Journal of Pediatric Surgery 53 (2018) 909–913

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg

# Liver damage, proliferation, and progenitor cell markers in experimental necrotizing enterocolitis

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ARTICLE INFO

*Article history:*
Received 11 January 2018
Accepted 1 February 2018

*Key words:*
Necrotizing enterocolitis
Liver injury
Hepatic progenitor cell
LGR5

ABSTRACT

*Background:* Necrotizing enterocolitis (NEC) is a disease known to cause injury to multiple organs including the liver. Liver regeneration is essential for the recovery after NEC-induced liver injury. Our aim was to investigate hepatic proliferation and progenitor cell marker expression in experimental NEC.
*Methods:* Following ethical approval (#32238), NEC was induced in mice by hypoxia, gavage feeding of hyperosmolar formula, and lipopolysaccharide. Breastfed pups were used as control. We analyzed serum ALT level, liver inflammatory cytokines, liver proliferation markers, and progenitor cell marker expression. Comparison was made between NEC and controls.
*Results:* Serum ALT level was higher in NEC ($p < 0.05$). The mRNA expression of inflammatory cytokines in the liver was also higher in NEC (IL6: $p < 0.05$, TNF-α: $p < 0.01$). Conversely, mRNA expression of proliferation markers in the liver was lower in NEC (Ki67; $p < 0.01$, PCNA: $p < 0.01$). LGR5 expression was also significantly decreased in NEC as demonstrated by mRNA ($p < 0.05$) and protein ($p < 0.01$) levels.
*Conclusions:* Inflammatory injury was present in the liver during experimental NEC. Proliferation and LGR5 expression were impaired in the NEC liver. Modulation of progenitor cell expressing LGR5 may result in stimulation of liver regeneration in NEC-induced liver injury and improved clinical outcome.
*Level of evidence:* Level IV.



Necrotizing enterocolitis (NEC) is one of the most severe gastrointestinal diseases in infancy. The incidence of NEC in very low birth weight (VLBW) infants remains high and the mortality and morbidity of this disease are among the highest in neonates [1–3]. Severe NEC can be associated with multi organ failures, resulting in worsened clinical outcome [4,5]. Therefore, to improve the outcomes of patients with NEC, investigation of the pathogenesis of organ injury is important to devise novel therapeutic strategies.

The liver is known to have a good regenerative capacity without any direct involvement of stem/progenitor cell populations [6–8]. However, when this regenerative capacity is overwhelmed by acute or chronic injury, hepatic progenitor cells (HPCs) are recruited to repair the damage. The leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) is the stem cell marker in the Wnt-driven self-renewing tissues. LGR5 has been shown to be present in the damaged liver and to generate functional hepatocytes and cholangiocytes in vivo [9]. The aim of this study is to verify whether liver injury is present in experimental NEC and to characterize the expression of both the proliferation markers and the HPC marker LGR5.

## 1. Methods

### 1.1. Experimental model

Following ethical approval (#32238), we studied experimental NEC in C57BL/6 mice pups. On postnatal day 5, pups were randomly assigned to either of control or NEC. Controls ($n = 10$) were kept with their mother to be breastfed without any stress. NEC ($n = 10$) was induced from postnatal day 5 to 9 using an established model [10,11]. This was done by giving hypoxia (10 min, three times a day), gavage administration of lipopolysaccharide (4 mg/kg/day) and gavage hyperosmolar formula feeding (15 g Similac Lower Iron (Abbott Laboratories, Ltd., Saint-Laurent, QC, Canada) +75 ml Esbilac Puppy Milk Replacer (PetAg, Inc., Hampshire, IL, USA), 152 kcal/100 ml: 50ul/g of body weight three times a day). The pups were sacrificed on postnatal day 9, and distal ileum and liver were harvested. A blood sample was also taken for analysis.

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https://doi.org/10.1016/j.jpedsurg.2018.02.006
0022-3468/

**Table 1**
List of primers.

| Gene | Forward sequence (5–3) | Reverse sequence (5–3) |
|---|---|---|
| IL6 | CCAATTTCCATTGCTCTCCT | ACCACAGTGAGGAATGTCCA |
| TNFα | TTCCGAATTCACTGGAGCCTCGAA | TGCACCTCAGGGAAGAATCTGGAA |
| Ki67 | CAACTTTGGTGATTCCATTA | TTAGGAGGCAAGTTTTCATC |
| PCNA | GCCGAGATCTCAGCCATATT | ATGTACTTAGAGGTACAAAT |
| LGR5 | CGAGCCTTACAGAGCCTGATACC | TTGCCGTCGTCTTTATTCCATTGG |
| GAPDH | TGAAGCAGGCATCTGAGGG | CGAAGGTGGAAGAGTGGGAG |
| RPLO | GGCGACCTGGAAGTCCAACT | CCATCAGCACCACAGCCTTC |

### 1.2. Intestinal morphology

Distal ileum samples were fixed in 4% formalin and embedded in paraffin, then stained with hematoxylin and eosin. The histology slides were blindly evaluated by three independent investigators (BL, CL and SS) using a published scoring system [11]. NEC was considered present when the intestinal injury severity score was ≧2.

### 1.3. Immunofluorescence staining for liver sample

Liver sections were fixed in 4% formalin and embedded in paraffin and sectioned. Immunofluorescence staining for myeloperoxidase (MPO) was performed. Primary antibody for MPO (RD systems, Inc., MN, USA) was diluted to 1:125. The second antibody (Goat anti-Mouse IgG, Alexa Flour 488) (Thermo Fisher Scientific, Inc., IL, USA) was diluted to 1:1000. Images were acquired using Nikon TE-2000 digital microscope (Nikon Instruments Inc., NY, USA) with Hamamatsu C4742–80-12AG camera (Hamamatsu Photonics K.K., Hamamatsu, Japan).

### 1.4. RT-qPCR

The expression of messenger RNA (mRNA) in the distal ileum and liver was examined by two-step RT-qPCR. The mRNA was extracted from the distal ileum and liver samples by TRIzol Reagent (Thermo Fisher Scientific, Inc., IL, USA). Complimentary DNA (cDNA) was made with qScript cDNA SuperMix (Quantabio, Beverly, MA, USA) and S1000 Thermal Cycler (Bio-Rad Laboratories, Inc). Real-time PCR was performed with Advanced qPCR Master Mix and CFX384 Real-Time System (Bio-Rad Laboratories, Inc). Interleukin 6 (IL6) and tumor necrosis factor α (TNF-α) gene expression were analyzed as inflammatory cytokine for both intestine and liver. Proliferating cell nuclear antigen (PCNA) and Ki67 gene expression were analyzed for cell proliferation in the liver and LGR5 was analyzed as a progenitor cell marker. The expression of each gene was normalized to the expression of the housekeeping gene GAPDH and RPLO. Sequence of primer in each gene is shown in Table 1.

### 1.5. Western blotting

Protein from liver samples was isolated by sonication in tissue extraction buffer (Invitrogen, CA, USA) containing protease inhibitor single-use cocktail (Sigma, MO, USA). Protein concentration was measured by Bicinchoninic Acid (BCA) protein assay (Thermo Fisher Scientific, Inc., IL, USA). Protein samples were separated by NuPAGE 4–12% Bis Tris gel and transferred to membrane using iBlot Gel Transfer Device (Thermo Fisher Scientific, Inc., IL, USA). The membrane was probed with primary antibodies, 1:400 diluted LGR5 (OriGene Technologies, Inc., MO, USA) and 1:2000 diluted β-actin (Thermo Fisher Scientific, Inc., IL, USA). Protein expression was captured using an Odyssey scanner (LI-COR Biosciences, Inc., NE, USA) and expression was quantified using Image Studio 5.0 (LI-COR Biosciences, Inc., NE, USA). The expression of LGR5 was normalized to the expression of β-actin.

### 1.6. Serum ALT

Blood samples were left at room temperature for 1 h, and then centrifuged (5000 rpm, 4 °C, 10 min) to isolate serum. ALT activity in isolated serum was analyzed using Alanine transaminase colorimetric activity assay kit (Cayman chemical, MI, USA), as instruction manual indicated.

### 1.7. Statistics

GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analyses. Categorical data were analyzed using the Chi-squared and Fisher's exact test. Continuous data were analyzed

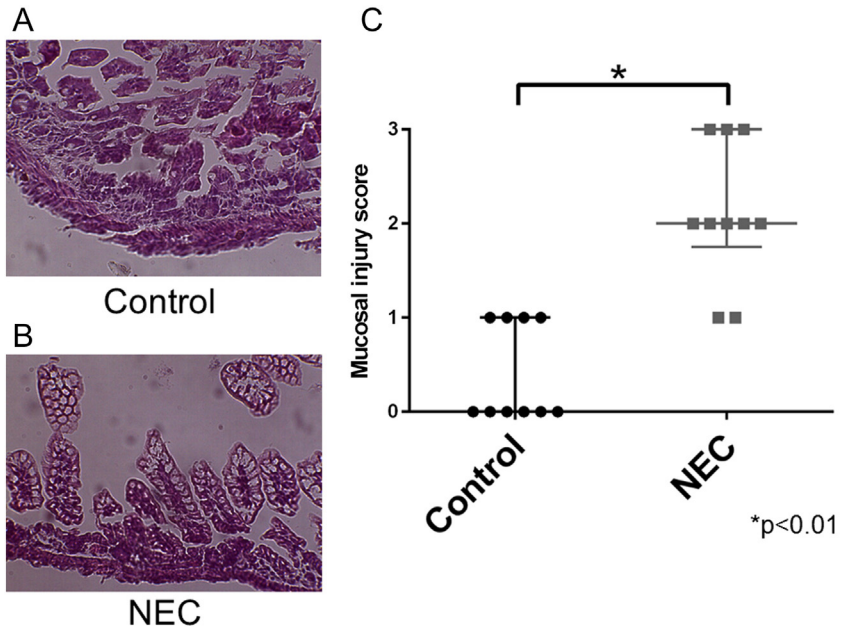


**Fig. 1.** Intestinal mucosal injury. A: HE staining of control ileum. B: HE staining of NEC ileum. C: Injury score. Bar indicate median and interquartile range.