Protein Malnutrition During Juvenile Age Increases Ileal and Colonic Permeability in Rats

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ABSTRACT

Protein malnutrition can lead to morphological and functional changes in jejunum and ileum, affecting permeability to luminal contents. Regarding the large intestine, data are scarce, especially at juvenile age. We investigated whether low-protein (LP) diet could modify ileal and colonic permeability and epithelial morphology in young rats. Isocaloric diets containing 26% (control diet) or 4% protein were given to male rats between postnatal days 40 and 60. LP-diet animals failed to gain weight and displayed decreased plasma zinc levels (a marker of micronutrient deficiency). In addition, transepithelial electrical resistance and occludin expression were reduced in their ileum and colon, indicating increased gut permeability. Macromolecule transit was not modified. Finally, LP diet induced shortening of colonic crypts without affecting muscle thickness. These data show that protein malnutrition increases not only ileum but also colon permeability in juvenile rats. Enhanced exposure to colonic luminal entities may be an additional component in the pathophysiology of protein malnutrition.

Key Words: epithelial morphology, gut barrier, young rats

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What Is Known

- Protein malnutrition enhances small intestinal permeability in humans and rodents.
- Morphological changes in the small intestine have been described in rats after long (>6 weeks) treatment with low-protein diet.

What Is New

- A 20-day low-protein diet induced shortening of colonic crypts in young rats.
- A reduction in mucosal tight junction protein expression and a decrease in transepithelial electrical resistance were induced by low-protein diet in the colon of young rats.

Profound effects in organ development and function can appear as a result of malnutrition. Health consequences of protein malnutrition depend on the severity, duration, and age/developmental stage of occurrence (1). Protein-energy malnutrition, which occurs more frequently in children than in adults, can present with wasting, edema, or both; it is accompanied by adipose degeneration of organs such as the liver and heart. Also, the loss of subcutaneous fat negatively affects temperature regulation and water storage (1). There is frequent overlap between protein-energy malnutrition and deficiency of 1 or more micronutrients such as vitamin A, iron, iodine, and zinc, leading to anemia, immune deficiency, and growth retardation among others (1,2).

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Nutritional alterations during the prenatal and early postnatal period, such as those induced by dietary restriction or by the use of parenteral nutrition, have been shown to impair digestive organ growth and maturation in humans (3) and rats (4), pigs (5), and sheep (6). In adults, several gastrointestinal symptoms, such as bloating, constipation, or diarrhea have been reported in anorexia nervosa, although these symptoms improve with refeeding (7).

Gut barrier function is sensitive to nutritional status (8). In severe protein-calorie malnutrition, mucosal atrophy may occur in addition to enhanced permeability to macromolecules and translocation of luminal bacteria (9,10). Furthermore, dietary changes modify the composition of gut microbiota (11,12) which on its own may result in multiple physiological effects (12) and moreover, such phenomena can be modeled in rodents.

In a standard laboratory rodent diet 18% to 26% of energy derives from proteins. Under severe protein malnutrition (0.03%), rodent small intestinal mucosa thickness is decreased after 3 weeks (13). When the reduction in protein supply is less severe (4%–8%), morphological changes in the small intestine are, however, delayed up to several months (14–16). Functional intestinal effects of low protein (LP) intake, such as increase in paracellular permeability or translocation of microorganisms precede morphological alterations (17–19).

Although most of these studies focused on jejunum and ileum, for the large intestine the data are scarce. A loss of distal colon mechanical resistance has been described for protein-malnourished rats (20). Regarding morphological or functional effects of protein malnutrition on the colon mucosal barrier, Marotta et al (21) fed rats a diet containing 2.5% protein, and found an increased

urinary excretion of a contrast medium instilled in the colon after 4 weeks. Further data are required considering the potential effect of increased permeability in the gut segment with the highest numbers of luminal bacteria. In the present study we aimed to establish whether a LP diet administered for 20 days could induce alterations in rat colonic and ileal barrier function and mucosal morphology.

METHODS

Animals

Male Sprague Dawley rats were maintained with food and water ad libitum, on a 12:12-hour dark-light cycle with temperature at 20°C ± 1°C. Killing was performed by thiopental overdose followed by decapitation. Protocols for animal care and use were approved by the institutional ethics committee of the Pontificia Universidad Católica de Valparaíso.

Diet

Control diet was a standard grain-based diet (ProLab RMH3000 from Purina, energy density: 4.1 kcal/g). Kilocalories were distributed as follows: protein 25.967%, fat 14.022%, and carbohydrates 60.011%. Experimental, LP diet was a custom-made purified diet (Purina, energy density: 4.05 kcal/g). Kilocalories were distributed as follows: protein 4.1%, fat 14.9%, and carbohydrates 81.0%. The LP food was stained blue for easy recognition.

After weaning at postnatal day (PND) 21, all animals were fed the control diet until PND 39. Thereafter, they were separated into the control group, which was kept under the same diet until PND 60, and the experimental group, which was fed the LP diet from PND 40 to PND 60. Body weight and health status were monitored daily. Intestinal tissue and blood plasma samples were obtained at PND 60.

Tissue Sampling

Intestinal tissue contents were rinsed with saline (0.9% NaCl). Both ileal and colonic tissue samples were distributed as follows: (starting from the proximal end of tissue) 1 cm was fixed in paraformaldehyde for morphological evaluation; the next 1 cm was used to obtain a mucosal scraping. The remaining tissue was divided into 2 pieces, for Ussing chamber and everted gut sac techniques, respectively.

Plasma Zinc Levels

Plasma samples treatment was performed as described before (22). Briefly, aliquots of approximately 1 g were weighed to 0.1 mg precision and transferred into glass vessels. Sixty-five percent nitric acid (6 mL) was added and the mixture was shaken for 24 hours. Samples were then heated at 90°C for 30 minutes. Thirty percent H_2O_2 (6 mL) was added and the mixture was heated in a stepwise fashion to avoid loss of sample from violent bubble formation. Also, blank solutions were prepared by applying the same digestion procedure.

Digested samples and standard calibration solutions were then analyzed through flame atomic absorption spectroscopy (GBC Avanta 932 Plus, GBC Scientific Equipment, Hampshire, IL) at 213.9 nm (direct air-acetylene flame).

Tissue Morphology

Tissue segments of 1 cm were fixed overnight at 4°C with 4% paraformaldehyde in phosphate-buffered saline (10 mmol/L) and dehydrated in 30% sucrose in phosphate-buffered saline. Cryosections (10 μm) were stained with hematoxylin and eosin and analyzed under a light microscope to measure intestinal crypt length and muscle wall thickness.

Transepithelial Electrical Resistance

Colon and ileum samples previously rinsed with saline were placed in oxygenated (95% O_2, 5% CO_2) Krebs buffer (1.2 mmol/L NaH_2PO_4, 117 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L $MgCl_2$, 25 mmol/L $NaHCO_3$, 2.5 mmol/L $CaCl_2$, and 11 mmol/L glucose). The muscle layers were stripped away, leaving only the mucosa and submucosa. Ileal preparations, which produce a substantial amount of mucus, were preincubated in oxygenated Krebs buffer at 37°C for 20 minutes to clear some of this mucus. Preparations were then placed in Ussing chambers (EasyMount from Warner Instruments, Whitehall, PA; exposed area of 0.3 cm^2) as described previously (23) with oxygenated Krebs buffer maintained at 37°C, and voltage clamped at 0 mV (World Precision Instruments, Sarasota, FL). In the case of colon preparations, these were immediately placed in the Ussing chambers (exposed area of 0.3 cm^2), as described before. Short-circuit current responses were continuously monitored using LabScribe 2 software (World Precision Instruments). After stabilization, a 2 mV challenge was applied for 5 seconds to calculate transepithelial electrical resistance (TEER).

Permeability to Macromolecules

Colon and ileum samples previously rinsed were placed in oxygenated Krebs buffer as described before. The tissues were everted using a crochet hook, thereby leaving the mucosa on the outer side. To prepare a sac, one end of a 2 to 3 cm everted gut segment was tied and the other end was affixed to a modified 2 mL cryovial, which allowed for repeated sampling. Each cryovial-intestine unit was then filled with a known volume of Krebs buffer and placed in a 15 mL centrifuge tube containing oxygenated Krebs buffer and 30 μg/mL TRITC-dextran 4.4 kDa (TD4.4) and 30 μg/mL FITC-dextran 40 kDa (FD40). Tissues were incubated at 37°C and 60 μL samples were taken from the internal medium every 60 minutes for up to 180 minutes. Internal medium samples were diluted 1:10 before performing fluorometric analyses. Fluorescence (TD4.4: λex546, λem575 and FD40: λex490, λem520) was measured in a FluoroMax-2 fluorometer (Jobin Yvon-Spex, Edison, NJ). Standard curves were used to obtain the concentration of each fluorophore (TD4.4 limit of detection: 71 ng/mL, limit of quantification: 238 ng/mL; FD40 limit of detection: 2 ng/mL, limit of quantification: 7 ng/mL). At the end of the assay, the mucosal area was measured. The volume of medium and the exposed mucosal area in each sac were used to express fluorophore transit as pmol/cm^2 at each sampling time.

Western Blot

Intestinal mucosa obtained by tissue scraping was homogenized in ice-cold lysis buffer (HEPES 10 mmol/L, pH 7.9, KCl 10 mmol/L, EDTA 0.1 mmol/L, EGTA 0.1 mmol/L, Triton X-100 0.06%, dithiothreitol 0.5 mmol/L, and protease inhibitors phenylmethanesulfonyl fluoride (PMSF) 0.1 mmol/L, Na_3VO_4 0.1 mmol/L, NaF 0.02 mmol/L, NaPPi 0.025 mmol/L, leupeptin 2 μg/mL, and aprotinin 2 μg/mL). Protein concentration was determined using Qubit protein assay (Life Technologies, Waltham, MA). Thirty micrograms of protein from each mucosal sample were separated by SDS-PAGE (12%, Sigma, St. Louis, MO) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica,

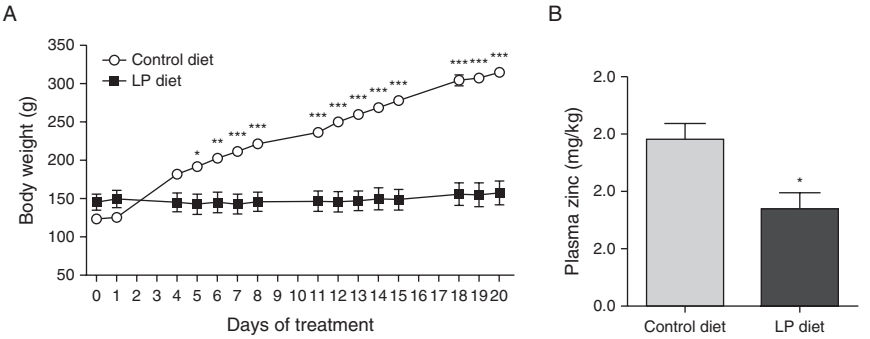

FIGURE 1. LP diet–induced alterations in physiology. A, Rat body weight was monitored daily. Asterisks indicate differences between experimental groups on a particular day ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ by 2-way analysis of variance and Bonferroni test). Data are expressed as mean ± standard error of the mean; n = 4. B, Plasma zinc determination was performed at the end of the experiment ($^{*}P<0.05$ by *t* test). Data are expressed as mean ± standard error of the mean; n = 5–8. LP = low protein.

MA). The membranes were blocked for 1.5 hours at room temperature (Tris-buffered saline containing 0.1% Tween-20 and 5% BSA). Mouse anti-occludin antibody (Invitrogen, Waltham, MA) was diluted 1:1000 in blocking solution and applied overnight at 4°C. After repeated washing, membranes were incubated for 2 hours at room temperature with peroxidase-conjugated anti-mouse secondary antibody (1:5000, Jackson, West Grove, PA). Signal was developed using Western-Lightning Plus-ECL (Perkin-Elmer, Waltham, MA). Autoradiographic films were scanned and band intensity was evaluated by densitometry. Data were expressed as the ratio of occludin to β-actin band intensity (1:5000).

Statistical Analyses

Values are expressed as mean ± standard error of the mean. Plasma zinc, morphological, TEER, macromolecule permeability, and Western blot data were analyzed by nonparametric *t* test. Weight data were analyzed by nonparametric 2-way analysis of variance, and any overall statistical differences were further examined using Dunn test (GraphPad Prism 5 Software Inc, La Jolla, CA). Significance was established at $P<0.05$.

RESULTS

Body Weight and General Health Status

The LP diet was generally well accepted by the rats. As reported by others (24), LP diet failed to induce a weight gain throughout the 20-day experiment, whereas control rats had a 134% increase in weight. The effect was significant for both treatment [$F(1,86)=482.8$, $P<0.0001$] and time [$F(14,86)=17.90$, $P<0.0001$], as shown in Figure 1A. In addition, there was significant interaction [$F(14,86)=14.88$, $P<0.0001$]. LP diet rats were active but did not show signs of disease or stress and plasma corticosterone levels were not different from control (data not shown). A noticeable sign, however, was an increase in fecal pellet softness during the first 5 days of exposure to the new diet. Protein-malnourished rats have been shown to absorb zinc at a lower rate than control rats after 4 weeks of treatment (25). Our model also induced a significant decrease in plasma zinc levels ($P<0.05$; Fig. 1B).

Intestinal Epithelial Morphology

Significant shortening of colonic crypts was observed in LP diet rats, whereas no diet effect was detected on the length of ileal crypts (Fig. 2). The thickness of muscle wall remained unaltered in ileum and colon of animals fed the LP diet.

Intestinal Permeability

Ileal and colonic TEER was reduced by LP diet (Fig. 3), indicating that the gut of these animals had an increased permeability compared with control rats. By 3 hours of culture, permeability of ileum to 4.4 and 40 kDa dextrans, and permeability of colon to 4.4 kDa dextrans showed a nonsignificant increase in LP diet rats compared with control rats (Fig. 4).

Occludin Expression

Ileal and colonic mucosal expression of occludin, a tight junction protein and marker of gut barrier integrity, was significantly reduced in animals that received the LP diet (Fig. 5).

DISCUSSION

As it has been previously shown for the small intestine, epithelial barrier in the rat large intestine is also susceptible to changes due to dietary protein deficiency. Here we show that a 20-day LP diet was able to induce a decrease in TEER both in ileum and colon. A decline in mucosal occludin protein expression was also detected in both tissues. Epithelial morphology was affected as well, because colonic (but not ileal) crypt length was reduced in young rats that received the LP diet.

Colonic permeability status is rarely reported in studies examining dietary effects on gut barrier function. Conventionally, these investigations have looked at perturbations in the small intestinal epithelium, where absorption of the majority of nutrients occurs. The amount of luminal bacteria in the large intestine is, however, the highest of the gastrointestinal tract, reaching up to 10^{12} colony-forming units/mL (26). A breach in colonic epithelial barrier may not only increase susceptibility to infection or induce inflammation: it has been suggested that gut epithelial permeability changes may represent a novel mechanism for visceral organ crosstalk (27).

In the present study we induced protein malnutrition in juvenile rats. Adolescence is recognized as a time of transition when eating habits may change, increasing the risk of malnutrition due to overeating, undereating, and/or nutrient inadequacy (28,29). Investigations addressing the question of physiological effects of adolescent malnutrition have focused on the endocrine and metabolic function, whereas our knowledge on the effects of adolescent malnutrition on other systems, including the gastrointestinal tract, is still limited. Protein malnutrition in combination with dysbiosis could predispose to alterations in gut-viscera communication.

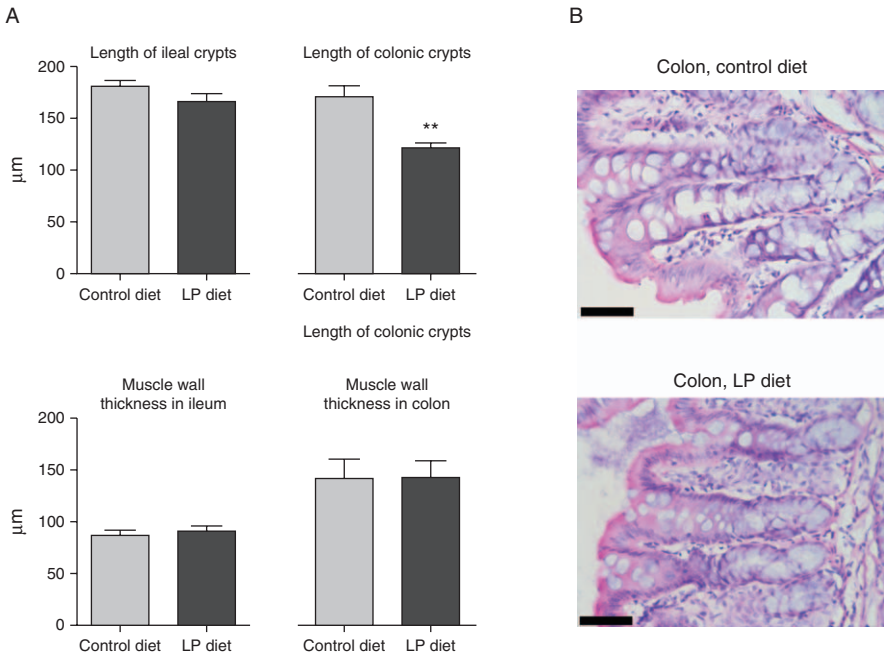

FIGURE 2. LP diet–induced alterations in colon morphology. A, The length of intestinal crypts and the thickness of the muscle wall was measured for both ileum and colon ($^{**}P < 0.01$ by *t* test). Data are expressed as mean ± standard error of the mean; n = 4–6. B, Representative microphotographs of colon sections stained with hematoxylin and eosin. Preparations from 1 control and 1 LP diet animal are shown (bar = 50 µm). LP = low protein.

Moreover, malnutrition-driven events occurring at the colonic lumen and epithelium may possibly modify the way enteric afferents signal to the central nervous system. This may be especially relevant during adolescence considering the wealth of colonic innervation and the emerging relevance of the microbiota-gut-brain axis in the pathophysiology of central nervous system disorders such as depression and anxiety (30).

Although macromolecule transepithelial transit was not affected, both ileal and colonic TEER was reduced by LP diet, suggesting these tissues were more permeable than controls to electrolytes and other entities smaller than 4.4 kDa. Interestingly, morphological changes were only observed in the colon mucosa. Other studies report a decrease in small intestinal mucosa thickness; however, this effect is only observed under more severe protein restriction (13) (0.03% vs 4% used in the present study) or after longer treatment times (15,16,31) (>6 weeks vs 20 days of LP diet

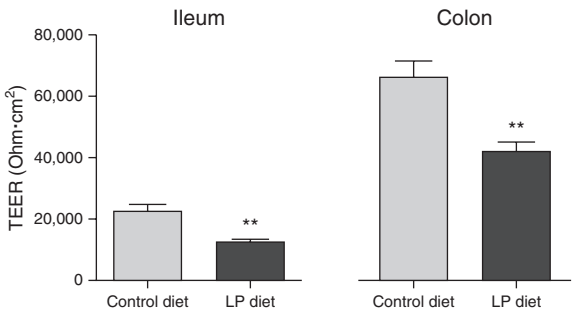

FIGURE 3. Intestinal TEER in rats subjected to LP diet. Basal TEER was measured for both ileum and colon ($^{**}P < 0.01$ by *t* test). Data are expressed as mean ± standard error of the mean; n = 4. LP = low protein; TEER = transepithelial electrical resistance.

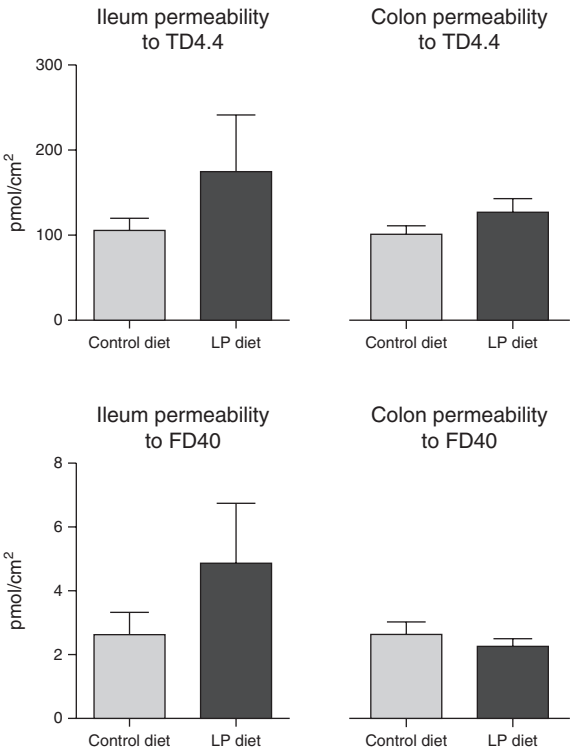

FIGURE 4. Gut permeability to macromolecules in rats subjected to LP diet. Graphs show the extent of mucosa-to-serosa permeation of TRITC-dextran 4.4 kDa (TD4.4) and FITC-dextran 40 kDa (FD40) in ileum and colon over a 3-hour incubation. Data are expressed as mean ± standard error of the mean; n = 4.

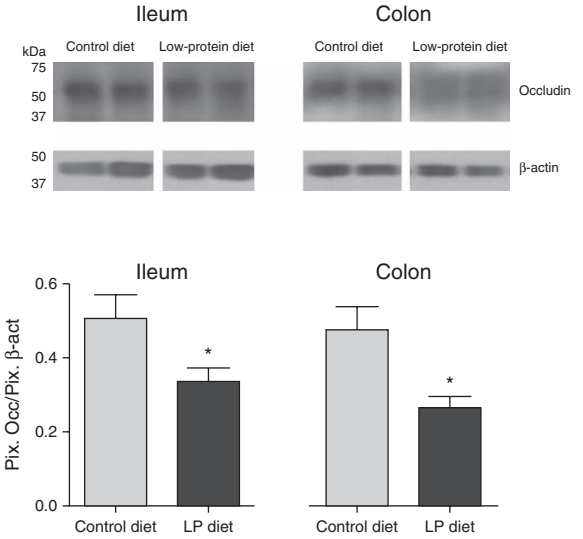

FIGURE 5. Mucosal expression of occludin in rats subjected to LP diet. The upper panel shows representative photographs of the Western blots for occludin and β-actin. The lower panel shows the result of densitometric analyses of the bands. Data are expressed as mean ± standard error of the mean; n = 6–8. LP = low protein.

used in the present study). Therefore, in juvenile rats colon mucosa thickness appears to be especially sensitive to dietary protein reduction; however, it is important to say that due to inadequate positioning of villi in our ileal histological sections we were not able to evaluate villi length, and therefore we can only state that ileal crypt morphology was unaffected by the LP diet.

Morphological changes alone do not explain the decreased TEER (which was also diminished in ileum). Interestingly, both ileum and colon showed significant reduction in the expression of the tight junction protein occludin indicating a potential change in epithelial cell differentiation. Taken together, these results suggest that protein-malnourished animals could have increased exposure to antigens not only in the small intestine, as previously reported (14), but also in the colonic lumen. Nonetheless, how LP diet affects colonic mucosal immunity and its potential neuroendocrine implications need to be further explored.

Decreased plasma levels of micronutrients such as zinc are observed in children with protein malnutrition (32) and in animals subjected to LP diet (25). Zinc is also used as an adjunct therapy to antidiarrheal rehydration solutions (33), and it has been proposed that it has antisecretory effects on the intestinal epithelium, by nonselective blockade of basolateral K channels (34). Moreover, the ex vivo actions of luminal zinc are enabled in ileum and colon when tight junctions are open (34). Therefore, under conditions of LP ingestion, enhanced colonic permeability may contribute to facilitate the therapeutic actions of exogenous zinc in the treatment of diarrhea.

The present data strongly suggest that the large intestine is another potential target for malnutrition-induced permeability changes. There is a need for clinical data in this regard and future efforts should focus on establishing colon permeability status in protein-malnourished adolescents and children.

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