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Hyperoxia disrupts the intestinal barrier in newborn rats

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ABSTRACT

Animal studies have demonstrated that neonatal hyperoxia injures the distal small intestine. This study aimed to determine the effects of neonatal hyperoxia exposure on the intestinal morphology and intestinal barrier integrity in newborn rats. Sprague–Dawley rat pups were exposed to either ambient air or hyperoxia. The ambient air and normobaric hyperoxia groups were maintained in room air and 85% O_2 for 2 weeks, respectively. The rats were euthanized on Postnatal Day 14, and the terminal ileum was collected for histological analyses and oxidative stress measurements. The generation of reactive oxygen species was evaluated by measuring the production of 8-hydroxy-2′-deoxyguanosine (8-OHdG). The expression and localization of epithelial injury markers [intestinal fatty acid binding protein (I-FABP)] and intestinal barrier proteins [occludin and zonula occludens (ZO)-1] were analyzed through immunofluorescence staining and western blotting. The body weight at birth was comparable between the two groups. On Postnatal Day 14, the rats in the hyperoxia group exhibited significantly lower body weight, a higher serum interleukin-6 level, a higher intestinal injury score, higher 8-OHdG and I-FABP expression, and lower occludin and ZO-1 protein expression than did those in the ambient air group. Hyperoxia exposure injured the distal small intestine and disrupted the intestinal barrier in newborn rats. This may be attributable to oxidative stress during the postnatal period.
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1. Introduction

Supplemental oxygen is often used to treat newborns with respiratory disorders. However, oxygen therapy provided to infants has not only beneficial effects but also adverse effects. Animal studies have demonstrated that neonatal hyperoxia injures the distal small intestine (Giannone et al., 2007; Li et al., 2012; Liu et al., 2010; Torbati et al., 2006). These injuries manifest as increased thickness of the ileal mucosa (Giannone et al., 2007), increased expression of the intestinal immunoglobulin (Ig) A secretory component (Liu et al., 2010), increased serosal and submucosal vasodilation and vascularization (Torbati et al., 2006), and distortion of villus structures (Li et al., 2012).

The intestinal epithelium covers the largest surface of the body and constitutes the largest and most crucial barrier against the external environment. The intestinal epithelium forms an intestinal barrier between the contents of the intestinal lumen and the intestinal mucosa (Clayburgh et al., 2004). The intestinal epithelium provides an effective barrier against luminal antigens and toxins in healthy people. Epithelial tight junctions are the most essential structures of the intestinal barrier. They connect adjacent enterocytes and thus determine paracellular permeability through the lateral intercellular space (Suzuki, 2013). Epithelial tight junctions are formed by transmembrane proteins, claudin and occludin, which are connected to the actin cytoskeleton through high-molecular-weight proteins called zonula occludens (ZOs) (Suzuki, 2013). Dysfunction of the intestinal barrier is associated with the pathogenesis of several diseases including necrotizing enterocolitis and hemorrhagic shock-, radiation-, and burn-induced intestinal injury in animals (Al-Ghoul et al., 2004; Anand et al., 2007; Bergmann et al., 2013; Lu et al., 2015; Shim et al., 2015; Yadav et al., 2014).

Preclinical studies have reported that neonatal hyperoxia exposure injures the small intestine. However, the effect of hyperoxia exposure on the intestinal barrier remains unknown. We hypothesized that the hyperoxia-induced intestinal injury is accompanied by the alteration of intestinal barrier components in newborn rats. This study aimed to determine the effects of neonatal hyperoxia exposure on oxidative stress and tight junction protein expression in the neonatal rat intestine.

2. Materials and methods

2.1. Animals and hyperoxia exposure

This study was performed in accordance with guidelines provided by and was approved by the Animal Care Use Committee of Taipei Medical University. Time-dated pregnant Sprague–Dawley rats were housed

Abbreviations: IL, interleukin; I-FABP, intestinal fatty acid binding protein; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; PBS, phosphate-buffered saline; RA, room air; ZO, zonula occludens.

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in individual cages with free access to laboratory food and water, maintained on a 12:12-h light–dark cycle, and allowed to deliver vaginally at term. Within 12 h of birth, litters were pooled and randomly redistributed to the newly delivered mothers, and the pups were then randomly assigned to receive room air (RA) or oxygen-enriched atmosphere (O_2) treatment. The pups in the O_2 treatment (normobaric hyperoxia) group were reared in an atmosphere containing 85% O_2 from Postnatal Days 1 to 14. The pups in the RA control (ambient air) group were reared in normal RA from Postnatal Days 1 to 14. To avoid oxygen toxicity in the nursing mothers, they were rotated between the O_2 treatment and RA control litters every 24 h. An oxygen-rich atmosphere was maintained in a transparent 40 × 50 × 60-cm plexiglass chamber receiving O_2 continually at 4 L/min. Oxygen levels were monitored using the ProOx P110 monitor (NexBiOxy, Hsinchu, Taiwan). Body weights were recorded at the time of euthanasia on Postnatal Day 14.

2.2. Tissue harvest

The pups from each group were deeply anesthetized with an overdose of isoflurane on Postnatal Day 14. The abdomen was opened through a midline incision, and the gastrointestinal tract was carefully removed. The last 2 cm of the terminal ileum was excised and fixed with formalin, embedded in paraffin, sectioned using a microtome at 5 μm, and stained with hematoxylin and eosin for histological evaluation. The part of the ileum of each rat was flushed with saline to remove residual fecal contents and immediately fresh frozen in liquid nitrogen for protein isolation. We chose the terminal ileum as this segment is most vulnerable to injury and intestinal pathologies such as necrotizing enterocolitis in the preterm.

2.3. Cytokine level

The serum interleukin (IL)-6 level was measured using an enzyme-linked immunosorbent assay kit (Cloud-Clone Corp., Houston, TX, USA).

2.4. Histological examination

The ileum was fixed in 4% paraformaldehyde overnight, washed in phosphate-buffered saline (PBS), and serially dehydrated in increasing ethanol concentrations before being embedded in paraffin. After sectioning, 5-μm tissue sections were rehydrated in decreasing ethanol concentrations, stained with hematoxylin and eosin, and examined using light microscopy for evaluating the intestinal morphology. The intestinal mucosal injury was scored using a scale of 0–5 with 0 = normal mucosal villi, 1 = subepithelial space at the villus tip and often with capillary congestion, 2 = extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria, 3 = massive epithelial lifting down the sides of villi with occasional denuded villi tips, 4 = denuded villi with the lamina propria and capillary dilation, and 5 = disintegration of the lamina propria, hemorrhage, and ulceration (Chiu et al., 1970). The villus height and crypt depth were measured from the baseline to the villus tip and from the baseline to the submucosa, respectively. The villus height and crypt depth were measured in 10 randomly selected villi and crypts, of which images were processed using Image Pro Plus 6.0 (Media Cybernetics, Silver Spring, USA).

2.5. Immunohistochemistry for 8-hydroxy-2′-deoxyguanosine and intestinal fatty acid binding protein

Immunohistochemical staining was performed on 5-μm paraffin sections by using immunoperoxidase visualization. After routine deparaffinization, heat-induced epitope retrieval was conducted by immersing the slides in 0.01 M sodium citrate buffer (pH 6.0). To block the endogenous peroxidase activity and nonspecific antibody binding, the sections were preincubated for 1 h at room temperature in 0.1 M PBS containing 10% normal goat serum and 0.3% H_2O_2. The sections were incubated for 20 h at 4 °C with the mouse monoclonal 8-hydroxy-2′-deoxyguanosine (8-OHdG) antibody (1:200 dilution; Abcam Inc., Cambridge, MA, USA) or goat polycolonal intestinal fatty acid binding protein (I-FABP) (1:50 dilutions; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) as primary antibodies. These sections were then treated for 1 h at room temperature with biotinylated rabbit antimouse IgG (1:200 dilution; Sigma-Aldrich Co., St. Louis, MO, USA) or biotinylated rabbit antigoat IgG (1:200 dilution; Jackson ImmunoResearch Lab., Inc., West Grove, PA, USA), respectively. After a reaction was produced using reagents from an avidin–biotin complex kit (Vector Laboratories, Inc., CA, USA), reaction products were visualized using a diaminobenzidine substrate kit (Vector laboratories) according to the manufacturer's recommendations. The sections were dehydrated in increasing ethanol concentrations, cleared using xylene, and coverslipped using Permount (Fisher, USA). All immunostained sections were viewed and analyzed using Nikon Eclipse E600. Positive 8-OHdG cell nuclei were scored in 10 randomly selected villi (length ranged from 260 to 360 μm) in each section at a 200× magnification. Semiquantitative analysis of the optical density values of the I-FABP-positive staining in the villi of each section at 400× magnification was performed using the Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, USA).

2.6. Immunofluorescence staining for occludin and ZO-1

Immunostaining was performed on 5-μm-thick ileum paraffin sections. After routine deparaffinization, heat-induced epitope retrieval was performed by immersing slides in 0.01 M sodium citrate buffer (pH 6.0). To block the endogenous peroxidase activity and nonspecific antibody binding, the sections were preincubated for 1 h at room temperature in 0.1 M PBS containing 10% normal goat serum and 0.3% H_2O_2. The sections were incubated for 20 h at 4 °C with rabbit polyclonal anti-ZO-1 tight junction protein antibody (1:100 dilutions; Abcam Inc.) or rabbit polyclonal antioccludin antibody (1:100 dilutions; Thermo Scientific, Rackford, IL, USA). The sections were then treated for 1 h at room temperature with fluorochrome-conjugated secondary antibodies or fluorescein AffiniPure goat antirabbit IgG (1:200 dilutions; Gene Tex Inc. Irvine, CA, USA). Nuclei were detected using 4′, 6-diaminidino-2-phenylindole (1:1000; Sigma-Aldrich Co.). The sections were then washed with PBS mounted, and examined under a fluorescence microscope.

2.7. Western blotting of tissue occludin and ZO-1

The ileum was homogenized, sonicated, and centrifuged at 12 000 ×g for 20 min at 4 °C to remove cellular debris. Proteins (30 μg) were resolved through 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis in reduced conditions and electroblotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). After blocking with 5% nonfat dry milk, the membranes were incubated with antioccludin (1:1000; Thermo Fisher Scientific, Waltham, MA, USA) and anti-ZO-1 antibodies (1:250; Abcam) or anti-β-actin (1:1000; Santa Cruz Biotechnology), and then incubated with horseradish peroxidase-conjugated goat antirabbit or antimouse IgG antibodies (GeneTex, San Antonio, TX, USA). Protein bands were detected using the SuperSignal Substrate from Pierce Biotechnology (Rockford, IL, USA). Densitometric analysis was performed to measure the intensity of occludin and ZO-1 expression and β-actin bands. Data were normalized to β-actin for each rat.

2.8. Statistical analysis

The data are presented as the mean ± SD. Statistically significant differences were analyzed by Kruskal-Wallis tests. Between-group comparisons were made by Mann-Whitney U tests. Spearman's correlation

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