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Cranberry proanthocyanidins improve the gut mucous layer morphology and function in mice receiving elemental enteral nutrition

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Abstract

Background—Lamina propria Th2 cytokines, IL-4 and IL-13, stimulate goblet cell (GC) proliferation and mucin2 (MUC2) production, which protects the intestinal mucosa. Elemental enteral nutrition (EEN) reduces tissue IL-4 and impairs barrier function. Since proanthocyanidins (PAC) stimulate oral mucin levels, we hypothesized that adding PAC to EEN would maintain Th2 cytokines – without stimulating Th1 cytokines - and preserve luminal MUC2 vs. EEN alone.

Materials and Methods—70 mice were randomized to 5 diet groups (14/group): Standard Chow, intragastric EEN, EEN+lowPAC (8 mg), EEN+midPAC (50 mg), or EEN+highPAC (100 mg PAC/kg body weight) for 5 days, starting 2 days after gastric cannulation. Ileal tissue was analyzed for histomorphology and the cytokines IL-4, IL-13, IL-1β, IL-6, TNF-α by ELISA. MUC2 was measured in intestinal washes by western blot.

Results—EEN lowered IL-13 ($p<0.05$) compared to Standard Chow, while IL-4 did not reach significance ($p<0.07$). However, EEN+lowPAC and EEN+midPAC increased IL-13 ($p<0.05$), while EEN+highPAC increased both IL-4 and IL-13 ($p<0.05$), compared with EEN alone. All EEN diets reduced ($P<0.05$) crypt depth compared to the Standard Chow group. Compared with Standard Chow, GC numbers and luminal MUC2 were reduced with EEN ($p<0.05$). These effects

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STATEMENT OF AUTHORS' CONTRIBUTIONS TO MANUSCRIPT

J.F.P., A.F.H, D.S., J.D.R., K.A.K., and C.G.K. designed research; J.F.P., A.F.H., R.P.F., and D.A.R. conducted research; J.F.P. analyzed data, and performed statistical analysis; J.F.P., D.S., J.D.R., and K.A.K wrote paper. J.F.P. had primary responsibility for final content; All authors have read and approved the final manuscript.

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were attenuated ($p<0.05$) with EEN+midPAC and EEN+highPAC. No changes were observed in tissue Th1 cytokines IL-1β, IL-6, and TNF-α.

Conclusions—Adding PACs to EEN reverses the impaired intestinal barrier resulting from EEN by improving the gut mucous layer morphology and function through increased size and number of GC as well as increased levels of MUC2 and ileal IL-4 and IL-13.

Keywords

enteral nutrition; proanthocyanidins; goblet cells; mucin; cytokines

INTRODUCTION

Elemental enteral nutrition (EEN) is a therapeutic option for inflammatory bowel disorders such as Crohns disease [1]. Unfortunately, EEN induces well-defined dysfunction of the mucosal immune system, specifically within the gut-associated lymphoid tissue (GALT), and suppresses mucosal barrier function when compared to normal nutrition [2–5]. The integrity of the mucosal barrier is critical for maintaining the physical and chemical barrier against food and environmental antigens, including microbes [6]. The mucosal barrier is partly dependent upon the physical and compositional characteristics of the mucous layer [7]. Dietary compounds that affect this layer may have implications in health through modulation of the intestinal barrier [8].

PAC are a class of polyphenolic compounds widely distributed in plant-derived foods and beverages [9–11] that are associated with the prevention of chronic diseases in epidemiological studies [12–14]. However, PAC are minimally absorbed due to non-hydrolyzable bonds between monomeric subunits and a propensity to bind proteins through hydrogen bonding [15]. PAC complex salivary glycoproteins, a process that causes astringency in the oral cavity when many fruits and beverages are ingested [16]. Complexation induces salivary excretion, hypertrophy of the parotid gland, and a shift in salivary composition to proline-rich glycoproteins in rodents [16, 17]. Because of poor absorption, greater than 95% of PAC remain in the intestinal lumen during transit [18, 19] suggesting beneficial dietary effects of PACs may occur through interactions at the mucosal surface of the gastrointestinal tract [8], for example, by influencing secretion of mucins, a class of glycoproteins, in the small intestine [14, 20].

Mucins are secreted by goblet cells (GC) and play a critical role in maintaining mucosal integrity [7]. GC, specialized intestinal epithelial cells, migrate up the villi after differentiating from crypt stem cells, turning over with the epithelial layer every 3–5 days. Mucin2 (MUC2) is the most abundant mucin secreted by intestinal GC. The importance of MUC2 is underscored in $MUC2^{-/-}$ mice, in which the deficiency leads to the development of lethal colitis [21]. MUC2 secretion is induced by cholinergic stimulation [22], while its production is regulated by the T-helper 2 (Th-2) cytokines IL-4 and IL-13, derived from lamina propria or intraepithelial lymphocytes [23–25].

In this study, we hypothesized that the addition of physiologically relevant doses [26, 27] of cranberry PAC (8–100 mg Gaelic Acid Equivalents (GAE) / kg body weight) to EEN would

attenuate the negative effects of EEN on intestinal barrier function as determined by changes in the Th-2 cytokines IL-4 and IL-13, GC number and size, and luminal MUC2. Additionally, we examined potential changes in pro-inflammatory Th1 cytokines (IL-1β, IL-6, and TNF-α) [28, 29] and histomorphometric parameters (e.g., villi length and crypt depth) [30].

MATERIALS AND METHODS

PAC Preparation and Characterization

The methodology for PAC preparation and characterization was previously published [31]. Briefly, Non-depectinized cranberry presscake was ground with liquid nitrogen and extracted with 70% acetone (Fisher Scientific, Fair Lawn, NJ). Samples were sonicated and centrifuged at for 10 minutes. The extraction was repeated twice. Acetone was removed by evaporation and the aqueous suspension was solubilized in ethanol (Decon Labs Inc., King of Prussia, PA), followed by centrifugation to eliminate ethanol insoluble material. Cranberry presscake crude extract was loaded on a Sephadex LH-20™ (GE Healthcare, Uppsala, Sweden) column and PAC were isolated by sequential elution with ethanol, ethanol/methanol (1:1) and 80% acetone. Acetone in the last fraction that contained PAC was removed by evaporation under vacuum and re-solubilized in methanol (Fisher Scientific, Fair Lawn, NJ). The total phenolic content of the PAC fraction was determined by the modified Folin-Ciocalteu method and reported as gallic acid equivalents (GAE).

An aliquot of the cranberry presscake PAC fraction was diluted tenfold and a sample was injected onto a Waters Spherisorb® 10 μm ODS2 RP-18 column. The solvents for elution were trifluoroacetic acid/water (0.1%) and methanol. The HPLC system consisted of a Waters automated gradient controller, two Waters 501 HPLC pumps, and a Rheodyne 7125 manual injector. The elution was monitored by a Waters 996 diode array detector using Waters Millennium software for collecting and analyzing three-dimensional chromatograms.

An aliquot of the cranberry presscake PAC fraction was mixed with 2,5-dihydroxybenzoic acid (Aldrich, Milwaukee, WI) and the mixture was applied onto a MALDI-TOF MS stainless steel target and dried at room temperature. Mass spectra were collected on a Bruker Reflex II MALDI-TOF-MS (Billerica, MA) equipped with delayed extraction and a N_2 laser (337 nm) in order to characterize the range in degree of polymerization (DP) and nature of interflavan bonds in the cranberry PAC. All preparations were analyzed in the positive ion linear and reflectron mode to detect $[M+Na]^+$ and $[M+K]^+$ molecular ions. MALDI-TOF MS is ideally suited for characterizing PAC because, unlike electrospray ionization in which multiple charge molecular ions create very complex spectral peaks that are often difficult to interpret, this mass spectral technique produces only a singly charged molecular ion for each parent molecule [11].

Animals

All animal experiment protocols were approved by Animal Care and Use Committee of the University of Wisconsin-Madison and the Middleton Veterans Administration Hospital, Madison. Male Institute of Cancer Research (ICR) outbred mice were purchased through

Harlan (Indianapolis, IN) and housed in an American Association for Accreditation of Laboratory Animal Care-accredited conventional facility on the V.A Williamson Hospital Campus. The mice were acclimatized for one week in a temperature and humidity controlled environment with a 12h/12h light/dark cycle. The mice were housed 5 per micro isolater-top cages and fed *ad libitum* Chow (Rodent Diet 5001, LabDiet, PMI Nutrition International, St. Louis, MO) and water for 1 week prior to initiation of study protocol. A description and detailed chemical composition of Rodent Diet 5001 is available at http://labdiet.com/pdf/5001.pdf. Once entering study protocol, the mice were housed individually in metal wire-bottomed cages to prevent coprophagia and ingestion of bedding.

Experimental design

Seventy male ICR mice (6 to 8 wk old) were randomized by weight (n = 14 / diet group) to receive Standard Chow, intragastric EEN or intragastric EEN+PAC [8 mg (EEN+lowPAC), 50 mg (EEN+midPAC) or 100 mg (EEN+highPAC) GAE of PAC/kg body weight]. Animals were anesthetized with intraperitoneal administration of ketamine (100 mg/kg) and acepromazine (10 mg/kg) and gastrostomy was performed. Catheters were tunneled subcutaneously from the gastrostomy site, over the back, finally exiting mid-tail. The mice were partially restrained by the tail for the remainder of the study to protect the catheter during infusions. This partial restraint technique does not induce significant stress in the mice [32]. The catheterized mice were connected to infusion pumps and allowed to recover for 48 h while receiving 4 mL/d of saline (0.9%) via the catheter. The mice also received *ad libitum* Chow (Rodent Diet 5001, LabDiet) and water.

Following the recovery period, animals received their assigned dietary treatments. The Standard Chow fed mice were given *ad libitum* chow diet and water, and continued to receive 0.9% saline at 4 mL/d via the intragastric catheter. EEN and EEN+PAC fed mice received solution at 4 mL/d (day 1), 7 mL/d (day 2) and 10 mL/d (days 3–5) as well as *ad libitum* water throughout the study. The EEN solution includes 6.0% amino acids, 35.6% dextrose, electrolytes, and multivitamins, with a non-protein calorie to nitrogen ratio of 126.1 (527.0 kJ/g nitrogen). This value meets the calculated nutrient requirements of mice weighing 30 to 35 g [33].

After 5 d of feeding (7 days post-catheterization), mice were weighed, anesthetized as before, and exsanguinated via left axillary artery transection. The small intestine from each mouse was removed and the lumen rinsed with 20 mL HBSS (Bio Whittaker, Walkersville, MD). The luminal rinse was centrifuged at 2,000 x *g* for 10 min, and supernatant was aliquoted and frozen at −80°C for MUC2 analysis. Ileal tissue samples were obtained from a 3 cm segment of ileum that excluded Peyer's patches. Samples for cytokine determination were flash-frozen in liquid N_2 with 1% protease inhibitor cocktail (p8340, Sigma-Aldrich, St. Louis, MO) and stored at −80°C until subsequent analysis, while samples for GC analysis were fixed in 4% paraformaldehyde overnight, transferred to 70% ethanol, and stored at 4°C until subsequent histology.

Analysis of ileal cytokines

The flash-frozen small intestine segment from each animal was homogenized in RIPA lysis buffer (Upstate, Lake Placid, NY) containing 1% protease inhibitor cocktail (Sigma-Aldrich). The homogenate was kept on ice for 30 min prior to centrifugation at 16,000 x *g* for 10 min at 4°C. The supernatant was then stored at −20°C until analysis. Prior to storage, the protein concentration of the supernatant was determined by the Bradford method using BSA as a standard.

Concentrations of IL-4, IL-13, IL-1β, IL-6, and TNF-α were determined in the supernatant using solid phase sandwich ELISA kits (BD Biosciences, San Diego, CA), according to manufacturer's instructions and identical to our previous work [5, 34]. The absorbance at 450 nm was determined using a Vmax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). The respective cytokine concentrations in the samples were determined by using a 4-parameter logistic fit standard curve (SOFTmax PRO software; Molecular Devices; Sunnyvale, CA) and normalized to total tissue protein content.

Analysis of luminal MUC2

Our method of MUC2 analysis was similar to previous work [35–38]. Proteins in the intestinal wash fluid (4μL) from each animal were separated by 10% agarose gel by electrophoresis at 150V for 80 min at room temperature. The resolved proteins were transferred to polyvinylidene fluoride membrane using tris-glycine buffer containing 20% methanol at 80V for 60 min at 4°C. The membrane was blocked with 5% nonfat dry milk prepared in Tris buffered saline containing Tween (0.05%) for 1 h at room temperature with constant agitation. Then, the membrane was incubated with mouse anti-human MUC2 (ab-11197, Abcam Inc, Cambridge, MA) primary antibody (diluted 1:2500) overnight at 4°C with constant agitation. The membrane was washed and incubated with stabilized goat anti-mouse IgG-HRP conjugate (sc-2005, Santa Cruz Biotechnology, CA) secondary antibody (diluted 1:20,000) for 1 h at room temperature with constant agitation. After washing, the membrane was incubated with HRP substrate (Super Signal West Femto substrate; Pierce, Rockford, IL) for 5 min and the protein of interest (MUC2) was detected using photographic film. The relative intensities of both the monomeric and dimeric forms of MUC2 were determined together for each sample using NIH ImageJ software (version 1.43, http://rsbweb.nih.gov/ij/); internal controls were used to normalize the densitometry across multiple films.

Histomorphometric analysis

The fixed ileal tissue sections were processed (Tissue-Tek V.I.P, Sakura Finetek, Torrance, CA), and embedded in paraffin. The embedded tissue was cut (5 μm thick), deparaffinized, rehydrated through graded ethanol washes (100% ethanol x 2, 95% ethanol x 2, 70% ethanol x 1, 2 min each) and placed into distilled H_20. Samples were stained with periodic acid-schiff (PAS) and counterstained with hematoxylin. GC number was determined by determining the average number of GC present in 15 individual villi per animal. GC size (μm^2) was obtained by imaging tissue sections and analyzing individual GC area with NIH ImageJ software (version 1.43, http://rsbweb.nih.gov/ij/). Villi length and crypt depth

measurements were determined in 15 villi and crypts. The histomorphometric measurements were performed by two independent, blinded researchers.

Statistical analysis

A fixed effects ANOVA model was fit for each measured parameter using the PROC MIXED function of the statistical software (SAS Software (Version 8), SAS Institute Inc, Cary, NC) to test for significant effects of diet. The correlations between observations between diet groups were modeled using a diagonal covariance structure. For each measured parameter, the model was fit using the untransformed data, and the residuals were evaluated to ensure that standard ANOVA assumptions of constant variance and normality were reasonably met. Transformations of the data were performed if required to improve adherence to these assumptions. Type III tests were then performed to evaluate the significance of the effects of interest for each measured parameter, and least-square means were calculated for the diet groups. Primary effects of interest were differences between the: (1) Standard Chow and EEN groups, (2) EEN and EEN+PAC groups (at each dose), and (3) Standard Chow and EEN+PAC groups (at each dose). The Standard Chow group was included in analysis as a positive control as done in all of our previous work. The data are reported as least-square mean ± standard error of mean (SEM). Statistical significance was accepted at $p < 0.05$.

RESULTS

PAC characterization by HPLC and MALDI-TOF MS

The cranberry presscake PAC eluted as two unresolved peaks that had absorbance at 280 nm and minor absorbance at 520 nm due to the presence of covalently linked anthocyanin-proanthocyanidin pigments. No peaks were observed with an absorbance max typical of the other classes of cranberry polyphenolic compounds (anthocyanins, hydroxycinnamic acids, and flavonols). The poorly resolved chromatogram at 280 nm is due to structural heterogeneity of cranberry presscake PAC [11].

Reflectron mode MALDI-TOF MS showed masses that correspond to PAC with at least 1A-type interflavan bond in trimers to undecamers. MALDI-TOF MS linear mode spectra had m/z peaks that correspond to cranberry presscake PAC with a range of 3 to 23 degrees of polymerization. The spectra also contained m/z peaks that correspond to covalently linked anthocyanin-proanthocyanidin molecules, ranging from monomers to heptamers (data not shown).

Body Weight Changes

Pre-experiment body weights did not significantly differ between treatment groups. Post-experiment body weights were significantly ($p < 0.05$) lower in all EEN fed groups compared with standard Chow [Table 2]. The decrease in body weight observed in EEN groups is partly due to absence of bowel fecal content, which we have measured previously at 1–1.5 grams. Post-experiment body weight between EEN fed groups did not differ.

Analysis of ileal cytokines

IL-4 level in the ileal tissue of the EEN group was lower than in the Standard Chow group, almost reaching statistical significance (P = 0.051) [Table 3]. IL-4 levels in the EEN +highPAC group was significantly higher than in the EEN group (P < 0.005), while levels in EEN+lowPAC nor EEN+midPAC groups significantly differed from the EEN group. Additionally, Tissue IL-4 was significantly greater in EEN+highPAC than EEN+lowPAC (P < 0.005).

EEN significantly reduced IL-13 in the ileal tissue compared to Standard Chow (P < 0.05). IL-13 levels in the EEN+lowPAC (P< 0.05), EEN+midPAC (P < 0.05), and EEN+highPAC (P < 0.005) were significantly higher than in the EEN group alone.

Compared with Standard Chow, EEN did not significantly affect the Th1 cytokines, IL-1β, IL-6, or TNF-α; the addition of PACs at any dose had no effect on these cytokines.

Analysis of GC density and size

While the length of villi were decreased in all EEN fed groups compared with Standard Chow, these changes were not significant. However, there was a significant reduction in crypt depth with all EEN diets (P< 0.05) compared with Standard Chow. The addition of PAC to EEN had no significant effect upon villi length or crypt depth compared with the EEN alone [Table 4].

EEN significantly reduced the number of GCs per villi compared with Standard Chow (P < 0.005). EEN+lowPAC (P < 0.05), EEN+midPAC (P < 0.01), and EEN+highPAC (P < 0.0001) significantly increased the number of GCs per villi compared with EEN alone. The number of GCs per villi in the EEN+highPAC was significantly greater than the EEN +lowPAC group (P < 0.05). When adjusted for villi length (GCs/μL villi length) in EEN, there were no significant differences between EEN and Standard Chow in the number of GCs (P = 0.12). However, there were more GCs/villi length in the EEN+midPAC (P = 0.05) and EEN+highPAC (P<0.01) compared with EEN alone. A representative histomorphometric image is shown for Standard Chow, EEN, and EEN+highPAC [Figure 1].

Although the GC size (μm^2) in the EEN group was smaller than in the Standard Chow group, this difference was not significant (P = 0.29) [Table 4]. The GC sizes in the EEN +lowPAC (P < 0.05), EEN+midPAC (P < 0.01), and EEN+highPAC (P < 0.05) groups were significantly greater than EEN alone.

Analysis of luminal MUC2

The monomer and dimer observed, at molecular weight markers 250 and 500 kDa respectively, were consistent with other reports of the highly oligomeric structure of intestinal MUC2 [35–38]. The relative luminal MUC2 [Figure 2] in the EEN and EEN +lowPAC groups was lower than the Standard Chow group, although these differences failed to reach significance (P = 0.057). However, the relative luminal MUC2 in the EEN +highPAC (P <0.005) group was higher than EEN alone, but the EEN+midPAC (P = 0.06)

group failed to reach significance. Additionally, the level of MUC2 in the EEN+highPAC was significantly greater than EEN+lowPAC ($P < 0.05$).

DISCUSSION

This study demonstrates that the addition of cranberry PAC to EEN solution improves ileal tissue IL-4 and IL-13 levels, GC number and size, and the secretion of intestinal MUC2, which likely contribute to the impairment of the mucosal barrier integrity previously observed by EEN alone [2, 39]. The gastrointestinal mucosa maintains a physical and chemical barrier against 100 trillion resident bacteria as well as food and environmental antigens [6]. A number of interrelated factors influence this function, including mucus glycoproteins, antimicrobial molecules, specific and non-specific antibodies, enterocyte tight-junctions, and colonization of a commensal microbiota [40, 41]. Dietary intake of the host affects the complex interplay between these factors [42, 43]. The route and complexity of nutrition profoundly influences the mucosal immune system, specifically the mucosal associated lymphoid tissue [4, 5, 44]. A reduction in dietary intake or complexity, such as those that occur with parenteral nutrition or administration of EEN, decreases the number of lymphocytes in Peyer's patches and lamina propria, reduces levels of IgA-stimulating Th-2 type cytokines in the gut wall, and reduces levels of intestinal immunoglobulins (primarily IgA) compared to the feeding of a Standard Chow diet or administration of a complex enteral diet containing complex carbohydrates, proteins and fats [4, 34, 44, 45]. EEN also increases barrier permeability and significantly suppresses bacterial diversity within the gut [2, 39]. While the influence of dietary intake or complexity on mucosal barrier and immunity is appreciated [41], very little is known of the influence of "non-nutritive" dietary compounds such as PAC.

PAC are complex oligomeric polyphenolic compounds widely distributed in fruits, including grapes, cranberries, and apples, and other foods and beverages such as chocolate and wine [9–11]. Epidemiological studies suggest PAC may have a wide range of beneficial health effects [12–14]. However, PAC are minimally absorbed across the enterocyte layer due to non-hydrolyzable bonds between flavan-3-ol monomeric units and their ability to complex both dietary and endogenous proteins [15]. Further, PAC oligomers range in DP from 3 to 30, or more, and therefore have higher molecular weight than other common plant polyphenols. Consequentially, greater than 95% of PAC remain in the intestinal lumen during transit through the gastrointestinal tract [46–48].

Since PAC are poorly absorbed, a number of mechanisms have been investigated to explain their potential beneficial effects. PAC have been shown to exert antioxidant and non-specific antimicrobial functions within the gut [20]. Recent animal studies also demonstrate the addition of dietary PAC palliates chemically-induced colitis, although the mechanism of this remains unclear [49–51]. Another important effect of PAC is their propensity to complex salivary glycoproteins when ingested, a process that causes the astringency of many fruits and beverages [10]. Astringency occurs when PAC crosslink and precipitate salivary glycoproteins and PAC with higher DP have greater effects on crosslinking and precipitation [52]. Several biological effects occur in response to astringency including increased salivary excretion, hypertrophy of the parotid gland, and shift in salivary composition to proline rich proteins [16]. Within the intestine, *in vitro* studies demonstrate that

intra-epithelial γδ T lymphocytes, in response to PAC, activate and proliferate [53]. Interestingly, the level of γδ T cell response also increases with greater DP of PAC. These observations not only suggest that PAC may play an influential role in context of mucosal barrier physiology and immunity, but that DP of PAC may be of importance when investigating their effects. Accordingly, we previously characterized the PAC used in this experiment [31]. This analysis allows for the characterization and reliable reproduction of chromatographic fractions for inclusion in experimental treatments.

In this study, we investigated the effects of addition of cranberry PAC to EEN solution on ileal tissue cytokine levels, morphology including GC number and size, and the secretion of the primary glycoprotein MUC2, and explored the effect of physiological doses of PAC on these parameters. We used a chemically defined EEN solution administered via a gastrostomy tube as a model of an elemental enteral diet that we have previously utilized. The EEN administration results in reproducible effects on intestinal (and respiratory) mucosal immunity allowing examination of changes induced with PAC.

Compared to Chow, EEN produces significantly fewer total GCs per villi. However, when normalizing the GC numbers over villi length (GC number/*μm)*, there were no differences between EEN and the Standard Chow diet. There were also no significant differences in GC size and villi length between Standard Chow and EEN, although EEN reduced the average measurement of both parameters. Interestingly, the number of GCs per villi length was significantly preserved in the EEN+midPAC and EEN+highPAC groups compared to EEN alone. GCs normally undergo hypertrophy and hyperplasia in response to IL-4 and IL-13, which act through the IL-4 receptor α and IL-13 receptor α1, respectively [23, 54, 55]. Our data shows that EEN lowered ileal IL-4 and IL-13 levels compared with Standard Chow. The addition of PAC to the EEN diet maintained IL-4 and IL-13, but did not significantly affect the cytokines IL-1β, TNF-α, or IL-6 [Table 3]. Since GC differentiate, migrate up the villi, and slough off every 3–5 days, these findings suggests the addition of PAC to the EEN diet alters the rate of cellular differentiation of progenitor crypt stem cells to GC likely via changes in Th-2 type cytokines observed [4]. The data also suggest PAC induce the observed effect through Th2 mediated immunity consistent with a previous study showing a similar IL-4 effect in colonic tissue following ingestion of proanthocyanidins [50]. Additionally, while the effects on the Th1 cytokine TNF-α were not significant across treatment groups, the trend of reduced tissue TNF-α level with increasing doses of PAC was consistent with previous work [56].

Simultaneously, EEN suppressed the concentration of MUC2 within the lumen, although this change did not reach significance. Functionally MUC2 forms the viscous mucin layer which overlays the intestinal surface, allowing smooth passage of digesta. From an immunological stand point, secreted antimicrobial proteins and peptides from Paneth cells as well as secretory IgA (sIgA) localize and are concentrated in this layer [57]. These mucin glycoproteins also provide endogenous flora with a consistent nutrient source. The observed decrease in luminal MUC2 may increase susceptibility to bacterial opportunistic pathogens or intestinal inflammation, since others have shown that $MUC2^{-/-}$ mice are at increased risk for spontaneous colitis [21]. The addition of PAC at the EEN+midPAC and EEN+highPAC doses maintained MUC2 to levels observed in the Standard Chow group.

Cranberry PAC administration at physiologic doses [26, 27] counteracts many of the changes associated with EEN administration. One limitation of the current study is that we do not address the source of the Th2 cytokines, although studies investigating changes to tissue lymphocytes and whether a mechanism similar to astringency is responsible for these observations effects are planned. Overall, this study supports the hypothesis that reduced enteral stimulation results in the impairment of mucosal integrity and gut barrier function through the reduction in the mucin component. The current work demonstrates that the administration of EEN produces lower levels of the Th2 stimulating cytokine IL-13, lower GC number and size, and lower luminal MUC2 levels in the ileum. The addition of cranberry PAC to this diet, at physiologic doses, attenuates these changes and likely normalizes mucosal integrity. This suggests that a non-nutritional dietary component such as PAC may influence health without being absorbed from the gastrointestinal tract.

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ABBREVIATIONS

DP	degree of polymerization
EEN	elemental enteral nutrition
GAE	gallic acid equivalents
GC	goblet cells
GALT	gut-associated lymphoid tissue
ICR	Institute of Cancer Research
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MUC2	mucin2
PAC	proanthocyanidin
PAS	periodic acid-schiff
sIgA	secretory IgA
Th-2	T-helper 2 lymphocytes

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CLINICAL RELEVANCY

Multiple components of the intestinal mucosal barrier including secreted mucus and antimicrobial compounds maintain the host-bacterial relationship within the gut lumen. Elemental enteral nutrition adversely affects mucus production and secretion impairing the most basic level of gut immunity – barrier function. The addition of a complex, unabsorbed phytochemical, proanthocyanidins, to elemental nutrition improves this aspect of mucosal defense.

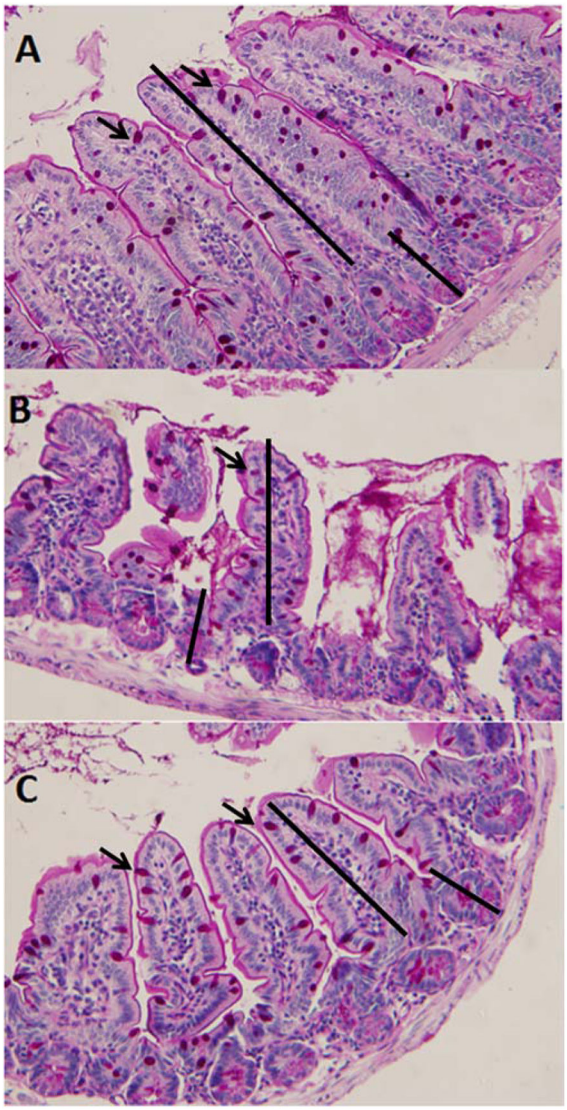

Figure 1. Representative Image of Periodic Acid Shiff-Base (PAS) Stained Ileum Tissue from (A) Chow, (B) EEN, and (C) EEN+highPAC

Goblet cells are stained pink (denoted by arrows). Measurements of villi length and crypt depth were made as indicated. 20x Zoom.

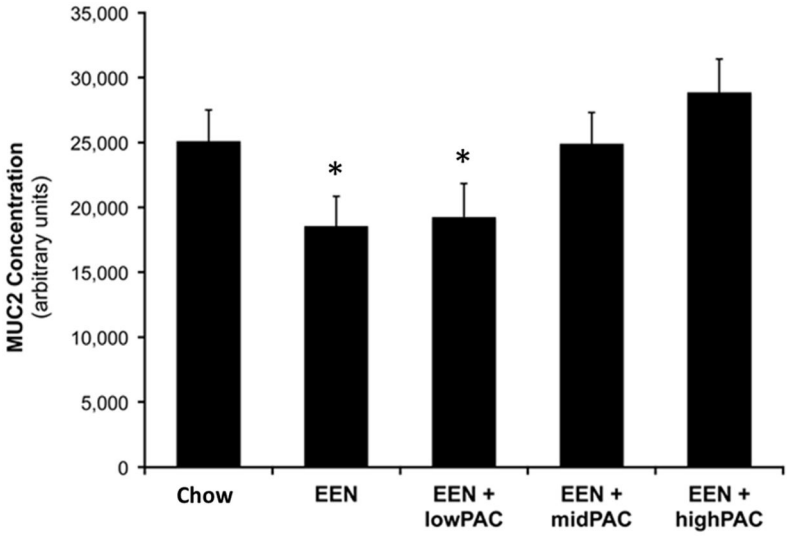

Figure 2.

Effects of Feeding Chow, EEN, EEN+lowPAC, EEN+midPAC, and EEN+highPAC Diets on Intestinal Lumen MUC2 Displayed in Arbitrary Units.

* denotes $P < 0.05$ vs EEN+highPAC.

Supplemental Table 1

Formulation of EEN Solution

Component	Amount (per 1 L)
Dextrose	356.0 g
Amino acids (Clinisol)	60.0 g
Sodium chloride	32.0 mEq
Sodium phosphate	36 mmol
Potassium chloride	16 mEq
Calcium gluconate	37.5 mEq
Potassium acetate	44.0 mEq
Magnesium sulfate	8.0 mEq
Manganese	0.8 mg
Copper	0.5 μg
Zinc	2.0 mg
Vitamin C	200 mg
Vitamin A	3300 IU
Vitamin D3	200 IU
Thiamine	6 mg
Riboflavan	3.6 mg
Pyridoxine HCl	6 mg
Niacinamide	40 mg
Folic Acid	600 mcg
Biotin	60 mcg
Cyanocobalamin	5 mcg
Vitamin E (*dl*-α-tocopheryl Acetate)	10 IU
Vitamin K1	150 mcg
Dexpanthenol	15 mg

Table 2

Effects of Feeding Chow, EEN, EEN+lowPAC, EEN+midPAC, and EEN+highPAC Diets on Intestinal Tissue Cytokines, IL-4, IL-13, IL-1β, IL-6, and TNF-α[1].

	Chow	EEN	EEN + lowPAC	EEN + midPAC	EEN + highPAC
IL-4 (pg/mg Protein)	6.01 ± 0.56	4.48 ± 0.52	4.98 ± 0.52	5.81 ± 0.52	6.99 0.52 ‡
IL-13(pg/mg Protein)	11.37 ± 1.63	7.54 ± 1.42 *	10.94 ± 1.42 ‡	11.83 ± 1.42 ‡	13.94 ± 1.79 ‡
IL-6 (pg/mg Protein)	7.55 ± 0.78 N/S	6.94 ± 0.75	7.19 ± 0.75	7.40 ± 0.75	7.04 ± 0.73
TNF-α (pg/mg Protein)	14.74 ± 2.67 N/S	18.29 ± 2.38	17.63 ± 2.46	17.69 ± 2.38	12.83 ± 2.38
IL-1β (pg/mg Protein)	167.7 ± 18.06 N/S	153.7 ± 16.8	129.3 ± 18.8	166.8 ± 16.8	114.7 ± 16.8

[1]Values are mean ± SEM, n = 6–14.

*denotes P < 0.05 vs Chow.

‡denotes P < 0.05 vs. EEN.

N/S, non-significant effect across groups.

EEN Elemental Enteral Diet.; EEN+lowPAC, EEN with 8 mg/kg BW PAC; EEN+midPAC, EEN with 50 mg/kg BW PAC; EEN+highPAC, EEN with 100 mg/kg BW.

Table 3

Effects of Feeding Chow, EEN, EEN+lowPAC, EEN+midPAC, and EEN+highPAC on Intestinal Histomorphometry[1].

	Chow	EEN	EEN + lowPAC	EEN + midPAC	EEN + highPAC
Villi Length, *μm*	171.5 ± 5.49 N/S	155.5 ± 5.14	152.5 ± 5.50	157.0 ± 5.50	155.4 ± 5.50
Crypt Depth, *μm*	79.99 ± 2.73	64.83 ± 2.55 *	66.14 ± 2.73 *	64.17 ± 2.73 *	66.17 ± 2.73 *
GC/Villi, N	9.72 ± 0.44	7.98 ± 0.36	9.15 ± 0.44 ‡	9.66 ± 0.48 ‡	10.37 ± 0.39 ‡,†
GC/Villi Length, N/*μm*	0.0598 ± 0.0036	0.0522 ± 0.0033	0.0598 ± 0.0036	0.0619 ± 0.0036 ‡	0.0657 ± 0.0036 ‡
GC area, μm^2	54.49	48.56	62.57 ‡	64.50 ‡	61.50 ‡

[1] Values are mean ± SEM, n = 8–14.

* denotes P < 0.05 vs Chow.

‡ denotes P < 0.05 vs. EEN.

† denotes P < 0.05 vs EEN+lowPAC.

N/S, non-significant effect across groups.

EEN Elemental Enteral Diet.; EEN+lowPAC, EEN with 8 mg/kg BW PAC; EEN+midPAC, EEN with 50 mg/kg BW PAC; EEN+highPAC, EEN with 100 mg/kg BW.