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Prenatal administration of heparin-binding epidermal growth factor-like growth factor in an experimental model of necrotizing enterocolitis decreased both incidence and severity of the disease

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ABSTRACT

Background Necrotizing enterocolitis (NEC) is the leading gastrointestinal cause of death in premature infants and causes long-term disabilities. Previously, enteral heparin-binding epidermal growth factor-like growth factor (HB-EGF) administered after birth demonstrated decreased incidence and severity of NEC in a neonatal animal model of NEC. We investigated the potential prophylactic strategy of preventing NEC using prenatally administered HB-EGF.

Methods An HB-EGF (800 µg/kg/dose) dose was injected into pregnant rats via tail vein or intraperitoneal route 2 hours prior to delivery. After cesarean section (C-section) at 21 days' gestation, the rat pups were subjected to the NEC protocol by inducing stressors: hypoxia, hypothermia, hypertonic feeds, and orogastric gavage of lipopolysaccharide (2 mg/kg). Postnatally, pups were monitored for 96 hours and assessed for the development of clinical and postmortem histological NEC.

Results The experimental NEC incidence in untreated, stressed rat pups was 66%. Compared with untreated pups, the maternal administration of HB-EGF correlated with a significant NEC incidence and severity decrease in rat pups. The strongest decrease was seen when HB-EGF was administered via the intraperitoneal route 2 hours prior to C-section (66% vs 31%, *p<0.05). Prenatal HB-EGF administration significantly increased pups' survival after NEC protocol exposure, with the greatest benefit observed in the group that received HB-EGF intraperitoneally 2 hours before delivery.

Conclusions Prenatal administration of HB-EGF decreases the incidence and severity of NEC, preserves gut barrier function and increases survival. This may represent a novel prophylactic clinical strategy for NEC offered to mothers at risk of delivering a premature infant.

Summary box

What is already known about this subject?
- Mortality of necrotizing enterocolitis (NEC) is 20%–30%.
- Intestinal heparin-binding epidermal growth factor-like growth factor (HB-EGF) mRNA expression is correlated to increased NEC resistance.
- Postnatally administered (HB-EGF) decreases the incidence and severity in an experimental animal model of NEC.

What are the new findings?
- Prenatally administered HB-EGF decreases NEC incidence and severity.
- Prenatally administered HB-EGF increases NEC survival.
- Intraperitoneal injection of HB-EGF is the best method for decreasing incidence and severity of NEC.

How might it impact on clinical practice in the foreseeable future?
- Decreased incidence and severity in this animal model of NEC with prenatal HB-EGF administration provides a convincing basis for continued work with HB-EGF. Using HB-EGF in the clinical setting for mothers delivering premature infants at high risk for developing NEC could be a novel prophylactic therapy added as a strategy to the armamentarium in the fight against NEC.

INTRODUCTION

Necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is a destructive disease that has a predilection to affect premature infants. In the group of neonates born weighing less than 1500 g, approximately 10% will develop NEC. The mortality rates for infants with the disease are reported to approach 30%.[1] While techniques to aggressively manage premature infants from a pulmonary standpoint have become a priority, the NEC incidence is rising and soon expected to supersede pulmonary insufficiency as the principle cause of death in premature infants.[2]

Because prematurity is the primary risk factor for NEC, normal expression of specific genes or factors occurring later in gestation have been considered to play an instrumental part in the pathogenesis of NEC.[3]

Heparin-binding epidermal growth factor-like growth factor

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) has been implicated as a member of the EGF superfamily and has been shown to increase resistance to NEC.[3] Feng *et al*[4] examined HB-EGF message ribonucleic acid (mRNA) expression in intestines resected from patients, comparing intestinal sample resection margins from normal intestine with areas afflicted by acute NEC. The authors found that NEC-diseased intestine had HB-EGF mRNA levels significantly lower compared with normal intestine.[4] Based on these findings suggestive that the NEC-afflicted intestine is relatively deficient in HB-EGF, this deficiency could potentially reveal NEC pathogenesis as described by Radulescu *et al*[3] that enterally administered postnatal HB-EGF decreases NEC incidence and severity. We speculated that prenatal administration of HB-EGF would potentially act as a prophylactic avenue against NEC.

HB-EGF was initially discovered in a human-cultured median among macrophages[5] and was later classified as a growth factor member of the EGF family.[6 7] Via binding and activating EGF receptor subtypes (ErbB-1 and ErbB-4), HB-EGF initiates mitogenesis.[7 8] The process continues as HB-EGF binds to the HB-EGF-specific receptor N-arginine dibasic convertase, stimulating chemotaxis.[9] Interestingly, endogenous HB-EGF plays a protective role in a myriad of pathologic conditions and plays a key part in mediating the basic cellular responses such as proliferative stimuli and cellular injury.[3]

Feng *et al*[10] previously explained that *Escherichia coli*-derived HB-EGF delivered enterally decreases the NEC-caused intestinal injury in the experimental neonatal rat model, with the ultimate injury decrease when using the 800 µg/kg/dose.[10] Furthermore, Radulescu *et al*,[11] using an HB-EGF knockout (KO) mouse pup model of NEC, provided evidence that while the loss of the HB-EGF gene increased, the exogenous HB-EGF reverses the susceptibility to NEC.[11] Using human amniotic fluid samples, Michalsky *et al*[12] showed amniotic fluid contains HB-EGF and it may be responsible in part of the gastrointestinal (GI) tract development in utero and gut mucosal injury protection after birth.[12]

Based on these lines of evidence, we tested the hypothesis that prenatal administration of HB-EGF acts as a prophylactic strategy to reduce the NEC incidence and severity.

METHODS

Rat pup model of NEC model

NEC was induced in neonatal rat pups using a modification of the experimental protocol described by Barlow *et al*.[13] Sprague-Dawley timed-pregnant rats were injected prenatally with 800 µg/kg HB-EGF by means of tail vein access or intraperitoneally. This route assures a reliable and reproducible method and is based on previous data demonstrating that HB-EGF can provide intestinal protective effects when delivered either intravenously or enterally.[14] The dose was chosen based on previous postnatal preclinical experiments and reflects the most efficient dose in preventing NEC in neonatal rat pups.[3] Cesarean section (C-section) occurred at 21 days' gestation to deliver newborn rat pups.

Following delivery, pups were nourished using a 1.9-French orogastric (OG) tube with a combination of Similac 15 g and Esbilac 75 cc (200 kcal/kg every 4 hours, receiving 0.1 mL/feed on day of life 0, advancing to 0.5 mL/feed by day of life 5). Pups were exposed to three stressors: hypoxia (100% nitrogen for 90 s), hypothermia (4°C for 10 min), and hypercaloric feeds. In addition, animals received oral lipopolysaccharide (2 mg/kg) during the first feed, replicating experiments previously explained by Mendez *et al*.[15] Pups were closely observed for clinical signs of NEC (abdominal distention, lethargy, bloody stool, respiratory distress). If they presented with any of the listed signs or survived to day 5, they were sacrificed and the intestines were harvested and fixed. The intestines were scored using a standardized histological injury grading system shown in figure 1. Animal experiments were carried out and comply with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines 2.0.[16]

Intestinal histological scoring of neonatal rat pups

Immediately after animals were sacrificed, the GI tract was resected and evaluated for histological signs of NEC. Tissue slides were created for each pup using sections of the duodenum, jejunum, ileum, and colon, fixed in formalin (10% solution), then stained with hematoxylin-eosin (H&E). Microscopic examination for histological presence and/or degree of NEC used a standard histological scoring system (see figure 1).[13 15] All tissue specimens were blindly graded by two board-certified pathologists. Tissues identified to have histological grade 2 or greater were classified as diseased with NEC.

Intestinal mucosal permeability

Fluorescein isothiocyanate (FITC)-labeled dextran (molecular weight 73 kDa, Sigma-Aldrich, St Louis, Missouri) was used as a probe to investigate mucosal permeability. Previous studies have shown that using a 73 kDa dextran molecule assists in reliable mucosal assessments 4 hours after enteral administration.[17] Pups were selected to receive FITC-labeled dextran (750 mg/kg) via OG gavage at 0 and 48 hours of life, followed by sacrifice for blood sample collection 4 hours later. FITC-dextran levels were measured in plasma by spectrofluorometry, with quantification based on a simultaneously established standard curve.[7] Following decapitation, blood (approximately 20 µL) from each rat pup was collected and stored in heparinized microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, Pennsylvania). Phosphate buffered saline (PBS) was used to dilute the volume of obtained

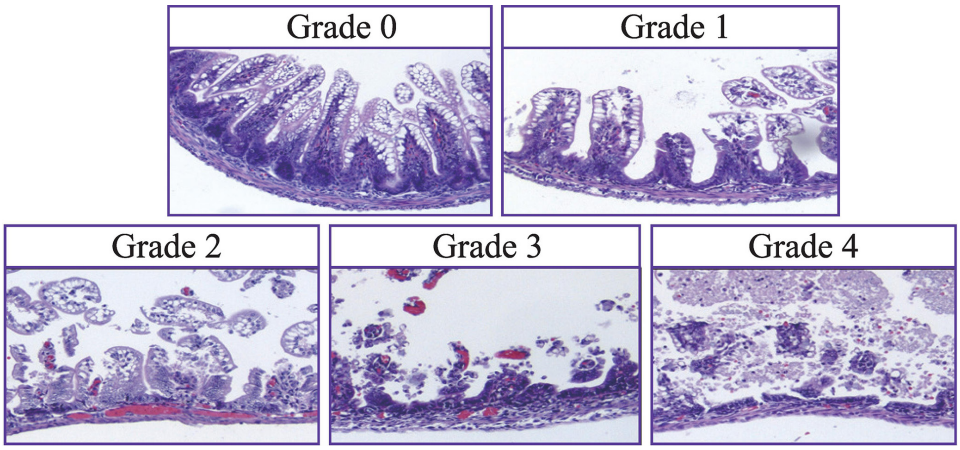

Figure 1 Histological injury grading system used in rat pups subjected to the experimental protocol to induce necrotizing enterocolitis (NEC). Here are representative hematoxylin-eosin (H&E)-stained sections showing: grade 0—normal intestine, grade 1—epithelial cell lifting or separation, grade 2—epithelial cells to the mid-villous level sloughing, grade 3—entire villous necrosis, and grade 4—transmural necrosis, where grade 2 or greater is consistent with NEC. Magnification ×40 (adapted from Mendez *et al*[15]).

blood to a total volume of 200 µL. Based on standard dilution curves, the plasma dextran amount was calculated and the concentration was adjusted by multiplying by 10 to compensate for the 1:10 dilution.

Amniotic fluid HB-EGF quantification

The analysis of the amniotic fluid samples detected the HB-EGF concentration using a commercial enzyme linked immunosorbent assay (ELISA) kit (Thermo Fisher, Waltham, Massachusetts). Prior to ELISA analysis, the amniotic fluid samples underwent ultracentrifugation (15 000 rpm for 15 min) to remove any debris. The optical density was measured at 450 nm using spectrophotometry. Standard curves were generated using recombinant HB-EGF and used to translate the rat amniotic fluid concentration of HB-EGF.

Statistical analysis

The incidence and mortality of NEC were compared between groups using the χ^2 test and the Fisher's exact test. The NEC grade severities were analyzed using the Mann-Whitney U test. Serum concentrations of FITC-dextran were expressed as mean±SE and were compared using the Student's t-test (p values <0.05 were considered statistically significant). Survival time was analyzed by Kaplan-Meier analysis. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software (V.27; 2020).

RESULTS

NEC incidence and histological injury severity

Rat pups originally subjected to the NEC protocol without any prenatal intervention (n=44) had a NEC incidence of 62.5% as well as increased intestinal injury severity when compared with all other groups (figure 2A,B). Animals that received a PBS tail vein injection 2 hours prior to C-section (n=31) had an incidence of 52.6% and had similar intestinal injury severity scores. When looking at the prenatal treatment groups, HB-EGF 800 µg/kg/dose administration by means of tail vein injection at the 24 hours (n=22) and 2 hours (n=25) before birth timelines decreased the incidence and severity of NEC to 36.3% and 31.5%, respectively, when compared with the non-treated control animals (62.9% vs 36.3%, p=0.17; 62.9% vs 31.5%, p=0.036). The most significant results were seen with prenatal delivery of intraperitoneal HB-EGF (n=31). This strategy significantly decreased the NEC incidence to 30% and lowered the severity of the intestinal injury. These results suggest that both the intravenous and intraperitoneal routes at the 2 hours before C-section timeline have similar efficacy. These results were replicated to compare the NEC (n=145), no prenatal intervention group, versus the HB-EGF intraperitoneal group (n=113) and again showed similar results with decreased incidence and severity (66% vs 31%, p=0.026) (figure 2C,D).

Survival

Prenatal HB-EGF administration also conferred an increase in survival with the best results seen in the group of animals that received intervention by means of intraperitoneal injection 2 hours prior to delivery (p=0.038) (figure 3A). Interestingly, the animals that received injection by tail vein at the 24 hours timeline had similar survival benefit (p=0.003). We could not correlate the decreased incidence of NEC results seen in the 2 hours' tail vein HB-EGF injection group with increased survival as these rat pups had similar results to the control animals (p=0.61). While we have no clear understanding why NEC incidence did not correlate with survival for the 2 hours' tail vein injection HB-EGF group, we hypothesize based on our experiment notes that it may have been related to increased aspiration at the time of feeding. During the repetition of this study with a focus on the NEC and HB-EGF intraperitoneal groups, again, there is a clear

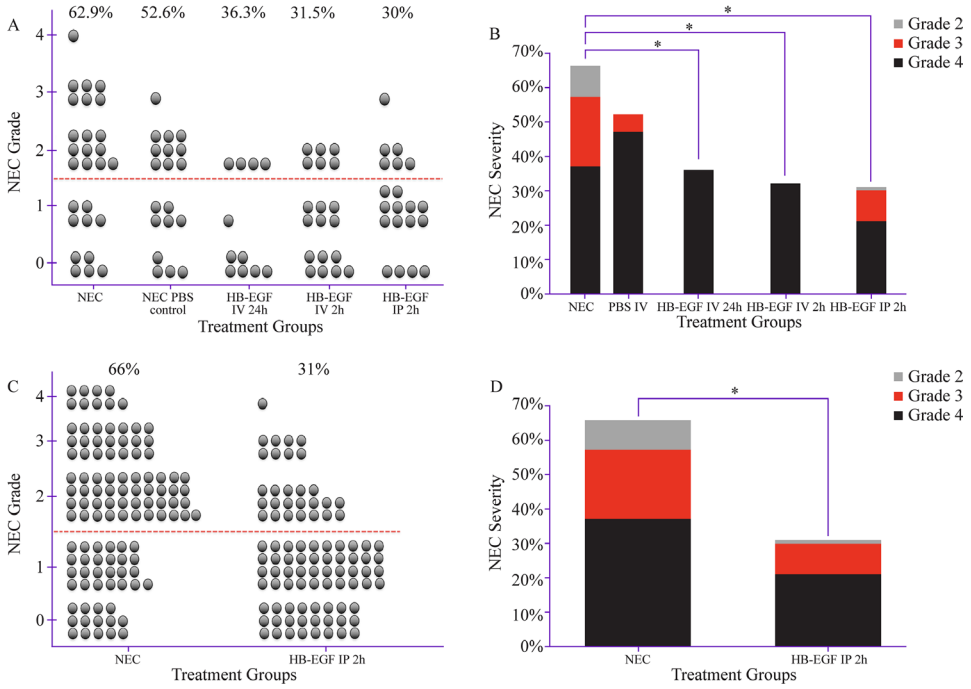

Figure 2 Incidence and severity of necrotizing enterocolitis (NEC) in control and heparin-binding epidermal growth factor-like growth factor (HB-EGF)-treated animals. (A) NEC incidence—NEC group rat pups subjected to NEC protocol without prenatal HB-EGF administration (n=44); phosphate buffered saline (PBS) intravenous—tail vein injection of PBS 2 hours before cesarean section (C-section) followed by NEC protocol (n=31); HB-EGF intravenous 24 hours—tail vein injection of HB-EGF 800 µg/kg 24 hours before C-section followed by NEC protocol (n=22); HB-EGF intravenous 2 hours—tail vein injection of HB-EGF 800 µg/kg 2 hours before C-section followed by NEC protocol (n=25); HB-EGF intraperitoneal 2 hours—intraperitoneal injection of HB-EGF 800 µg/kg 2 hours before C-section followed by NEC protocol (n=31). Each dot represents an individual rat pup. (B) NEC severity. The percent of animals with NEC grade 2—black, grade 3—red, and grade 4—gray bars (*p<0.05). (C) NEC incidence; control versus HB-EGF intraperitoneal. Replication of the study to directly compare NEC control (n=145) and HB-EGF intraperitoneal (n=113) incidence (*p<0.05). (D) NEC severity; control versus HB-EGF intraperitoneal. Replication of the study to directly compare NEC control and HB-EGF intraperitoneal severity (p<0.05).

survival benefit in the HB-EGF intraperitoneal group (p=0.009) (figure 3B). When taken together, intraperitoneal route of HB-EGF administration at 2 hours prior to delivery correlates with the greatest decrease observed for NEC incidence and histological severity.

Gut barrier function

Based on our best NEC incidence and severity as well as survival data, we wanted to further offer mechanistic evidence that intraperitoneal injection prenatally achieves these results by preserving gut permeability. We evaluated this at the 0 and 48 hours' time points by comparing the control animals that received no prenatal intervention to the rat pups that received HB-EGF prenatally by means of maternal intraperitoneal injection. Our results show that HB-EGF intraperitoneal group was able to preserve gut barrier function at the 0 and 48 hours when compared with control NEC animals (p=0.014; p=0.01) (figure 4).

Amniotic fluid

The intraperitoneal injection proved to be the best method for decreasing incidence and severity while increasing survival; thus, the amniotic fluid of intraperitoneal HB-EGF-treated pregnant rats was compared with the control, non-HB-EGF-treated rats. The repeated experiments used to confirm the results account for the increased number of animals in this group. In the case of intraperitoneal injection, the average concentration of HB-EGF was significantly higher, 156.3 vs 52.8 pg/mL (figure 5).

DISCUSSION

In this paper, we have provided evidence that prenatal administration of HB-EGF can reduce the severity of NEC in a rat pup model. Growth factors play an important role in the development and maintenance of the GI tract as well as its response to injury. Through a variety of in vitro, in vivo, and human experiments, we have learnt what growth factor influences demonstrate. Specifically, EGF and HB-EGF demonstrate some favorable effects in both experimental and clinical intestinal injuries, including NEC.[18 19]

HB-EGF has a protective effect on the intestines by protecting them from injury, such as that caused by NEC. The mouse and rat animal models have shown beneficial effects which may be translated to humans. Human small intestine resected for suspected NEC showed higher HB-EGF mRNA levels at the healthy edge of the resection

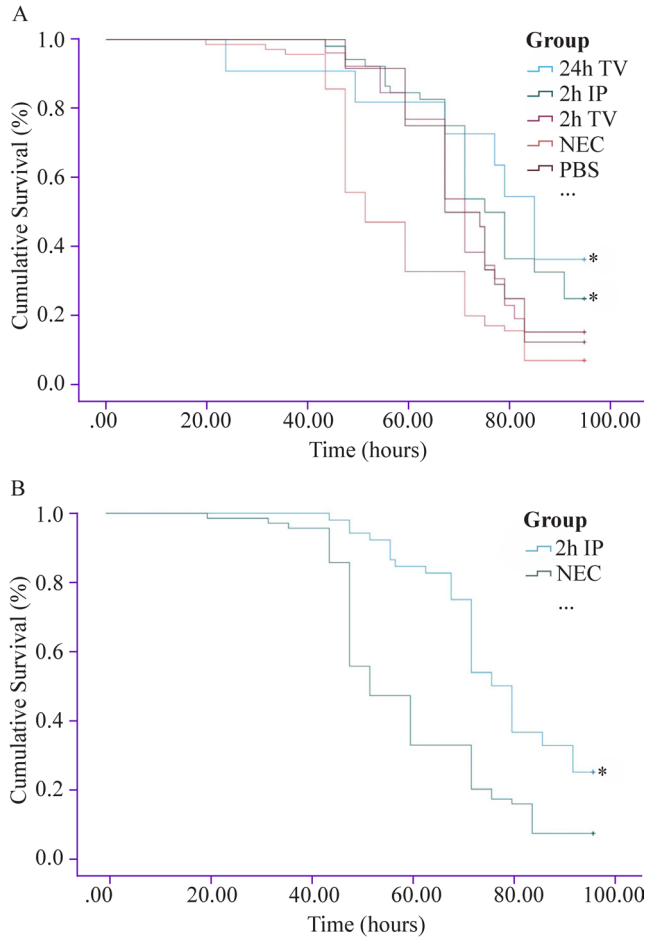

Figure 3 Kaplan-Meier survival analysis for time. (A) The median survival time in the heparin-binding epidermal growth factor-like growth factor (HB-EGF) prenatally treated animals by means of tail vein injection 24 hours prior to delivery and intraperitoneal injection 2 hours prior to delivery was significantly longer than that of the necrotizing enterocolitis (NEC) control animals. (B) When the experiment was replicated, controls were compared against intraperitoneal injection. Again, the median survival time in the HB-EGF-treated rats was significantly longer than controls (*p<0.05). PBS, phosphate buffered saline.

margins compared with that which was adjacent to the NEC-afflicted tissue. This suggests that an absence or decreased HB-EGF expression may contribute to the pathogenesis of NEC, or healing after NEC intestinal injury.[4 19]

Both amniotic fluid and breast milk contain growth factors yielding potential effects to bathe intestinal cells before and after birth.[19] Fetuses are naturally exposed to HB-EGF in utero during intestinal tract development, and postnatally, infants exposed are exposed to HB-EGF via breast milk.[12] Michalsky *et al*[12] showed HB-EGF is present in amniotic fluid obtained from pregnant females less than 36 weeks of gestation. These results, combined with the data from the postnatal HB-EGF administration paper where NEC incidence and severity were significantly reduced by OG administration, led us to investigate a prenatal prophylactic pathway.[3]

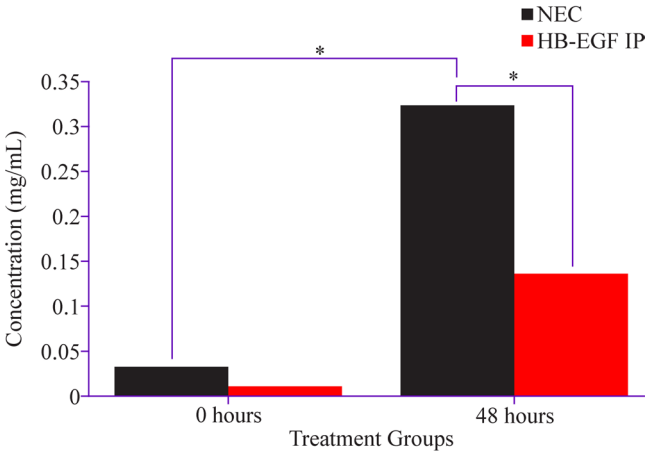

Figure 4 Effect of prenatal heparin-binding epidermal growth factor-like growth factor (HB-EGF) on gut barrier function. Gut barrier function was determined by measuring serum fluorescein isothiocyanate (FITC)-dextran levels 4 hours after enteral administration of FITC-dextran compared with the necrotizing enterocolitis (NEC) control animals at 0 and 48 hours after injection (*p<0.05). Animals that received prenatal HB-EGF by means of intraperitoneal injection were labeled as HB-EGF intraperitoneal (NEC vs HB-EGF intraperitoneal at 0 hour *p=0.016, NEC vs HB-EGF intraperitoneal at 48 hours *p=0.01).

A prenatal administration of HB-EGF can be a prophylactic strategy for high-risk pregnancies that result in premature deliveries of newborns at high risk for NEC. The envisioned strategy would resemble the prenatal administration of corticosteroids prenatally to prevent respiratory distress syndrome and to accelerate fetal lung maturation[20]; however, it is unclear what

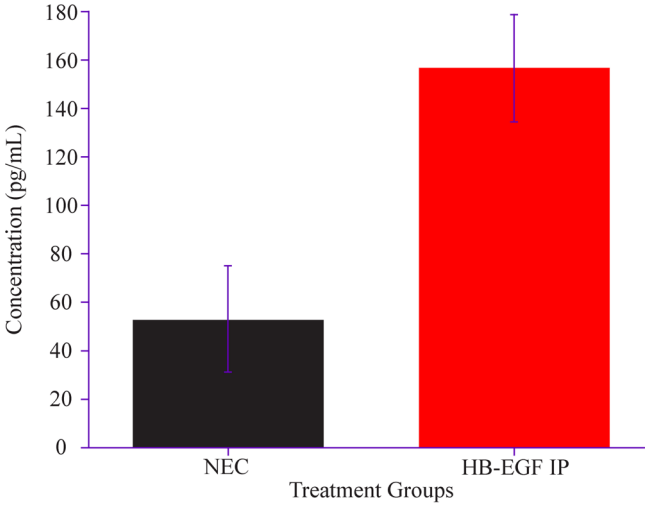

Figure 5 Amniotic fluid. After maternal intraperitoneal injection of heparin-binding epidermal growth factor-like growth factor (HB-EGF), amniotic fluid was collected 2 hours later during the cesarean section. The HB-EGF concentration in these treated rats was compared with the maternal amniotic fluid in the control, non-HB-EGF-treated rats. The concentration was significantly higher in the therapy group than non-therapy group, 156.3 vs 52.8 pg/mL. NEC, necrotizing enterocolitis.

dose, timing, and frequency would be needed for such prenatal approach and, most importantly, what, if any, is the potential maternal toxicity. Identifying premature infants that will develop NEC is very difficult, but based on the known predisposing factors for NEC development, it is possible to target a certain population. This novel prophylactic strategy could be offered to this population; mothers who are delivering a premature infant less than 28 weeks of gestation with a higher risk of developing NEC. While compared with ovarian cysts and normal ovaries, HB-EGF expression is more enhanced in ovarian cancer tissue, it remains to be determined if a single dose or a time point of HB-EGF administration to a pregnant mother will have postnatal short or long-term maternal risks.[21 22]

The injury of NEC on neonatal intestine is associated with increased wall permeability, decreased intestinal wall barrier function, and subsequent bacterial translocation.[23] In mice HB-EGF KO experiments, HB-EGF was essential for demonstrating gut barrier function preservation largely due to the inhibition of cell interactions between neutrophils and endothelial cells.[24] In the experimental animal models inducing intestinal injury in NEC, exogenous administration of HB-EGF given enterally preserves the gut barrier function.[19 25] Our results show that HB-EGF is able to preserve gut barrier function when administered prenatally, which we believe may explain, in part, the mechanism behind decreased NEC incidence.

With regard to the route of administration, we chose the intravenous and the intraperitoneal routes based on previous data demonstrating that HB-EGF can serve to have protective effects in the intestine when administered.[14] The importance of testing the amniotic fluid from the rats with intraperitoneal injection is to prove the efficacy of the delivery route. Of note, the intravenous route would not result in an increased HB-EGF concentration in the amniotic fluid because HB-EGF is limited to the systemic circulation in the pup. Thus, this would be meaningless to investigate the amniotic fluid after intravenous injection. Intragastric and intravenously administered HB-EGF has had well-described pharmacokinetics[26]; however, the absorption and distribution of intraperitoneally administered HB-EGF remain a mystery.

In an experimental 125I-labeled HB-EGF intravenous bolus delivery, the distribution half life was noted to be 0.8 min and an elimination half life was 26.67 min.[26] After gastric 125I-labeled HB-EGF administration, the absorption phase was noted to have a 2.38-hour half life and the elimination phase had an 11.13-hour half life, with an overall bioavailability of 7.8%. The rank order of normalized HB-EGF tissue in decreasing distribution was solid abdominal organs (liver, kidney, spleen) and lungs, then heart and intestines, followed by the testes and brain. Two hours following the administration coincided with increased radioactivity in intestine, brain, and testes was increased, the liver, lungs, kidneys, heart, and spleen showed gradually decreased radioactivity from 0.5 to 8 hours.[26]

Large multicenter assessments of the mortality of surgical NEC showed a mortality of approximately 35%.[27 28] A prophylactic strategy for NEC would make most sense in terms of reducing severity and incidence to ultimately result in increased survival. Our data align with previous reports that have shown HB-EGF administration in a postnatal fashion by means of OG gavage significantly increases the survival in the neonatal rat NEC model.[10] Our laboratory is in the process of correlating these improved survival data with long-term neurodevelopmental benefits.

This study provides evidence that HB-EGF administered prenatally decreases the incidence and severity of NEC and increases the survival rate in the neonatal rat model. Additionally, the mechanisms responsible for this effect are potentially rooted in gut barrier function preservation. Based on these results, these advantages of HB-EGF administration could potentially represent a viable prophylactic strategy for high-risk pregnancies.

In conclusion, we have shown that prenatal HB-EGF administration decreases the NEC incidence and severity, preserves gut barrier function, and increases survival. The fundamental goal of this research is to find a clinical use of HB-EGF for mothers at risk of delivering a premature infant at high risk for NEC as a novel prophylactic therapy. Our current work adds a novel strategy to the armamentarium in the fight against NEC. We hope that clinical trials will emerge to investigate the translational use of HB-EGF therapy in humans.

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Contributors MAS and YSM contributed to investigation, data curation, and writing–original draft, review and editing. FAK carried out investigation and writing–review and editing. RP and CWZ performed data curation and validation. CGW was involved in conceptualization, methodology, writing–review and editing. AR was responsible for conceptualization, methodology, supervision, validation and writing–review and editing. MAS and AR serve as the guarantors for this manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval All experiments were conducted according to the guidelines for the ethical treatment of experimental animals after ethical approval by the Institutional Animal Care and Use Committee (IACUC 18-041) at the Loma Linda University.

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Data availability statement Data are available upon reasonable request.

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