Epidermal Growth Factor Reduces the Development of Necrotizing Enterocolitis in a Neonatal Rat Model


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Running Title: Enteral EGF Reduces NEC

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Abstract

Necrotizing enterocolitis (NEC) is the most common gastrointestinal disease of prematurely born infants. Maternal milk plays an important protective role against NEC development and is the major source of epidermal growth factor (EGF) for neonates. The aim of this study was to examine the effect of orally administered EGF on the incidence of NEC in a neonatal rat model. Newborn rats were artificially fed either with growth factor-free rat milk substitute (RMS) or RMS supplemented with 500 ng/ml of EGF (RMS+EGF). Experimental NEC was induced by exposure to asphyxia and cold stress. Development of NEC was evaluated by gross and histological scoring of damage in the ileum. Ileal EGF-receptor (EGF-R), EGF and transforming growth factor-alpha (TGF-α) mRNA expression was assessed by reverse-transcription competitive-PCR and the EGF-R was localized by immunohistochemistry. EGF supplementation of formula reduced the incidence and severity of NEC in rats (NEC, 13/16 RMS vs. 4/13 RMS+EGF). Ileal EGF-R mRNA expression was markedly increased in the RMS group compared to RMS+EGF. Enhanced EGF-R expression in the RMS group was localized predominantly in the epithelial cells of injured ileum. These data suggest a new potential therapeutic approach for the prevention and treatment of NEC.
Keywords: intestinal injury; inflammation; neonatal intestine; artificial feeding

Abbreviations: EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; NEC, necrotizing enterocolitis; PCR, polymerase chain reaction; RT, reverse transcription; TGF-a, transforming growth factor-alpha
Neonatal necrotizing enterocolitis (NEC) is the most common gastrointestinal (GI) disease of premature infants affecting 3-5000 neonates in the USA each year (31). Prematurity, enteral feeding, bacterial colonization, and intestinal hypoxia-ischemia are considered the major risk factors for the development of this disease (6). In spite of extensive epidemiological, clinical and basic research, the pathogenesis of NEC is unknown and there is no effective preventative treatment for this disease (40). During the last three decades, several animal models have been developed to study the etiology of NEC, (9) with the neonatal rat model considered one of the best means to study this disease (5, 9, 45). In this model, NEC is induced in newborn rats by enteral feeding of artificial formula coupled with asphyxia stress. (2, 5, 7). This treatment simulates the most common conditions for NEC in humans; formula feeding, immature gastrointestinal tract, and hypoxia. Since the majority of infants with NEC are fed enterally with formula before disease onset, it has been suggested that the composition of the milk is the key factor in preventing the development of NEC (24). Indeed, the protective role of maternal milk in NEC pathogenesis has been reported both in human (27, 41) and animal studies (2, 10). These findings stimulated the search for various components of milk that might be responsible for protection against NEC.

Mammalian milk contains a large number of biologically active substances that directly affect gut maturation and mucosal protection (14). One of these substances, epidermal growth factor (EGF), is a potent peptide that produces a variety of biologic responses, such as enhanced proliferation and differentiation of epithelial cells. In addition, significant effects of EGF on the healing of damaged gastrointestinal mucosa or on intestinal adaptation after injury have been reported in a number of studies (see review (23)). EGF is detected in many body fluids, including colostrum (60) and milk (18) and during the early postnatal period, colostrum and maternal milk are the major sources of EGF for the developing neonate (42). The biological actions of EGF are mediated through binding to its specific receptor, EGF-R, which is distributed throughout the fetal
and neonatal gastrointestinal tract (8). Diminished levels of salivary and serum EGF in human babies with NEC (19, 48) and the development of hemorrhagic enteritis pathologically similar to NEC in EGF-R knockout mice (28), suggest an important role of EGF insufficiency in the pathogenesis of NEC. In addition, a critically ill child with NEC-like symptoms was successfully treated by continuous intravenous infusion of EGF (51).

The aim of the present study was to test the effects of enteral administration of EGF on the development of NEC in neonatal rats. Our hypotheses were that enteral administration of EGF would reduce the development and incidence of neonatal NEC and the healing effect of EGF would be mediated through interaction with the EGF-R at the site of intestinal injury. We induced experimental NEC in neonatal rats using artificial formula feeding coupled with exposure to asphyxia/cold stress (2, 5). We evaluated the effects of enteral administration of EGF on the development and incidence of NEC, gene expression and cellular localization of the EGF-receptor at the site of injury, and changes in ileal endogenous synthesis of two major EGF-R ligands - EGF and transforming growth factor-alpha (TGF-α) peptides.
Material and Methods

Animal Model and Diets

The study protocol was approved by the Animal Care and Use Committee of the University of Arizona (A-324801-95081). Sixty Sprague-Dawley neonatal rats (Charles River Labs, Pontage, MI), originating from 9 different litters, were used in three separate experiments. Newborn rats were collected from their mothers immediately after birth to prevent suckling of maternal milk. Animals were weighed, then placed in an infant incubator to control body temperature and assigned to 3 experimental groups: artificially fed with growth factor-free rat milk substitute (RMS), artificially fed with RMS supplemented with 500 ng/ml rat EGF (Harlan Bioproducts, Indianapolis, IN) (RMS+EGF), or littermates fed by their mothers (Dam Fed). In addition, non-stressed dam fed littermates served as physiologic controls in these studies. To develop clinical and pathological signs of NEC, rat pups from all 3 experimental groups (RMS, RMS+EGF, Dam Fed) were stressed twice daily with asphyxia by breathing 100% nitrogen gas for 60 sec followed by cold stress at 4°C for 10 minutes as originally described by Barlow et. al (1, 2) and recently modified by Caplan et al. (5).

For the first 48 hours of life, rat pups were hand-fed every 3-4 hours using a silicone rubber tube (0.2 mm) with 0.1 ml of RMS prepared as described previously (13, 50). This method is time and labor demanding yet essential because gastrostomy of newborn rats is associated with very high surgery-related death rate. After 48 hours, the hand-feeding method was replaced with mechanized artificial feeding for additional 48 hours as described previously (13). Body weights were recorded daily. Two times daily, urination and defecation were induced by gentle stimulation of the anogenital region and stool was collected for future measurements. Animals that developed abdominal distention, respiratory distress and lethargy during first 96 hours of experiment were terminated. After 96 hours, all surviving animals were terminated via decapitation.
NEC Evaluation

After decapitation, the gastrointestinal tract was removed. The small intestine was visually evaluated for typical signs of NEC such as intestinal discoloration, intestinal hemorrhage, ileal distention, and ileal stenosis. Results of macroscopic visual evaluation were recorded and pictures of small intestine and colon was taken for objective comparison of samples from different studies using a digital still camera MVC-FD91 (Sony, Japan). The small intestine was then divided into two halves - jejunum and ileum. A 3 cm section of distal ileum 4 cm proximal to the ileocecal valve was cut, fixed in 70% ethanol, embedded in paraffin, sectioned (4-6 µm/section), and counterstained with hematoxylin and eosin (H&E) for histological evaluation of NEC. The rest of the ileum was snap frozen in liquid nitrogen for DNA, protein, and mRNA measurements. Histological changes in the ileum were scored by a blinded evaluator and graded as follows: normal (0) - no damage; mild(1+) – slight submucosal and/or lamina propria separation ; moderate (2+) – moderate separation of submucosa and/or lamina propria, and/or edema in submucosal and muscular layers; severe (3+) – severe separation of submucosa and/or lamina propria, and/or severe edema in submucosal and muscular layers, regional villus sloughing ; or necrosis (4+) – loss of villi and necrosis(30).

Stool Occult Blood

The detection of occult blood in the stool was performed using the guaiac test. A small quantity of stool was smeared on filter paper and mixed with 100 µl of glacial acetic acid. Guaiac solution (100 µl of 1 g of gum guaiac in 5 ml of ethanol) and 100 µl of 3% H₂O₂ were added and mixed. The appearance and intensity of any blue or blue-green color was evaluated within 1 to 5 min. and scored on a scale of 0 to 4 by two independent, blinded evaluators. Deep blue color appearing within 1 min. was graded 4. Lesser degrees of color development occurring within 1 to 5
min. were graded 3 to 1 (3 - strong signal, 2 - moderate signal, and 1 - small traces of blood in stools). No blood in stools (no color development) was scored as 0. To minimize the variability in analysis of grading, a no blood sample (0) and a systemic blood sample (4) were assayed as a standard range.

**DNA and Protein Measurements**

Tissue DNA and protein content was measured as we described previously (13). Briefly, total DNA content in the ileum was assayed by the diphenylamine method of Burton (4) and determined by spectrophotometry. Assays for total protein content in the ileum (26) were determined by spectrophotometry (SpectraMAX Plus, Molecular Devices, Sunnyvale, CA).

**Immunohistology of the EGF-R**

Samples of distal ileum (2-3 cm) were fixed overnight in 70% ethanol, processed, paraffin-embedded, and microtome-sectioned at 4-6 µm. Sections were deparaffinized in xylene and rehydrated in serial dilutions of 100% - 50% ethanol and water. Antigen unmasking was achieved using 0.05% saponin (Sigma, St. Louis, MO) for 30 minutes, followed by blocking of endogenous peroxidase using 0.1% hydrogen peroxide for an additional 30 minutes. Sections were blocked with 1.5% goat serum (Vector Laboratories, Burlingame, CA) in PBS for 30 minutes, then incubated with 1 µg/ml rabbit anti-EGF-R polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 30 minutes washed with PBS 3x, and incubated with a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) for 30 minutes. After 3 PBS washes, the Vectastain Elite ABC reagent (Vector Laboratories) was then applied for 30 minutes, slides were washed 3 x with PBS, then DAB (Sigma) was utilized as the substrate. Sections were then counterstained with hematoxylin, dehydrated, and coverslipped (58). Control
sections were treated with the same procedure except that they were incubated with 5µg/ml rabbit Ig (Sigma) instead of the anti-EGF-R antibody. No staining was detected in control slides. Sections from all three experimental groups were stained for EGF-R at the same time and stained sections were evaluated by a blinded observer.

**RNA Extraction and RT Competitive-PCR Assay**

Total RNA was isolated from tissue using the RNeasy Mini Kit (Qiagen, Santa Clarita, CA) as described in the manufacturer's protocol. All samples were incubated with RNase-free DNase (20 U per reaction) for 10 min. at 37°C to eliminate DNA contamination. RNA concentration was quantified by UV spectrophotometry at 260 nm (A$_{260}$) and the purity was determined by the A$_{260}$/A$_{280}$ ratio (SPECTRAmax PLUS, Molecular Devices, Sunnyvale, CA). The integrity of RNA samples was verified by electrophoresis on 1.2% agarose gel containing formaldehyde (2.2 M) and ethidium bromide in 1 x MOPS buffer {40 mM MOPS (pH 7.0), 10 mM sodium acetate and 1 mM EDTA (pH 8.0)}. The reverse transcription (RT) competitive-PCR assay was used to quantify intestinal EGF-R, EGF, and TGF-$\alpha$ mRNA levels as previously described in detail (12, 15).

**Statistics**

Statistical analysis of the results was performed by one-way analysis of variance (ANOVA) followed by Fisher PLSD using the statistical program Statview for Macintosh computers (Abacus Concepts Inc., Berkeley, CA). A value of p < 0.05 was considered significant at the 95% confidence level. All data in the figures are means ± SEM.
Results

Effects of EGF on Body Weight Gain and Ileal Parameters

Body weights in the RMS group gradually decreased during the study period while rats fed RMS+EGF were able to maintain the same body weight during the same time period (Figure 1). Differences in body weight between RMS and RMS+EGF groups were statistically significant at 72 hours (p < 0.05) with the difference increasing by 96 hours (p < 0.01). Dam Fed pups showed steady increases in body weight throughout the experiments. There were no differences in body weight gain of pups in the asphyxia/cold stressed Dam Fed group compared to their non-stressed dam fed littermates (results not shown). Ileal protein content was similar in all three experimental groups (Table 1). DNA content was significantly higher in Dam Fed rats compared to both RMS and RMS+EGF groups, but there was no statistical difference between the RMS and the RMS+EGF groups (Table 1).

Effects of EGF on the Incidence of Necrotizing Enterocolitis

Animals developed visual signs characteristic of intestinal injury between 72 - 96 hours after the beginning of the experiments. These signs included abdominal distention, discoloration of the abdominal wall and occult blood in the stool. Because progression of intestinal damage typically resulted in death, we performed guaiac measurements at 72 rather than 96 hours. Results from these measurements are summarized in Table 1. The RMS group exhibited a significantly stronger signal (p<0.0001) for the presence of blood in the stool compared to RMS+EGF group. Dam Fed animals had no or minimal blood in the stool.

Macroscopic and microscopic examination of the gastrointestinal tract showed clear evidence of intestinal damage similar to neonatal NEC. Macroscopically, the jejunum and ileum exhibited
severe inflammation, hemorrhage and discoloration (Figure 2). The degree of damage varied between pink/red to dark purple/black discoloration in NEC rats (Figure 2B) compared to the yellow/green appearance of normal gut from healthy rat pups (Figure 2A). The ileum was generally more severely affected than the jejunum; typical intestinal distention and stenosis were located predominantly in ileum (Figure 2B, detail). In addition, luminal contents of the ileum were dark and bloody. In animals with moderate progression of NEC, pathological changes in the small bowel were patchier with scattered areas of hemorrhage, distention, and stenosis in the ileum. Histologic analyses of ileal segments were performed using a scoring system from 0 to 4+ (Figure 3) to determine the severity of NEC. Results from these measurements are shown in Figure 4. In the RMS group, histological findings in most animals showed significant pathological changes in ileal structure (mean NEC score = 2.6). The degree of ileal damage was significantly reduced (p < 0.001) in rats fed with RMS+EGF (mean NEC score = 1.3) compared to the RMS group. Dam Fed pups showed no or minimal pathological changes in ileal morphology (mean NEC score = 0.43). Comparison of the overall incidence of NEC revealed 81% of RMS pups developed significant intestinal abnormalities described as moderate, severe or full necrosis. Supplementation of EGF into formula resulted in a dramatic, 50% reduction in the incidence of NEC. Dam Fed animals showed no changes or abnormalities in the small intestinal structure resembling the development of NEC (Table 2).

**Ileal EGF-R, EGF and TGF-α mRNA levels**

To evaluate changes in gene expression of the EGF-R, ileal steady state mRNA levels were measured using RT Competitive-PCR (Figure 5A). Ileal EGF-R mRNA levels were markedly increased (approximately 7-8-fold) in the RMS group compared with the Dam Fed group. Supplementation of the RMS diet with EGF was associated with a statistically significant decrease in
ileal EGF-R content compared to RMS alone (p < 0.01), with the mRNA level in this group intermediate between the mRNA levels of Dam Fed and RMS fed rats. There was no difference in the EGF-R mRNA level between Dam Fed and non-stressed dam fed littermates (results not shown). Changes in endogenous synthesis of two major EGF-R ligands – EGF and TGF-α - revealed no statistically significant changes in either EGF and TGF-α mRNA levels in the ileum of RMS animals compared with either RMS+EGF or Dam Fed pups (Figure 5B, 5C).

**Histologic Localization of the EGF-R**

Histologic examination of ileal samples from Dam Fed pups revealed low levels of EGF-R expression in the apical and basolateral membranes of the villi (Figure 6A, 6D). The pattern of EGF-R localization in the RMS group was similar to that seen in Dam Fed animals, except there was very strong staining of the apical membrane and moderate staining of the basolateral membrane of the villi enterocytes (Figure 6B, 6E). In contrast, animals given RMS + EGF had markedly decreased expression of the EGF-R in the apical and basolateral membranes of the villi compared to the RMS group (Figure 6C, 6F).
**Discussion**

Our present study provides, for the first time, evidence that enteral administration of EGF reduces the development and the incidence of NEC in a neonatal rat model. Moreover, the increased expression of EGF-R in damaged ileum indicates that the healing effect of exogenous EGF is mediated directly at the site of intestinal injury. Interestingly, the development of NEC has no effect on the endogenous production of EGF and TGF-α ligands in damaged ileum.

Necrotizing enterocolitis is a disease of premature babies. The reasons for a predilection for prematurity are unclear, but an immature mucosal barrier and immune response likely contribute to the premature neonates susceptibility. Amniotic fluid concentrations of EGF gradually increase during pregnancy, with the highest level achieved at the end of normal gestation period (22). EGF is also present in high concentrations in colostrum and breast milk of many mammalian species (25, 34) and during the suckling period, milk-borne EGF is the major source of EGF for the developing neonate (42). In contrast, EGF is absent in all commercial formulas. In suckling animals, supplementation of EGF into formula enhances the growth of stomach and the small intestine (3), induces precocious maturation of intestinal brush-border disaccharidase activities (32), and modulates intestinal nutrient transport (33). Because the incidence of NEC is, in the majority of cases, related to formula feeding of newborn babies, we proposed that the absence of EGF in developing gut increases the potential to develop this disease. Results from the present study support this hypothesis and suggest a critical role of milk-borne EGF in the maintenance of gut integrity and healthy development.

The first report relating EGF and intestinal necrosis was reported in England where an 8-month old child with intestinal necrosis similar to NEC was successfully treated with continuous infusion of EGF (51). Scott et al. (46) have shown significant elevation of urinary EGF levels in neonates diagnosed with NEC that they suggest might result from increased absorption of EGF.
through the damaged intestinal mucosa. Further, markedly diminished serum and salivary EGF levels have been reported in premature infants with NEC (19, 48). These data suggest administration of exogenous EGF may provide an effective means to prevent or treat this disease. In this study, we demonstrate the supplementation of milk formula with EGF markedly reduces the development and severity of NEC. The importance of this finding is particularly significant because EGF was given orally. The use of growth factors for the treatment of gastrointestinal disease is always associated with concerns over their potential risks. Systemically administered growth factors could induce proliferation in other regions of the body that harbor premalignant cells (34). Thus, enteral administration of EGF enables use of a higher dose of EGF and helps deliver growth factor directly into the site of intestinal injury. Rat milk contains 30-50 ng/ml of EGF (43, 55). Our preliminary study has indicated that supplementation of formula with physiological dose of EGF (100 ng/ml) has markedly lower efficiency on the prevention and severity of NEC (data not shown). In addition, maternal milk contains a variety of proteins, such as casein, which can act as competitive substrates for proteolytic enzymes and specific proteolytic inhibitors, that can protect luminal peptide growth factors from degradation (35, 38). Moreover, intestinal proteolytic activity is significantly lower in the early postnatal period compared to adulthood (20). Therefore, supplementation of higher dose of EGF into milk formula can protect EGF against this inherent proteolytic degradation in the stomach and in the small intestine and improve the efficiency of injury treatment during the perinatal period of life.

The mechanisms underlying the protective effects of EGF in injured mucosa are not clearly understood. However, the involvement of the EGF receptor in the biological action of EGF has been extensively studied (8). The presence of EGF-R in human fetal intestine was first demonstrated in crypt cells, epithelial cells at the base of the villus and in the inner circular layer of the intestinal muscle (37). In rats, EGF-R expression was detected throughout the entire small intestine (39) and
localized originally to the basolateral membrane of the crypt and villus epithelium only (44, 53, 54). However, recent studies have shown the presence of EGF-R also on the apical membrane of villus epithelium in rats (29) and rabbits (56). Indeed, results from our study indicate that in neonatal rats low expression of EGF-R is localized in both apical and basolateral membrane of ileal enterocytes. In addition, during mucosal injury EGF-R localization and expression in intestinal mucosa is changed. In mice and rats that have undergone small bowel resection, an increase in EGF-R production and increased receptor activation was noted (47, 57). Increased EGF-R production was reported in epithelial cells of the ulcer margins (59) with EGF-R expression localized on the basolateral and apical surfaces (52). These results indicate that during mucosal injury, the EGF-R can effectively be reached by its ligands (EGF or TGF-α) from the luminal side. In addition, during the early postnatal period high intestinal permeability enables rapid transport of EGF across the jejunal epithelium (39) and therefore EGF-R on the basolateral side of the epithelium can be reached as well. Our results complement the findings of these previous studies. Gene expression of EGF-R at the site of injury (distal ileum) is increased and treatment with orally administered EGF down-regulates EGF-R mRNA levels toward normal levels. The presence of EGF-R in NEC tissues is localized predominantly in the apical and basolateral membranes of the villi enterocytes, whereas in healthy tissue EGF-R is minimally detected. Changes in EGF-R expression are observed as soon as 24 hours after asphyxia/cold stress exposure, before any histopathological changes in intestinal architecture can be detected (data not shown). Taken together, these data support the idea that the EGF-R belongs to an essential defense mechanism of intestinal epithelial integrity where peptide growth factors such as EGF or TGF-α play critical protective and healing roles (34, 36).

In suckling rats, intestinal contents of EGF and TGF-α peptides are similar but the origins are different. The major source of intestinal EGF is maternal milk (42). TGF-α, however, is not detectable in rat milk and its intestinal content is likely the result of endogenous synthesis by the
small intestine and pancreatic secretion (11). Recently, we have shown that endogenous intestinal EGF and TGF-α production can be regulated nutritionally in suckling rats. Feeding growth factor-free RMS diet for 4 days to neonatal rats resulted in significant increases of TGF-α and EGF mRNA levels in the developing duodenum and ileum (15). Studies have shown increased mucosal TGF-α expression in adult rabbits (49) and rats (21) with experimental colitis. Mice lacking TGF-α exhibited increased susceptibility to dextran sulfate-induced colitis (17), while mice over expressing TGF-α had markedly decreased susceptibility to experimentally induced colitis (16). Our current observations that ileal EGF and TGF-α mRNA levels were unaffected by either NEC injury or EGF treatment may indicate that highly immature intestinal tissue does not have the potential to initiate the reparative process by increased endogenous production of EGF-R ligands.

In the present study, we have shown that oral administration of EGF has beneficial effects in the prevention of NEC in an experimental rat model. Supplementation of commercial milk formula with recombinant EGF to stimulate intestinal repair processes may significantly reduce the incidence of neonatal NEC in prematurely born neonates and suggests new therapeutic approaches in the treatment of gastrointestinal diseases of pediatric patients.

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Figure legends

**Figure 1.** Body weight gain in neonatal rats that were fed by mother (Dam Fed), artificially reared with rat’s milk substitute (RMS), and artificially reared with RMS supplemented with 500 ng/ml of EGF (RMS+EGF). The values are mean ± standard error of 10 Dam Fed, 16 RMS-fed, and 13 RMS+EGF-fed rats. P < 0.05 and p < 0.01 RMS rats vs RMS+EGF.

**Figure 2.** Macroscopic appearance of the gastrointestinal (GI) tract of a normal, healthy neonatal rat compared to the GI tract from a rat with fully developed NEC. The jejunum and ileum from healthy rats has no visual pathological changes in the structure (A). The jejunum and ileum from rats with NEC show severe inflammation, hemorrhage and discoloration (B). Significant pathological changes are detected predominantly in the ileum. Ileal luminal contents are dark and bloody, typical intestinal distention and stenosis are located in distal ileum (B, detail).

**Figure 3.** Histological changes in intestinal architecture of rats with NEC. Neonatal rat ileum stained with hematoxylin and eosin showing representative sections for each morphological grading score. (A) Normal ileum, NEC score 0. Note there is no separation in the submucosa or lamina propria. (B) NEC score +1, slight submucosal and lamina propria separation. (C) 2+, Increased submucosal and lamina propria separation with edema of the submucosa. (D) 3+, Severe separation of the submucosa and lamina propria. (E) 4+, necrosis and loss of villi structure. (Magnification x 200).

**Figure 4.** Mean NEC scores from ileal tissue from Dam Fed, RMS, and RMS+EGF neonatal rats. The values are mean ± standard error from histological scoring of 10 Dam Fed, 16 RMS-fed,
and 13 RMS+EGF-fed rats. * P < 0.001 Dam Fed vs. RMS or RMS+EGF rats. # P < 0.001 RMS rats vs. RMS+EGF rats.

**Figure 5.** Expression of EGF-R, EGF and TGF-α mRNA in the ileum of Dam Fed, RMS, and RMS+EGF neonatal rats. Steady-state mRNA levels were quantified using RT competitive-PCR, n=10-15 samples per group. * P < 0.001 Dam Fed vs. RMS or RMS+EGF rats. # P < 0.001 RMS rats vs. RMS+EGF rats.

**Figure 6.** EGF-R localization in neonatal ileum. Representative sections from (A, D) Dam Fed, low levels of EGF-R signal in apical and basolateral membranes of enterocytes; (B, E) RMS, increased expression in apical and basolateral membranes of enterocytes; and (C, F) RMS + EGF, decreased expression on the apical and basolateral membranes of the villus. Magnification x 200 (A, B, C) and x 1000 (D, E, F).
Table 1. Ileal protein and DNA content and blood in stool values.

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<th>Dam Fed</th>
<th>RMS</th>
<th>RMS+EGF</th>
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<tr>
<td>Protein, mg/g of tissue</td>
<td>108.4 ± 6.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95.5 ± 4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>102.4 ± 4.6&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>DNA, mg/g of tissue</td>
<td>6.0 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.0 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.0 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Protein:DNA ratio</td>
<td>18.4 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.2 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.2 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Blood in stool*</td>
<td>0.2 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.3 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.3 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
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* 0 - no blood in stools; 1 - small traces of blood; 2 - moderate presence of blood; 3 - strong signal for the presence of blood; 4 - very strong signal for the presence of blood.

All data are means ± SE. Numbers of animals are given in parentheses. Mean values within a row not sharing a common superscript letter were significantly different, P < 0.01 (ANOVA).
### Table 2. Effect of Enteral EGF on Incidence of NEC in Neonatal Rat Model

<table>
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<tr>
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<th>Dam Fed</th>
<th>RMS</th>
<th>RMS+EGF</th>
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<tr>
<td>NEC Positive*/Total</td>
<td>0/7</td>
<td>13/16</td>
<td>4/13</td>
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<tr>
<td>% NEC</td>
<td>0</td>
<td>81</td>
<td>31</td>
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* To compare the incidence of NEC from all conducted studies, animals with ileal changes histologically scored 2+ were considered NEC positive. The definition for each histological grade was as follows: normal (0) - no damage; mild (1+) – slight submucosal and/or lamina propria separation; moderate (2+) – moderate separation of submucosa and/or lamina propria, edema in submucosal and muscular layers; severe (3+) – severe separation of submucosa and/or lamina propria, severe edema in submucosal and muscular layers, regional villi sloughing; and full necrosis (4+) – loss of villi and necrosis.
Fig 1.

![Graph showing body weight over days with comparison of Dam Fed, RMS, RMS+EGF groups with statistical significance indicated by p < 0.05 and p < 0.01.]
Fig. 3
Fig. 4

Mean NEC score

- Dam Fed
- RMS
- RMS+EGF

* #
Fig. 5

A  EGF-R mRNA

Mean EGF-R mRNA (amol/µg total RNA)

Dam Fed  RMS  RMS+EGF

B  EGF mRNA

Mean EGF mRNA (amol/µg total RNA)

Dam Fed  RMS  RMS+EGF

C  TGF-a mRNA

Mean TGF-a mRNA (amol/µg total RNA)

Dam Fed  RMS  RMS+EGF
References:


