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Does Abnormal Bile Acid Metabolism Contribute to NEC?

Melissa D. Halpern, Ph.D.[1] and **Bohuslav Dvorak, Ph.D.**[1,2]

1 *University of Arizona Department of Pediatrics and Steele Children's Research Center*

2 *University of Arizona Department of Cell Biology and Anatomy*

Abstract

Bile acids (BAs) facilitate emulsification, absorption and transport of fats and sterols in the intestine and liver and are essential for normal digestion. However, accumulation of BAs in the intestine can result in damage to the intestinal epithelium. Using the neonatal rat model of NEC, we have recently shown that BAs accumulate in both the ileal lumen and enterocytes of neonatal rats with NEC and the increased BA levels are positively correlated with disease severity. Importantly, when BAs are not allowed to accumulate, neonatal rat pups develop significantly less disease. In addition, BA transporters are altered during disease development. These data indicate that BAs play an important role in the development of experimental NEC, and suggest that the inability of neonatal rats to adequately regulate BA transporters may be a mechanism by which ileal damage occurs.

Index Words

necrotizing enterocolitis; bile acids; enterohepatic circulation

Introduction

Necrotizing enterocolitis (NEC) affects thousands of newborns each year in the United States alone, and with a mortality rate ranging from 10–50%, this disease remains a major cause of morbidity and mortality in prematurely born infants [1–3]. Severe NEC is characterized by an extensive hemorrhagic inflammatory necrosis of the distal ileum and proximal colon [4]; the disease can be mild to severe with clinical presentation ranging from abdominal distension, pneumatosis intestinalis, occult or frank blood in stools, intestinal gangrene, bowel perforation, sepsis and shock [5–7]. Survivors of a severe episode of NEC frequently suffer the effects of short bowel syndrome [3, 8, 9], resulting in prolonged medical expenses and chronic gastrointestinal difficulties [10]. While prematurity, enteral feeding, intestinal hypoxia-ischemia, and bacterial colonization are considered major risk factors for development of NEC, the pathophysiology of this disease remains poorly understood and no predictive tests are currently available.

Bile acids (BAs) are physiologic compounds that facilitate emulsification, absorption and transport of fats and sterols in the intestine and liver. Enterohepatic circulation of BAs is an essential process involving coordinated regulation of BA synthesis in the liver, transport of

Corresponding Author: Melissa D. Halpern, Ph.D., University of Arizona, Department of Pediatrics, PO Box 245073, Tucson, AZ 85724-5073, 520-626-2809 (P), 520-626-5009 (F), mhalpern@peds.arizona.edu.

BAs from the liver to the intestine, and transport of BAs back to the liver. When enterohepatic circulation is altered, accumulation of hydrophobic BAs in the intestine can result in damage to the intestinal epithelium [11–13]. Importantly, when exogenous BAs are introduced into the GI tract of neonatal rats via intragastric gavage, histologic damage to ileal architecture is similar to what is observed in animals with experimental NEC (Figure 1) [14].

During gestation, biliary BA levels are markedly increased [15], and meconium contains significant levels of BAs. Total BA levels in feces decreases between birth and day 7, then begins to increase [16]. In addition, the composition of BAs changes after birth and in the months to follow [17–20]. The bile salt pool size in premature infants is significantly reduced compared to term infants, but bile salt synthesis is high [19]. Studies in animals have shown that many of the key processes of BA homeostasis are immature in newborns [21–27] and reach maturity by the time of weaning. It has been proposed that breast-fed newborns may not have need for high levels of BAs, as human milk fat is digested differently than solid foods or cow's milk-based formula [22, 28–32]. Diet can also affect levels and composition of BAs. Fecal deoxycholic acid (DCA) and lithocholic acid (LCA) were significantly lower in breast-fed infants than in formula-fed infants and the percent of secondary BAs excreted in feces was significantly higher in the formula-fed group by 11 months of age [33]. Significantly, the incidence of NEC is 6–10 times higher in formula-fed infants compared to those that are breast-fed [34].

Ileal BAs in Experimental NEC

To determine if BA play a role in NEC pathogenesis, total BA levels were evaluated in ileal luminal flushes in neonatal rats using the neonatal rat model of NEC. This model, developed by Barlow [35, 36] and modified by Caplan [37], reproduces the major risk factors for NEC – prematurity of the gastrointestinal tract, enteral formula feeding, hypoxia/ischemia and bacterial colonization. NEC is developed by feeding prematurely born, never suckled rats with cow's milk-based formula coupled with exposure to asphyxia and cold stress. Dam-fed (DF) pups undergoing the same schedule of asphyxia and cold stress do not develop NEC and are utilized as controls. In the neonatal rat model, many clinical and pathological changes are similar to those found in humans: the abdomen is distended, blood is detected in stool, and the ileum and proximal colon are the most affected parts of the intestine. In addition, the key risk factors for human NEC (intestinal immaturity, enteral formula feeding, bacterial colonization and hypoxia/ischemia) are essential factors to develop disease [37–39]. When neonatal rats were subjected to the NEC protocol, total BAs were significantly increased in the ileal luminal contents compared to DF controls (Table 1) and these elevated values were positively correlated to disease severity [14]. If intestinal BAs are required for development of NEC injury, then reduction of BAs should result in decreased incidence and severity of NEC. Cholestyramine (Chol) binds BA in the intestine and the resulting Chol/BA complex passes out of the body without being absorbed [40–42]. When neonatal rats subjected to the NEC protocol were given Chol, both the incidence and severity of ileal damage was significantly decreased. In addition, pharmacologic sequestration of BAs with Chol resulted in increased survival during NEC development [14].

In the classic BA synthetic pathway, the primary BAs cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized from cholesterol in the liver by the enzyme CYP7A1 [43]. After synthesis, CA and CDCA are secreted into bile via the bile salt excretory pump (BSEP). BAs can be deconjugated by bacteria in the small intestine to form secondary BAs, principally DCA and LCA. Bile acids can be further characterized as either hydrophilic or hydrophobic. The hydrophobic BAs (e.g. CA and DCA acid) are considered toxic when they accumulate in the intestine [11–13]. During experimental NEC, CA and DCA acid levels are significantly increased in the luminal contents of neonatal rats with NEC (Table 1), indicating ileal luminal BAs consist mainly of the more toxic, hydrophobic BAs [14]. These data are also consistent with our

understanding that intestinal microflora are essential for the development of NEC as the formation of DCA requires the presence of intestinal bacteria.

These data strongly suggest that BAs play a critical role in the development of experimental NEC. However, a basic question remained - does BA accumulation in the ileum cause ileal injury or is it a consequence of damage to ileal tissue from other mediators? Neonatal rats subjected to the NEC protocol were sacrificed post-birth at 24, 48, 72 or 96 hours. A section of distal ileum from each animal was flushed to evaluate total luminal BA levels and an adjacent section of distal ileum was evaluated for histological damage by a blinded observer. [38, 39, 44–46]. By 48 hours, ileal luminal BA levels were elevated in animals with NEC, but histological damage did not significantly increase until the 72 hour observation point (Figure 2A). Similar results were also obtained when intra-enterocyte BA levels were compared to histologic damage over the course of disease development (Figure 2B) [47]. Thus, it appears that BA levels increase prior to ileal damage further supporting the concept that BAs play a crucial role in disease development.

Ileal BA transporters in NEC

Both primary and secondary BAs are reclaimed in the distal ileum - the site of injury during NEC - via the apical sodium-dependent bile acid transporter (ASBT) [48, 49]. BAs are transported into the enterocyte via ASBT and are thought to be bound to the ileal bile acid binding protein (IBABP) [50]. At the basolateral surface of enterocytes, the heteromeric organic solute transporter (OSTα-β) removes BAs from the cell and transports them into portal circulation [51]. The transport of BA from the ileal lumen to portal circulation is crucial for BA homeostasis and to insure that BAs do not accumulate in the enterocytes. In the intestine, ASBT, IBABP and OSTα-β are expressed predominantly in the terminal ileum [51–54]. In rat ileum, Asbt, Ibabp and OSTα-β undergo biphasic regulation; they are expressed in the fetus, down-regulated in the neonate, then up-regulated at weaning [21, 48, 50, 55–58]. These variations likely reflect the changing BA levels described during fetal development, the neonatal period and weaning.

Asbt, Ibabp, Ostα-β protein levels from distal ileum were evaluated during the development of experimental NEC. As expected, Asbt was not present in most 4 day-old, DF neonates. However, Asbt levels in animals with NEC were significantly increased (Figure 3A) and decreased when luminal BA levels were reduced with Chol [14]. While Asbt protein levels in neonatal rats with NEC were only approximately 10% of that seen in weanling rats [14], the Asbt appears to be functional, as intra-enterocyte levels of BAs are also significantly elevated compared to control pups [47]. These data also suggest that intra-enterocyte rather than luminal BA accumulation contributes to ileal injury during NEC pathogenesis.

In contrast to Asbt, Ibabp was consistently detected in the DF group only and was produced only minimally in animals with NEC (Figure 3B) [14]. For Ostα-β, similar protein levels were observed in both DF and NEC groups (Figure 3C) but its was found primarily in the crypts and not in the upper villi as seen in weanling animals [14]. This suggests a possible mechanism by which BAs accumulate in enterocytes during experimental NEC; elevated luminal BAs are transported into the enterocytes by Asbt, but cannot be moved through the cell to be transported into portal circulation. The observation that portal BA levels are decreased in neonatal rats with NEC supports this hypothesis [14].

Inflammatory Mediators and Hepatic BA Transporters

There are mechanisms by which BAs affect and are affected during the development of intestinal inflammation that are not directly related to alterations in enterohepatic circulation of BAs. TNF-α, a pro-inflammatory cytokine which is known to affect hepatic BA transporters

[59–61], can alter BA uptake in hepatoma cell lines [62], and is a crucial component of NEC pathogenesis [46, 63, 64]. Further, TNF-α is known to contribute to compromised epithelial barrier functions and affect intestinal permeability [65–67]. Thus, inflammatory mediators could affect BA levels during NEC by influencing BA transport or altering intestinal permeability. If inflammation causes elevation of BAs, then infiltration of macrophages - a hallmark of inflammation in experimental NEC - should be apparent prior to elevation of BAs. Intra-enterocyte BA levels increase prior to ileal macrophage infiltration during the development of experimental NEC [47]; data that strongly suggest that inflammation does not cause elevation in BA levels.

Completion of enterohepatic circulation of BAs is mediated primarily by the sodium-dependent taurocholate co-transporting polypeptide (NTCP)[68] which is located on the basolateral membrane of hepatocytes. Like the ileal BA transporters, NTCP displays ontogenic regulation ; in rats, Ntcp mRNA is expressed at adult levels by day 7 post birth [69, 70], but it functions at only 75% of the adult rate until approximately 4 weeks of age. Ntcp is significantly decreased in rats with NEC compared to DF controls [71]. It has not been established if the decrease in Ntcp is specific for this disease process, or if it is simply in response to lower levels of BAs re-circulating through portal circulation. However, TNF-α has been shown to down regulate Ntcp and hepatic TNF-α is elevated during experimental NEC [46, 63]. Neonatal rats subjected to the NEC protocol were either injected with anti-TNF-α or vehicle. Ntcp mRNA was significantly increased in pups with NEC given anti-TNF-α (Figure 4). Thus, elevated hepatic TNF-α in NEC may contribute to the down-regulation of Ntcp [71]. In the rat, sodium-independent uptake of BAs is mediated by members of the organic anion-transporting polypeptide (Oatp) family, Oatp1, Oatp2 and Oatp4 and dianionic conjugated BAs are secreted into bile via the multi-drug-resistance-associated protein 2 (Mrp2). It has been shown that TNF-α can down-regulate both Oatp4 and Mrp2 mRNA [72–74]. To determine if Oatp and/or Mrp2 expression is altered during NEC pathogenesis, we examined mRNA in 4 day-old DF, NEC and NEC + anti-TNF-α groups. Oatp4 and Mrp2 mRNA were significantly decreased in NEC and NEC + anti-TNF-α groups compared to DFs. There was no difference in Oatp2 expression between groups (Figure 4). Thus, down-regulation of Oatp4 and Mrp2 in NEC does not appear to be a consequence of elevated TNF-α and suggests that another factor may down-regulate these transporters in NEC [71].

The decreased levels of BAs returned to the liver via portal circulation and/or because of decreased Ntcp should result in continued production of BAs. Because direct cannulation of the bile duct is not possible in 4 day-old rats, total BA levels were evaluated in proximal jejunal contents to indirectly determine BA production from the liver. Not unexpectedly, the overall levels of jejunal BA were similar between DF control animals and those with NEC [14]. In addition, levels of Bsep, the transporter responsible for exporting BAs from the liver, were unchanged (Figure 4).

Nuclear Receptors and BA Accumulation

Regulation of BA metabolism involves multiple steps and the nuclear receptors farnesoid X receptor (FXR) [75] and liver X receptor (LXR) [76] are essential components that regulate BA and cholesterol. Oxidized metabolites of cholesterol are involved in positive feed-forward pathways involved in cholesterol homeostasis via LXR along with and the retinoid X receptor (RXR)[76]. FXR, for which BAs are natural ligands, operates in an opposing fashion to LXR to repress BA synthesis [75]. BA-activated FXR inhibits CYP7A1 transcription in the liver (via the small heterodimer partner, SHP[77]) and activated FXR can also repress NTCP (via SHP) [77], but can up-regulate BSEP expression [78]. In enterocytes, BA bound to ileal FXR induces expression of IBABP [79, 80]. Previous research has shown that unlike rabbits, mice and humans, the rat Asbt gene is not regulated via a negative feedback mechanism by BAs [81–85]. BA

responsiveness of ASBT is mediated by FXR-dependent activation of SHP and subsequent inhibition of LRH-1 (liver receptor homologue-1) [84, 85]. The rat Asbt promoter lacks a functional LRH-1 *cis*-acting element, and rat ileum does not express the LRH-1 protein [86]. The genes encoding human OSTα-β have also been shown to be induced by BA and FXR [87, 88]. Thus, BA responsiveness can be regulated by positive and negative feedback mechanisms via interactions between BAs and FXR [89, 90]. Ileal FXR mRNA levels were examined in DF and NEC groups and there were no differences observed. However FXR expression was significantly lower than what was observed in weanling rats [14]. In contrast, hepatic FXR mRNA was significantly lower in animals with NEC. Further studies to evaluate the contribution of FXR and to assess if the low levels of ileal FXR are sufficient to regulate BA transporters during the development of disease are crucial to understanding the overall process of BA homeostasis during NEC.

Tying Together the Complex Regulation of BAs in NEC

Our working paradigm for the role of BAs in NEC pathogenesis is as follows (Figure 5): In dam-fed (DF) neonatal rats, low levels of ileal BA transporters can effectively transport naturally low levels of hydrophilic, luminal BAs through the enterocyte and out into portal circulation. During NEC, toxic, hydrophobic ileal luminal BA levels are significantly increased and Asbt and Ost α-β are precociously up-regulated, perhaps in an attempt to deal with elevated luminal and intra-enterocyte BAs, respectively. However, Ibabp is significantly decreased. Our data suggests that without adequate levels of Ibabp, BAs are not efficiently transported into portal circulation, and may accumulate in the enterocytes where they contribute to ileal damage. Weanling animals express high levels of Asbt, Ibabp, and Ostα-β compared to neonatal animals, and efficiently transfer BAs from the lumen into portal blood. Decreased portal blood BA levels in NEC result in diminished levels of Ntcp, with less BAs extracted from portal circulation. This leads to continued production of BAs which contributes to more accumulation of BAs in the intestine.

The contribution of BAs to NEC pathogenesis represents a shift in the way this disease has traditionally been viewed. While further study is essential, there is strong evidence in animal models that BAs play a critical role in the initiation of ileal damage during disease development. The inability of neonatal intestine to adequately regulate ileal BA transport may be a mechanism by which BAs accumulate and could help explain why the disease predominates in premature infants. This paradigm can also encompass two other important risk factors for NEC, formula feeding and bacterial colonization: formula feeding elicits more toxic, potentially tissue damaging BAs than breast feeding [33] and the formation of secondary BAs, which require bacterial-induced deconjugation, occurs only in animals with NEC [14]. Additional studies will clarify the role of BAs in both experimental and human disease.

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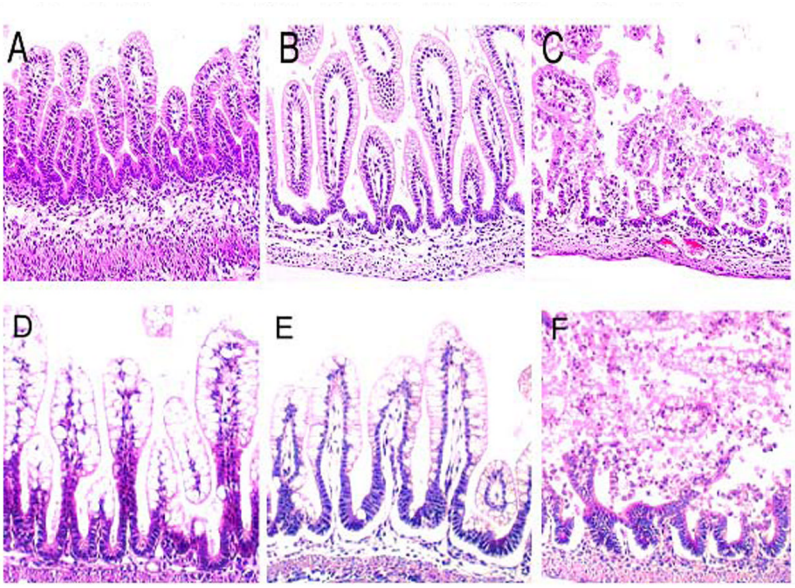

Figure 1. Ileal damage in BA gavaged rats resembles damage in experimental NEC

In the upper panels, representative slides from neonatal rats gavaged with **(A)** PBS, **(B)** one dose of 50 mM sodium deoxycholic acid, and **(C)** two doses of 50 mM sodium deoxycholic acid. In the lower panels, representative slides from **(D)** DF, histologic score = 0; **(E)** NEC, histologic score +2; **(F)** NEC animals with full necrosis, histologic score +4. In contrast to the ileal tissue shown in (A), the large enterocytes visible in the DF ileum (D) are seen because these animals were not fasted prior to removal of ileal tissue. Magnification 100X. Taken from [14].

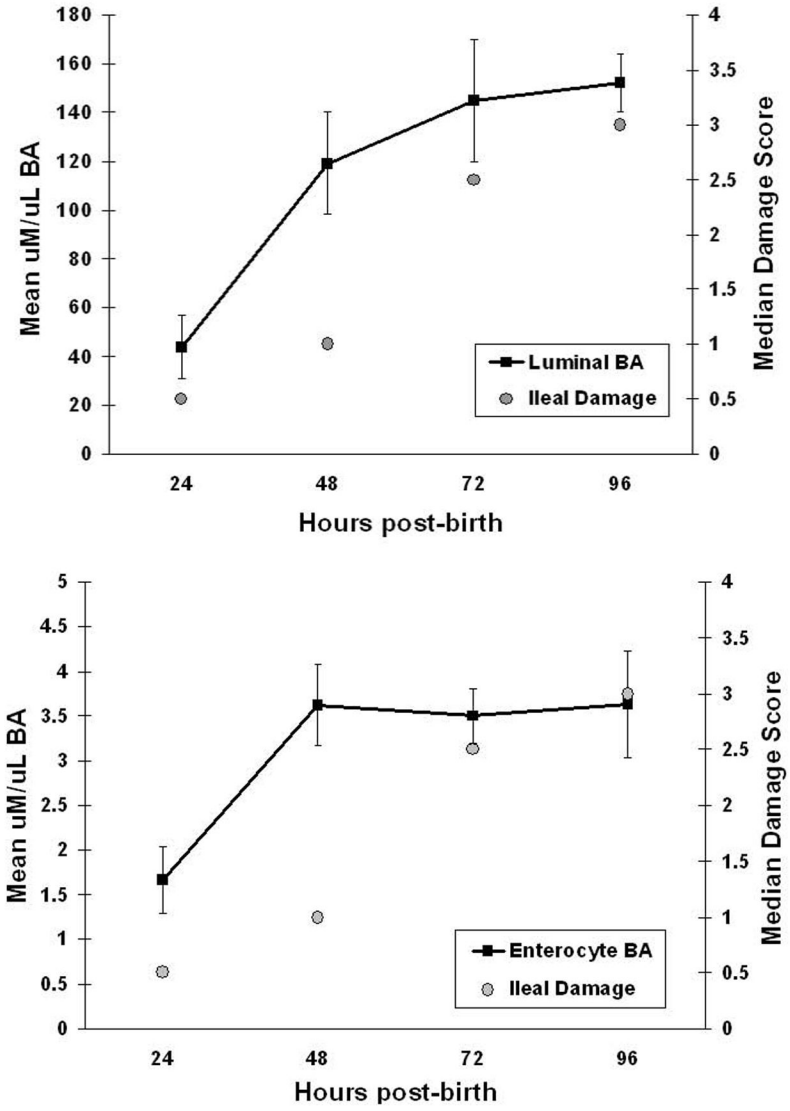

Figure 2. Temporal relationship between BAs and ileal injury

To determine if BA dysregulation causes or is a consequence of ileal injury, never-suckled, prematurely born rats were hand-fed with cow's milk-based formula and exposed to asphyxia and cold stress twice a day to develop NEC. Animals were sacrificed post-birth at 24 (n=6), 48 (n=6), 72 (n=6) or 96 hours (n=5). A 3 cm section of distal ileum from each animal was flushed with 400ul PBS to evaluate total luminal BA levels **(A)**. After flushing with additional PBS, this section of ileum was homogenized in 400ul PBS to determine intra-enterocyte BA levels **(B)**. Total BA levels were determined using the Diazyme Total BA Kit. An adjacent section of distal ileum was evaluated for histological damage by a blinded observer using our previously published NEC scoring system where 0 is normal and 4 indicates full necrosis.

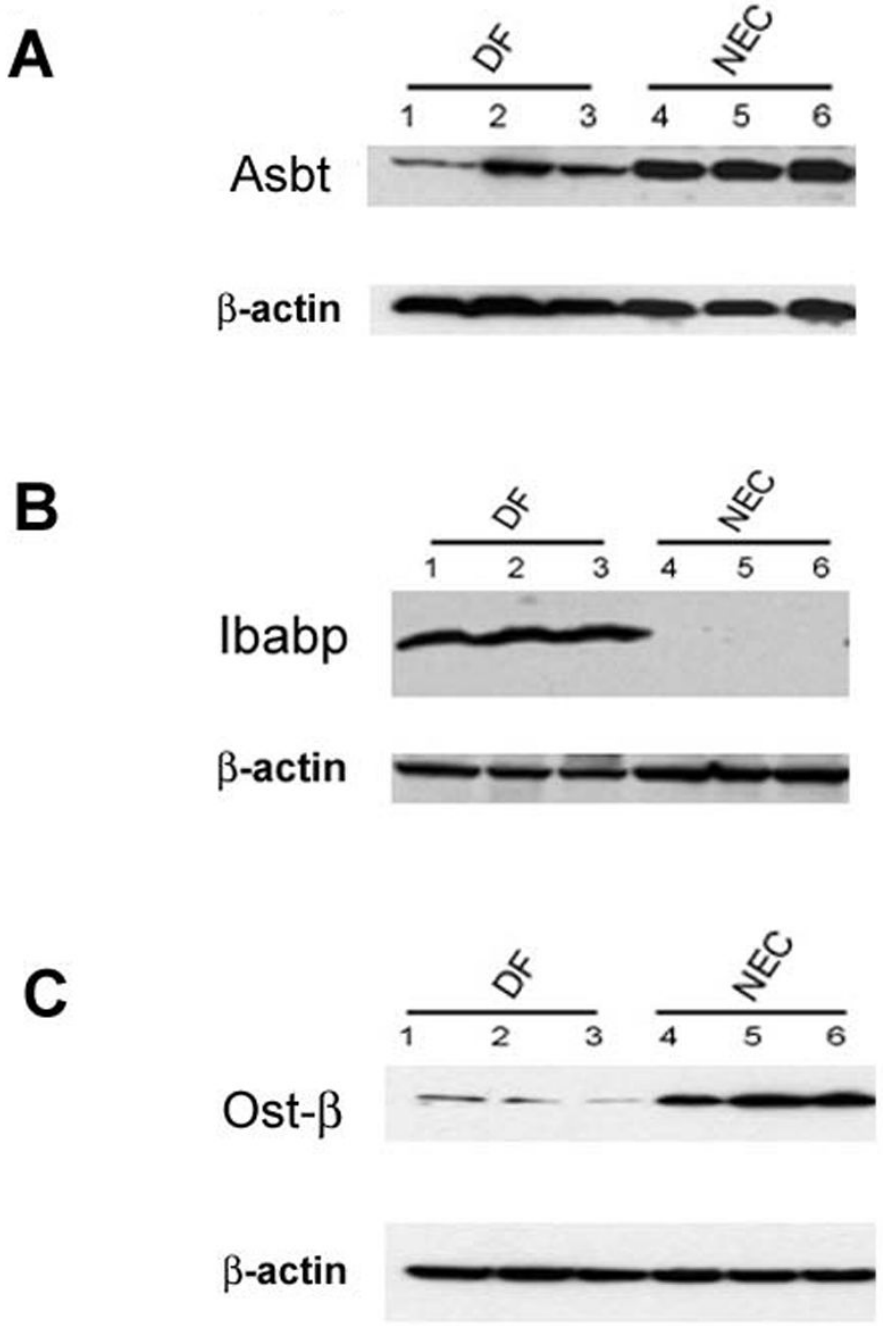

Figure 3. Ileal Asbt, Ibabp and Ost-β

A: Representative ileal protein samples from DF and NEC groups subjected to Western blotting to detect Asbt. **B:** Representative ileal protein samples from DF and NEC groups subjected to Western blotting to detect Ibabp. **C:** Representative ileal protein samples from DF and NEC groups were subjected to Western blotting to detect Ost-β. Data taken from [14].

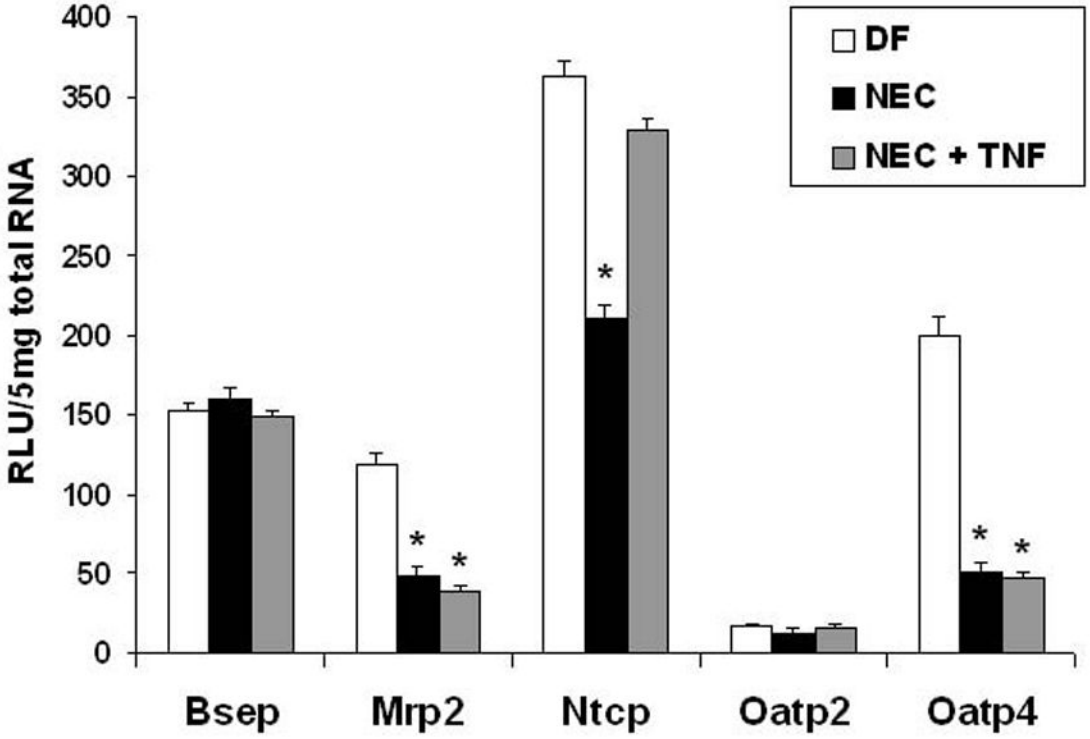

Figure 4. Hepatic BA transporter regulation in NEC

Hepatic mRNA expression of Bsep, Ntcp, Mrp2, Oatp2 and Oatp4 were assessed from DF, NEC and NEC + anti-TNF groups using the Quantigene^{tm} signal amplification assay (because Oatp1 is not expressed until postnatal day 15, Oatp1 expression was not evaluated). * $p \leq 0.01$ versus DF.

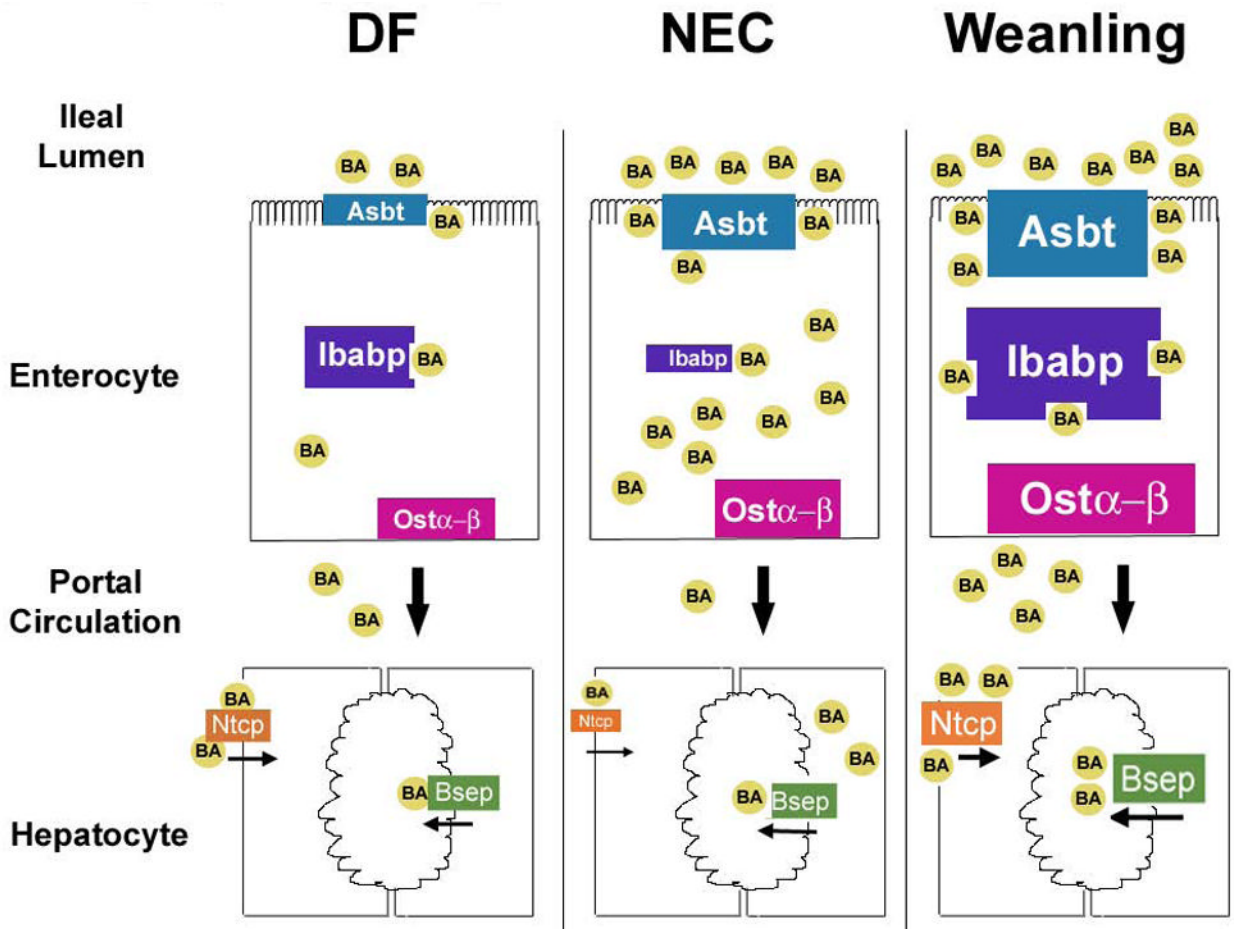

Figure 5. Working Paradigm for BA dysregulation during NEC Pathogenesis

During development of experimental NEC, BAs in the terminal ileum increase which leads to precocious expression of Asbt, the BA transporter responsible for moving BAs from the ileal lumen into enterocytes. This allows BAs to be transported across the apical surface of enterocytes, but transport is compromised because Ibabp is not sufficiently induced in the neonatal intestine. BAs accumulate within the enterocytes and the unsuccessful transport of BAs is reflected in the diminished levels of BAs in portal circulation. The accumulation of toxic BA promotes accelerated cellular damage to the terminal ileum. Diminished BAs reclaimed by the liver promotes continued production of BAs, which exacerbates ileal BA accumulation. In normal, DF animals, BA levels remain low because they consume mother's milk and low levels of ileal BA transporters are adequate to effectively transport these BAs maintaining physiologic BA metabolism. When weaned, rats produce larger amounts of BAs but BA homeostasis is maintained because ileal BA transport accommodates the increased levels of BAs.

Table 1

Ileal Luminal BAs in Experimental NEC.

Group	Total BA (uM/L)	CA (ug/ml)	DCA (ug/ml)
DF	148 ± 54	0.14 ± 0.02	Not Detected
NEC*	722 ± 143[#]	1.07 ± 0.41[*]	0.37 ± 0.002[*]

Total BA levels were determined from ileal luminal flushes using the Diazyme Total BA kit; BA composition of luminal flushes was determined using LC/MS/MS.

[#] $p \leq 0.01$;

$p \leq 0.05$.

Data taken from [14].