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JPEN J Parenter Enteral Nutr published online 28 January 2013
DOI: 10.1177/0148607112473654

Original Communication

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Journal of Parenteral and Enteral Nutrition
Volume XX Number X
Month 2012 1-8

DOI: 10.1177/0148607112473654
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Abstract

Background: Elemental enteral nutrition (EEN) decreases gut-associated lymphoid tissue (GALT) function, including fewer Peyer's patch lymphocytes and lower levels of the tissue T helper 2 (Th2) cytokines and mucosal transport protein polymeric immunoglobulin receptor (pIgR), leading to lower luminal secretory immunoglobulin A (sIgA) levels. Since we recently demonstrated that cranberry proanthocyanidins (PACs) maintain the Th2 cytokine interleukin (IL)–4 when added to EEN, we hypothesized the addition of PACs to EEN would normalize other GALT parameters and maintain luminal levels of sIgA. *Methods*: Institute of Cancer Research mice were randomized (12/group) to receive chow, EEN, or EEN + PACs (100 mg/kg body weight) for 5 days, starting 2 days after intragastric cannulation. Ileum tissue was collected to measure IL-4 by enzyme-linked immunosorbent assay, pIgR by Western blot, and phosphorylated STAT-6 by microarray. Intestinal wash fluid was collected to measure sIgA by Western blot. *Results*: Compared with chow, EEN significantly decreased tissue IL-4, phosphorylated STAT-6, and pIgR. The addition of PACs to EEN prevented these alterations. Compared with chow, EEN resulted in significantly lower levels of luminal sIgA. The addition of PACs to EEN increased luminal sIgA levels compared with EEN alone. *Conclusions*: This study suggests the addition of PACs to EEN may support GALT function and maintain intestinal sIgA levels compared with EEN administration alone. (*JPEN J Parenter Enteral Nutr.* XXXX;xx:xx-xx)

Keywords

proanthocyanidins; elemental enteral nutrition; secretory IgA; JAK-STAT

Clinical Relevancy Statement

Reduced residual diets are necessary in certain patients. Unfortunately, the decreased complexity and bulk provided with these diets decrease function and responsiveness of the gut-associated lymphoid tissue (GALT), which leads to lower intestinal and respiratory secretory immunoglobulin A (sIgA). This work describes improvements in GALT and intestinal sIgA levels during administration of a reduced residual diet with the addition of proanthocyanidins, a class of oligomeric flavonoid tannins.

Introduction

Elemental enteral nutrition (EEN) is a useful therapy option for conditions requiring a reduced residual diet, including inflammatory bowel diseases and pancreatitis.[1-3] Decreased dietary bulk and complexity provided with EEN attenuate mucosal agitation and painful symptoms. Unfortunately, reduced dietary complexity, such as provided with EEN or parenteral nutrition (PN), alters the structure and function of the gut-associated lymphoid tissue (GALT). Ultimately, reduced dietary complexity manifests as decreased secretory immunoglobulin A (sIgA) in the gut lumen compared with enteral feeds.[4-7] sIgA is the primary protective compound of acquired immunity secreted by the host mucosa, which among other notable functions exclude enteric bacteria from attachment to the host.[8,9] EEN also results in increased bacterial translocation, reduced microbiome diversity, and decreased release of antimicrobial compounds.[9-11] To address EEN-induced susceptibilities, various interventions, including the

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The contents of this article do not represent the views of the U.S. Department of Veterans Affairs or the U.S. government.

Received for publication October 26, 2012; accepted for publication December 12, 2012.

Funding: Supported by award I01BX001672 from the Biomedical Laboratory Research & Development Service of the Veterans Affairs Office of Research and Development and the Reed Research Group.

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use of natural products, have been investigated to provide anti-inflammatory and protective effects in the gut.[7] Since our established feeding model employing intragastric administered EEN results in the reproducible loss of intestinal (and respiratory) sIgA, this work investigated whether a class of natural compounds isolated from cranberries, proanthocyanidins (PACs), supports mucosal protection by stimulating luminal sIgA levels when added to EEN.

PACs are a class of polyphenols that are found in many dietary sources, including fruits, tea, chocolate, and wine.[12,13] Epidemiological studies suggest that PACs may prevent the onset of chronic pathologies, such as cardiovascular disease and cancer.[14,15] Interestingly, >95% of ingested PACs remain in the gut lumen during transit, limiting their interaction with systemic compartments and making benefits of PAC ingestion upon health unique compared with other polyphenols. We recently investigated the effect of adding PACs to EEN at 3 physiologic concentrations in our intragastric feeding model and observed improved tissue T helper 2 (Th2) cytokines, including interleukin (IL)–4 and IL-13, with increasing PAC doses.[16] Increased tissue levels of IL-4 have also been observed in experimental colitis models following PACs supplementation.[17]

Our previous work demonstrates that reduced luminal sIgA levels following EEN or PN are multifactorial, including fewer lymphocyte numbers in both Peyer's patches (PP) and lamina propria compartments; suppressed Th2 cytokines, IL-4, and IL-10 in the lamina propria; and reduced expression of mucosal polymeric immunoglobulin receptor (pIgR), which is the primary transport protein for sIgA.[4,18-20] Expression of pIgR is regulated in part through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, a cytokine signaling cascade for a wide array of cellular events.[21-24] IL-4 binds the IL-4 receptor-α, inducing intracellular STAT-6 phosphoyrlation, dimerization, and migration into the cell nucleus targeting transcription products, including pIgR.[25] Since EEN decreases sIgA levels and our recent work suggests PACs support intestinal Th2 cytokines, we hypothesized that the addition of the highest PAC dose from our previous work[16] would increase GALT function and luminal sIgA when added to EEN compared with EEN alone.

Materials and Methods

PAC Preparation and Characterization

The PAC preparation and characterization for use in experimental diets for this study were previously published.[26] Briefly, non-depectinized cranberry press cake was ground with liquid nitrogen and extracted with 70% acetone 3 times (Fisher Scientific, Fair Lawn, NJ). 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 press cake crude extract was loaded on a Sephadex LH-20 (GE Healthcare, Uppsala, Sweden) column, and PACs 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 resolubilized in methanol (Fisher Scientific). The total phenolic content of the PAC fraction was determined by the Folin-Ciocalteu method and reported as gallic acid equivalents (GAEs).

An aliquot of the cranberry press cake PAC fraction was diluted 10-fold, and a sample was injected onto a Waters Spherisorb (Waters Corporation, Milford, MA) 10-µm ODS2 RP-18 column. The solvents for elution were trifluoroacetic acid/water (0.1%) and methanol. The high-performance liquid chromatography (HPLC) system consisted of a Waters automated gradient controller, 2 Waters 501 HPLC pumps, and a Rheodyne 7125 manual injector (Sigma-Aldrich, St Louis, MO). The elution was monitored by a Waters 996 diode array detector using Waters Millennium software for collecting and analyzing 3-dimensional chromatograms.

An aliquot of the cranberry press cake PAC fraction was mixed with 2,5-dihydroxybenzoic acid (Aldrich, Milwaukee, WI), and the mixture was applied onto a matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) stainless steel target and dried at room temperature. Mass spectra were collected on a Bruker Reflex II MALDI-TOF-MS (Bruker Corp, Billerica, MA) equipped with delayed extraction and an N_2 laser (337 nm) to characterize the range in degree of polymerization and nature of the 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.[27]

Animals

The Animal Care and Use Committee of the University of Wisconsin–Madison and Middleton Veterans Administration (VA) Hospital, Madison approved all animal experimental protocols. Male Institute of Cancer Research (ICR) mice were purchased through Harlan (Indianapolis, IN) and housed in an American Association for Accreditation of Laboratory Animal Care–accredited conventional facility on the VA Williamson Hospital Campus. Mice were acclimatized for 1 week in an environment controlled for temperature and humidity with a 12/12-hour light/dark cycle. Mice were housed 5 per covered/filtered box and fed ad libitum chow (LabDiet; PMI Nutrition International, St Louis, MO) and water for 1 week prior to initiation of the study protocol. After entering the study protocol, mice were housed individually in metal cages with wire grid floors to prevent coprophagia and bedding ingestion.

Experimental Design

Male ICR mice, ages 6 to 8 weeks, were randomized to chow with a gastric catheter (n = 12), intragastric EEN (n = 12) via gastrostomy, or EEN + PACs via gastrostomy (100 mg/kg body weight [EEN + PACs]) (n = 12). Animals were anesthetized by

intraperitoneal injection of ketamine (100 mg/kg) and acepromazine (10 mg/kg). Catheters were tunneled subcutaneously from the gastrostomy site over the back and exited mid-tail. Mice were partially immobilized by tail fixation to protect the catheter during infusion. This technique does not induce significant physical or biochemical stress as was previously shown.[28]

Catheterized mice were connected to infusion pumps and allowed recovery for 48 hours while receiving 4 mL/d saline (0.9%) and ad libitum chow (Agway, Inc, Syracuse, NY) and water. Following the recovery period, experimental diets were given. Chow mice continued to receive 0.9% saline at 4 mL/d as well as ad libitum chow and water throughout the study. The EEN solution included 6.0% amino acids (Clinisol, Baxter, Deerfield, IL) and 35.6% dextrose, electrolytes, and multivitamins, with a nonprotein calorie/nitrogen ratio of 126.1 (527.0 kJ/g nitrogen). This value meets the calculated nutrient requirements of mice weighing 25–30 g.[29] 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.

After 5 days of feeding (7 days postcatheterization), mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and acepromazine (10 mg/kg) and exsanguinated via left axillary artery transection. The small intestine was removed and the lumen rinsed with 20 mL Hank's balanced saline solution (HBSS; Bio Whittaker, Walkersville, MD). The luminal rinse was centrifuged at 2000 *g* for 10 minutes, and supernatant aliquots were frozen at −80°C for sIgA analysis. Tissue samples were taken by removing a 3-cm segment of ileum excluding PPs. PP lymphocytes were assessed by counting on a hemocytometer. Samples were frozen in liquid N_2 and stored at −80°C until processing.

PP Lymphocytes

PPs from the entire length of the small intestine were removed (9–11/animal) into 1.5-mL tubes of calcium and magnesium-free (CMF)-HBSS. PPs were strained through a 100-μm mesh with a total volume of 15 mL CMF-HBSS. The effluent was collected and spun at 1700 rpm at 5°C for 10 minutes. The supernatant was removed and the pellet resuspended in 15 mL CMF-HBSS; this step was repeated. Cells were counted on a hemocytometer with trypan blue.

Tissue Cytokine Quantitative Analysis

The flash-frozen small intestine segment from each animal was homogenized in RIPA lysis buffer (Upstate, Lake Placid, NY) containing 1% protease inhibitor cocktail (P8340; Sigma-Aldrich, St Louis, MO). The homogenate was kept on ice for 30 minutes prior to centrifugation at 16,000 *g* for 10 minutes 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 bovine serum albumin as a standard.

Concentration of IL-4 was determined in the supernatant using solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, San Diego, CA), according to manufacturer's instructions. The absorbance at 450 nm was determined using a V_{max} Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). The IL-4 concentrations in the samples were determined by using a 4-parameter logistic fit standard curve (SOFTmax PRO software; Molecular Devices) and normalized to total tissue protein content (ρg/mg protein).

JAK-STAT Profiling by the JAK-STAT Antibody Microarray

The Phospho Explorer antibody microarray (Full Moon Biosystems, Inc, Sunnyvale, CA) contains 42 antibodies. Each of the antibodies has 6 replicates that are printed on coated glass microscope slides, along with multiple positive and negative controls. The antibody array experiment was performed according to an established protocol.[30] In brief, mid-ileum mucosa lysates (n = 8/group) were biotinylated with the Antibody Array Assay Kit (Full Moon Biosystems, Inc, Sunnyvale, CA). The antibody microarray slides were first blocked in a blocking solution for 30 minutes at room temperature, rinsed with Milli-Q grade water (Millipore, Billerica, MA) for 5 minutes, and dried with compressed nitrogen. The slides were then incubated with the biotin-labeled cell lysates (~80 μg protein) in coupling solution at room temperature for 2 hours. The array slides were washed 5 times with 1× Wash Solution (Full Moon Biosystems, Inc, Sunnyvale, CA) and rinsed extensively with Milli-Q grade water before detection of bound biotinylated proteins using Cy3-conjugated streptavidin. The slides were scanned on a GenePix 4000 scanner and the images analyzed with GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA). The fluorescence signal of each antibody was obtained from the fluorescence intensity of this antibody spot after subtraction of the blank signal (spot in the absence of antibody), and we used the signal of the phosphorylated protein to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping protein expression.

Analysis of pIgR expression by Western Blot

Solubilized protein from small intestinal tissue homogenate was denatured at 95°C for 10 minutes with sodium dodecyl sulfate and β-mercaptoethanol, and 20 μg of protein from each sample was separated in a denaturing 10% polyacrylamide gel by electrophoresis at 150 V for 1 hour at room temperature. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane, and Western blot was performed as previously described.[31] Densitometric measurements of protein bands were analyzed and quantified with the National Institutes of Health (NIH) Image J software. pIgR standard (cat. 2800; R&D, Minneapolis, MN) was used to compare multiple gels. The combined value of the 120-kDa and 94-kDa bands was

determined for the quantitation of the pIgR protein expression in the sample.

Analysis of IgA by Western Blot

Luminal wash sIgA was measured by Western blot since we observed that the addition of PACs to control animal luminal wash samples rapidly decreases sensitivity and total signal measured by IgA ELISA (unpublished observation), likely through the complexation between IgA and PACs.[32] In total, 4 μL of luminal fluid was denatured at 95°C for 10 minutes with sodium dodecyl sulfate and β-mercaptoethanol. Proteins were separated in a denaturing 10% polyacrylamide gel by electrophoresis at 150 V for 1 hour at room temperature and transferred to a PVDF membrane using Tris-glycine buffer plus 20% methanol at 80 V for 50 minutes at 4°C. The membrane was blocked with 5% nonfat dry milk prepared in Tris-buffered saline (TBS)–Tween for 1 hour at room temperature with constant agitation. Membranes were incubated with goat anti-mouse IgA, α-chain specific (Sigma-Aldrich), diluted 1:7000 for 1 hour at room temperature with constant agitation. Then, membranes were washed and incubated with stabilized donkey anti-goat IgA–horseradish peroxidase (HRP)–conjugated secondary antibody diluted 1:20,000 for 1 hour at room temperature. After washing, membranes were incubated with HRP substrate (Super Signal West Femto maximum sensitivity substrate; Pierce, Rockford, IL) for 5 minutes, and bands were detected using photographic film. Densitometric measurements of immunoglobulin α-chain protein bands (~55 kDa) were analyzed and quantified with the NIH Image J software. IgA heavy chain standard (M-1421; Sigma-Aldrich) was used to normalize across multiple gels.

Statistical Analysis

Experimental values were compared using analysis of variance (ANOVA) and Fisher protected least significance difference (PLSD) corrected for multiple comparisons, with $\alpha = 0.05$ considered significant (Statview 5.0.1; SAS, Cary, NC). Numerical results are presented as mean ± standard deviation of the mean.

Results

PAC Characterization by HPLC and MALDI-TOF MS

The cranberry press cake PAC eluted as 2 unresolved peaks that had absorbance at 280 nm and minor absorbance at 520 nm due to the presence of covalently linked anthocyanin-proanthocyanidin pigments (see Figure 1 in Feliciano et al[26]). No peaks were observed with an absorbance max typical of the other classes of cranberry polyphenolic compounds (anthocyanins, hydroxycinnamic acids, and flavonols), and the PAC extract was determined to be 99.0% ± 1.3% pure.[26] The poorly resolved chromatogram at 280 nm is due to structural heterogeneity of cranberry press cake PAC.[27]

Reflectron mode MALDI-TOF MS showed masses that corresponded to PAC with at least a 1A-type interflavan bond in trimers to undecamers. MALDI-TOF MS linear mode spectra had m/z peaks that corresponded to cranberry press cake PAC with a range of 3–26 degrees of polymerization (see Figure 2 in Feliciano et al[26]). The spectra also contained m/z peaks that corresponded to covalently linked anthocyanin-proanthocyanidin molecules, ranging from monomers to heptamers.

PP Lymphocytes

Compared with chow ($4.533 \times 10^6 \pm 1.226 \times 10^6$ cells), EEN significantly lowered total PP lymphocytes ($2.428 \times 10^6 \pm 0.574 \times 10^6$ cells, $P < .0001$) (Figure 1). Compared with EEN alone, total PP lymphocytes were significantly higher in EEN + PAC ($3.957 \times 10^6 \pm 1.291 \times 10^6$ cells, $P < .001$). There were no significant differences between chow and EEN + PAC ($P = .19$).

Ileum Tissue IL-4

EEN significantly lowered ileum IL-4 (4.15 ± 1.44, $P < .01$) compared with chow (6.5 ± 1.11 ρg/mg protein) (Figure 2). Compared with EEN alone, ileum IL-4 was significantly higher in EEN + PAC (5.8 ± 2.2, $P < .05$), and the difference between the level of ileum IL-4 for chow and EEN + PAC was not significant ($P = .42$).

Ileum Tissue Phosphorylated STAT-6

Phosphorylated STAT-6 (PSTAT-6) was measured at 2 phosphorylation sites, Tyrosine 641 (Tyr641) and Threonine 645 (Thr645), and normalized to GAPDH expression. Compared with chow (8.66 ± 1.5 PSTAT-6 [Tyr641]/GAPDH), PSTAT-6 at the Tyr641 site was significantly reduced with EEN (6.08 ± 1.3, $P < .001$). The addition of PAC to EEN significantly elevated PSTAT-6 at Tyr641 (8.11 ± 0.7, $P < .01$). There was no difference between chow and EEN + PAC ($P = .37$) (Figure 3A).

Similarly, compared with chow (8.97 ± 1.6 PSTAT-6 [Thr645]/GAPDH), PSTAT-6 at Thr645 was significantly lower with EEN (6.60 ± 1.0, $P < .01$). The addition of PAC to EEN significantly elevated PSTAT-6 at Thr645 (7.99 ± 0.9, $P < .05$), but there was no significant difference between chow and EEN + PAC ($P = .13$) (Figure 3B).

Ileum Tissue pIgR

EEN (10.23 ± 5.23) lowered tissue pIgR (relative concentration/20 μg protein) compared with chow (20.71 ± 7.63, $P < .001$) (Figure 4). PAC + EEN (16.13 ± 5.97, $P < .03$) levels of

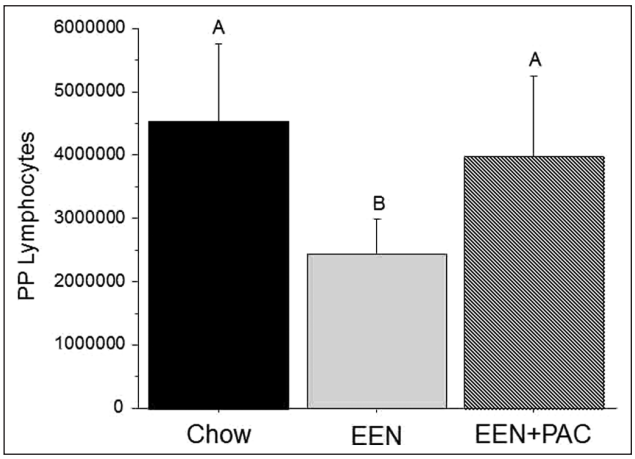

Figure 1. The total number of Peyer's patch (PP) lymphocytes in chow-, EEN-, and EEN + PAC–fed mice. Means without a common superscript differ significantly, $P < .05$. EEN, elemental enteral nutrition; PAC, proanthocyanidin.

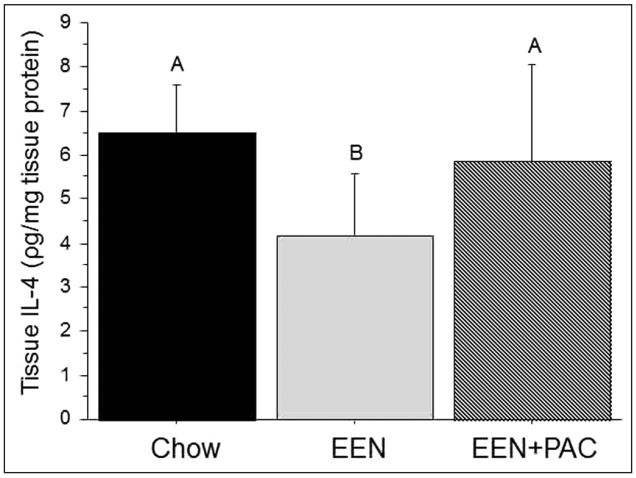

Figure 2. Ileum tissue interleukin (IL)–4 levels in chow-, EEN-, and EEN + PAC–fed mice. Means without a common superscript differ significantly, $P < .05$. EEN, elemental enteral nutrition; PAC, proanthocyanidin.

tissue pIgR were significantly higher than EEN alone. There was no significant difference between chow and EEN + PAC ($P = .08$).

Luminal sIgA

Compared with chow (17.62 ± 6.52), the level of luminal sIgA (relative concentration/4 μL luminal wash) was significantly lower following EEN (10.33 ± 4.23, $P < .001$) (Figure 5). The addition of PAC to EEN (14.67 ± 5.86, $P < .05$) significantly elevated luminal sIgA compared with EEN alone. There was no significant difference between EEN + PAC and chow ($P = .15$).

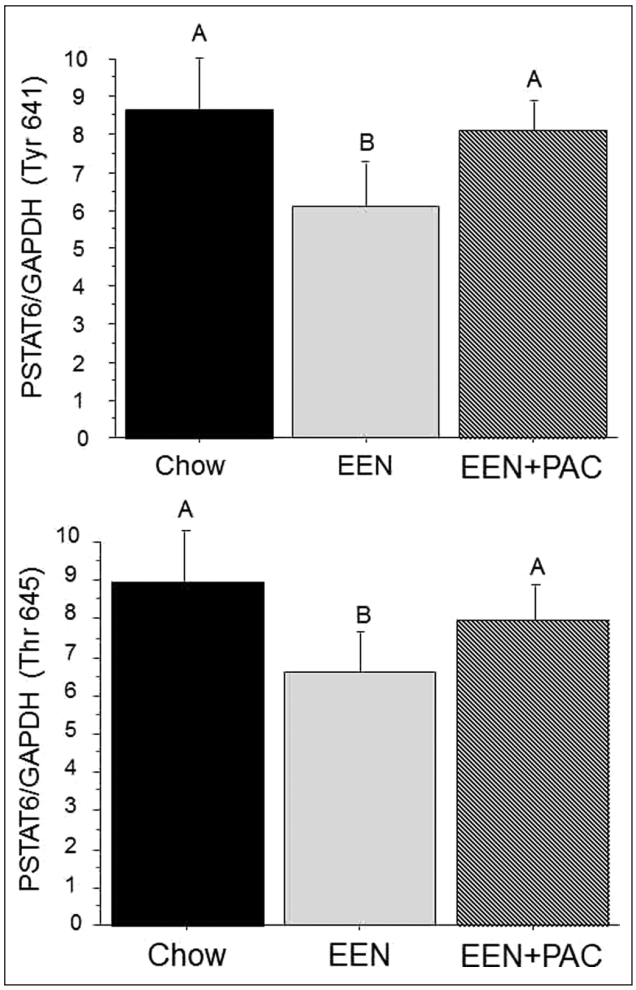

Figure 3. (A) Phosphorylated STAT-6 (Tyr641) and (B) phosphorylated STAT-6 (Tyr645) ileum tissue levels in chow-, EEN-, and EEN + PAC–fed mice. Means without a common superscript differ significantly, $P < .05$. EEN, elemental enteral nutrition; PAC, proanthocyanidin.

Discussion

EEN allows alimentation to patients with contraindication to normal feeding by administering a liquid diet directly into the gastrointestinal (GI) tract.[33] EEN formulas are usually used in clinical conditions involving intestinal or pancreatic inflammation.[2] Our previous work demonstrates that the administration of a glucose–amino acid infusion (EEN) administered via gastrostomy decreased several aspects of GALT function, including fewer PP and lamina propria lymphocytes; reduced tissue IL-4 and IL-10; reduced pIgR, the sIgA mucosal transport protein; and decreased levels of luminal sIgA.[4-7,34] Unfortunately, these changes result in increased susceptibility to infection and inflammation since sIgA is the primary protective molecule of specific (acquired) immunity that is secreted onto mucosal surfaces.[34,35] sIgA opsinizes bacteria,

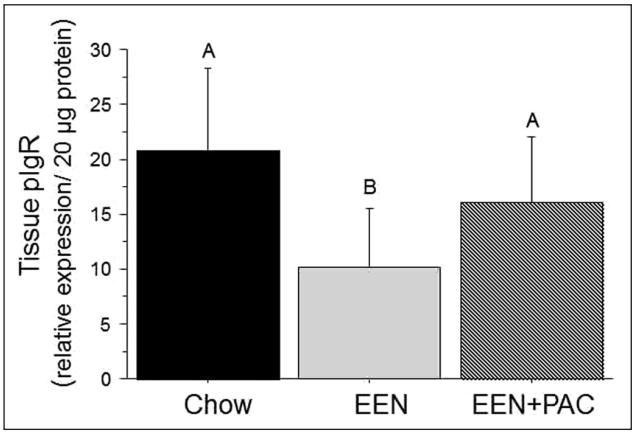

Figure 4. Ileum tissue levels of polymeric immunoglobulin receptor (pIgR) in chow, EEN-, and EEN + PAC–fed mice. Means without a common superscript differ significantly, $P < .05$. EEN, elemental enteral nutrition; PAC, proanthocyanidin.

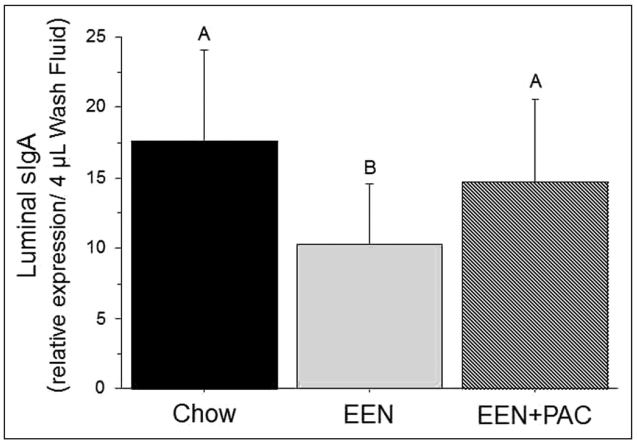

Figure 5. Concentration of secretory IgA in small intestine luminal wash samples in chow, EEN-, and EEN + PAC–fed mice. Means without a common superscript differ significantly, $P < .05$. EEN, elemental enteral nutrition; PAC, proanthocyanidin.

preventing their attachment to the mucosa,[36] and reduces virulent expression in enteric pathogens.[37] Consistent with its negative effect on luminal sIgA, EEN also increases mucosal barrier permeability and decreases microbiome diversity.[10,35] Since EEN is the only enteral formula tolerated in certain patients, EEN supplements that improve host immune and barrier function are of particular value. In this work, we investigated the effect of PACs, isolated from cranberry press cake, on GALT function leading to the release of sIgA in the intestinal lumen. We previously characterized the PACs used in this experiment, which showed the PACs ranged from 3–26 degrees of polymerization, and the extract was 99.0% ± 1.3% pure.[26] This analysis allows for the characterization and reliable reproduction of chromatographic fractions for use in experimental treatments.

PACs are complex oligomeric polyphenolic compounds distributed in fruits, including grapes, cranberries, and apples, and other foods and beverages such as chocolate and wine.[12,13] Epidemiological data suggest PACs may have beneficial health effects by preventing multiple chronic diseases.[38] However, PACs do not appear to leave the gut lumen for a variety of reasons, including nonhydrolysable bonds between flavan-3-ol monomeric units and their ability to complex both dietary and endogenous proteins. Furthermore, PAC oligomers range in degree of polymerization from 3–25+ and therefore have a higher molecular weight than other common plant polyphenols. Due to these characteristics, rodent models demonstrate that >95% of PACs remain in the intestinal lumen during transit through the GI tract,[39,40] and a recent human study demonstrated ingested PACs do not contribute to circulating flavanol levels.[41] Furthermore, in this study, we observed a pink/purple residue lining the intestinal mucosa in PAC-fed animals, consistent with the accumulation of PACs in the lumen. These observations suggest that PACs may exert beneficial health effects through their interaction at the gut mucosa.

Investigations demonstrate that PACs and precursors exert antioxidant and nonspecific antimicrobial functions in the gut[38] and are capable of palliating chemically induced colitis and gastric ulceration while increasing Th2 cytokines, including IL-4, in GI tissue. [17,42-44] We recently reported the effects of 3 increasing doses of PACs in EEN that maintained the Th2 cytokines, IL-4 and IL-13, compared with EEN alone.[16] In this study, we used the highest PAC dose from our previous work and observed increased PP cellularity, suggesting that PACs may influence GALT lymphocyte populations. Since PACs largely remain in the intestinal lumen, others have investigated mucosal intraepithelial lymphocyte populations, which are in close proximity to the lumen. Accordingly, those studies demonstrate that PACs stimulate mucosal γδ intraepithelial lymphocyte activation and proliferation.[45] However, the protective effects of orally administering polyphenols during colitis were lost in TCR$\alpha^{-/-}$ mice but not TCR$\gamma\delta^{-/-}$,[46] suggesting that αβ T lymphocytes play a more important role within intestinal tissue under the context of experimental colitis. PACs also strongly promote $CD3^+CD25^+FOXP3^+$ T-regulatory lymphocyte expansion, while suppressing IL-17 lymphocyte subsets, effects that support the production and release of sIgA.[47,48] Together, these observations suggest that PACs may influence mucosal barrier physiology and immunity that is mediated in part through lymphocyte interactions. Future work aims to determine the effect of PACs on leukocyte subsets from GALT compartments, including the PP, intraepithelial space, and lamina propria, to assess the potential role of these cells.

The expression of pIgR in the intestinal epithelium is regulated through the Th2 cytokine IL-4, which influences the nuclear factor STAT-6, a member of the JAK/STAT signaling

cascade.[21,49] STAT-6, in part, regulates luminal sIgA through regulation of the mucosal transport protein pIgR.[19] We established the importance of STAT-6 during PN with a lack of enteral stimulation showing that lower IL-4 levels correlated with levels of phosphorylated STAT-6, pIgR, and luminal sIgA. Administration of exogenous cytokines that stimulate STAT-6 phosphorylation during PN significantly increased levels of pIgR expression and luminal IgA levels, suggesting a cause-and-effect relationship.[3] In this work, EEN decreased intestinal tissue levels of IL-4 and phosphorylated STAT-6, correlating with decreased pIgR and luminal sIgA. The addition of PACs to EEN at physiological levels (100 mg GAE/kg body weight) resulted in increased tissue IL-4, STAT-6 phosphorylation, pIgR, and luminal sIgA, supporting our hypothesis that PACs may influence health by interacting with GALT tissue.

Previous work demonstrated that polyphenolic supplementation, including curcumin or a polyphenolic-rich diet, increases sIgA levels when added to normal diets.[50,51] However, our research is the first to demonstrate that PAC supplementation may improve luminal sIgA during EEN. PACs pose a significant challenge for accurately quantifying luminal sIgA since PACs form complexes with endogenous and dietary proteins, including immunoglobulins, through hydrophobic interactions and hydrogen bonding.[32] During our analysis, we observed that the addition of small concentrations of PACs to luminal wash fluid from control animals significantly decreased the detectable levels of sIgA via ELISA quantification (unpublished observation). Specifically, PAC concentrations <700 ng/mL decreased the measured sIgA by approximately 50% using ELISA. For this reason, measurement of luminal sIgA in this study was achieved by first denaturing and reducing intestinal wash fluid samples with heat, sodium dodecyl sulfate, and β-mercaptoethanol and performing Western blot analysis to detect the sIgA heavy chain directly. Future work with PACs should take the complexation and masking effect into consideration when investigating intestinal sIgA. There were no differences in body weight or signs of toxicity with the current dose of 100 mg/kg body weight. It should be noted that since a principal characteristic of PACs is complexation of dietary and endogenous proteins, consumption of elevated levels of these compounds can inhibit digestion and nutrient absorption, which can lead to decreased growth efficiency.[40] However, the EEN formula used in this study contains only simple macronutrients that are unlikely to complex with PACs sufficiently to impair nutrient absorption.

One limitation to this study is that it remains unclear if the effects of PAC are dependent on direct GALT stimulation, such as through PP or intraepithelial lymphocyte interactions at the mucosa, or if changes to the GI luminal environment, including increased goblet cell mucin secretion[16] and microbiome diversity, are responsible for maintained GALT function. Other work in our laboratory demonstrated that the addition of PACs to EEN also maintains microbiome diversity in the gut lumen compared with the reduced diversity that both we and others have observed in EEN alone.[35]

In summary, this work supports the hypothesis that decreased enteral stimulation, such as EEN or parenteral feeding, suppresses GALT function—including total PP and lamina propria lymphocyte numbers, Th2 cytokine levels, and the mucosal sIgA transport protein, pIgR—that leads to reduced luminal sIgA levels. Consistent with the hypothesis of the current study—that PACs may provide immunoprotective effects through interactions with the GALT and intestinal mucosa—the supplementation of physiological doses of PACs to EEN elevates GALT function and luminal sIgA compared with EEN feeding alone. This study suggests that moderate levels of PACs may be beneficial when added to enteral diets by promotion of adaptive immune function.

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