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BASIC SCIENCE ARTICLE

Neonatal intermittent hypoxia, fish oil, and/or antioxidant supplementation on gut microbiota in neonatal rats

Darren Bodkin[1], Charles L. Cai[1], Alex Manlapaz-Mann[1], Ghassan Mustafa[1], Jacob V. Aranda[1,2,3] and Kay D. Beharry[1,2,3]✉

BACKGROUND: Preterm infants frequently experience intermittent hypoxia (IH) episodes, rendering them susceptible to oxidative stress and gut dysbiosis. We tested the hypothesis that early supplementation with antioxidants and/or fish oil promotes gut biodiversity and mitigates IH-induced gut injury.
METHODS: Newborn rats were exposed to neonatal IH from birth (P0) to P14 during which they received daily oral supplementation with: (1) coenzyme Q10 (CoQ10) in olive oil, (2) fish oil, (3) glutathione nanoparticles (nGSH), (4) CoQ10 + fish oil, or (5) olive oil (placebo control). Pups were placed in room air (RA) from P14 to P21 with no further treatment. RA controls were similarly treated. Stool samples were assessed for microbiota and terminal ileum for histopathology and morphometry, total antioxidant capacity, lipid peroxidation, and biomarkers of gut injury.
RESULTS: Neonatal IH induced histopathologic changes consistent with necrotizing enterocolitis, which were associated with increased lipid peroxidation, toll-like receptor, transforming growth factor, and nuclear factor kappa B. Combination of CoQ10 + fish oil and nGSH were most effective for preserving gut integrity, reducing biomarkers of gut injury, and increasing commensal organisms.
CONCLUSIONS: Combination of antioxidants and fish oil may confer synergistic benefits to mitigate IH-induced injury in the terminal ileum.

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IMPACT:

- Antioxidant and fish oil (PUFA) co-treatment was most beneficial for reducing neonatal IH-induced gut injury.
- The synergistic effects of antioxidant and fish oil is likely due to prevention of IH-induced ROS attack on lipids, thus preserving and augmenting its therapeutic benefits.
- Combination treatment was also effective for increasing the abundance of the non-pathogenic Firmicutes phylum, which is associated with a healthy gastrointestinal system of the newborn.
- Extremely low gestational age neonates who are at high risk for frequent, repetitive neonatal IH and oxidative stress-induced diseases may benefit from this combination therapy.

INTRODUCTION

Extremely low gestational age neonates (ELGANs) experience frequent, repetitive episodes of intermittent hypoxia (IH) resulting in oxygen radical diseases of the newborn including necrotizing enterocolitis (NEC).[1–3] Neonatal IH consists of brief cycles of arterial oxygen desaturations followed by re-oxygenation either in normoxia or hyperoxia.[4] A neonatal IH event is usually defined as a decline in SaO_2 by 5% lasting <3 min in duration.[5] Prolonged IH lasting >20 s may lead to induction of reactive oxygen species (ROS), inflammation, lipid peroxidation, and organ injury. Studies show that the pattern, duration, and severity of IH is indicative of the severity of oxidative stress.[6,7] Preterm infants are deficient in antioxidants and thus are highly vulnerable to ROS attack and lipid peroxidation, which are associated with NEC.[8–10]

In addition to poor antioxidant defenses, preterm infants are prone to increased non-ferritin-bound free iron,[11] a major catalyst for lipid peroxidation.[12,13] Studies show the importance of the glutathione (GSH) redox system for preventing iron-induced ferroptosis and lipid peroxidation.[14–18] Coenzyme Q10 (CoQ10) is a potent antioxidant that has been shown to suppress lipid peroxidation and ferroptosis by inducing GSH.[19,20] While both CoQ10 and GSH are potent antioxidants, limited intestinal penetration and possible destruction by intestinal microorganisms contribute to their low bioavailability. Nanoparticle technology improves delivery to the tissues, increases intracellular penetration, and protects against premature degradation. In these experiments, we utilized a novel GSH nanoparticle formulation, which was shown to be safe, and improve absorption, delivery, and blood concentrations of GSH.[21]

ELGANs are often supplemented with fish oil lipid emulsions to improve growth and neurodevelopmental outcomes.[22] Lipids have been shown to decrease the abundance of pathogenic bacteria

[1]Department of Pediatrics, State University of New York, Downstate Medical Center, Brooklyn, NY, USA. [2]Department of Ophthalmology, State University of New York, Downstate Medical Center, Brooklyn, NY, USA. [3]SUNY Eye Institute, Brooklyn, NY, USA. ✉email: kbeharry@downstate.edu

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in preterm infants[23] and reduce oxidative stress.[24] ELGANs are predisposed to a delay in acquiring non-pathogenic commensal bacteria and to a loss of microbiota diversity and richness, which makes them more susceptible to inflammatory diseases and NEC.[25–28] A number of studies have shown that chronic IH alters gut microbiota in adult humans[29,30] and animals.[31–33] Whether neonatal IH contributes to gut dysbiosis and injury in neonatal rats and whether early supplementation with antioxidants and/or lipids during IH is protective remains to be determined. We therefore tested the hypotheses that: (1) neonatal IH negatively impacts the gut microbiota leading to dysbiosis and gut inflammation and (2) supplementation with antioxidants and/or fish oil prevents IH-induced injury and preserves commensal microbiota in the neonatal gut. Our hypothesis was tested with the following objectives: (1) to examine the effects of neonatal IH on the gut microbiota in rats and (2) to determine whether early postnatal supplementation with antioxidants and/or fish oil preserves gut integrity, antioxidant capacity, and commensal microbiota.

MATERIALS AND METHODS

Animals

All experiments were approved by the State University of New York, Downstate Medical Center Institutional Animal Care and Use Committee, Brooklyn, NY. Certified infection-free, timed-pregnant Sprague Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) at 18 days gestation. The animals were housed in an animal facility with a 12-h day/12-h night cycle and provided standard laboratory diet and water ad libitum until delivery of their pups.

Experimental design

Within 2–4 h of birth, newborn rat pups delivering on the same day were pooled and randomly assigned to expanded litters of 18 pups/litter (9 males and 9 females). Sex was identified by the anogenital distance. The expanded litter size was used to simulate poor nutrition and relative postnatal malnutrition of ELGANs who are at increased risk for NEC. Animals were exposed to neonatal IH from birth (P0) to P14 and then allowed to recover from IH in room air (RA) until P21. Neonatal IH was induced with the use of an oxycycler to simulate brief arterial oxygen desaturations experienced by preterm infants who are at a high risk for developing NEC. During neonatal IH (P0–P14), pups were administered daily oral doses of: (1) CoQ10 (0.35 mg in 50 µL extra virgin olive oil) purchased from Sigma Aldrich (St. Louis, MO); (2) 50 µL fish oil containing 22 mg eicosapentaenoic acid and 13 mg docosahexaenoic acid. Fish oil capsules (Nature's Bounty, Bohemia, NY) containing 360 mg of total omega 3 fatty acids were used; (3) 50 µL CoQ10 + fish oil; (4) oral glutathione nanoparticle (nGSH) sublingual drops (Nanoceutical Solutions (San Antonio, TX), 200 mg/mL (optimized for instant absorption), diluted to 24 µg in 50 µL with extra virgin olive oil. nGSH was previously shown to be safe and resulted in improved absorption, delivery, and blood concentrations of GSH[21]; and (5) 50 µL extra virgin olive oil (OO, placebo controls). The choice for extra virgin oil as a control was based on previous reports.[34–38] The dose of nGSH was based on the manufacturer's recommended dose adjusted for body weight. The doses of fish oil and CoQ10 were based on results of our previous findings.[39] Supplementation occurred only from P0 to P14 and not during the reoxygenation/recovery period. RA littermates were raised in atmospheric oxygen from P0 to P21, were similarly supplemented, and served as age-matched controls.

Neonatal IH profiles

Animals randomized to neonatal IH were placed with the dams in specialized oxygen chambers attached to an oxycycler (BioSpherix, NY). The IH profiles consisted of an initial exposure of hyperoxia (50% O_2) for 30 min followed by three brief, 1-min, clustered hypoxic events (12% O_2), with a 10-min re-oxygenation in 50% O_2 between each hypoxic event. Recovery from IH occurred in 50% O_2 following each clustered IH event for 2.5 h for a total of 8 clustering IH episodes per day for 14 days, as previously described.[6,39,40]

Sample collection and processing

At euthanasia (P21), stool samples were collected from the large bowels, placed in specialized barcoded tubes, and sent to Transnetyx Microbiota (Cordova, TN) for blinded microbiota analyses. For enzyme-linked immunosorbent assays (ELISAs), biopsies from the terminal ileum were freshly harvested, rinsed in ice-cold phosphate-buffered saline (PBS, pH 7.4) on ice, and placed in sterile Lysing Matrix D 2.0 mL tubes containing 1.4 mm ceramic spheres (MP Biomedicals, Santa Ana, CA) and 1.0 mL sterile PBS then snap-frozen in liquid nitrogen. Samples were stored at −80 °C until analysis on the same day for lipid peroxidation (malondialdehyde (MDA) assay), total antioxidant capacity, toll-like receptor (TLR)-4, and transforming growth factor β1 (TGFβ1) levels ($n = 8$ samples/group).

Histopathology and morphometry

For histopathology, biopsies of fresh terminal ileum from 4 rats per group were placed in 10% neutral buffered formalin and sent to the Pathology Dept. at SUNY Downstate Medical Center for processing, embedding in paraffin, and standard hematoxylin–eosin (H&E) staining. Images were captured at ×40 magnification (scale bar = 20 µm). Morphometric analyses of the H&E stains were quantitatively determined using the count and measure tool of the CellSens software (Olympus America, Inc., Center Valley, PA).

Lipid peroxidation

Lipids are susceptible to oxidative attack resulting in the production of end products, such as MDA. Lipid peroxidation (MDA assay) was determined using assay kits purchased from Sigma-Aldrich, according to the manufacturer's protocol.

Total antioxidant capacity

Total antioxidant capacity was analyzed using assay kits purchased from Sigma-Aldrich (St. Louis, MO), according to the manufacturer's protocol.

TLR-4 and TGFβ1 assays

TLR-4 and TGFβ1 levels were determined using commercially available ELISA kits purchased from MyBiosource, Inc. (San Diego, CA), according to the manufacturer's recommendations.

Total cellular protein levels

Data from all assays were standardized using total cellular protein levels. On the day of assays, an aliquot (10 µL) of the terminal ileum homogenates was utilized for total cellular protein levels using the Bradford method (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

Immunohistochemistry

Unstained sections were de-paraffinized with xylenes and alcohols prior to unmasking of antigens. Sections were washed in PBS containing triton X-100 and incubated in blocking solution for 1 h, prior to incubation with primary antibodies for TLR-4, TGFβ1, phospho nuclear factor kB (pNFkB), and IkB (Santa Cruz Biotechnology (Dallas, TX) overnight. IHC-Tek antibody diluent (pH 7.4) purchased from IHC World (Woodstock, MD) was used for negative controls. The sections were incubated with Alexa Fluor fluorescent secondary antibodies (ThermoFisher Sci/Life Technologies, Grand Island, NY) and counterstained with 4,6-diamidino-2-phenylindole (DAPI). Images were captured at ×20 magnification (scale bar = 50 µm) using an Olympus BX53 microscope, DP72 digital camera, and CellSens imaging software attached to a Dell Precision T3500 computer (Olympus America, Inc., Center Valley, PA). Quantitative analysis of the stain intensity was conducted using the count and measure on region of interest tool of the CellSens software.

Microbiota analysis

One Codex sample kits containing barcoded sample collection tubes were provided by Transnetyx Microbiota (Cordova, TN). Fecal samples ($n = 2$ samples per group) were placed in individual tubes containing DNA stabilization buffer and shipped for DNA extraction, library preparation, and sequencing by One Codex (San Francisco, CA). Shallow shotgun whole-genome sequencing for microbiota analysis was performed by Transnetyx with classification performed by One Codex. Shotgun metagenomics generates whole-genome sequencing for accurate taxonomic identification.[28]

Statistical analysis

To determine differences among the treatments, a test for normality of variances were conducted using Bartlett's test. Normally distributed data

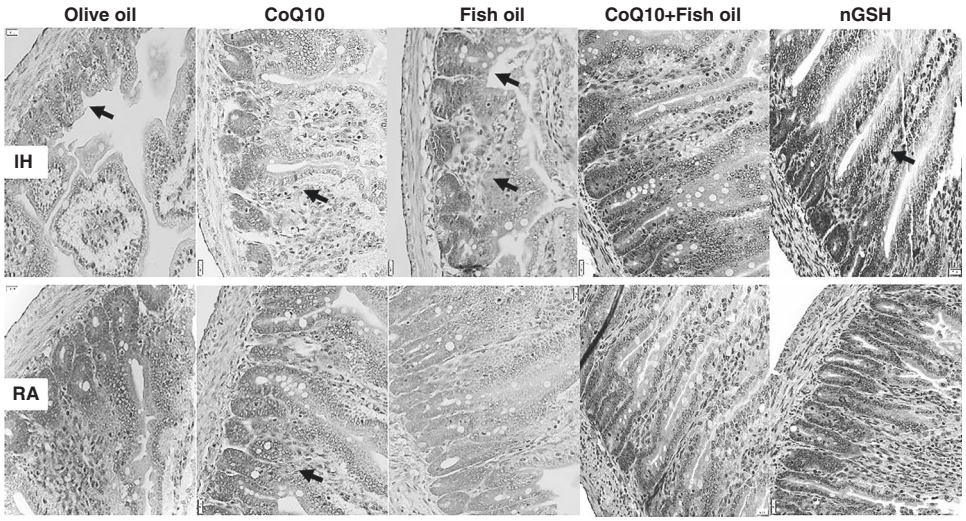

Fig. 1 Representative H&E-stained sections from the terminal ileum of 21-day-old (P21) neonatal rats exposed to neonatal intermittent hypoxia (IH, upper panel) and room air (RA, lower panel). Arrows indicate location of pathology. OO olive oil, CoQ10 coenzyme Q10, fish oil omega 3 polyunsaturated fatty acids, nGSH glutathione nanoparticles. Images are ×40 magnification, scale bar is 20 µm.

were analyzed using two-way analysis of variance with Dunnett's post hoc tests. Non-normally distributed data were analyzed using Kruskal–Wallis test with Dunn's multiple comparison test. Data are presented as mean ± SEM and a p value of <0.05 was considered as statistically significant; data were analyzed using SPSS version 16.0 (SPSS Inc., Chicago, IL). $^{*}p < 0.05$, $^{**}p < 0.01$ vs OO RA; $^{§}p < 0.05$; $^{§§}p < 0.01$ vs OO IH; and $^{†}p < 0.05$, $^{‡}p < 0.01$ vs RA. Graphs were prepared using GraphPad Prism version 7.03 (GraphPad, San Diego, CA).

RESULTS

Histopathology

Figure 1 represents the H&E stains showing histopathology of the terminal ileum in all groups. The upper panels represent the IH groups, and the lower panels represent the RA groups. OO in IH had a thinner outer layer of muscularis externa and submucosa, more distortion of the villi mucosa, and increased space between the base of adjacent villi (arrow). The OO treatment group in RA had a thick layer of muscularis externa, very little submucosa, prominent and packed villi, and intact epithelial mucosa. Many lacteals are seen in the RA and IH groups. Interestingly, CoQ10 in IH also had tightly packed villi, but the appearance of hemorrhage was noted in both RA and IH (arrows), although CoQ10 in RA resulted in prominent, well-formed villi. Fish oil treatment in IH resulted in shorter, denuded, and abnormal villi (arrows), compared to normal appearance in RA. The muscularis externa was also thinner than that in RA. Further images showing more details of damage in the terminal ileum with fish oil treatment in neonatal IH compared to RA are presented in the Supplemental Fig. S1. Combination of CoQ10 + fish oil treatment showed normal muscularis mucosa and similar diameter in IH and RA. The villi appear densely packed with few denuded villi in IH. nGSG treatment in IH and RA also had similar lumen muscularis mucosa thickness but treatment in IH had less densely packed villi and appearance of hemorrhage (arrow). These histopathology findings confirm that lipids are prone to oxidation in the setting of neonatal IH, and thus co-administration with antioxidants may be essential to preserve its integrity.

Morphometry

Figure 2 shows the morphometric analysis of the H&E-stained sections using the count and measure tools of the CellSens software. Overall, IH significantly decreased the wall thickness and villi length, with no effect on the number of villi and number of denuded villi in the OO control group. CoQ10 treatment in RA was associated with a significant reduction in wall thickness, but this effect was not seen with treatment in IH. Lumen area was increased, while villi length was decreased with CoQ10 treatment in both RA and IH when compared to the OO controls. CoQ10 treatment in IH also resulted in a higher number of denuded villi and reduced villi diameter compared to CoQ10 treatment in RA and to the OO IH group. Fish oil treatment in IH resulted in increased villi lumen and number of denuded villi but decreased villi length and overall number of villi compared to the OO and its RA counterpart. Fish oil treatment in RA decreased wall thickness and villi length and increased villi lumen and number of denuded villi compared to OO. Interestingly, combination of CoQ10 + fish oil treatment in IH and RA resulted in markedly reduced wall thickness, villi length, and villi diameter, but increased villi lumen compared to OO. In IH, nGSH increased villi lumen and number of denuded villi, but decreased villi length and number of villi compared to OO treatment in IH. Treatment in RA reduced wall thickness, increased villi lumen, number of denuded villi, and number of villi but decreased villi length and diameter compared to OO treatment in RA. Substantial differences between the nGSH treatment in IH and RA were noted for villi length, number of denuded villi, villi diameter, and number of villi. Together, these findings confirm that combination of CoQ10 + fish oil was the most effective treatment for preserving terminal ileum integrity in neonatal IH.

Lipid peroxidation and antioxidant capacity

Figure 3a shows lipid peroxidation and Fig. 3b total antioxidant capacity in the terminal ileum homogenates. Lipid peroxidation was significantly increased with IH, an effect that was suppressed with all treatments, although both combination of CoQ10 + fish oil and nGSH were most effective in RA and IH (Fig. 3a). Total antioxidant capacity remained unchanged with CoQ10 and fish oil in RA and IH. However, combination of CoQ10 + fish oil in IH increased total antioxidant capacity compared to OO treatment in IH. Conversely, nGSH treatment in RA and IH resulted in reduced total antioxidant capacity compared to OO treatment (Fig. 3b). While all treatments were effective for reducing IH-induced lipid peroxidation, the findings show that CoQ10 + fish oil and nGSH were most effective. However, only CoQ10 + fish oil increased total antioxidant capacity.

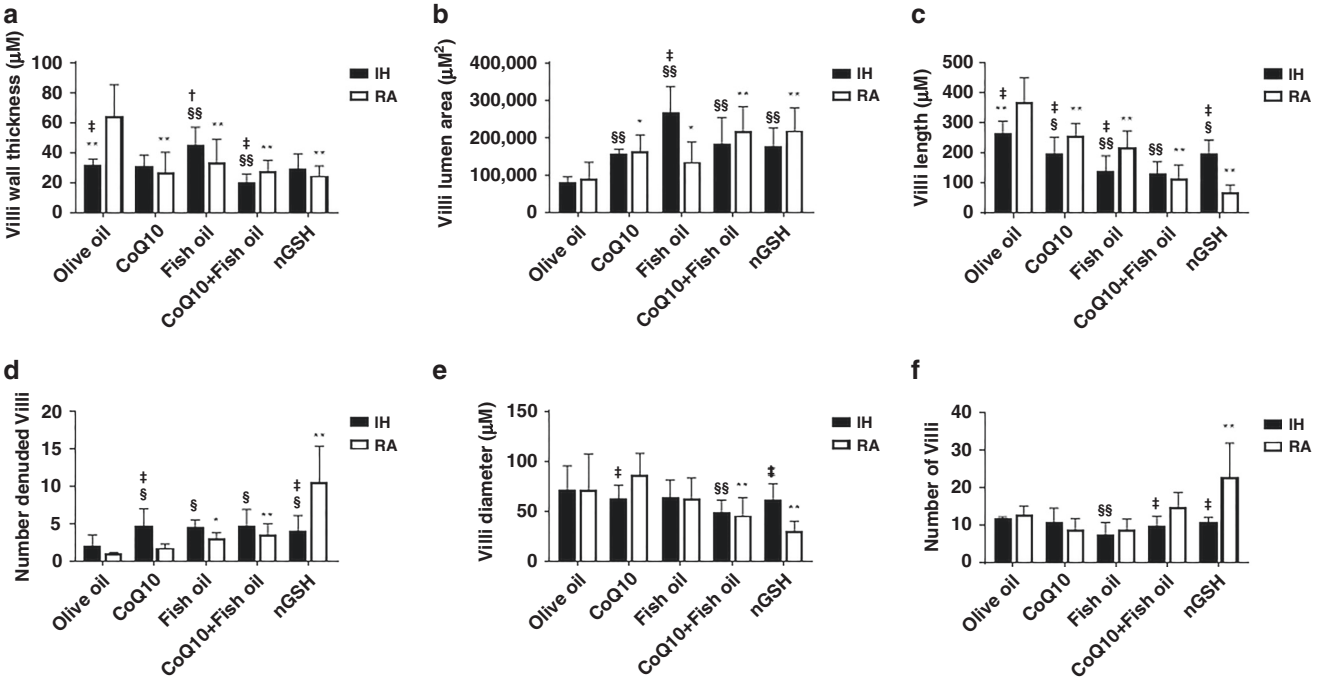

Fig. 2 Morphometric analysis of the terminal ileum H&E stains presented in Fig. 1. a Villi thickness, **b** villi lumen area, **c** villi length, **d** number of denuded villi, **e** villi diameter, and **f** number of villi. Data were analyzed using two-way ANOVA. Data are mean ± SEM. *$p < 0.05$; **$p < 0.01$ vs Olive Oil RA; $^{§}p < 0.05$; $^{§§}p < 0.01$ vs Olive Oil IH; $^{†}p < 0.05$, $^{‡}p < 0.01$ vs RA littermates.

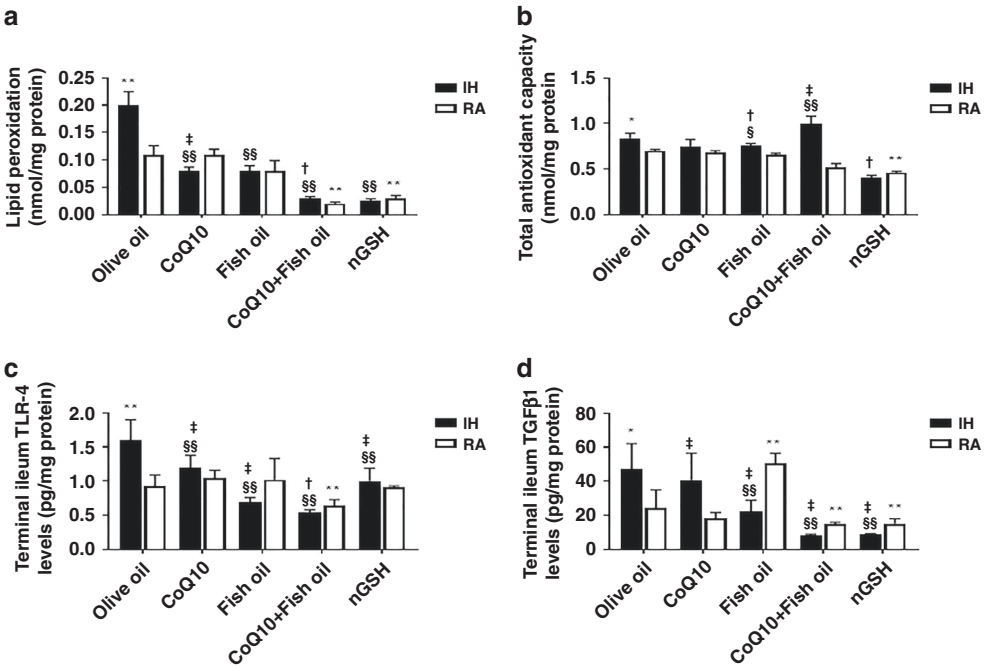

Fig. 3 Effect of antioxidants and/or fish oil on lipid peroxidation, total antioxidant capacity, toll-like receptor (TLR)-4, and transforming growth factor (TGF) β1 in the terminal ileum homogenates. A Lipid peroxidation/malondialdehyde, **B** total antioxidant capacity, **C** TLR-4C, and **D** TGFβ1. Rats were exposed to neonatal IH from P0 to P14 and allowed to recover in room air (RA) from P14 to P21. Groups are as described in Fig. 1. Data was analyzed using two-way ANOVA. Data are mean ± SEM ($n = 8$ samples/group). *$p < 0.05$; **$p < 0.01$ vs Olive Oil RA; $^{§}p < 0.05$; $^{§§}p < 0.01$ vs Olive Oil IH; $^{†}p < 0.05$, $^{‡}p < 0.01$ vs RA.

TLR-4 and TGFβ1 levels

Figure 3 shows the mean levels of TLR-4 (Fig. 3c) and TGFβ1 (Fig. 3d) in the terminal ileum homogenates. TLR-4 was significantly increased in the OO group exposed to IH. All treatment groups decreased IH-induced TLR-4 but the most effective treatments were fish oil and CoQ10 + fish oil. In RA, CoQ10 + fish oil was the only treatment that decreased TLR-4 compared to OO (Fig. 3c). TGFβ1 levels were also elevated in response to neonatal IH and reductions were noted with all treatments. However, combination of CoQ10 + fish oil and nGSH were the most effective. Surprisingly, treatment with fish oil in RA increased TGFβ1 levels compared to OO treatment in RA and to the IH counterparts

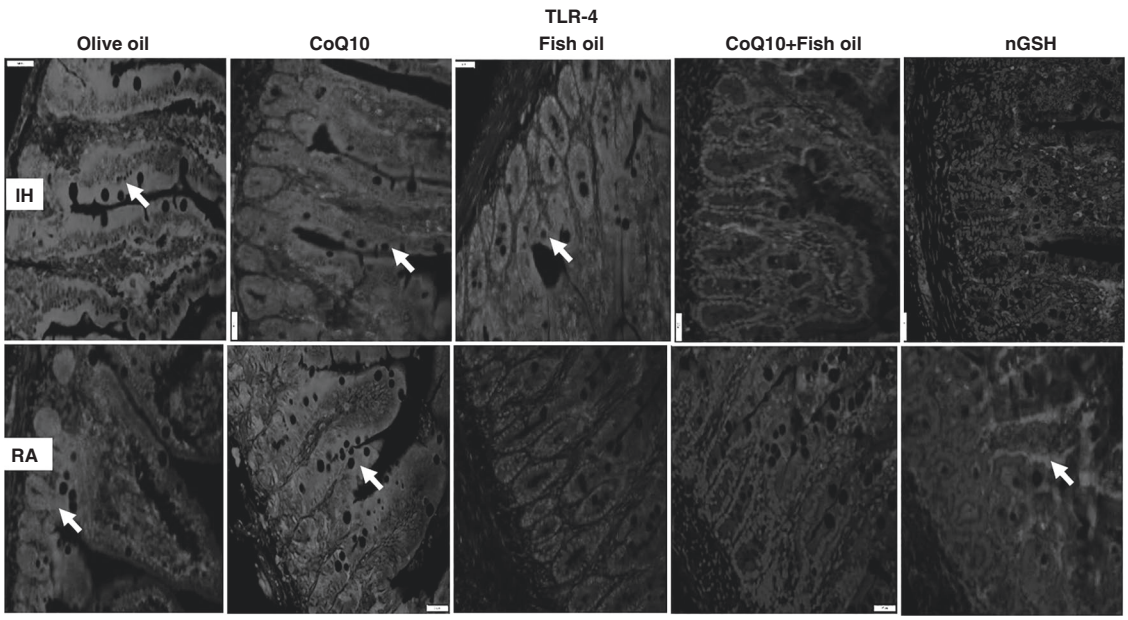

Fig. 4 Representative immunoreactivity of TLR-4 (red), counterstained with DAPI (blue) in the terminal ileum of P21 neonatal rats exposed to RA (upper panel) and neonatal IH (lower panel). Images are ×20 magnification, scale bar is 50 μm.

(Fig. 3d). Overall, CoQ10 + fish oil selectively suppressed both TLR-4 and TGFβ1 suggesting an anti-inflammatory effect.

TLR-4 immunoreactivity

Representative TLR-4 immunostaining (red) counterstained with DAPI (blue) in the terminal ileum is presented in Fig. 4. The upper panel represents samples in IH, and the lower panel represents samples in RA. TLR-4 was predominantly expressed in the villi epithelium, lamina propria, crypts, and mucosa. Similar to the ELISA findings (Fig. 3), TLR-4 was significantly increased with IH, an effect that was decreased with all treatments. The most effective treatments for suppressing TLR-4 were combination CoQ10 + fish oil and nGSH. The nGSH finding differs from the ELISA findings presented in Fig. 3. The nGSH group had the highest number of denuded villi. ELISA measures protein in homogenized samples, including lumen contents, and immunofluorescence examines localization within the tissue, which may account for the differences. TLR-4 was not appreciably expressed with fish oil, particularly in the RA, combination of CoQ10 + fish oil, and nGSH treatment groups in RA and IH. In these groups, staining was observed particularly in the lamina propria and villi crypts. Of all treatments in neonatal IH, the most significant reduction in TLR-4 occurred with combination of CoQ10 + fish oil and nGSH, suggesting antioxidant and anti-inflammatory effects.

Immunostaining intensity quantitation

Quantitative assessments of immunoreactivity of TLR-4, pNFkB, IkB, and TGFβ1 are presented in Fig. 5a–d, respectively. Data correspond to Fig. 4 (TLR-4) and Supplemental Figs. S2–S4 (pNFkB, IkB, and TGFβ1, respectively). All treatments reduced IH-induced TLR-4 and pNFkB, although the most effective treatments for TLR-4 suppression were CoQ10 + fish oil and nGSH. Similar findings were noted in RA (Fig. 5a). All treatment equally suppressed pNFkB expression in RA and IH (Fig. 5b). In contrast, IkB was decreased with CoQ10 in both RA and IH and with fish oil in IH (Fig. 5c). Similar to TLR-4, all treatments suppressed IH-induced TGFβ1 but the most effective treatments were CoQ10 + fish oil and nGSH. Only CoQ10, CoQ10 + fish oil, and nGSH suppressed TGFβ1 in RA (Fig. 5d).

Gut microbiota

Microbiota results are presented in Fig. 6. Unidentified and low abundance (<1%) organisms are not shown. Neonatal IH induced a wide variety of mostly pathogenic organisms with a significant reduction in the abundance of commensal species. In the OO group exposed to RA, there was a high abundance of the Firmicutes phyla at the order level of Lactobacillus, which was decreased from 100% to undetectable levels in IH. The most common organisms in the OO group exposed to IH were Bacteroidetes, Proteobacteria, and Firmicutes phyla and an unspecified 33% in the low abundance group that has not been identified (not shown). CoQ10 RA produced the highest abundance of Lactobacillus species (95.3%), but this declined to 60.4% in IH. Low abundance phyla in CoQ10 IH were Firmicutes, Proteobacteria, Verrucomicrobia, and 2.8% unidentified organisms (not shown). Fish oil treatment in RA produced a higher variety of Lactobacillus, which was altered in IH. Compared to RA, *Lactobacillus* sp. increased from 20 to 69% in IH, *Lactobacillus reuteri* decreased from 40.6 to <1% (not shown) in IH, *Lactobacillus johnsonii* decreased from 31 to 23% in IH, and the overall abundance declined from 100 to 92% in IH. CoQ10 + fish oil treatment in IH produced the highest percentage of *Lactobacillus* sp. (84%) although the total Firmicutes phylum of the Lactobacillus order was 98.5%. In contrast, CoQ10 + fish oil in RA resulted in 92.7% *Lactobacillus* subspecies. Treatment with nGSH in IH produced 24% *Romboutsia ilealis*, compared to 2.2% in RA, 23.8% *Lactobacillus* sp. compared to 81.4% in RA, 28.4% *Lactobacillus murinus* compared to 4% in RA, 5.5% *Lactobacillus johnsonii* compared to 7.5% in RA, and 12% *Escherichia coli* compared to 2% in RA. Overall, the data show that neonatal IH results in a significant decline in commensal organisms and an increase in the variety and abundance of pathogenic organisms.

pNFkB, IkB, and TGFβ immunoreactivity

Immunoreactivities of pNFkB, IkB, and TGFβ (red) counterstained with DAPI (blue) are presented in the Supplemental Figs. S2–S4, respectively. Figure S2 shows that pNFkB was strongly present in the epithelial cells of the villi in the OO group exposed to neonatal IH and minimally present in the other treatment

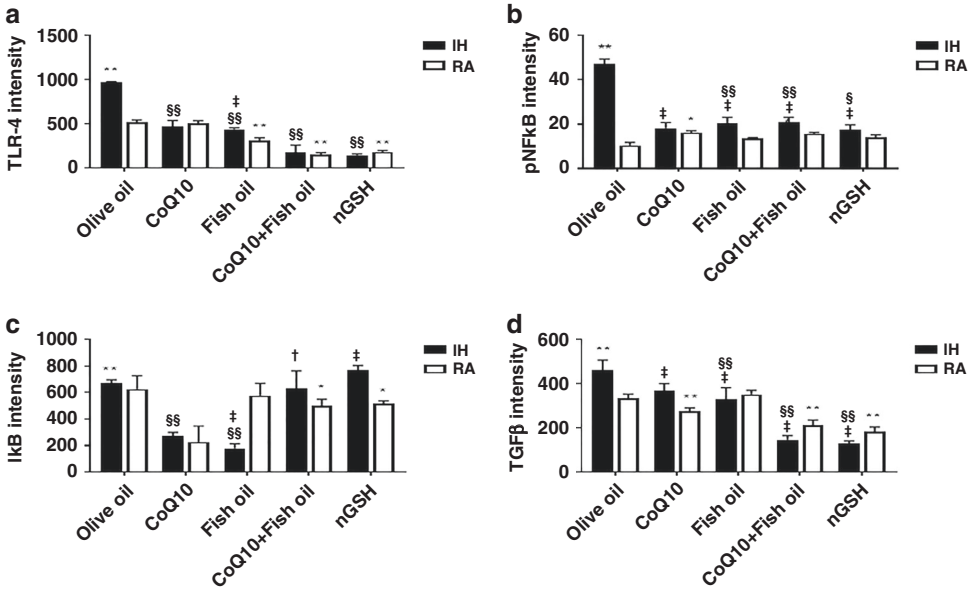

Fig. 5 Quantitative analysis of phosphorylated nuclear factor kappa B (pNFkB), inhibitor of NFkB (IkB), toll-like receptor (TLR)-4, and transforming growth factor (TGF)β1 immunoreactivity in the terminal ileum represented in Fig. 4. A pNFkB, **B** IkB, **C** TLR-4, and **D** TGFβ1. Rats exposed to neonatal IH from P0 to P14 and allowed to recover in (RA) from P14 to P21. Groups are as described in Fig. 1. Data are mean ± SEM ($n = 12$ measurements/group). $^{*}p < 0.05$; $^{**}p < 0.01$ vs Olive Oil RA; $^{§§}p < 0.01$ vs Olive Oil IH; $^{†}p < 0.05$, $^{‡}p < 0.01$ vs RA.

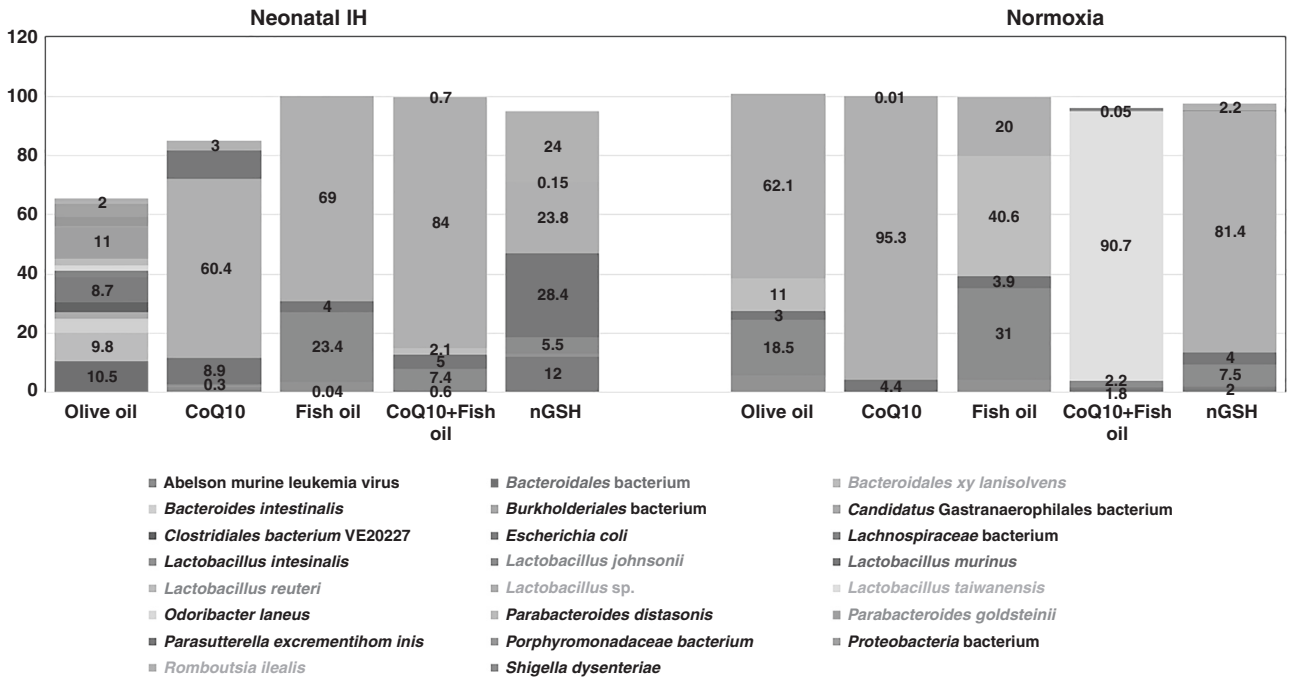

Fig. 6 Changes in the relative proportion of the most abundant microorganisms in response to neonatal IH and antioxidant and/or fish oil supplementation. Unspecified organisms and organisms that are <1% of the total are not shown.

groups. Figure S3 shows that IkB was highly expressed in the villi absorptive epithelium, lamina propria, mucosa, and intestinal crypts in both RA and IH and was robustly expressed in the OO group. Figure S4 shows that TGFβ immunostaining was robust with OO treatment in RA, particularly in the villi lamina propria and some crypts, and was more prominent in the OO RA and IH groups. All supplements decreased TGFβ1 immunoreactivity. Negative controls for TLR-4, pNFkB, IkB, and TGFβ1 in IH and RA conditions are presented in Figure S5 confirming antibody specificity.

DISCUSSION

This study used a clinically relevant neonatal animal model to test the hypotheses that neonatal IH leads to intestinal dysbiosis and injury, and treatment with antioxidants and/or fish oil can mitigate the adverse effects. To the best of our knowledge, this is the first study examining the effects of neonatal IH with antioxidant and/or fish oil (polyunsaturated fatty acid (PUFA)) supplementation on gut microbiota. The clinical significance of this investigation is that almost all ELGANs experience neonatal IH averaging 50–100 episodes per day, which may escalate the proinflammatory

cascade.[41] The major findings of this report are: (1) IH produces changes in the gut microbiota profile in the neonatal rat model that may lead to a predominance of pathogenic bacteria; (2) our model of neonatal IH produces histopathologic characteristics in the gut, which were associated with increased TLR-4 and TGFβ1, known biomarkers of NEC; (3) combination CoQ10 + fish oil was associated with a high abundance of organisms in the Firmicutes phyla *Lactobacillus* species and an impressive reduction of the percentage of Proteobacteria; and (4) combination of CoQ10 + fish oil and nGSH were most effective for reducing IH-induced lipid peroxidation, TLR-4, and TGFβ1 while preserving ileum architecture. It is likely that the adverse outcomes noted with fish oil alone may be due to the susceptibility of lipids to ROS attack, particularly in the setting of neonatal IH, and thus the use of antioxidants can preserve their known benefits, as previously suggested.[42] These findings support our hypothesis and suggest a novel therapeutic approach of combining PUFA lipids with antioxidants to mitigate IH-induced injury in the terminal ileum and possibly reduce the risk of NEC.

In our model, rat pups were exposed to neonatal IH from the first day of life. Although they were suckling, it is likely that neonatal IH causes changes in milk production and possibly alterations in commensal organisms. Prior to the onset of NEC, the microbiota is initially dominated with organisms of Firmicutes and Bacteroidetes phylum, then shifts toward Proteobacteria: *Escherichia coli*, *Citrobacter*, *Klebsiella* spp., and other more pathogenic organisms.[43] Fundora et al.[44] reported that a Proteobacteria bloom is common among preterm infants who develop NEC, but it is only seen in a minority of these infants. We did not observe an increase in the Proteobacteria phylum in any IH group. This may be a result of the gut colonization pattern in this animal model. In general, preterm infants with NEC have low gut microbial diversity compared to term infants and a reduced relative proportion of Firmicutes and *Bacteroides* preceding NEC.[28] Our study showed that neonatal IH is also associated with these reductions suggesting that IH may be a risk factor for gut dysbiosis and may be a primary effect. However, it is important to note that these changes were noted during the reoxygenation/reperfusion period and not during the actual IH period. Other studies show significant alterations in gut microbiota in mice exposed to IH.[33] Flemer et al.[45] found a high abundance of Firmicutes phyla, and a much lower abundance of Bifidobacterium of the Actinobacter phyla in Sprague Dawley rats at P21. In our animal model, the microbes from Firmicutes and Bacteroidetes phyla increased, with the greatest improvement seen in the CoQ10 + fish oil group, compared to control and single treatment. This suggests beneficial synergy and support the hypothesis that antioxidants preserve the beneficial effects of lipids in oxidative stress conditions.[42] However, regardless of treatment, neonatal IH produces significant changes in the abundance of the gut microbiota.

CoQ10 is an important factor in oxidative phosphorylation, which has also been shown to induce GSH peroxidase.[19] The effects of single CoQ10 administration on gut microbiota resulting in elevated phylum Bacteroidetes, Firmicutes, and Proteobacteria suggests that reduction in ROS makes a more favorable anaerobic environment or changes to the luminal pH that allows the bloom of Bacteroides. On the other hand, fish oil was associated with a larger increase in the *Lactobacillus* sp. of Firmicutes phyla, which suggest a preferential impact on microbiota composition.[46] nGSH treatment in IH produced the lowest abundance of microbes from Firmicutes and Bacteroides phyla. Studies show that intestinal mucosal production of GSH is upregulated by microbes *Lactobacillus acidophilus*, Bifidobacterium, and B Lactum.[47] Nanoparticle technology improves delivery to the tissues, increases intracellular penetration, and protects against premature degradation.[21] However, nGSH did not increase the abundance of species in IH, suggesting that higher doses may be needed. Nevertheless, nGSH was effective for suppressing lipid peroxidation and agrees with previous reports,[13,15–18,48] as well as TGFβ1 and TLR-4, albeit to a lesser degree than combination of CoQ10 + fish oil.

Neonatal IH produced histopathological and morphometric characteristics that were similar to previous reports[49] and was associated with the well-known biomarker of NEC, TLR-4.[50–52] Although our model was not a NEC model, the data showed that neonatal IH significantly induces TLR-4. We also noted an impressive reduction of TLR-4 expression with combination of CoQ10 + fish oil and nGSH treatments. The significant reduction in TLR-4 noted in the combination of CoQ10 + fish oil group was associated with increased microbiota of the Firmicutes phylum, confirming its benefits. These important findings could have clinical implications for reducing the risk and/or severity of NEC. Our study also showed reductions in TLR-4 with fish oil, confirming previous reports.[53] However, the finding of elevated TLR-4 in the CoQ10 RA and IH groups were surprising. It is likely that the suppressive effect occurred only during treatment and was not sustained. This suggests that higher doses may be needed.

TGFβ1 is a key regulator of immunosuppressive response in the intestine, involving T and B cells.[54] The production of TGFβ1 is increased from the microbiota such as *Clostridium* species, mostly occurring in the colonic lamina propria. The observation from our data shows significant activation of the TGFβ1 in the IH, with significant reductions in the fish oil, CoQ10 + fish oil, and nGSH groups. Although we did not detect changes in the microbiota order of Clostridiales, we did see increases in the members of the same phylum Firmicutes order level Lactobacillales. The inflammatory response in the gut occurs due to the complex interaction of several mediators, including NFkB, a potent transcription activator of the inflammatory cascade.[55,56] Our data showed that CoQ10 + fish oil combination resulted in a significant reduction of NFkB expression and increase in IkB, supporting a role for combining the two treatments to prevent IH-induced gut injury. IkB is an inhibitory protein that is bound to NFkβ, preventing its release and subsequent translocation to the nucleus for cytokine transcription.[57] Increased IkB is likely explained by the change in the microbiota pattern, favoring a predominance of non-pathogenic bacteria. The location of IkB expression gives important information of areas with ongoing inflammatory insults.

Although our study has significant clinical implications, there are limitations. We did not examine the microbiota immediately post neonatal IH exposure at P14. Pups are weaned at P21 and studies show that weaning itself can cause oxidative stress in the gut.[58] We also did not combine nGSH with fish oil to determine whether this combination would provide superior benefits. Although combining the two antioxidants may result in increased total antioxidant capacity, previous reports show that CoQ10 induces GSH and this effect may confound the levels of GSH. In addition, CoQ10 is lipid soluble while nGSH is water soluble. Since CoQ10 is a lipid-soluble substance, it is usually prepared in lipid carriers, such as olive oil.[34–38] Despite these limitations, our data show that neonatal IH increases the risk for gut injury and induces pathogenic organisms, coincident with lipid peroxidation, TGFβ1, and TLR-4, which may contribute to the incidence and severity of NEC. Combination treatment effectively increased the abundance of the non-pathogenic Firmicutes phylum, important for a healthy gastrointestinal system of the newborn. Since lipid emulsions are widely used in preterm infants who experience significant IH episodes, the use of antioxidants to preserve and/or improve the benefits of lipids may be warranted.

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COMPETING INTERESTS

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Correspondence and requests for materials should be addressed to K.D.B.

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