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Mucosal loss with increased expression of IL-6, IL-8, and COX-2 in a formula-feeding only neonatal rat model of necrotizing enterocolitis

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Abstract

Introduction: The aim of our study is to establish a reliable neonatal rat model by formula feeding only for evaluation of early surgical intervention on the course of experimental necrotizing enterocolitis (NEC).

Material and methods: Newborn Sprague–Dawley rats were divided into 50 breast-fed (group 1) and 38 formula fed (Similac/Esbilac, group 2) animals. The pups were sacrificed on the 4th, 5th, and 6th day of life and the terminal intestine examined for macroscopic and histologic changes as well as cytokine expression.

Results: The histological mucosal damage was significantly higher of group 2 compared to group 1. The area of the vital mucosa of group 2 was significantly (58.57%, $p < 0.001$) lower compared to group 1 (75.12%). The mRNA expression of the inflammatory cytokines IL-6, IL-8 and COX-2 was significantly 2-, 5- and 10-fold increased in group 2 compared to group 1.

Discussion: Formula fed newborn rats displayed an inflammatory enterocolitis similar to human NEC. Our study demonstrates a significant loss of mucosa in animals with NEC having increased expression levels of IL-6, IL-8 and COX-2. Mucosal loss appears to be a distinct feature of experimental NEC and has to be correlated with the human disease.

Necrotizing enterocolitis (NEC) is an acute inflammatory intestinal disease of infants. Its course varies from mild symptoms to life threatening necrosis of the middle to distal ileum leading to bacteremia, peritonitis, septic shock and demise [1]. NEC remains a leading cause of morbidity in neonatal intensive care units, with a reported incidence of approximately 10% among very low birth weight infants and a mortality of 26% [2]. Although several predisposing factors of NEC have been identified - such as prematurity, enteral feeding and infection – its pathogenesis still remains elusive [3].

The symptoms of NEC have been staged according to widely used criteria by Bell [4]. The mainstay treatment of

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NEC is its prevention [5]. Mild forms (Bell I) are treated with antibiotics and food restriction. The fulminant course of NEC (Bell III), which often follows the less severe forms, requires urgent surgical intervention for abdominal decompression and intestinal salvage. Unfortunately, no adequate data exists whether early surgical intervention (in Bell stage II or even I) is able to ameliorate the often disastrous progressive course of NEC [6,7].

The overall aim of our project is to assess the effect of the timing of surgical treatment to the course of NEC. Therefore, a reliable animal model of NEC needs to be established. The successful creation of progressive NEC was reported in newborn rats by the use of hyperosmolar formula-feeding combined with hypoxia, hypothermia and bacterial lipopolysaccharides by various authors [8–11]. The primary goal of this study is to create an animal model of NEC with as less stress factors (hypoxia, hypothermia, LPS, formula-feeding) as possible which will ultimately be used to evaluate the impact of early versus late surgery on the course of NEC. As we hypothesize unphysiological enteral formula feeding to be the main contributor for the development of NEC, the aim of this study was to establish an animal model of experimental NEC by formula feeding alone and assess the clinical and intestinal damage between animals with experimental NEC and controls for further surgical trials [8–11].

1. Material and methods

1.1. Neonatal rat model of necrotizing enterocolitis

All experiments were approved by the Hamburg State Administration for animal research (57/08).

In timed pregnant Sprague Dawley rats, birth was induced by intraperitoneal injection of 1 unit oxytocin (Pitocin) on day 21 of gestation [10,12,13]. Directly after birth, the litter was divided into two groups.

Group 1 consisted of 50 newborn rats left with their mother as breast-fed controls.

Animals of group 2 (n = 38) were separated from their mother and housed in a heated incubator at a temperature of 37 °C and 50–60% air humidity. They were gavage fed four times per 24 hours with 0.2 ml of a hyperosmolar formula (15 g of Similac SMA Gold (SMA Nutrition, Berkshire, UK) in 75-mL Esbilac canine supplement (Pet-Ag Inc., Hampshire, USA)) as originally described by Barlow [8].

The animals in both groups were weighed and examined daily. A clinical sickness score was applied as described by Zani [11]. The pups were euthanized by intraperitoneal injection of ketamine and xylazine followed by decapitation on the 4th, 5th and 6th day of life.

1.2. Evaluation of animals and intestine

The terminal ileum was harvested and evaluated. Specimens were formalin-fixed, embedded in paraffin, routinely processed, and stained with hematoxylin–eosin for blinded evaluation.

A macroscopic score for grading of the intestine on gross observation was adapted from Zani (Color: rosy, patchy discoloration or extensive discoloration; consistency: elastic/normal, thinned/moderately friable, extremely friable; dilatation: not dilated, patchy dilatation/mildly dilated/peristalsis seen, extensive dilatation, no peristalsis seen) [11]. The histological grading of the intestine was adapted from Meyer and Chiu (0 = Mucosa with no alterations, 1 = Well-formed villi with no cell lyses or inflammatory process, formation of Gruenhagen's subepithelial spaces, 2 = Presence of cell lysis, increased formation of Gruenhagen's subepithelial spaces, increased spacing between villi, 3 = Destruction of the villi free portion, presence of dilated capillaries, presence of inflammatory cells, 4 = Structural destruction of villi (which in some cases appear only roughly and are formed by inflammatory cells and necrotic material, bleeding, basal glandular ulceration, 5 = Destruction of the entire mucosal tunic, with no glandular structure found, only amorphous material deposited on the submucosal layer, perforation of the intestine) [14,15].

The percentage of mucosal area of a representative transverse section of the terminal ileum was compared to the whole area of the intestine under microscopic magnification (×100) using the analySIS Digital Image Evaluation Program (Soft Imaging System GmbH, Muenster, Germany, Version 3.2).

1.3. RNA Extraction, cDNA Synthesis and quantitative real-time PCR

Total RNA was isolated from the terminal ileum of pups of both groups using the NucleoSpin® RNA II Kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's protocol. Equal amounts of total RNA was reverse transcribed using Oligo p(dT)15 primers (Roche, Mannheim, Germany) and SuperScript III Reverse Transcriptase (Life Technologies, Darmstadt, Germany) as per the manufacturer's recommendations. Quantitative real-time PCR (qRT-PCR) was performed using LightCycler® 480 SYBR Green Master and a LightCycler® 480 instrument (Roche, Mannheim, Germany). Specific amplification of cDNA was detected with SYBR® Green and melt curve analysis. Relative expression of target genes was calculated as a ratio of the expression level of the target gene to that of beta-2 microglobulin determined in the same sample using the comparative threshold ($\Delta\Delta$Ct) method.

1.4. Statistical tests

Statistical tests were performed using SPSS version 12.0. Skewed numerical and ordinal data is given as median and interquartile range (IQR) and the Wilcoxon–Mann-Whitney test was used to test for difference of medians. In normally