng/ml) and group 2 (mean 15.1 ng/ml, range 6.8-29.2 ng/ml) compared with those in the control group (mean 18.05 ng/ml, range 13-25.1 ng/ml). We did not observe a statistically significant difference between group 1 and group 2 data of the mean total NO, ET-1, HBD2, and MUC2 concentrations. With regard to mean NO/ET-1 ratio in the newborns, this ratio was lower in group 1 infants ($p< 0.05$) compared with group 2 and control infants (Table 2).

To determine the correlation between the vascular tone regulators and the markers of intestinal defense, we analyzed NO and ET-1 data in both HIE groups (37 cases). Spearman's rank-order correlation analysis showed significant negative relation of ET-1 with MUC2 (r=-0.499; p=0.015) and HBD2 (r=-0.597; p=0.002), while NO were not significantly related to MUC2 and HBD2 (Fig. 3). As shown in Figure 4, it was also found a significant positive

correlation of NO/ET-1 ratio with fecal HBD2 (r=0.611, p=0.005) and plasma MUC2 concentrations (r=0.643, p=0.006).

DISCUSSION

Hypoxic and ischemic complications during the pre- and perinatal period cause acquired neonatal brain damage associated with different grades of poliorgan insufficiency [21]. Although the neonatal brain is one of the most vulnerable organs due to its high energy and oxygen consumption, hypoxic ischemia has been implicated in the breakdown of the intestinal epithelial barrier, which can lead to bacterial translocation [22-23]. In our study, increased expression of fecal HBD2 in both HIE groups early after birth suggested the activation of acute repair mechanisms at the site of injury. Hypoxia has been reported to increase the expression of different intestinal mucosa defense factors in previous studies. It was interpreted this process as the mechanism for maintenance of barrier function when oxygen levels are low [24-25]. Increased expression of defensive factors in the intestines of rats exposed to intrauterine asphyxia increased 72 h after birth, followed by the rapid proliferation of the mucosa with the recovery of intestinal function [26]. We found that HBD2 synthesis in early days of postnatal life was not sufficiently high in infants diagnosed with NEC compared to neonates who did not show NEC, and was accompanied with decreased MUC2 concentrations in peripheral blood. MUC2, the major colonic gel-forming protein, is known to be a critical factor for the establishment of goblet cell morphology and plays an important role in mucosal protection by preventing bacterial pathogens from gaining access to the epithelium [27-29]. The previous study confirms that MUC2-knockout mice show increased susceptibility to the development of inflammatory bowel diseases, suggesting a potential role for mucins in epithelial protection [30]. We speculate that severe/moderate hypoxic-ischemic injury and systemic hypoperfusion in group 1 infants was associated with more serious alterations in goblet cell function, leading to reduction of MUC2 and HBD2, compared with that in control infants. According to the literature, brain injury can induce significant damage to gut structures and impairment of barrier function due to relative hypoperfusion and interactions of inflammatory mediators with their receptors located on gut epithelial cells [31-32]. Considering the possibility of intestinal injury as a result of an imbalance between vasoconstriction and vasodilatation [33-35], significant positive correlation between ET-1 concentrations and intestinal mucosa defense markers in our study might point to relationship of the balance shifting towards increased vasoconstriction with

different structural and pathological changes of the gastrointestinal tract of LBW infants diagnosed NEC in early neonatal period.

We have not determined the significant difference between the main baseline characteristics (NO, ET-1, HBD2, and MUC2) of both HIE groups, and in that case, the relationship of NEC development with the levels of vasoregulatory and intestinal injury markers is quite conflicting. The determination of NO/ET-1 balance is the important finding, which gives the possibility for interpretation of this question. We found significantly lower NO/ET-1 ratio in group 1 infants, compared to newborns that have not suffered from NEC. Therefore, the impaired NO/ET-1 balance in the peripheral blood of NEC diagnosed infants during early postnatal life was the main cause of intestinal ischemia and might alter the protective functions of the intestinal mucosa, manifesting as a depressed synthesis of serum MUC2 and fecal HBD2 concentrations. The statistically significant positive correlation between NO/ET-1 ratio and intestinal mucosa defense markers (HBD1 and MUC2) corresponds to the aforementioned result (Fig. 4). Thus, the high ET-1 concentration without adequate NO synthesis causes an imbalance between vasoconstriction and vasodilatation and leads to serious injury of intestinal mucosa of LBW infants with severe/moderate HIE.

There is currently no perfect method for predicting the NEC from the early days of neonatal period in preterm infants with HIE. The lack of sensitive markers for the early prediction of intestinal ischemia and a wide spectrum studies justify the need for further investigations in this area. The present study confirms that systemic vascular tone activity plays an important role in mediating the functional activity of the gut mucosa through maintenance of intestinal blood circulation. We also propose that decreased NO/ET-1 ratio in the background of impaired intestinal mucosa defense can serve as a predictive marker for NEC, and may be accepted as a sensitive marker for intestinal injury in preterm infants with HIE. The early assessment of the severity of intestinal injury in preterm infants may provide a useful tool for the prevention of further complications in these infants.

Author contributions

N.P. and R.B. initiated the project, S.H., and N.P. carried out the theoretical design and analysis, designed the devices and the studies, and wrote the manuscript. A.H., S.Sh. and N.H. collected the patient data and contributed the computing resources. S.H. contributed to the biomarker analyses and participated in the analysis of the results.

Conflict of interest: The authors declare no conflict of interest.

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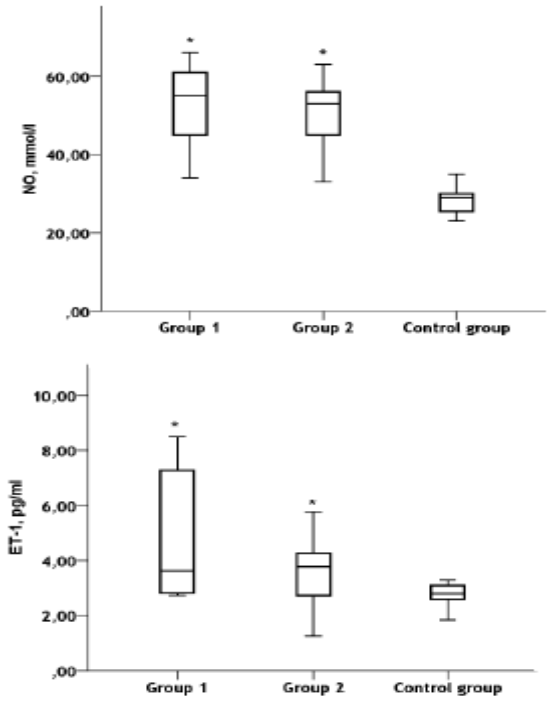

Fig. 1. Mean total NO and ET-1 concentrations of peripheral blood in the study groups. *$p < 0.05$ versus the control group.

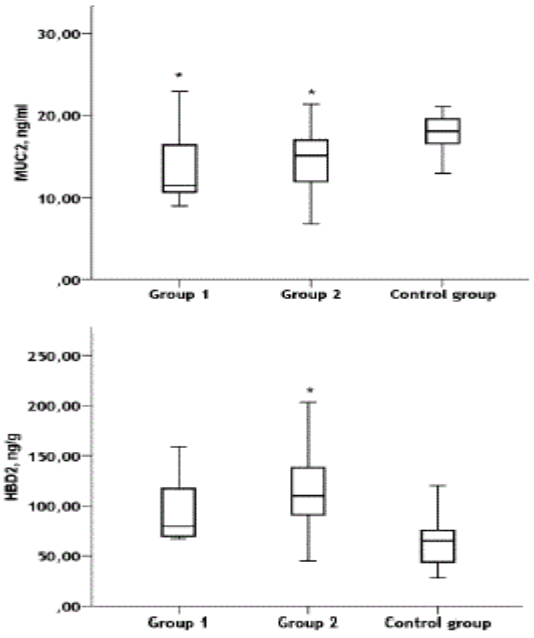

Fig. 2. Mean fecal HBD2 and plasma MUC2 concentrations in the study groups. It is described HBD2 concentrations in 12–24 h before the first clinical diagnosis of NEC in group 1, and inappropriate day samples of group 2. *$p < 0.05$ versus the control group.

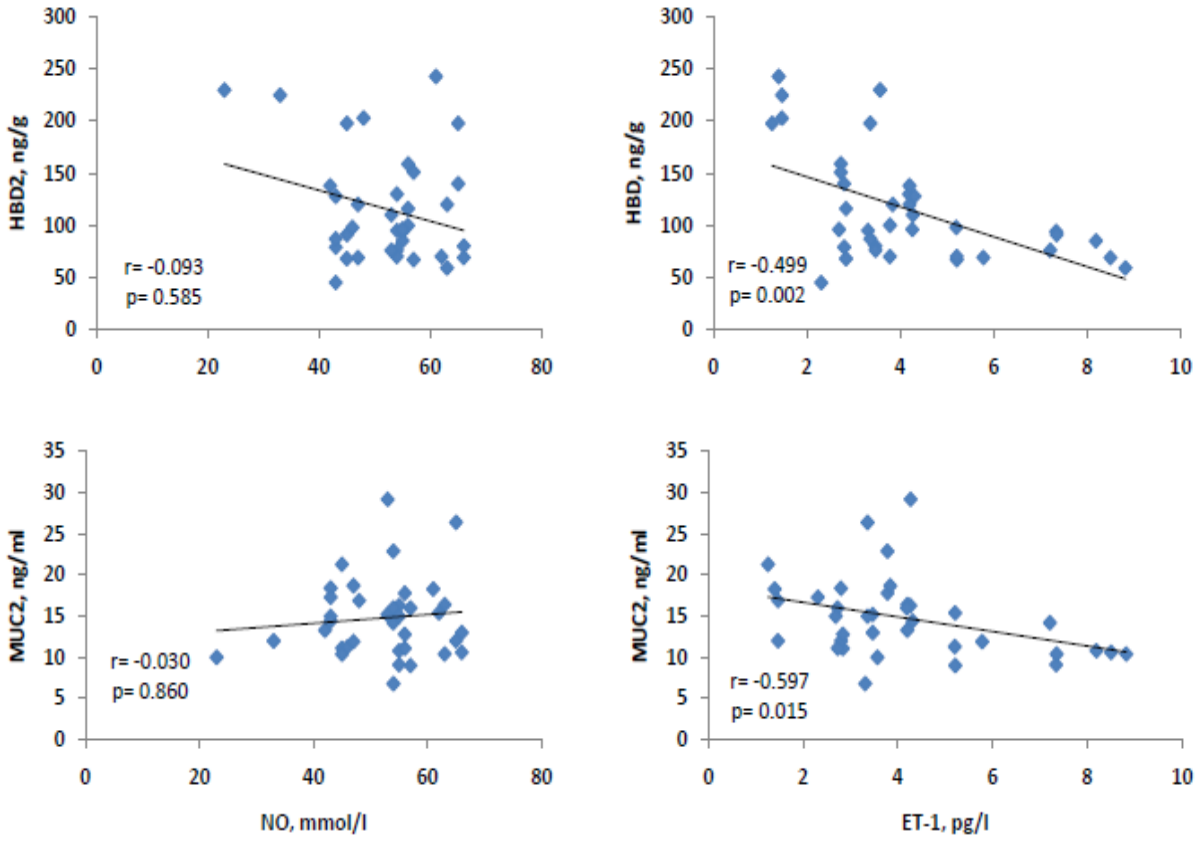

Fig. 3. Spearmen's rank-order correlation between vascular tone regulator and intestinal mucosa defense markers in LBW infants with moderate/severe HIE. Each correlation analysis includes group 1 and group 2 parameters (n=37).

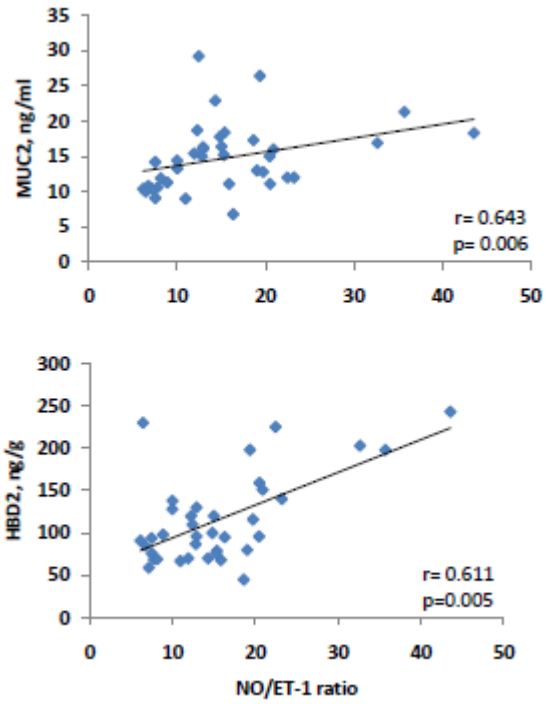

Fig. 4. Spearmen's rank-order correlation between NO/ET-1 ratio and intestinal mucosa defense markers in LBW infants with moderate/severe HIE. Each correlation analysis includes group 1 and group 2 parameters (n=37).

Table 1. Epidemiological Characteristics of the Study Groups. Data are shown as the mean (range) or n (%).

	Group 1 (n = 12)	Group 2 (n = 25)	*p* value
Maternal characteristics			
Age (y)	25.2 (19–31)	26.4 (19–34)	0.865
Premature rupture of membranes	4 (33,33%)	7 (28%)	0.740
Preeclampsia	3 (25%)	5 (20%)	0.729
Anaemia	4 (33,33%)	10 (40%)	0.695
Caesarean section	5 (41,67%)	7 (28%)	0.406
Antenatal steroids	3 (25%)	5 (20%)	0.729
Neonatal characteristics			
Gestational age, weeks	33.4 (29–35)	32.9 (29–35)	0.231
Male	5 (41.7%)	9 (36%)	0.088
Birth weight, g	1745 (1520–2160)	1898 (1501–2450)	0.543
Small for gestational age	3 (25%)	6 (24%)	0.947
Apgar 1 min	4.2 (3–5)	4.5 (3–5)	0.325
Apgar 5 min	6.3 (4–6)	6.2 (4–7)	0.620
pH at birth	7.15 (6.85–7.29)	7.21 (6.98–7.32)	0.102
C-reactive protein at birth, mg/dl	1.24 (0.7–2.49)	0.98 (0.3–2.41)	0.230
Breast milk feeding	4 (33,33%)	10 (40%)	0.695
Days on antibiotics of 1st week	4.2 (2-6)	3.9 (2-6%)	0.542
RDS	3 (25%)	6 (24%)	0.947
Moderate HIE	7 (58.3%)	16 (64%)	0.739
Severe HIE	5 (41.7%)	9 (36%)	0.088

Table 2. Mean nitric oxide/endothelin-1 (NO/ET-1) ratio in study groups. *p < 0.05 vs group 2 infants; ^.p < 0.05 vs control group. Data are shown as the mean (range).

Study groups	NO/ET-1 ratio
Group 1 (n=12)	10,3*^ (8,2-20,6)
Group 2 (n=25)	14,5 (11,8-25,3)
Control group (n=20)	16,1 (19,1-25,5)