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Original Research Article

Small intestinal lactoferrin and calprotectin levels in different stages of necrotizing enterocolitis in a rat model

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ABSTRACT

Purpose: Necrotizing enterocolitis (NEC) is a severe disease of mostly premature infants with high morbidity and mortality rates. There is no reliable biomarker for detecting newborns at risk for NEC development. We aimed to investigate small intestinal lactoferrin (LF) and calprotectin (CAL) levels as predictors and indicators of disease severity in an experimental newborn rat model.
Materials and methods: Newborn pups were randomly divided into two groups, NEC and control. The NEC group pups were decapitated on the second, third and fourth days of the experiment for an assessment of the different stages of NEC. In the study group, hypoxia-reoxygenation model used to induce NEC. As biochemical parameters, small intestinal LF and CAL levels were measured with an enzyme-linked immunosorbent assay technique and intestinal injury scoring was evaluated as a pathologic parameter.
Results: Small intestinal levels of both LF and CAL increased in the second and the third day groups, but began to decrease by the fourth day. The first, second and third day levels of LF and CAL were higher than controls. The intestinal injury scores of all NEC groups were significantly higher than the control group.
Conclusion: Small intestinal lactoferrin and calprotectin were good markers for demonstrating NEC. However, instead of spot testing, monitoring the levels of these markers may be more informative.

1. Introduction

Necrotizing enterocolitis (NEC) is one of the most common gastrointestinal (GI) emergencies afflicting predominantly premature infants in neonatal intensive care units (NICU) [1]. The disease is often observed in premature infants with low birth-weight and gestational age. Low APGAR scores, chorioamnionitis, exchange transfusion, prolonged rupture of membranes, congenital heart disease, and neural tube defects are among other predisposing factors [2]. Its incidence varies between 5% and 10% among infants born at less than 32 weeks of gestation and with a birth weight of less than 1500 g [3]. The mortality of NEC ranges from 10% to 50% and survivors suffer from significant morbidity, such as long-term requirement for parenteral nutrition, malabsorption and malnutrition related to short bowel syndrome, and neurodevelopmental, neurosensory, and functional disabilities [4]. Despite prematurity, enteral feeding, intestinal hypoxia-ischemia, and bacterial colonization have been hypothesized to cause NEC. Besides being difficult to treat, there is no effective preventative strategy of NEC [5].

The diagnosis of NEC is based on modified Bell criteria proposed by Walsh and Kleigman, who developed the criteria with systemic, abdominal and radiologic findings to more clearly classify NEC [6]. There is no diagnostic laboratory findings, but only supportive in diagnosis of NEC [7]. The presenting signs are nonspecific, such as feeding intolerance, abdominal distention or tenderness, occult or gross blood in the stool, lethargy, apnea, respiratory distress, or poor perfusion that resembles diseases like sepsis and respiratory distress syndrome [8]. Thus, detecting infants at risk for NEC or those in early stages of the disease would offer opportunities for early intervention.

The fecal proteins calprotectin (FCAL) and lactoferrin (FLF), which are derived from neutrophils, have the potential to be ideal

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markers of intestinal inflammation because their measurement is non-invasive, easy to obtain, and inexpensive. Measurement can be done in small intestinal contents/feces with less than 5 g of stool in the case of intestinal inflammation [9]. Fecal calprotectin, a calcium- and zinc-binding neutrophilic cytosolic protein, is found in proportion to the degree of inflammation present, is resistant to colonic bacterial degradation, and is stable in stools for up to 1 week at room temperature. Fecal lactoferrin is an iron-binding protein that is similar to fecal calprotectin regarding that it is released upon neutrophil activation and is also resistant to proteolysis in the feces for up to 1 week [9].

Both of the fecal biomarkers have been previously established as screening tests for use in diagnosing inflammatory bowel disease in children (Crohn's disease, ulcerative colitis, etc.) [7,9,10] and are now taking on an expanding role in disease monitoring and relapse detection. They have also been evaluated as potential diagnostic tools for NEC [11–18]. This research has concluded that the utility of these biomarkers for early diagnosis and assessment of resolution of NEC needs to be studied in wider NEC series.

In this study, we were able to investigate small intestinal LF and CAL levels in different stages of NEC as predictors and indicators of disease severity in an experimental newborn rat model and their correlation with intestinal injury score.

2. Materials and methods

2.1. Animals and experimental design

The study protocol was approved by the Animal Care and Use Committee of Gulhane Military Medical School (Ankara, Turkey). Forty-six Sprague-Dawley neonatal rats, originating from five different litters, were used in four separate groups in the experiment. Newborn pups were randomly divided into one of the following four groups: NEC (assigned to three groups; G1, G2, G3) and control (group C). Group C rats (controls; $n = 7$) were left with their mothers, breast fed ad libitum, and not submitted to stress. In the NEC groups, newborn rats were collected from their mothers immediately after birth to prevent suckling of maternal milk and kept at 37 °C in a humidified incubator.

2.2. Necrotizing enterocolitis procedure

All the pups in NEC groups were gavage fed 0.2 mL of special rodent formula using a 24 gauge thickness silicon catheter 3 times a day (Fig. 1A). A hyperosmolar rat milk substitute was prepared using 15 g Similac 60/40 (Abbott-Turkey) in 75-ml Puppy-milk canine milk replacement (Beaphar-bogena, B.V. Sedel, Netherland). To induce clinical and pathological signs of NEC, rat pups from all three NEC groups (G1, for 48 h; G2, for 72 h; G3, for 96 h) were stressed twice daily with asphyxia by breathing 100% CO_2 gas for 5 min followed by cold stress at 4 °C for 5 min, and 100% O_2 inhalation for 5 min in a sealed airtight plastic container.

2.3. Sample collection

All rats were killed on the day as previously described for each group (for G1 [$n = 9$], G2 [$n = 7$], G3 [$n = 6$], and C groups, at the end of the 2nd day, 3rd day, and 4th day, respectively) via decapitation. The abdomen was opened and the whole intestine were inspected for evidence of NEC, such as intestinal discoloration, fragility, weakness of tissue integrity, edema, intestinal hemorrhage, ileal distension, pneumatosis intestinalis, perforation, and necrosis, and fixed length of intestinal specimens such as a 2 cm section of the distal ileum and a 3 cm to the ileocaecal valve were harvested for histopathologic and biochemical evaluation, respectively. Tissue specimens were flushed with cold saline solution and fixed in 10% buffered formalin for histopathologic evaluation. Materials from all of the newborn rats included in the study were collected from the resected intestinal segments. Tissue specimens were taken away from 3 cm-fixed length to the ileocaecal valve placed in 0.5 mL of saline for providing standardization procedure in the measurements of the biomarkers. Then all materials was frozen in liquid nitrogen and stored at −80 °C for biochemical examination of small intestinal lactoferrin and calprotectin.

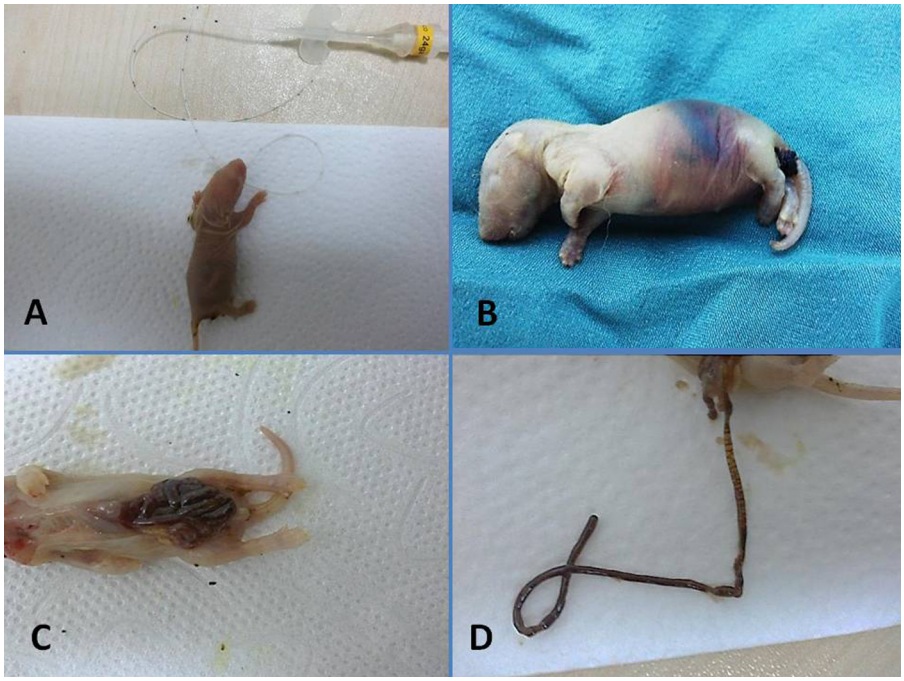

Fig. 1. (A) A 24 gauge-thickness silicon catheter was used to feed the pups, (B) Blackly abdomen with hyperemia, and distension, (C) Dark purple/black discolorated intestines, (D) Dilated intestines as a result of extensive gas.