Total parenteral nutrition adversely affects gut barrier function in neonatal piglets

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Running title: Gut barrier function in neonatal piglets
Abbreviations used in this paper: TPN, total parenteral nutrition; GLP-2, glucagon-like peptide-2; IEL, intraepithelial lymphocyte; GC, goblet cell; MPO, myeloperoxidase; TJ, tight junction
ABSTRACT

Sepsis is the most common morbidity in preterm infants, who often receive total parenteral nutrition (TPN). We hypothesized that gut barrier function is compromised in TPN-fed compared to enterally fed newborn piglets. Colostrum-deprived, newborn pigs were implanted with jugular venous and bladder catheters under general anesthesia. Pigs were either administered TPN (n=15) or fed formula (ENT, n=15). After 6 d, pigs were gavaged a solution of mannitol, lactulose, and polyethylene glycol-4000 (PEG 4000) and urine was collected for 24 h. At 7 d, small bowel samples were assayed for myeloperoxidase (MPO) activity, morphometry, and tight junction (TJ) protein abundance. Intestinal contents and peripheral organ sites were cultured for bacteria. Urinary recovery (%dose) of mannitol (53 vs. 68) was lower, whereas that of lactulose (2.93 vs. 0.18) and PEG4000 (12.78 vs. 0.96) were higher in TPN versus ENT pigs (p<0.05). The incidence of translocation was similar in TPN and ENT pigs. MPO activity was increased in TPN versus ENT pigs in the jejunum (p<0.001) and was weakly correlated with lactulose (R^2=0.32) and PEG 4000 (R^2=0.38) recovery. Goblet cell (GC) counts did not change, but intraepithelial lymphocyte numbers decreased with TPN. Only claudin-1 protein abundance was increased in the TPN group. We conclude that TPN is associated with impairment of neonatal gut barrier function as measure by permeability, but not translocation.
INTRODUCTION

Late-onset sepsis (LOS) is the number one morbidity in the neonatal infants. Many factors have been implicated in the increased incidence of infection in preterm infants, especially total parenteral nutrition (TPN) (32). The risk factors associated with TPN that may increase the incidence of LOS in neonates are multifactorial, and include the presence of central venous catheters, very (<1500 g) low birthweight, and mechanical ventilation. However, TPN results in deprivation of luminal nutrition that also adversely affects the mucosal integrity and may compromise barrier function of the neonatal gut resulting in a phenomenon described as “gut-derived” sepsis. Gut-derived sepsis results, in part, from deterioration in the mucosal epithelial barrier and subsequent translocation of luminal bacteria and toxins into the blood (4).

Studies have shown that gut mucosal atrophy occurs in the absence of total or partial (<60% total caloric intake) enteral nutrition in neonatal and adults animals (6, 33) and, to a lesser extent, in human infants (44). TPN-induced mucosal atrophy is characterized by morphological changes, such as reduced villus height and mucosal surface area, that are secondary to decreased crypt cell proliferation and protein synthesis and to increased apoptosis (7). TPN not only reduces the population of enterocytes, but also has been associated with expansion of mucosal goblet cells and some lymphocyte subtypes (11, 30). Furthermore, newborn (11, 19) and adult (17) animal studies indicate that TPN-induced activation of lymphocytes may induce mucosal inflammation, based on increased ICAM-1 expression and myeloperoxidase activity. Studies in TPN-fed mice demonstrated that increased expression of the proinflammatory cytokine, interferon-γ (INF-γ), in activated intraepithelial lymphocytes (IELs) is linked to increased apoptosis and may contribute to the breakdown of epithelial barrier integrity (26, 63).
This breakdown in barrier integrity during TPN administration has been evaluated experimentally via the assessment of bacterial translocation, defined as the passage of enteric bacteria to the mesenteric lymph nodes and other extra-nodal sites. Alverdy et al. found TPN was associated with increased bacterial translocation in adult rats (1). The increase in bacterial translocation has been associated with higher rates of sepsis in human neonates/infants (42) and adult surgery patients (41) receiving TPN.

Parenteral nutrition appears to negatively impact another measure of gut barrier function, paracellular permeability. Permeability has long been considered an indicator of gut dysfunction in many disease states, from trauma and burns to malnutrition (59) to inflammatory bowel disease (IBD) (43). Cell monolayer experiments have shown increased permeability when exposed to INF-γ (35). A study in adult rats noted a concomitant increase in in vitro intestinal permeability as well as bacterial translocation in TPN fed animals (12). Cell culture experiments and adult clinical studies have also supported this finding of increased permeability in critically ill patients on TPN (20, 35).

As far as the authors are aware, a cellular mechanism for the increase in intestinal permeability during TPN has not been completely elucidated, especially in the newborn. A potential explanation can be a breakdown in TJ integrity. Tight junctions are a group of proteins intimately involved in intercellular adhesion. Patients with IBD, known to have increased permeability, have also been found to have down-regulation of TJ proteins, particularly occludin (29).

These various observations have not been evaluated simultaneously and prospectively in an in vivo newborn model. Therefore, we hypothesize that parenterally fed neonatal piglets have compromised gut barrier function as measured by bacterial translocation and paracellular
permeability and this deterioration is related to increased gut inflammation and impaired TJ integrity.

MATERIALS AND METHODS

Animals

The study protocol was approved by the Animal Care and Use Committee of Baylor College of Medicine and was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* [DHHS publication no. (NIH) 85-23, revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205]. For the study, pregnant, crossbred sows (Large White x Hampshire x Duroc) were obtained from the Texas Department of Criminal Justice (Huntsville, Texas) approximately one week prior to estimated date of confinement. They were housed, fed, and kept under surveillance in the animal facility at the Children’s Nutrition Research Center (Houston, TX) until delivery. The sow delivered the piglets vaginally. Remote video cameras were used to monitor the sows around the clock, allowing collection of the piglets prior to suckling. The newborn piglets were kept in heated cages and fed only water until surgery.

Study Design

Surgical Procedure

Figure 1 illustrates an overview of the study. Preoperatively, each animal received only one systemic dose of enrofloxacin (2.5 mg/kg; Bayer, Shawnee Mission, KS). We gave only one dose to prevent confounding of culture data, although previous studies indicate that systemic antibiotics affects intestinal microflora minimally (23, 24, 31). The piglets were surgically catheterized within 12 hours of birth under isoflurane general anesthesia. Catheters were
inserted into the jugular vein, carotid artery, and bladder. Silastic vascular catheters were inserted via a cutdown procedure as previously described (49). Bladder catheter insertion initially involved a midline (females) or para-abdominal (males) vertical incision caudal to the umbilicus. The peritoneal cavity was entered and the bladder isolated. A purse-string monofilamentous suture was placed in the anterior wall of the bladder. Next, a 16- or 18-gauge needle was used to create a vesicotomy. A silastic catheter was inserted into the vesicotomy and secured with the purse-string. A second stitch was used to anchor the catheter to the abdominal wall. The catheter was the tunneled subcutaneously and brought out through the skin dorsally. A two-layered closure was utilized to close the abdominal incision. Bactericidal ointment was placed over the incision and covered with gauze. Catheters were secured in a jacket that was attached to a tether. The tether allowed safe and secure administration of TPN to the piglets once in their cages. Postoperatively, the piglets received one dose of analgesic (0.1 mg•kg⁻¹ butorphenol tartrate, Fort Dodge Labs, Fort Dodge, IA).

Nutritional Protocol

Pigs were divided into two equal groups, one of which was placed on TPN (240 mL•kg⁻¹•d⁻¹; glucose, 25 g•kg⁻¹•d⁻¹; protein (amino acids) 13 g•kg⁻¹•d⁻¹; lipid (Intralipid 20% (Fresenius Kabi, Bad Homburg, Germany)) 5 g•kg⁻¹•d⁻¹) and the other fed liquid milk replacer (Advance®Liqui-Wean, Milk Specialties, Dundee, IL, 240 mL•kg⁻¹•d⁻¹ mixed 1:5, powder to water) for six days. The enteral feedings were divided evenly 5-6 times per day. The liquid milk replacer did not contain antibiotics. The nutritional support was continued until euthanasia. Total caloric intake was not different between the 2 groups (~ 840 kJ•kg⁻¹•d⁻¹).
Outcomes Measurements

Intestinal Permeability

On day 6, both groups received an intragastric bolus of three permeability marker molecules (mannitol [50 mg•kg⁻¹; Sigma, St. Louis, MO]: lactulose [500 mg•kg⁻¹; Sigma, St. Louis, MO]: polyethylene glycol (PEG) 4000 [3 g•kg⁻¹; Fluka Chemika, Steinheim, Switzerland]) at Time 0. These markers were dissolved in sterile water to make a 10 mL•kg⁻¹ solution, which was filtered through a 0.2 micron filter (Nalgene Filtration Products, Nalge Nunc International, Rochester, NY) prior to administration. Mannitol is absorbed via the transcellular route, while lactulose and PEG are markers of paracellular permeability. We administered mannitol to control for a potential concern of delayed gastric emptying in the TPN piglets. Baseline urine samples were obtained prior to administering the marker solution. Subsequent urine samples were collected at Time 3, 6, 12, and 24 hours. Specifically, the urine was collected via the implanted catheters, which were allowed to empty by gravity into a beaker kept on the floor at a level below that of the pig. Total collection the urine was obtained during each interval.

The urine was analyzed as previously described (8, 46). In brief, the urine is centrifuged in a 1.5 mL microcentrifuge tube at 3,000 rpm for 5 min. A 20 μL aliquot of the urine is injected onto an Aminex HPX 87C 300 7.8 mm cation-exchange column protected with a precolumn of BioRad Deashing system (BioRad Laboratories, Richmond, CA) and eluted with degassed pure water at a flow rate of 0.6 mL•min⁻¹ at 85°C. The column effluent is monitored with a differential refractometer (Millipore Corporation, Medford, MA) with the sensitivity setting at 128, scale factor at 25, and internal temperature at 50°C. The column is calibrated using PEG-4000, lactulose, and mannitol as standards. The coefficient of variation with this method is ≤ 5%.
Bacterial Translocation

At the end of the collection period, the piglets were euthanised with an intravenous injection of pentobarbital sodium (50 mg•kg\(^{-1}\)) and sodium phenytoin (5 mg•kg\(^{-1}\); Beutanasia-D, Schering Plough Animal Health, Union, NJ). Subsequently, under sterile conditions, samples of blood (via direct cardiac puncture), liver, spleen, mesenteric lymph nodes, and intestinal lumens were cultured for aerobic and anaerobic bacteria. Anaerobic transport medium was used for transport of anaerobic samples (Anaerobe Systems, Morgan Hill, CA). Tissue samples were diluted 1:1 with PBS before plating. Blood was streaked directly onto plates, and gut contents were diluted from log10\(^{-2}\) to log 10\(^{-5}\) before plating. Restrictive media used for bacterial isolations were: Anaerobic Brucella blood agar for \textit{Clostridium perfringens}, (Anaerobe Systems, Morgan Hill, CA); \textit{Bacterioides} bile esculin agar for \textit{Bacterioides} (Anaerobe Systems, San Jose, CA); and, cycloserine-cefoxitin fructose agar for \textit{Clostridium difficile} (Anaerobe Systems, Morgan Hill, CA). Plates were incubated under anaerobic conditions for up to 4 days at 37°C. Media for aerobic isolation isolations included: Rogosa SL Agar for lactobacilli (Difco, Detroit, MI); MacConkey agar for \textit{E. coli}, Klebsiella, and Enterobacter (Difco, Detroit, MI); enterococcus agar for Enterococcus (Difco, Detroit, MI); and mannitol salt agar for Staphylococcus (Difco, Detroit, MI). Plates were incubated at 37°C for 48 hours under aerobic conditions. Further identification (species) of isolates was done by api biochemical Test Kits (bioMerieux, Inc., Hazelwood, MO).

Myeloperoxidase Activity

As an indicator of inflammation we assayed tissue myeloperoxidase activity using a modification of the method by Suzuki et al. (51) for use in a 96-well microplate reader.
(Molecular Devices Corp, Sunnyvale, CA). Full thickness tissue samples were homogenized at 20,000 g twice, with the second centrifugation step being preceded by addition of a detergent, hexadecyltrimethylammonium bromide (HTAB), to disrupt the cell membranes, as previously described (28). Activity was based on a standard curve of human myeloperoxidase standards (Sigma-Aldrich Corporation, Saint Louis, MO) with dilutions from 1:4 to 1:256.

**Intestinal Morphology and Cell Counts**

The small bowel was measured for wet weight and length after cold saline rinse to remove intestinal contents upon collection. Sections were placed in 10% formalin for histology. Further assessment of villous height and area and crypt depth were made on 5 µm, hematoxylin and eosin (H & E) stained sections using standard light microscopy (Axiophot, Carl Zeiss Inc., Werk Göttingen, Germany) and NIH Image software, version 1.60 (National Institutes of Health, Bethesda, MD). Ten well formed villi from each sample were used for analysis. A blinded observer counted intraepithelial lymphocytes and GC counts morphologically on H & E stained sections using a high power objective.

**Tight Junction Protein Abundance**

One gram of pulverized tissue was homogenized in 2 mLs of buffer (50 mM HEPES, 150 mM NaCl, 5 mM NaEDTA) and protease inhibitor “cocktail” (Bacitracin (2 mg•mL\(^{-1}\)), PMSF (348 mg•L\(^{-1}\); 18 mg•50 mL\(^{-1}\) buffer), Leupeptin (25 µg•mL\(^{-1}\); 1.25 mg•50 mL\(^{-1}\) buffer), Aprotinin (25 µg•mL\(^{-1}\); 1.25 mg•50 mL buffer\(^{-1}\)). Homogenate was centrifuged at 8,000 g (10,000 rpm) for 10 min at 4°C. Supernatant was removed and centrifuged at 100,000 g (39,000 rpm) for 30-45 min at 4°C. Supernatant was discarded and membrane pellet was resuspended in buffer + 1% triton X-100 (2 mL). The pellet was homogenized and solubilized for 24 h with gentle rocking at 4°C.
Equal protein amounts were loaded into SDS-PAGE gels and run at 100 V for 60 min then transferred to nitrocellulose (100 V for 60 min). Membrane was blocked with 3% nonfat dry milk (NFDM) in tris-buffered saline (TBS). Rabbit polyclonal antibody (Zymed, South San Francisco) was used for claudins 1 and 2 in concentrations of 1:250 and 1:500, respectively. For occludin and ZO-1, rabbit polyclonal antibodies (Santa Cruz, Santa Cruz, CA) were also used in concentrations of 1:500 and 1:400, respectively. After washing with TBS-Tween 20 buffer, the membrane was incubated with a secondary antibody (anti-rabbit IgG conjugated with biotin, Santa-Cruz Biotech Inc.) at room temperature for 1 h. Then the membrane was enhanced with Neutravidin-HRP (Pierce) in the NFDM-TBS solution for 1 h at room temperature and allowed to react with horseradish peroxidase substrate (SuperSignal, Pierce) for 5 min. Finally, the membrane was exposed to X-ray film for 30-180 sec, and the image was scanned and quantified by ImageQuant 5.0 software (Molecular Dynamics, Amersham Biosciences, Sunnyvale, CA).

**Statistical Analyses**

One-way ANOVA was used for all normally distributed data comparisons and χ²-square (for BT) for non-parametric data. Pearson correlations were used when indicated. All data shown are mean±SEM, unless otherwise stated. Statistical significance was determined to be $p<0.05$. Statistical calculations were carried out using Minitab v.13.32 (Minitab, Inc., State College, PA).
RESULTS

Pig body growth and urine output

There was no difference in birthweight (1.56 vs. 1.59 kg) or weight gain (44.8 vs. 42.3 g•kg•d⁻¹) in TPN versus ENT over the course of the study period. Urine output (mL•kg⁻¹•d⁻¹), as measured during the final 24 hours of the study was also not different (81.94 vs. 84.75; TPN vs. ENT).

Gut morphometry and cell counts (Tables 1 & 2)

In the jejunum, wet weight (g•kg BW⁻¹), villus height (µm) and area (µm²) were significantly reduced in the TPN group when compared with the enterally fed controls. Total protein content (mg) and DNA content (mg), expressed in absolute amounts as body weights were similar, were decreased in the TPN piglets. Crypt depth (µm) was similar in both treatment groups.

Ileal measurements follow a similar trend. Wet weight, protein and DNA contents were less in the TPN versus enteral groups. Unlike the jejunum, no difference was noted with regards to villus height and area, and crypt depth. Cell counts were corrected for villus size differences between treatment groups. IELs showed a marked reduction in number when the animals received TPN; however, there seemed to be no effect of enteral nutrition on the number of goblet cells per villus.

Intestinal permeability (Figure 2)

As a measure of paracellular permeability, we assessed pooled 24 h urinary recovery of markers expressed as a percent of the dose recovered. Twenty-four hour collections were used to obtain adequate marker recovery. We found 75% and 88% of the total recovered amount of mannitol within 12 h in the TPN and ENT groups, implying that collecting urine beyond 24 h
would have resulted in insignificant changes to the data. Lactulose recovery was significantly greater at 2.931±0.67 versus 0.179±0.11% between TPN and enteral groups, respectively. Similarly, PEG 4000 recovery was 12.78±4.11 versus 0.96±.24% between the same groups. In order to assure pre- and post-intestinal factors did not affect recovery of the paracellular markers, we used mannitol, absorbed via the transcellular route, as a controlling factor and recovery was notably reduced in the TPN group when compared to the enteral group, 52.99±3.86 versus 68.35±3.94%, respectively. Mannitol is often used with lactulose to correct for the latter’s recovery, as the two markers are not metabolized before or in the small bowel, empty similarly from the stomach, and are cleared the same from the kidneys (5). When lactulose/mannitol ratios were used to corroborate recovery data, the results were no different (0.0504±0.0100 vs. 0.0023±0.0014, TPN vs. ENT).

**Intestinal inflammation (Figures 3)**

Myeloperoxidase activity, expressed as millunits of activity per milligram of protein (mU•mg protein⁻¹), was used as an indirect indicator of inflammation. One unit of activity equals one micromole of substrate catalyzed in one minute at 25°C. Our findings showed a significant increase in enzyme activity in the proximal jejunum, 4.63±0.47 vs. 2.16±0.39 mU•mg protein⁻¹, and a trends towards increased activity in the distal ileum, 5.66±0.58 vs. 4.03±0.66 mU•mg protein⁻¹ (p=0.08), of the TPN group when compared to controls.

Weak, but significant, correlation estimates were found for myeloperoxidase activity and recovery of lactulose and PEG 4000, $R^2=0.32$ (p=0.003) and $R^2=0.38$ (p=0.003), respectively, when the animals were assessed collectively. No such correlation was found for bacterial translocation and either myeloperoxidase activity or permeability.

**Bacterial translocation (Table 3)**
We assessed translocation of bacteria from the lumen of the jejunum, ileum, or cecum to peripheral sites, which included liver, spleen, mesenteric lymph nodes, and peripheral blood. We defined a translocation event as the presence of the same bacterium in both the lumen and in one or more peripheral sites. Based on our definition, we found no difference in bacterial translocation between TPN and ENT groups (62% vs. 69%). Also, we noted that a larger variety of organisms translocated in the ENT, although the prevalence of translocation did not differ.

Tight junction protein abundance (Figure 4)

Western blot analysis of gut mucosal samples, evaluated by densitometry, revealed only an increased expression of claudin-1 in the TPN group when compared with enterally fed controls. We found no difference in the amounts of claudin-2, occludin, and ZO-1 as assessed by Western blotting.
DISCUSSION

Parenteral nutrition has been a life-saving therapy used in hospitalized patients, especially those admitted to neonatal intensive care units. Newborns admitted to neonatal intensive care units (NICU) and receiving TPN are at greater risk for late onset sepsis. TPN can increase the risk for infection in many ways, but we focused on its effects on gut barrier function. Despite being clinically beneficial, TPN is the deprivation of enteral nutrition and is associated with intestinal changes in structure and function. Adult animal studies have demonstrated increases in bacterial translocation and intestinal permeability, but the degree to which these are altered in the newborn model has not been described. Therefore, we hypothesized that barrier function would be compromised in the parenterally fed piglets. Interestingly, we did see that gut barrier function was diminished in the TPN group, but only as measured by intestinal permeability, not bacterial translocation.

*Mucosal Atrophy.* Consistent with many previous studies, we have shown that TPN (i.e., the lack of enteral nutrition) leads to gut atrophy, specifically, mucosal atrophy (6, 33). The current study showed notable decreases in jejunal mass (34.8%), villus height (44.4%), and villus area (56.1%) of parenterally fed piglets when compared to controls. However, in the ileum, only tissue mass (33.9%), protein and DNA content were reduced by TPN, whereas villus height and area were unaffected. These findings are not necessarily novel and highlight the fact that the proximal mucosa is more susceptible to lack of enteral nutrients than the distal gut (33).

*Intestinal permeability.* This study was designed to examine, in breadth, neonatal gut barrier function. Although there is no single “ideal” test for quantifying barrier function, measures of intestinal permeability have been used frequently by many investigators. Intestinal
permeability generally implies paracellular permeability and is measured *in vitro* via ion flux, electrical resistance/voltage changes, or, *in vivo*, via excretion of inert markers into the urine.

In the present study, both paracellular permeability and bacterial translocation (see below) were measured. As expected, we found an increased urinary recovery of the paracellular markers, lactulose and PEG 4000, in piglets fed parenterally. The improved recovery with the enterally fed gut was similar to that of a 4-week-old pig, having very little marker being absorbed from the bowel lumen (60). These previous studies in pigs and those in humans (46) have shown that intestinal permeability is high at birth and declines with advancing postnatal age. The relative amount of PEG 4000 recovered compared to lactulose was greater, which may be due to a larger dose of PEG 4000 and the molecular structure of PEG 4000. Although having an average molecular weight of 4000 kD, the shape of a PEG 4000 molecule is linear, as compared to a lactulose molecule, which is bulky (5, 50). Therefore, the ability for a narrow PEG molecule to pass through openings in the TJs may be greater than lactulose. Nonetheless, the pattern of recovery for the two markers was similar. We also used mannitol, a transcellular marker, to confirm paracellular marker recovery accuracy. The ratio with which they are recovered should not change, and if a change is noted, it is due to alterations in lactulose recovery (3, 58).

The cellular mechanisms involved in the increased permeability during TPN have not been completely established, however, gut inflammation may play a role. In rodents, Fukatsu et al. (17), found that myeloperoxidase activity was increased in animals fed TPN. Furthermore, they found an increase in intercellular adhesion molecule-1 expression in the intestinal tissue of the animals fed parenterally. Given that in the proximal small bowel we saw a significant increase in myeloperoxidase activity, which can be a surrogate for polymorphonuclear
leukocytes (PMN) infiltration or inflammation, we looked for a possible link to the increased permeability. Our correlation estimates showed a significant positive relationship between the recovery of lactulose and PEG 4000, explaining 32 and 38% of the change in marker recovery. Moreover, the increase in inflammation proximally was inversely related to mucosal villus height and area.

Inflammatory bowel disease (IBD) is a clinical disorder including Crohn’s disease and ulcerative colitis, in which PMNS infiltrate into the intestinal mucosa and lead to clinical manifestations (21, 25). A portion of the pathophysiology involving the increased permeability seen in IBD may relate to alterations in the TJ and PMN infiltration (29). Tight junctions are a complex of proteins forming essential structures that function to keep mucosal domains separate (e.g., apical and basolateral areas of enterocytes) and also to create a barrier to the passage of paracellular solutes. They are made up of intracellular scaffolding components intimately linked to the cell membrane and also consist of intercellular strands or fibrils which actually span the space between cells. ZO-1 is a scaffolding protein and occludin and the claudins are strand proteins (52, 53). Thus we examined the abundance of TJ proteins to help clarify the molecular mechanism of increased paracellular permeability in neonates fed TPN.

As seen in Figure 5, abundance was notably increased for the TJ protein, claudin-1, in the TPN group. This was an unexpected finding, given that paracellular permeability was increased in that group. Studies have demonstrated that transepithelial resistance (a measure of epithelial integrity) increases with increasing strand number, but exceptions have been noted in cell culture models (9, 10, 48). Investigators have suggested TJ fibrils are heteropolymeric with differing claudin superfamily subtypes combining to form varying degrees of “adhesiveness” (54). Also mentioned, TJ strands are repeatedly broken and reannealed to give the junction a
certain tightness (55). Because so little is known about the molecular regulatory mechanisms of TJs, it would be difficult to speculate on the nature of the current finding. The remaining proteins, although demonstrating no difference in abundance, may change their localization. Thus, electron microscopy and confocal microscopy may be required to determine changes in the localization of TJ integral proteins in the face of parenteral feeding (16).

The underlying cellular mechanisms linking mucosal inflammation to epithelial permeability and TJ alterations are not fully established. However, recent studies have demonstrated a potential connection between IEL function and loss of mucosal integrity and permeability in TPN-fed mice. This study quantified IELs in an attempt to assess the effects of lack of enteral nutrition on their numbers. Intraepithelial lymphocytes are the first immune cells to be exposed to foreign antigens and have the potential to be critical in host defense. A previous experiment in neonatal piglets demonstrated increased cytotoxic T lymphocytes in the lamina propria, as well as increased goblet cells, implying that inflammation may play a role in the pathogenesis of TPN associated mucosal changes (19). In addition, Li, et al., found that lymphocytes play an important role in the regulation of mucosal immunity in a mouse model. Also, they noted that a change in the ratio of CD4+/CD8+ T cells in TPN-fed mice are related to decreases in secretory IgA, a key player in the prevention of bacterial adherence to epithelium (34).

However, recent studies in mice have shown that TPN decreases IEL numbers (30). Moreover, these changes in IELs increased INF-γ, which has been shown to decrease TJ integrity (27). Previous cell culture experiments confirm this loss of integrity due to INF-γ and other cytokines through alterations in the TJ (36), (57), (64). Kiristioglu et al. also found that the CD8αβ+ IELs, responsible for IEL proliferative activity, declined with TPN administration. Moreover, the CD44+ IELs, which are mature lymphocytes, also decrease with TPN
administration (26). These findings support the current finding of decreased IELs in the TPN group, and may even help explain the increased apoptosis of enterocytes associated with TPN or the lack of enteral nutrients (62).

*Bacterial translocation.* Another commonly used assessment of barrier function is bacterial translocation. Bacterial translocation is the passage of enteric bacteria from the bowel lumen to peripheral sites. This is generally reported as positive cultures from peripheral sites containing “enteric” bacteria, with the reasonable supposition that the bacteria were indeed from the gut (41). It is important to note, that in the current study we verified positive peripheral cultures with concurrent cultures from the gut lumen. Translocation has been described as early as the 1960’s and was reported to be increased in adults receiving TPN (1, 61). In addition, a study of infants on TPN demonstrated an association between translocated gut bacteria and blood culture-confirmed septicemia and concluded that sepsis in infants receiving TPN may be a gut-related phenomenon (42). Another report found increased post-operative sepsis in adult surgical patients showing evidence of bacterial translocation (41% vs. 14%; BT vs. non-BT; p<0.001) (41).

Investigators have found that the primary site for translocation, in adult guinea pigs, is the small bowel (18). This finding was corroborated with a significant association of small intestinal bacterial colonization and translocation in newborn rabbits (56). Unlike the former study, the latter experiment was not designed to test parenteral versus enteral nutrition. The authors also speculated that this may explain why sepsis and necrotizing enterocolitis, an inflammatory condition strongly associated with prematurity, occur after neonates are 3-5 days of age, once the small bowel has been populated.
Uniquely evident in this experiment is that TPN, despite being associated with increased permeability and gut atrophy, did not increase translocation in the newborn piglet. When compared to the changes previously noted, there does not appear to be a correlation with the incidence of bacterial translocation. One group attempted to correlate bacterial translocation with plasma levels of PEG 4000 (2). Although the authors attempted to provide a practical method to assess translocation, the limitations of the study prevented extrapolation to widespread use. The assumption was made that increases in macromolecule permeability indicate a greater propensity for translocation. This presumption overlooked the fact that the mechanisms responsible for paracellular permeability and bacterial translocation are most likely not the same. Adult animal and human studies and the current newborn model confirm that these measures of gut barrier function are not strongly related (22, 40). Furthermore, despite finding slightly over half the piglets with translocation in both groups, we did not observe any clinical signs of sepsis.

Another factor that may affect bacterial translocation is the innate immune function of the GC. As part of the innate immune system, GC alterations may impact on gut barrier integrity. Goblet cell mucin production occurs via two processes, simple and compound exocytosis, describing baseline and enhanced secretion in response to stimuli, respectively (13). Many factors can impact on GC number and mucin production, including changes in diet and local microflora (15, 45). Also, TPN administration has been shown to increase jejunal sulfomucin producing GCs, a finding that is consistent with the GC population of the fetal intestine and indicative of a more bacteriostatic mucus (11). In contrast to the previous study, we found no difference in the number of GCs between enterally and parenterally fed piglets. A possible reason for this discrepancy is because we did not specifically identify GC type based on mucin composition. Perhaps specific stains will show that the sulfomucin numbers did change despite
total GC numbers remaining constant. However, we did find that *Clostridium* species was one of only two bacteria that were found to translocate in the TPN group. This finding is consistent with recent reports from DePlancke et al. showing that TPN is associated with selective colonization of mucolytic bacteria, namely *Clostridium* species (14). A link between *Clostridium* and epithelial dysfunction also has been demonstrated through alteration of the TJ (39).

*Perspectives.* In summary, TPN administration in newborn piglets led to mucosal atrophy and increased paracellular permeability and decreased intraepithelial lymphocytes, but bacterial translocation and goblet cells were not different between the two groups as was TJ abundance. We propose that TPN or the lack of enteral nutrition leads to impaired gut barrier function, as assessed by intestinal permeability, and that bacterial translocation and permeability are not interchangeable entities in the measurement of gut integrity. Investigators had previously shown in preterm neonates that early feeding with human milk reduces gut permeability (46). Although the importance of human milk to premature infants cannot be overstated, not all babies are able to receive mother’s milk for a variety of reasons. Therefore, beginning enteral feeding as early as possible may enhance gut barrier function, in addition to other functional improvements as previously described (38, 37, 47). Although TPN is an important development in the care of newborns, enteral nutrition appears to offer benefits to the gut barrier and deserves further study.
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FIGURE LEGENDS

Figure 1. Piglets received enteral or parenteral (TPN) nutrition for 7 days with intestinal permeability measurements made during the last 24 hours prior to tissue sampling.

Figure 2. Values are mean ± SEM. Urinary recovery of permeability markers (percent of dose administered [%]) in 24 hours in ENT versus TPN piglets were determined via HPLC.

Figure 3. Mean ± SEM. Myeloperoxidase activity (milliUnits activity per milligram protein) in the proximal and distal small bowel of ENT versus TPN piglets was increased in the letter group.

Figure 4. Densitometric measurement of TJ protein abundance in jejunal tissues of ENT and TPN groups. Claudin-1 (22 kD) abundance was significantly increased in the TPN group, but the remainder of tested TJ proteins did not change.
Table 1. *Jejunal and ileal gut morphometry*

<table>
<thead>
<tr>
<th></th>
<th>Jejunum</th>
<th></th>
<th></th>
<th></th>
<th>Ileum</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TPN</td>
<td>ENT</td>
<td>p-value&lt;sup&gt;1&lt;/sup&gt;</td>
<td>TPN</td>
<td>ENT</td>
<td>p-value&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mass&lt;sup&gt;1&lt;/sup&gt;</td>
<td>(g·kg BW&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>10.92 ± 0.78</td>
<td>16.75 ± 0.80</td>
<td>&lt;0.001</td>
<td>12.24 ± 0.62</td>
<td>18.51 ± 0.69</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Villus height†</td>
<td>(µm)</td>
<td>542 ± 95</td>
<td>975 ± 51</td>
<td>0.003</td>
<td>617 ± 73</td>
<td>722 ± 53</td>
<td>0.273</td>
<td></td>
</tr>
<tr>
<td>Villus area‡</td>
<td>(10&lt;sup&gt;3&lt;/sup&gt; µm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>35.8 ± 8</td>
<td>81.6 ± 5</td>
<td>0.001</td>
<td>47.3 ± 8</td>
<td>56.8 ± 7</td>
<td>0.386</td>
<td></td>
</tr>
<tr>
<td>Crypt depth‡</td>
<td>(µm)</td>
<td>93.8 ± 5.2</td>
<td>107.9 ± 5.3</td>
<td>0.085</td>
<td>92.9 ± 5.2</td>
<td>103.7 ± 4.0</td>
<td>0.133</td>
<td></td>
</tr>
<tr>
<td>Protein content‡</td>
<td>(mg)</td>
<td>4094 ± 1182</td>
<td>7868 ± 838.3</td>
<td>0.001</td>
<td>2905 ± 327.7</td>
<td>6489 ± 666.3</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>DNA content‡</td>
<td>(mg)</td>
<td>151 ± 12</td>
<td>195 ± 17</td>
<td>0.043</td>
<td>136 ± 8</td>
<td>239 ± 20</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> One-way ANOVA

<sup>†</sup>n=6

<sup>‡</sup>n=12

<sup>§</sup>n=15
Table 2. *Jejunal IEL and GC counts*

<table>
<thead>
<tr>
<th></th>
<th>TPN</th>
<th>ENT</th>
<th>p-value$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goblet cells/villus (#)</td>
<td>9.14±1.53</td>
<td>7.28±1.7</td>
<td>0.272</td>
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<tr>
<td>IELs/villus ( #)</td>
<td>3.72±0.79</td>
<td>11.1±1.2</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Values corrected for villus size. GC, goblet cells. IELs, intraepithelial lymphocytes. N=6. $^1$One-way ANOVA.
Table 3. *Prevalence of bacterial translocation and frequency of individual translocating bacterial species*  

<table>
<thead>
<tr>
<th></th>
<th>Frequency of positive (n)</th>
<th>TPN (Total Events = 62%*)</th>
<th>ENT (Total Events = 69%*)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Enterococcus</em></td>
<td></td>
<td>7/13</td>
<td>7/13</td>
</tr>
<tr>
<td><em>Klebsiella</em></td>
<td></td>
<td>NT</td>
<td>5/13</td>
</tr>
<tr>
<td><em>Staphylococcus</em></td>
<td></td>
<td>NT</td>
<td>2/13</td>
</tr>
<tr>
<td><em>Enterobacter</em></td>
<td></td>
<td>NT</td>
<td>1/13</td>
</tr>
<tr>
<td><em>Pediococcus</em></td>
<td></td>
<td>NT</td>
<td>1/13</td>
</tr>
<tr>
<td><em>Clostridium</em></td>
<td></td>
<td>1/13</td>
<td>NT</td>
</tr>
</tbody>
</table>

One translocation event was defined as one or more bacterial species found in peripheral and intestinal sites of the same animal. Bacterial species listed in order of decreasing frequency. They are for descriptive purposes only and individual comparisons have not been made. *χ²* analysis for assessing difference in bacterial translocation prevalence. NP, not translocating.
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Reference List


41. **O'Boyle CJ, MacFie J, Mitchell CJ, Johnstone D, Sagar PM and Sedman PC.**


42. **Pierro A, van Saene HK, Donnell SC, Hughes J, Ewan C, Nunn AJ and Lloyd DA.**


