# Mechanism of necrotizing enterocolitis in preterm infants through the hypoxia signaling pathway, neuronal-glial signaling pathway, and intestinal fatty acid signaling pathway

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## ABSTRACT

The etiology of necrotizing enterocolitis (NEC) is influenced by many factors including hypoxia, intestinal immaturity, bacterial colonization, reactive oxidants, and imbalanced inflammatory response; therefore, the pathogenesis of NEC is considered multifactorial. However, the pathogenesis of NEC has not been fully elucidated and requires further investigation. This study aimed to analyze the association between hypoxia inducible factor-1alpha (HIF-1alpha), glial fibrillary acidic protein (GFAP), glial derived neutrophic factor (GDNF), fatty acid binding protein-2 (FABP-2), peroxime proliferator activated receptor-gamma (PPAR-gamma), interleukin-6 (IL-6), and interleukin-8 (IL-8) with the incidence of NEC in preterm infants. All preterm infants with birth weight <1500 grams or gestational age <34 weeks were included in this study. After the umbilical cord was removed, 1 mL of umbilical blood was taken for HIF-1alpha, GFAP, GDNF, FABP-2, PPAR-gamma, IL-6, and IL-8 examination. Examination of HIF-1alpha, GFAP, GDNF, FABP-2, PPAR-gamma, IL-6, and IL-8 was repeated in infants with NEC symptoms using peripheral venous blood specimen. Infants were observed for 2 weeks. NEC was diagnosed based on clinical symptoms and abnormal abdominal radiographs. Of the 30 infants, there were 9 (30%) infants who experienced NEC. Logistic regression analysis showed significant results on GFAP with Odds Ratio (OR)=15.629 (95% confidence interval=1.697-143.906) $P$=0.015 and FABP-2 with OR=1.008 (1.001-1.015) $P$=0.033. Multivariate analysis using Backward LR logistic regression model showed significant results on GFAP with adjusted OR=15.629 (1.697-143.906) with $P$=0.015. This study demonstrated that GFAP and FABP-2 were significantly associated with the incidence of NEC. This may explain the pathogenesis of NEC through a hypoxic mechanism.

## CCS CONCEPTS

• **Applied computing**; • **Health informatics**;

## KEYWORDS

## 1 INTRODUCTION

Preterm birth has a major contribution to death in children under five years of age worldwide. It is estimated that every year, 11% of all deliveries in the world are preterm, and one million out of six million preterm infant deaths are caused by complications of prematurity [1]. Necrotizing enterocolitis (NEC) is a digestive tract emergency that often occurs in preterm infants and often requires surgery [2]. NEC mortality is quite high, ranging from 15-40% and it is possible to reach 50% in developing countries [3]. Symptoms of sequelae of surviving NEC infants include short bowel syndrome, failure to thrive, and neurodevelopmental disorders [4]. Since clinical manifestations of NEC were observed in 1965, mortality and morbidity of NEC are still high [5]. The pathogenesis of NEC is still not fully elucidated and requires continuous research developments. However, some studies have demonstrated an association between hypoxia and NEC [6].

Previous studies have demonstrated that activation of hypoxia inducible factor-1 (HIF-1) is required for intestinal injury [7]. HIF-1alpha can be detected in the intestinal tissue of infants with NEC [8]. Hypoxia can affect the structure and function of the fetal gut where it will inhibit the adaptation of the newborn's digestive tract and increase the risk of NEC [9]. An increase in HIF-1alpha can be found in the umbilical blood of premature infants, which indicates that the baby is hypoxic [10]. Hypoxia causes brain damage and results in glial cell death which is characterized by an increase in glial fibrillary acidic protein (GFAP). GFAP is known to have high accuracy in detecting perinatal asphyxia where increased GFAP levels correlate with the severity of asphyxia [11]. Another study showed a decrease in the number of glial cells in vitro (expressed by GFAP) in NEC. This proves that there is damage to the intestinal nervous system in NEC which is correlated with brain nerve damage [12]. Glial derived neurotrophic factor (GDNF) signals are expressed

in peripheral glial cells [13]. GDNF activation can inhibit intestinal glial cell apoptosis and can inhibit the increase in intestinal permeability. The role of GDNF is to preserve the integrity of the intestinal epithelium and inhibit the inflammatory process including inhibiting the occurrence of NEC [14]. Fatty acid binding protein-2 (FABP-2) is found on the cell membrane of enterocytes, has a role in fat transport, and has a small molecular mass, so it is easy to move into the circulation if there is intestinal damage. Besides being used to detect NEC, FABP-2 can predict the extent of the severity of intestinal damage in NEC with fairly good accuracy [2, 3]. Peroxime proliferator activated receptor-gamma (PPAR-gamma) is a transcriptional gene in fat metabolism. An in vitro study found that intestinal ischemia can stimulate PPAR-gamma; however, activation of PPAR-gamma is required to repair intestinal damage in NEC [15]. Cytokines in the adaptive immune response are believed to have key roles in the NEC mechanism, including interleukin-6 (IL-6) and interleukin-8 (IL-8) [16]. IL-6 levels were detected to be elevated in preterm infants where this increase was correlated with a lower Apgar score. Increased levels of IL-6 were also detected to be associated with the incidence of NEC [17]. Furthermore, IL-8 RNA expression is also known to be increased in gut resection of infants experienced NEC [18].

The results of the above study indicate that the association between HIF-1alpha, GFAP, GDNF, FABP-2, PPAR-gamma, IL-6, and IL-8 can explain the pathogenesis of NEC through hypoxic mechanisms. This study aims to analyze the mechanism of the occurrence of NEC involving the roles of HIF-1alpha, GFAP, GDNF, FABP-2, PPAR-gamma, IL-6, and IL-8.

## 2 METHODS

This research design was an observational study. The sample of this study used preterm infants with gestational age <34 weeks or birth weight <1500 grams. Infants will be observed since birth until 2 weeks of age. Exclusion criteria were infants with intestinal atresia, spontaneous intestinal perforation, and sepsis. Research ethics were obtained from the Health Research Ethics Committee, Faculty of Medicine, Universitas Airlangga, Soetomo Academic General Hospital (Reference number. 0123/KEPK/I/2021). Informed and signed consent was acquired from the parents or authorized representatives of each infant.

At the time the infant was born, shortly after the umbilical cord is removed by the doctor, the placenta will be placed in a sterile container. Subsequently, blood collection was performed on the umbilical cord of the placenta. The volume of blood taken was 1 mL for examination of HIF-1alpha, GFAP, GDNF, FABP-2, PPAR-gamma, IL-6, and IL-8 (referred to as the first sample). Examination of HIF-1alpha, GFAP, GDNF, FABP-2, PPAR-gamma, IL-6, and IL-8 was repeated in infants with NEC symptoms using peripheral venous blood specimen with a blood volume of 1 mL (referred to as the second sample). Samples were examined using the enzyme-linked immunosorbent assay method (Elabscience Biotechnology; Texas, United States). Abdominal X-ray examination was performed on infants who showed symptoms of NEC. Biomarker and radiological examinations are carried out by competent specialists.

Infants were diagnosed as NEC if gastrointestinal symptoms were found (abdominal distension, vomiting, increased gastric aspirate >20%, bloody stools, abdominal tenderness) and abnormal abdominal radiographs (dilated bowel, ileus, ascites, pneumatosis intestinalis, persistent bowel loops, portal venous gas, pneumoperitoneum). The association between HIF-1alpha, GFAP, GDNF, FABP-2, PPAR-gamma, IL-6, and IL-8 on the incidence of NEC was analyzed statistically.

## 3 RESULTS

A total of 35 infants met the inclusion criteria of the study, however, there were 5 samples that could not be analyzed due to defects so that as many as 30 infants were included in the final analysis. Of the 30 infants, there were 9 (30%) infants who experienced NEC. Most of the infants with NEC were male, gestational age 30-<34 weeks, birth weight 1000-<1500 grams, and had a history of cesarean delivery. The group of infants with NEC had a minimum gestational age of 27 weeks, a maximum of 33 weeks, a mean of 30 weeks, and a standard deviation (SD) of 2.4 weeks. The group of infants with NEC also had a minimum birth weight of 950 grams, a maximum of 1450 grams, an average of 1116 grams, and an SD of 183.7 grams. All NEC infants in this study were singleton pregnancies. All infants with NEC also had a history of asphyxia at birth. A total of 2 out of 9 infants (22.2%) with NEC died. The onset of NEC was seen on average at 10 days of age, minimum 7 days, maximum 12 days, with SD of 1.5 days. The distribution of biomarker was described in table 1. There was no significant difference in the levels of biomarkers in the first and second samples ($P$>0.05).

Univariate analysis using logistic regression test showed significant results on GFAP with Odds Ratio (OR)=15.629 (95% confidence interval=1.697-143.906) P=0.015 and FABP-2 with OR=1.008 (1.001-1.015) $P$=0.033. Multivariate analysis using Backward LR logistic regression model showed significant results on GFAP with adjusted OR=15,629 (1,697-143.906) P=0,015. Figure 1 showed the receiver operator characteristic (ROC) curve between GFAP and the incidence of NEC. From the figure, the area under curve = 0.783 (0.573-0.993) with $P$ value=0.015. The cut-off level of GFAP = 0.95 ng/mL has a sensitivity of 66.7% and a specificity of 95.2%.

## 4 DISCUSSION

Our study found that the majority of NEC occurred at 30-<34 weeks' gestation and birth weight of 1000-<1500 g. Similar studies reported that NEC occurred mostly in infants <34 weeks or birth weight <1500 grams, or even lower. The incidence of NEC can occur in term infants, which is around 7-25% or 0.05 cases per 1000 live births. The occurrence of NEC in term infants is usually related with a history of asphyxia and congenital abnormalities of the heart, gastrointestinal, and nervous organs.

In this study, 2 out of 9 infants (22.2%) with NEC died. Previous studies stated that NEC mortality is still quite high, ranging from 15-40% and could be higher in developing countries. NEC mortality increases at high NEC grades (grade 3) and at very low gestational ages. The use of ventilators, unavailability of pediatric surgeons, and the presence of sepsis increase NEC mortality [19].

Our study analyzed the significance of the association between HIF-1alpha, GFAP, GDNF, FABP-2, PPAR-gamma, IL-6, and IL-8

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**Table 1: Distribution of biomarkers in preterm infants with NEC and without NEC**

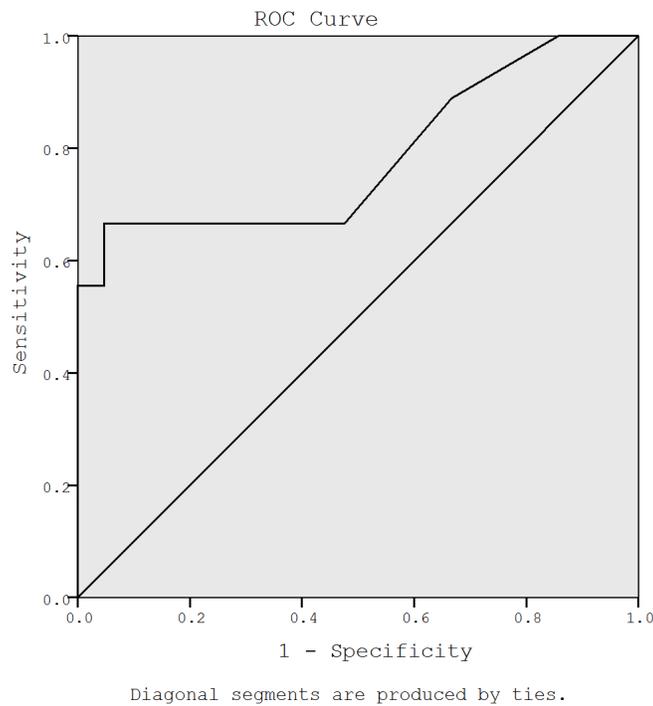| Biomarker(unit) | With NEC (n=9) | | | | Without NEC (n=21) | | | |
|---|---|---|---|---|---|---|---|---|
| | Minimum | Maximum | Mean | SD | Minimum | Maximum | Mean | SD |
| HIF-1alpha (ng/mL) | | | | | | | | |
| First sample | 1.10 | 10.50 | 3.31 | 3.08 | 0.90 | 2.70 | 1.32 | 0.41 |
| Second sample | 0.70 | 8.00 | 3.26 | 2.44 | N/A | N/A | N/A | N/A |
| GFAP (ng/mL) | | | | | | | | |
| First sample | 0.20 | 6.50 | 2.00 | 2.25 | 0.10 | 1.30 | 0.38 | 0.27 |
| Second sample | 0.30 | 3.10 | 1.74 | 1.29 | N/A | N/A | N/A | N/A |
| GDNF (ng/mL) | | | | | | | | |
| First sample | 1.05 | 14.11 | 4.39 | 4.19 | 1.07 | 3.48 | 1.69 | 0.56 |
| Second sample | 0.56 | 10.30 | 3.94 | 3.26 | N/A | N/A | N/A | N/A |
| FABP-2 (ng/L) | | | | | | | | |
| First sample | 278.40 | 2207.00 | 844.44 | 677.04 | 211.40 | 597.50 | 335.48 | 104.39 |
| Second sample | 179.20 | 1571.00 | 807.37 | 534.03 | N/A | N/A | N/A | N/A |
| PPAR-gamma (ng/mL) | | | | | | | | |
| First sample | 23.40 | 207.10 | 66.74 | 61.44 | 21.30 | 45.70 | 27.57 | 5.98 |
| Second sample | 8.80 | 142.30 | 63.23 | 45.62 | N/A | N/A | N/A | N/A |
| IL-8 (ng/L) | | | | | | | | |
| First sample | 45.30 | 683.80 | 216.58 | 233.33 | 42.30 | 112.70 | 60.18 | 17.29 |
| Second sample | 39.60 | 504.60 | 218.46 | 159.54 | N/A | N/A | N/A | N/A |
| IL-6 (ng/L) | | | | | | | | |
| First sample | 8.00 | 247.60 | 81.31 | 92.12 | 0.10 | 61.20 | 13.63 | 12.97 |
| Second sample | 13.60 | 495.50 | 113.71 | 151.28 | N/A | N/A | N/A | N/A |

N/A, not available



**Figure 1: ROC curve between GFAP and the incidence of NEC**

with the incidence of NEC. The results of statistical analysis showed significant results on GFAP and FABP-2. GFAP is a protein encoded by the human GFAP gene and belongs to the type III intermediate filament (IF) protein. Type III IF protein forms the cytoskeleton structure of muscle, brain, and mesenchymal tissue. GFAP is immediately released into the circulation when there is damage or death of astroglial cells [20]. Other studies have shown that an increase in GFAP is present from birth and correlates with the severity of asphyxia. The increase in GFAP can last up to 96 hours after the baby is born [11]. There is a new evidence that enteric glia participated actively in gut pathology associated with barrier dysfunction. Changes in intestinal permeability contribute to the pathology of NEC. There is a relationship between neuronal cell death and decreased GFAP expression in NEC [21].

A systematic review study conducted by Cheng in 2015 showed that FABP-2 examination has high accuracy in the diagnosis of NEC where accuracy increases according to the severity of NEC [3]. Apart from being a diagnostic tool, FABP-2 can be used as a tool to predict disease severity. FABP-2 can predict NEC cases that require surgery and which do not require surgery [22]. Other studies have also demonstrated that FABP-2 can be used to predict the extent of intestinal damage severity in NEC [23].

## 5 CONCLUSION

This study demonstrated that GFAP and FABP-2 were significantly associated with the incidence of NEC. This may explain the pathogenesis of NEC through hypoxic mechanisms, particularly in hypoxia associated with brain damage. The limitation of this study is that it does not consider maternal factors, therefore further research is needed.

## 6 FUTURE WORK

Cord blood has an abundance of immunomodulatory molecules that provide early information regarding efforts to diminish intestinal injury, primarily through hypoxic mechanisms. In the future, we would like to explore the potential mechanisms of which cord blood immunomodulatory molecules can be used to reduce NEC.

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