ELSEVIER

Archives of Medical Research ■ (2018) ■

Archives of Medical Research

ORIGINAL ARTICLE

Vitamin A and Retinoic Acid Exhibit Protective Effects on Necrotizing Enterocolitis by Regulating Intestinal Flora and Enhancing the Intestinal Epithelial Barrier

Sa Xiao,[a,b,c] Qiuping Li,[a,b,c] Kun Hu,[a,b,c] Yu He,[a,b,c] Qing Ai,[a,b,c] Liuhong Hu,[a,b,c,d] and Jialin Yu[a,b,c,d]

[a]*Chongqing Key Laboratory of Child Infection and Immunity, Chongqing, China*
[b]*China International Science and Technology Cooperation Base of Child Development and Critical Disorders, Chongqing, China*
[c]*Department of Neonatology, Children's Hospital of Chongqing Medical University, Ministry of Education Key Laboratory of Child Development and Disorders, Chongqing, China*
[d]*Department of Pediatrics, Shenzhen University General Hospital, Shenzhen, China*

Received for publication November 9, 2017; accepted April 6, 2018 (ARCMED-D-17-00639).

Background. Exaggerated inflammation that characterizes necrotizing enterocolitis (NEC) is caused by the invasion of pathogens through an immature intestinal barrier. Vitamin A (VA) and retinoic acid (RA) play important roles in the growth of epithelial tissue and in modulating immune function.

Objective. To investigate the roles of VA and RA in the development of NEC.

Methods. Levels of serum retinol in patients and in a NEC mouse model were detected with high-performance liquid chromatography. Bacterial communities of NEC mice treated with VA or PBS were detected by high-throughput sequencing. *In vitro* and *in vivo*, levels of inflammatory factors were measured by ELISA and RT-PCR, and expression levels of claudin-1, occludin, and ZO-1 were detected by Western blotting. Transepithelial electrical resistance (TEER) was measured in Caco-2 cell monolayers.

Results. The level of VA in the NEC patients was lower than in the control patients. In the NEC mice that were treated with VA versus PBS, the proportion of *Escherichia-Shigella* was lower, while the abundance of *Bacteroides* was markedly higher. Both *in vivo* and *in vitro*, the levels of inflammatory factors were significantly reduced, while the expression levels of claudin-1, occludin, and ZO-1 were increased, after the VA and RA treatments. Meanwhile, TEER was increased and lipopolysaccharide-induced damage was reduced in Caco-2 cell monolayers after RA treatment.

Conclusions. These results suggest that VA may regulate intestinal flora, alleviate inflammatory reactions, and enhance the intestinal epithelial barrier in NEC. Thus, VA may be an effective drug for providing protection against NEC in newborns.

Key Words: Vitamin A, Retinoic acid, Necrotizing enterocolitis, Microbial communities, Inflammatory factors, Tight junction proteins.

Introduction

Necrotizing enterocolitis (NEC) is an acute and serious intestinal disease that affects newborns and it is associated with high rates of mortality and complications after surgery. Generally, NEC is caused by an invasion of bacteria and exaggerated inflammation due to an immature intestinal barrier and immune system in newborns (1). The intestinal epithelial surface is the primary barrier against invasions by pathogens and it has three main functional components: commensal bacteria that colonize on the surface of the gut; an epithelium layer that is formed by epithelial cells and intercellular junctions; and gut-associated lymphoid tissue that includes various immune cells (2). An invasion

Address reprint requests to: Jialin Yu, Department of Neonatology, Children's Hospital of Chongqing Medical University, No. 136, Zhongshan Road, Yuzhong District, Chongqing 400014, China; Phone: +86-023-63633050; FAX: +86-023-63622754; E-mail: yujialin486@126.com

0188-4409/$ - see front matter.
https://doi.org/10.1016/j.arcmed.2018.04.003

of pathogenic bacteria and an immature intestinal barrier are crucial factors that predispose an individual to the pathogenesis of NEC. Consequently, many studies have been conducted to identify effective therapies that are able to target an immature gut barrier to cure or prevent NEC (3–5). It has been well-established that vitamin A (VA) is essential for maintaining good health and is particularly important for development of the visual system and epithelial tissue (6). Retinoic acid (RA) is a metabolite of VA and is responsible for most of the activities of VA. Both VA and RA have been found to contribute to normal metabolism, resistance to infection, and enhanced immunity (7,8). It has also been reported that newborns are prone to vitamin A deficiency (9,10), and this may be an important factor which leads to an immature intestinal barrier and NEC in neonates.

In order to further explore the relationship between VA and NEC, we compared the levels of VA in NEC and control newborns. The potential for VA and RA to provide protection from NEC was also examined in a mouse model of NEC and in the Caco-2 cell line.

Materials and Methods

Ethics Statement

This study was approved by the ethics commission of the Children's Hospital of Chongqing Medical University. Informed consents were obtained from the parents of the neonates included in this study and investigations were conducted according to the principles expressed in the Declaration of Helsinki.

Patients and the Collection of Samples

A total of 24 preterm infants in the Department of Neonatology at the Children's Hospital of Chongqing Medical University were enrolled in this study. Twelve of these neonates met the diagnostic criteria of Bell stage II and III (11) were selected to be included in the NEC group. Meanwhile, the remaining twelve neonates that had pneumonia or hyperbilirubinemia were included as a control group. Gestational age, birth weight, day-age, delivery mode, feeding practices, and medical conditions for each patient in the control group were matched to cases in the NEC group. A venous blood sample was collected from each neonate for routine tests once the neonate was transferred to the neonatal ward. The blood remaining after testing was centrifuged and serum samples for each neonate were collected in sterile tubes and frozen at −80°C for subsequent measurements of VA.

Animal Experiments

Newborn C57BL/6J mice were purchased from the Animal Experiment Center of Chongqing Medical University (Chongqing, China) and were randomly divided into two groups with their mothers. Half of the mice (the VAS group) received an intragastric administration of 20 IU VA once a day from postnatal 1−7 d via a 1.9 Fr silicone tube (12). The remaining mice received PBS (the PBS group) at the same time as the control group. Eight days after birth, the mice were placed in individual neonatal incubators and were established as an NEC model with formula feeding performed every 4 h with a silicone tube (1.9Fr) administering substitute formula (Similac Advance Infant Formula (Abbott Laboratories, USA)/Esbilac canine milk replacer (Pet-Ag, USA); 2:1) for 3 d. These pups were also stressed twice a day with hypoxia treatments (100% nitrogen gas for 1 min), followed by a cold stress (4°C for 10 min).

Tissue Harvest and NEC Evaluation

Decollation was performed 11 d after birth and the blood samples collected were centrifuged and reserved. In addition, the intestinal tract was removed and a 1 cm section of the distal ileum was cut and stored in 4% neutral buffered formalin solution. Tissue specimens were subsequently paraffin embedded and 4 μm tissue slices were stained with hematoxylin and eosin (HE). Histologic changes in the structure of the ileum were graded by a blinded evaluator in order to evaluate the presence of NEC, as previously described (13).

Detection of Serum Retinol

Levels of serum retinol in patients and in NEC mice were detected with high-performance liquid chromatography (HPLC). Briefly, each serum sample (200 μL) was deproteinized with dehydrated alcohol before the retinol present was extracted with hexane and evaporated with nitrogen gas. The resulting residue was dissolved in 100 μl of a mobile phase mixture (methanol: water ratio, 97:3). Twenty μl of each sample was transferred to an Agilent 1200 series HPLC apparatus (Agilent Technologies, USA). Retinoids were separated by chromatography on an analytical column (Waters SunFire C18, 150 mm × 4.6 mm, Waters, USA) with a gradient mobile phase. Eluant was detected by a liquid chromatograph equipped with a 315 nm ultraviolet photodiode array detector.

Analysis of Intestinal Microbial Community

Contents from the intestinal tracts of fourteen mice in each group were collected. Due to an insufficient quantity of feces, each sample for high-throughput detection contained the feces from two mice of the same group. Fecal DNA was extracted using a QIAamp FAST DNA Stool Mini-Kit (Qiagen, Germany). The V3-V4 region of the bacterial 16S rRNA gene was amplified by PCR with the following degenerate primers: 338F (5′-barcode-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The PCR