Research Paper

Expression of Aryl Hydrocarbon Receptor in the Intestinal Tissue of Preterm Infants with Necrotizing Enterocolitis

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To observe the expression of aryl hydrocarbon receptor in the intestinal tissues of premature infants who had necrotizing enterocolitis is the main objective of the study. From January 2018 to July 2021, 14 intestinal tissue samples of preterm infants with necrotizing enterocolitis at the Children's Hospital of Chongqing Medical University were selected as the necrotizing enterocolitis group. 14 intestinal tissue samples of preterm infants with congenital intestinal atresia during the corresponding period were selected as the control group. Hematoxylin-eosin staining was performed on pathological specimens from the two groups. The expression of aryl hydrocarbon receptor in the intestinal tissue was analyzed by immunohistochemical methods. Gross morphology, histopathology grade and aryl hydrocarbon receptor expression in the intestinal tissue samples were compared between the two groups. Intestinal wall hyperemia and edema, villus structure destruction, reduced crypts, higher inflammatory cell infiltration and histopathology grade were higher in the necrotizing enterocolitis group than in the control group and the difference was statistically significant ($p<0.05$). The expression level of aryl hydrocarbon receptor was lower in the necrotizing enterocolitis group than in the control group ($p<0.05$). Meanwhile, the aryl hydrocarbon receptor expression level was negatively correlated with the histopathology grade. The lower the expression of aryl hydrocarbon receptor, the more severe the intestinal injury, thus, suggesting that aryl hydrocarbon receptor activation may be a protective factor against necrotizing enterocolitis.

Key words: Necrotizing enterocolitis, aryl hydrocarbon receptor, immunohistochemical staining, histopathology grade

Necrotizing Enterocolitis (NEC) is one of the most common severe critical diseases in the neonatal period, especially in premature infants. However, the cause of NEC is not completely clear and it is considered that an immature intestinal barrier, intestinal flora imbalance and other factors result in an excessive inflammatory reaction and intestinal epithelial cell death[1]. Although many scholars have continued to conduct in-depth research in recent years, no much progress has been made in the prevention and treatment of this disease.

The Aryl Hydrocarbon Receptor (AHR) is a ligand-dependent transcription factor, which is widely expressed in a variety of immune and non-immune cells[2]. The regulation of AHR is highly correlated with the process of cellular, tissue and organism development, and maintenance of homeostasis[3]. Studies have shown that AHR can regulate the gastrointestinal barrier and immune function, the AHR signaling pathway has anti-inflammatory effects in the intestine, and AHR deletion will aggravate the intestinal inflammation in experimental colitis in mice[4]. Increased expression of AHR in the intestinal mucosa of mice can reduce the expression of pro-inflammatory markers in the NEC process and reduce NEC-induced intestinal damage[5]. Therefore, in this study, immunohistochemistry was used to determine the expression level of AHR in the intestine of children with NEC and a correlation analysis with histopathological scores was carried out to explore the role of AHR in NEC among neonates.

MATERIALS AND METHODS

Collection of research objects and their data and specimens:

From January 2018 to July 2021, 14 postoperative

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pathological specimens of children with NEC admitted to the Children's Hospital of Chongqing Medical University were selected as the NEC group and 14 postoperative pathological specimens from children with congenital intestinal atresia were selected as the control group. The clinical data of the two groups of children, including gestational age, birth weight, age of specimen sampling, whether the mother had diabetes during pregnancy, whether the mother had cholestasis during pregnancy, whether hormones were used before delivery, whether milk was prescribed before surgery, single/twin births, specimen location and length of the resected intestine, were collected.

Hematoxylin-Eosin (HE) staining and tissue damage scoring of tissue specimens:

The intestinal tissues from the two groups were sliced at a thickness of 4 μm, stained by HE and pictures were taken under a microscope and analyzed. The scoring methods of Nadler *et al.*[6] and Pisano *et al.*[7] were used. Pathologists performed histopathological scoring of pathological specimens from the two groups of children. The scoring method was as follows. 0 points-Normal, the intestine is not damaged and the epithelium and villus structure of the intestinal tissue are clear and complete; 1 point-Focal mild injury confined to the tip of the villi, slight swelling and separation of the submucosal or lamina propria and minimal inflammatory cell infiltration; 2 points-Partial loss of villi, moderate swelling and separation of the submucosa or propria of the intestine, and more inflammatory cell infiltration; 3 points-Necrosis extends to the submucosa, severe swelling and separation of the submucosa or propria of the intestine, and infiltration of a large number of inflammatory cells; 4 points-Transmural necrosis, almost complete disappearance of the intestinal villi and full-thickness necrosis of the intestinal wall.

Immunohistochemical staining and AHR expression analysis:

AHR immunohistochemical staining of pathological sections in the NEC group and control group was performed by the pathology laboratory of Chongqing Medical University. The specimens were subjected to xylene dewaxing; soaking in 100 %, 95 %, 85 % and 75 % ethanol for 2-3 min; rinse with Phosphate Buffered Saline (PBS); placing in sodium citrate for high-pressure repair and heating at about 95° for 13 min. Then, the specimens were removed and cooled naturally at room temperature. The samples were then incubated in 3 % hydrogen peroxide for 10 min and washed 3 times with PBS for 1 min at a time. Dropwise addition of the primary antibody, i.e. the rabbit anti-human AHR polyclonal antibody (Proteintech, United States) was performed and the antibody working concentration was 1:200. The samples were incubated in a 37° incubator for 1.5 h and rinsed 3 times with PBS after removal for 2 min each time. Dropwise addition of the secondary antibody i.e. polymer anti-rabbit Immunoglobulin G (IgG)-Horseradish Peroxidase (HRP) as the secondary antibody (GBI, United States) was performed. The samples were incubated at 37° in an incubator for 15 min and PBS rinses were performed 3 times for 2 min each. The 3,3′-Diaminobenzidine (DAB) chromogenic working solution was added dropwise and incubated for 5 min at room temperature. Hematoxylin counterstaining was performed for 30 s and the samples were washed twice with distilled water for 2 min each; dehydrated; soaked in 75 %, 85 %, 95 % and 100 % ethanol for 2-3 min and placed in fresh xylene was for 10 min. Neutral gum seal was used and the samples were stored at room temperature.

The Image-Pro plus 6.0 professional image analysis software was used to analyze the average optical density values of positive products in tissues under a 200× field of view in each group for statistical analysis.

Statistical analysis:

Statistical Package for the Social Sciences (SPSS) 22.0 software was used for statistical analysis. The Shapiro-Wilk test was used to test the normality of the measurement data and the measurement data showing normal distribution were tested by two independent samples t-test and expressed as x±s. Non-normal distribution of the measurement data was tested by the Mann-Whitney test of the independent sample and the data were expressed as the median. The chi-square test was used for the count data and $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Comparison of clinical data between the two groups was shown in Table 1. There were no significant differences in gestational age, birth weight, age at specimen collection, Gestational Diabetes Mellitus (GDM), Intrahepatic Cholestasis of Pregnancy (ICP), Antenatal Corticosteroid Treatment (ACT),

preoperative milk prescription, single/twin births, specimen location and length of resected bowel segments, as shown in Table 1.

HE staining and histopathological grade was shown in fig. 1 and fig. 2. There were obvious differences in the intestinal tissue structure between the NEC group and control group. Destruction of the intestinal mucosal villus structure, submucosal and muscular layer hyperemia and edema, gland reduction and higher inflammatory cell infiltration in children in the NEC group are shown in fig. 1. The histopathological grade in the NEC group was 3.28±0.61 and the histopathological grade in the control group was 1.50±0.51, and the difference was statistically significant ($p<0.05$), as shown in fig. 2.

AHR immunohistochemical analysis results were compared here. AHR was widely expressed in both groups, with its expression in absorptive cells, goblet cells and intraepithelial lymphocytes being the most pronounced, as shown in fig. 3. Under 200× microscopy, the mean optical density in the NEC group was 0.101±0.028 and the mean optical density in the control group was 0.171±0.044, and the difference between the two groups was statistically significant ($p<0.05$), as shown in fig. 4. Correlation analysis of the AHR expression level and histopathological grade in the two groups showed a negative Pearson correlation ($p<0.0001$, $r=-0.717$), as shown in fig. 5.

TABLE 1: COMPARISON OF CLINICAL DATA BETWEEN THE TWO GROUPS

Clinical data	NEC group	Control group	t/χ^2	p
Gestational age (w)	35.04±1.18	35.38±1.09	-0.804	0.429
Birth weight (g)	2272±300	2421±383	-1.146	0.262
Sampling day age (d)	4.64±1.33	4.07±2.09	0.861	0.397
GDM (Yes/No)	3/11	2/12	0.235	0.628
ICP (Yes/No)	2/12	1/13	0.360	0.549
ACT (Yes/No)	3/11	2/12	0.235	0.628
Preoperative feeding (Yes/No)	11/3	7/7	1.400	0.237
Single/gemellary pregnancy	12/2	12/2	-	1
Sample position (small intestine/colon)	12/2	13/1	0.373	0.541
Length of enterectomy (cm)	23.30±14.75	19.42±19.10	0.601	0.553

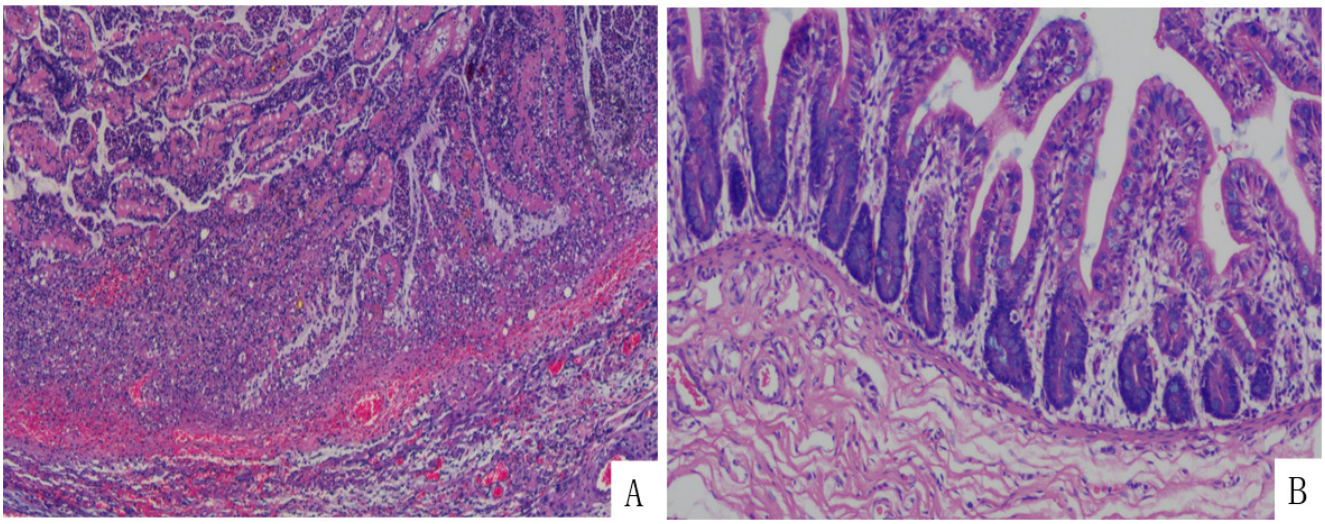

Fig. 1: HE staining, (A) In the NEC group (40×) and (B) In the control group (40×)

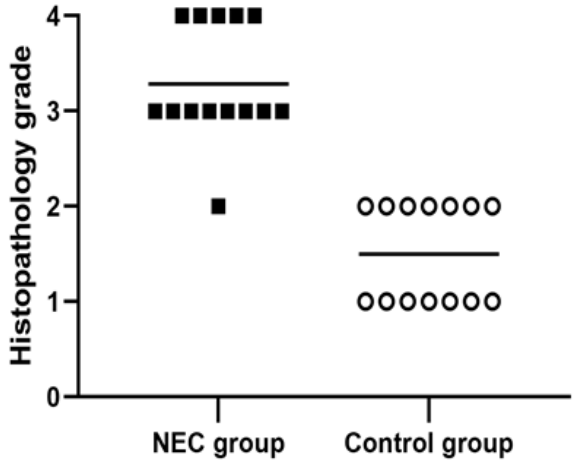

Fig. 2: Histopathology grade in NEC group and control group
Note: The histopathology grade in the NEC group was significantly higher than that in the control group and the difference was statistically significant (p<0.05)

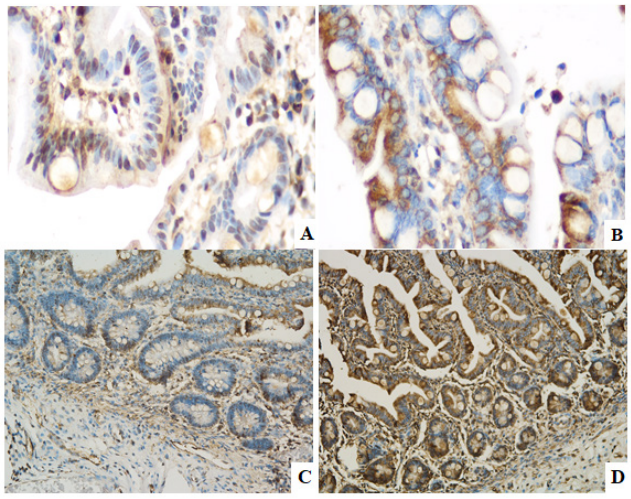

Fig. 3: AHR immunohistochemical analysis, (A) In the NEC group (400×); (B) In the control group (400×); (C) In the NEC group (200×) and (D) In the control group (200×)

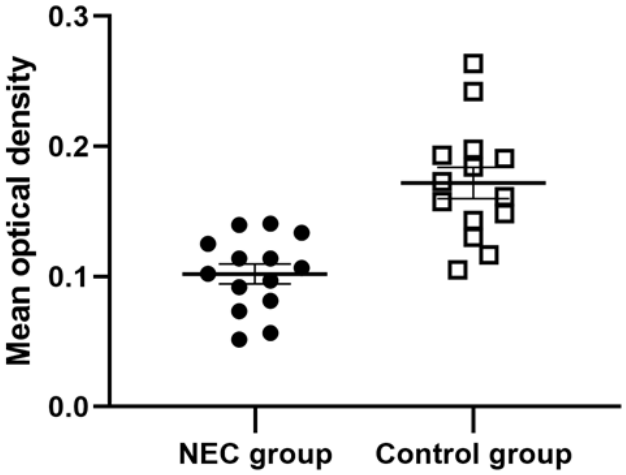

Fig. 4: AHR represents the mean optical density results
Note: The mean optical density of AHR expression was significantly different between the two groups (p<0.05)

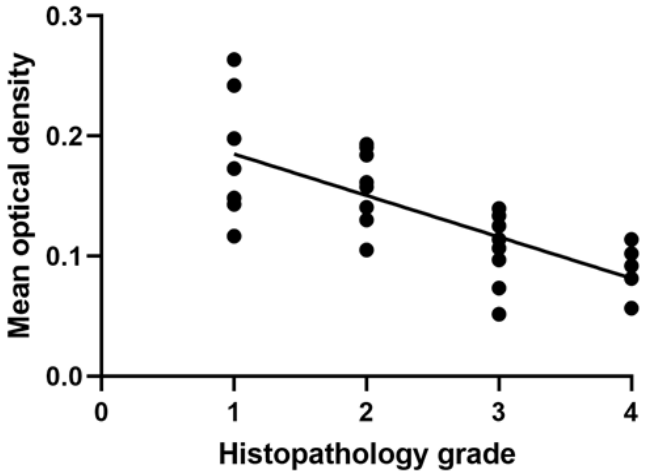

Fig. 5: Correlation analysis of the mean optical density and histopathology grade
Note: The mean optical density of AHR in the two groups was negatively correlated with the histopathology grade

NEC is a common neonatal emergency that occurs predominantly in preterm infants and its pathogenesis has not been fully understood; but it is related to a variety of factors, such as genetic predisposition, intestinal immaturity, abnormal colonization of intestinal microorganisms and intestinal mucosa with a high immune response[1]. NEC can occur anywhere in the intestine, most commonly in the small intestine and colon[8]. Intestinal mucosal injury plays an important role in the pathogenesis of NEC[9]. The pathological characteristics of NEC are intestinal mucosal bleeding, edema, destruction of the villous structure and inflammatory cell infiltration, which result in destruction of the intestinal epithelial barrier, allow pathogen invasion, trigger an inflammatory cascade and eventually lead to further intestinal mucosal damage, amplification of the inflammatory response, complicated by systemic sepsis and intestinal perforation[10]. This study found that HE staining in the NEC group showed submucosal and muscular layer hyperemia and edema, villous structure destruction, gland reduction and high inflammatory cell infiltration.

AHR is a ligand-activated transcription factor. In the inactive state, AHR is located in the cytoplasm and binds to various chaperones, such as heat shock protein 90 and human p23 protein (p23). When AHR binds to ligands, AHR separates from the chaperone and enters the nucleus, forming a dimer with the AHR nuclear transcription factor, resulting in transcriptional activation of the target gene[11]. Target genes for AHR include Cytochrome P450 family 1 subfamily A1 (CYP1A1), Cytochrome P450 family 1 subfamily A2 (CYP1A2), Cytochrome P450 family 1 subfamily B1 (CYP1B1), 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD)-Inducible Poly-Adenosine Diphosphate (ADP)-Ribose Polymerase (TIPARP) and Aryl Hydrocarbon Receptor Repressor (AHRR). Activation of the AHR signaling pathway plays an important role in maintaining intestinal homeostasis[12,13], but its role in NEC has not been fully understood.

This study found that AHR was widely expressed in the intestinal tissues of preterm infants, mainly in intestinal epithelial cells, including absorptive cells, goblet cells and intraepithelial lymphocytes. Intestinal epithelial cells play an important role in the establishment of intestinal immune barriers and resistance to microbial invasion[14]. Thus, AHR may be involved in maintaining normal intestinal mucosal barrier function. Previous studies have shown impaired intestinal barrier in AHR−/− mice, suggesting that AHR may be important for intestinal barrier maintenance or developmental health[15]. AHR deficiency impairs goblet cell and mucus production and increases microbial translocation to other anatomical sites[16]. The deletion of AHR in intestinal epithelial cells disrupts the integrity of the intestinal barrier and the number of intestinal intraepithelial lymphocytes decreases[17,18]. At the same time, it affects the differentiation of intestinal crypto vascular stem cells to epithelial cells, which is more likely to lead to bacterial displacement and $AHR^{\Delta IEC}$ (IEC is Intestinal Epithelial Cell) is more severe than NEC in wild-type mice[19]. AHR barrier maintenance function may be driving the cytokine Interleukin (IL)-22, which regulates epithelial production of antimicrobial defensins, intestinal repair and stem

cell populations to strengthen the intestinal epithelial tight junctions, thereby promoting epithelial barrier integrity and microbial homeostasis[20,21].

The results of this study revealed that the average optical density of AHR in intestinal tissues was significantly reduced in the NEC group and the expression of AHR was significantly reduced, suggesting that AHR may be involved in the pathogenesis of NEC. AHR can affect the expression of a variety of cytokines (e.g., IL-1 beta (β), IL-4, IL-6, IL-17, IL-10, IL-23, IL-27 and Interferon-gamma (IFN-γ)[15,22,23], and this regulation of the cytokine environment in the intestinal lamina propria determines the activation or inhibition phenotype, thereby affecting the integrity of the intestinal barrier and the community structure of the microbiota. Intestinal bacteria or their metabolites may also trigger AHR reactions. In the absence of chronic inflammation, intestinal homeostasis maintains a balance between commensal and pathogenic microorganisms, and microbial translocations and trigger adaptive responses occurs when local dysbiosis and epithelial barrier rupture occur, amplifying local tissue damage and inflammation. Bacterial or microbial products, such as *Lactobacillus reuteri* and *Lactobacillus bulgaricus*, may modulate an excessive inflammatory response after AHR activation[24].

In addition, this study also showed that reduction in the AHR expression was inversely correlated with the histopathological score and the lower the AHR expression, the heavier the intestinal damage, suggesting that AHR activation may be a protective factor for NEC. AHR exerts biological effects through activation with ligands, both exogenous and endogenous. A large number of AHR ligands can be accommodated in the human intestine, which can also be produced through the metabolism of the microbial communities present in the gut. The expression of AHR has been shown to maintain a specific proportion of various bacterial populations in the cecum[25]. Both endogenous and microbiota-derived tryptophan metabolites in the cecum and fecal samples from mice and humans can induce AHR activation[24]. There was no special diet for all patients included in this study and there was no statistically significant difference in preoperative milk prescription. Hence, it is presumed that AHR activation in preterm infants is derived from binding to endogenous ligands. Common endogenous ligands include 6-Formylindolo[3,2-b]carbazole (FICZ), 2-(1'H-Indole-3'-carbonyl)-Thiazole-4-carboxylic acid methyl Ester (ITE) and Kynurenine (KYN). FICZ has been shown to reduce IL-7 production and activate dendritic cells by activating intraepithelial lymphocytes to improve experimental colitis[26]. FICZ-exposed mice exhibit a significant reduction in T helper 1 (Th1) cytokine synthesis, upregulation of IL-22 and a reduction in colonic inflammatory lesions[27]. Administration of ITE is able to inhibit effector cells *in vitro*, induce regulation of Cluster of Differentiation 39 (CD39) and granzyme B, and re-establish immune tolerance in the gut by regulatory T cells (Tregs); thus, improving experimental colitis in human-derived mouse models[28].

Due to the protective effect of AHR activation on the intestine, supplementation of AHR exogenous ligands makes it possible to reduce the inflammatory response in the intestine. In mouse experiments, AHR activation prevents NEC by limiting Toll-Like Receptor 4 (TLR4) signaling and expression in the intestinal epithelium. Supplementation with the AHR ligand Indole-3-Carbinol (I3C) reduces the severity of NEC by reducing the histologic damage and decreasing the expression of IL-6, Tumor Necrosis Factor alpha (TNF-α) in intestinal epithelial cells. In addition, studies have shown that supplementation with I3C to activate AHR expression can protect the structure of the intestinal epithelium during NEC and the expression of pro-inflammatory factors, Lipocalin-2 (LCN2) and Macrophage Receptor with Collagenous structure (MARCO) is reduced; thus, indicating that ligand-mediated AHR function is a protective mechanism of the pro-inflammatory response during NEC, possibly reducing the inflammatory response of NEC through activation of AHR in CD11c1 immune cells[5].

In conclusion, with respect to the type of endogenous ligand, the mechanism through which supplementation with this ligand can increase AHR activation and have a protective effect in NEC among preterm infants which needs to be further studied.

Conflict of interests:

The authors declared no conflict of interest.

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