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Chronic oral exposure to glycated whey proteins increases survival of aged male NOD mice with autoimmune prostatitis by regulating gut microbiome and anti-inflammatory responses

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Abstract

Glycated whey proteins have been shown to be protective against type 1 diabetes in our previous studies, suggesting a potential application as medical food. To determine if the protection could be extended to other autoimmune diseases, aged male non-obese diabetic (NOD) mice that develop a wide spectrum of autoimmune pathologies, including spontaneous autoimmune prostatitis, were used. After a 6-month oral exposure to whey protein-derived early glycation products (EGPs), EGP-treated NOD mice had an increased survival rate, decreased macrophage infiltration in anterior lobe and decreased inflammation in the prostate when compared to the mice that received non-reacted control. The systemic immunity was regulated towards anti-inflammation, evidenced by an increase in serum IL-10 level and decreases in total splenocytes, splenic M1 macrophages, CD4⁺ T cells, CD8⁺ T cells and B cells. Consistent with an overall anti-inflammatory status, the gut microbiome was altered in abundance but not diversity, with increased *Allobaculum*, *Anaerostipes*, *Bacteroides*, *Parabacteroides* and *Prevotella*, and decreased *Adlercreutzia* and *Roseburia* at the genus level. Moreover, increased *Bacteroides acidifaciens* correlated with most immune parameters measured. Collectively, chronic oral exposure to EGPs produced an anti-inflammatory effect in aged male NOD mice, which might contribute to the protective effects against spontaneous autoimmune prostatitis and/or other organ specific autoimmune diseases.

Graphical Abstract

Glycated whey proteins modulate gut microbiome and are anti-inflammatory in aged male NOD mice.

Authors' contributions

Y.C. and T.L.G. designed the study. Y.C. performed the animal experiments and analyzed the data; T.N. performed the histological assessments; T.L.G. conducted the flow cytometry; K.M.G and T.L.G did the microbiome analysis. Y.C. and T.L.G wrote the manuscript.

Conflicts of interest

There are no conflicts to declare.

Introduction

Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS), the Category III prostatitis classified by NIH, has a prevalence of over 90% among the symptomatic patients with prostatitis¹, and it can be further divided into IIIA and IIIB. The IIIA CP/CPPS is the inflammatory subtype characterized by the presence of leukocytes in expressed prostatic secretions, post prostatic massage urine or semen¹. Autoimmune process and local pro-inflammation were proposed as two important mechanisms for CP/CPPS, although other reasons (e.g., endocrine dysfunctions) also matter^{2,3}. Traditional nonsteroidal anti-inflammatory drugs are effective in inhibiting the inflammatory process and managing pain for CP/CPPS patients⁴, but anti-inflammatory therapies are not recommended as the monotherapy for treating prostatitis, and a multimodal therapeutic regimen may be used⁵. Herein, dietary supplements with anti-inflammatory properties are gaining popularity in the treatment of CP/CPPS⁴.

Glycated food proteins or dietary early glycation products (EGPs) are the Amadori or Heyns compounds produced during rearrangement in the Maillard reaction/glycation. They have enhanced functional properties, such as increased solubility and thermostability⁶. Along with the well-established physiochemical properties, their immunoregulatory effects have been under investigation. Our previous studies showed that whey protein derived early glycation products were able to polarize macrophages toward the anti-inflammatory M2 both *in vitro*⁷ and *in vivo*⁸. Sub-chronic oral exposure to these EGPs reduced the type 1 diabetes (T1D) incidence in female non-obese diabetic (NOD) mice, and the mechanisms were through increasing anti-inflammatory responses and decreasing autoantibody generation⁹. These findings suggest EGPs are anti-inflammatory and may have potential as a functional food for patients with chronic prostatitis caused by inflammation.

The animal model employed in this study was male NOD mouse. This murine strain spontaneously develops autoimmune T1D at an early age and shares similarities with human T1D development in early adolescence. However, aging male NOD mice exhibit a wide spectrum of autoimmune pathologies distributed through a variety of organs¹⁰. The intra-prostatic inflammatory infiltration is well established by about 20 weeks-of-age¹¹. In a comparison of NOD to other five stains (i.e., BALB/c, B10, NZB, MRL, SWR) regarding prostatic inflammation, NOD males exhibit the highest inflammation score spontaneously¹². In this study, non-diabetic NOD males (> 4 months) were orally exposed to EGPs or non-reacted (NR) control for up to 6 months to determine their effects on prostatic inflammation and associated mechanisms (e.g, cytokines/chemokines, gut microbiome).

Materials and methods

EGP preparation

The whey protein derived EGPs were prepared as described before ⁷. In brief, whey protein isolate (WPI, Fonterra (USA) Inc, Rosemont, IL) and glucose (Sigma-Aldrich, St. Louis, MO) were dissolved in distilled water at the molar ratio of free amino groups and reducing ends at 1:2. The solution was freeze-dried and incubated in a desiccator containing a saturated aqueous Mg(NO₃)₂ solution at 55 °C for 8 h. LC-MS showed that all the whey proteins were modified by glucose, and the reaction had not progressed to later stages ⁹.

Mouse model of autoimmune prostatitis and the treatment regimen

NOD males were obtained from Taconic Biosciences (Rensselaer, NY), and housed in the Coverdell Vivarium at the University of Georgia (UGA). The room was maintained on a 12-h dark/light cycle at 21–24 °C with 20–60% relative humidity. Mice had access to food (Diet 5053, PicoLab[®] Rodent Diet 20, LabDiet, MO) and filtered tap water *ad libitum*. Blood glucose levels were measured from sampling caudal venous tail blood with a Bayer's CONTOUR blood glucose meter (Bayer Health Care LLC, Mishawaka, IN). Diabetic mice were identified as those with a blood glucose level > 200 mg/dL in two consecutive weeks. At the study termination, the mice were euthanized by CO₂ inhalation followed by cervical dislocation. The use of the animals was performed in accordance with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals, and approved by UGA Institutional Animal Care and Use Committee (IACUC).

Age-matched non-diabetic NOD males (approximately 4-month-old) were randomly separated into 2 groups and gavaged daily with NR and EGPs at 600 mg/kg body weight (BW) for up to 6 months. This dose was physiologically relevant as discussed previously ⁷, and EGPs at this dose protected NOD mice from developing T1D ⁹. Mice were euthanized at month 3 and 6 following the first dosing. Aged NOD males have decreased incidences of diabetes, and none of them developed T1D during the experimental period. However, for the survival study, some mice had to be euthanized during the study when the thresholds of humane endpoints were reached due to other autoimmune diseases and cancer. They were recorded as moribund events.

Histological assessment of the prostatic inflammation

The urogenital organs without seminal vesicles and partial anterior prostates were dissected and placed in 10% buffered formalin. Longitudinal cuts at every 1–2 mm were conducted, and the sections were processed for hematoxylin and eosin (H&E) staining by the Histology Laboratory (College of Veterinary Medicine, UGA). The inflammation in 4 prostatic lobes (dorsal, lateral anterior and ventral) was scored by a board-certified veterinary pathologist. The scoring system was described in detail by Haverkamp et al.: 0, no inflammation; 1, mild inflammation; 2, moderate inflammation; 3, severe inflammation ¹³. The mouse prostatic lobes were identified according to the histological guidelines introduced by Oliveira et al ¹⁴.

Flow cytometric analysis

The preparation of single-cell suspensions were similar to the procedures described before⁹. A section was dissected from the anterior prostate (AP) and placed into PBS, and digested by 0.05 mg/ml collagenase (Sigma-Aldrich) and 5 U/ml DNase (Sigma-Aldrich) at 37 °C for 30–45 min. The mixture was centrifuged at $300 \times g$ at 4 °C for 8 min, and the pellet was resuspended in PBS and passed through a 40 μ m-cell strainer with a fresh tube placed underneath to collect the single-cell suspension. Spleen was mashed using the frosted ends of two microscope slides, with the red blood cell lysed by adding ACK lysing buffer (Gibco by Life Technologies, Orand Island, NY) for 5 min.

Splenocytes and AP cells were stained for the following surface markers conjugated with fluorophores: CD3 (145–2C11, PerCP-Cy5.5, BD Pharmingen), CD4 (RM4–5, V450, BD Horizon), CD8 (53–6.7, APC-H7, BD Pharmingen), F4/80 (BM8, FITC, eBioscience), CD80 (16–10A1, PE-CD594, BD Horizon), CD209 (LWC06, Alexa Fluor, Novus Biologicals), and B220 (RA3–6B2, FITC, eBioscience). After adding the antibodies, cells were incubated at 4 °C in the dark for 30 min. The cells were washed, and acquired on a Becton Dickinson LSR II Flow Cytometer (BD Biosciences). The flow cytometric data were analyzed using FlowJo v10 (FlowJo, LLC, Ashland OR). Leukocytes were characterized as CD4⁺ T cells (CD3⁺CD4⁺CD8[−]), CD8⁺ T cells (CD3⁺CD4[−]CD8⁺), macrophages (F4/80⁺), M1 (F4/80⁺CD80⁺CD209[−]), M2 (F4/80⁺CD80[−]CD209⁺), and B cells (B220⁺).

Hematology

Hematology was conducted after 6-month treatment. A volume of ~200 μ l venous blood sample was drawn from a tail nick, collected in tubes with K2EDTA (BD Microtainer, Franklin Lakes, NJ), and kept on ice. The hematology analyses with differentials were performed on the same day in the Veterinary Diagnostic Laboratory at the UGA Veterinary Teaching Hospital using the HESKA Element HT5 Veterinary Analyzer (Loveland, Co).

Cytokine/chemokine quantitation

Following EGP treatment for 6 months, blood was collected from the orbital sinus of mice after being deeply anesthetized. The sera were separated and stored at −80 °C prior to use. Thirty-one cytokines/chemokines in the sera were detected using the MILLIPLEX MAP Mouse Cytokine/Chemokine Kit (Millipore, Billerica, MA) following the manufacturer's instructions, and the data were collected and analysed using Bio-Plex Manager 6.1.1. The working rang was 12.8–40,000 pg/ml for IL-13, and 3.2–10,000 pg/ml for the rest with IL-15 being out of range.

16S rRNA gene sequencing and bioinformatics analysis

The feces were collected from individual mice 6 months after initial EGP dosing, transferred to the 500 μ L Eppendorf tubes and kept in a −80 °C freezer. DNA was extracted using QIAamp DNA stool mini kits (Qiagen, Valencia, CA) following manufacturer protocols. For library preparation, DNA was normalized to 20 ng/ μ L at Georgia Genomic Facility (GGF) and the V3–V4 region of 16S rRNA was targeted using locus-specific primers for the first round of PCR. Illumina-specific iTru_R1_5' fusion and iTru_R2_5' fusion Read 1 and Read 2 sequencing primers (forward: S-D-Bact-0564-a-S-15, and reverse: S-D-Bact-0785-a-

A-21) were used with 20 internal tags (8 forward fusion primers and 12 reverse fusion primers) ranging from 5 nucleotides (NT) to 8 NTs long¹⁵. The PCR mix was from Kapa Biosystems, Inc. (Boston, MA). Next, the PCR amplicon aliquot was purified and quantified using AMPure beads. The second round PCR was run using Illumina i5 and i7 primers, and sequencing was done on Illumina Miseq (Illumina Inc., San Diego, US). Bioinformatics analysis was performed as previously described¹⁶. The subsequent analysis was performed on Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.0, a pipeline that worked with high-throughput 16S rRNA sequencing data¹⁶. Linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to identify significantly different taxa following the conditions on the website: <http://huttenhower.sph.harvard.edu/galaxy>. Features that had an LDA score > 2 were plotted.

Statistical analysis

The survival curve was compared using Gehan-Breslow-Wilcoxon test, and all the immune parameters were tested using Student's t-test on GraphPad Prism 6 (GraphPad Software, San Diego, CA). Non-parametric t-test was used to test the statistical significance for α diversity, and Analysis of Similarities (ANOSIM) for β diversity. Correlational analysis was assessed by XLSTAT (Addinsoft Inc., Long Island City, NY) using Spearman's correlation test.

Results

Chronic exposure to EGPs increases the survival rate and moderately reduces the prostatic inflammation

The survival rate of mice was recorded to indicate the overall effects of NR/EGPs. As shown in Fig 1, chronic oral consumption of EGPs had an overall beneficial effect on the mice when compared to NR treatment: after 6 months of dosing, EGP treatment maintained the survival rate of the male NOD mice at ~ 80%, while those dosed with NR only had a survival rate below 40%. No animals developed diabetes during the dosing period (data not shown).

To evaluate the effect of EGPs on the intraprostatic inflammation, aging NOD males (> 4 months) were euthanized after dosing for 3 or 6 months. Organs including AP were harvested, and none of them showed significant difference in weight between the NR and EGP treatment groups (Supplementary table 1). Longitudinal sections of the urogenital organs containing 4 prostatic lobes were H&E stained, and the inflammations associated with the 4 lobes were scored. Immune infiltration was detected in both dorsal and lateral prostates (Fig 2 A&B black arrows). After 3 months of dosing, EGP treatment limited the inflammation in the dorsal prostate at the mild stage for all mice, while 2/6 mice treated with NR developed moderate inflammation. After 6 months of dosing, EGPs kept the inflammation in dorsal prostate mostly at the mild stage (5 mild and 1 moderate), whereas the NR group had half mice at the mild stage and the other half at the moderate stage (Fig 2A last panel). For the lateral prostate (Fig 2B), one animal had mild inflammation after 3 months of EGP treatment, which would most likely be due to the immune infiltration prior to the experiment, which could have occurred as early as 7 weeks-of-age¹¹. Prolonged treatment with EGPs for up to 6 months protected the lateral prostate from developing

inflammation: no mice in the EGP group developed inflammation, while 1 mild and 1 moderate inflammatory incidence occurred in the NR group.

No inflammation was observed for anterior and ventral prostates using histopathological analysis (data not shown). In our previous studies, however, H&E staining was shown to be less sensitive than flow cytometric analysis⁹, evidenced by the subtle differences in the immune infiltration of the pancreas being successfully detected using the latter, but not the former. Therefore, the infiltrating immune cells in the anterior prostate, after being treated for 6 months, were analyzed by flow cytometry (Fig 2C). Macrophages, CD4⁺ T cells, CD8⁺ T cells and B cells were analyzed because they were frequently reported as being associated with inflamed prostates in NOD mice^{11, 13}. Among the analyzed cells, macrophages were significantly decreased by EGPs, while CD8⁺ T cells and B cells were numerically decreased. Taken together with the histopathological analysis, oral exposure to EGPs generated beneficial effects by controlling or slowing down the inflammatory progression in dorsal, lateral and anterior prostates. The inflammatory status within the ventral prostate had not been further studied due to technical issues (e.g., unable to clearly separate from other lobes). However, a previous study comparing the prostatic inflammation upon ovalbumin-specific CD8⁺ T cell transfer showed that the anterior, dorsal and ventral prostates responded similarly¹³.

EGPs alter the systemic immunity

The WBC number in the blood was slightly increased from 6.4 to 7.3 ($\times 10^3/\mu\text{l}$) by EGP treatment for 6 months. Neutrophil and monocyte numbers were only numerically increased, while the eosinophil number was significantly increased. On the other hand, exposure to EGPs slightly decreased the number of lymphocytes, with more differences detected for the percentage (NR 48.3% vs. EGPs 40.8% of WBC, Fig 3).

The spleen weights were not significantly different between the two groups (Fig 4A), but total splenocytes and all the measured immune populations (i.e., macrophages, M1, M2, CD4⁺ T cells, CD8⁺ T cells, B cells) were decreased in the EGP group (Fig 4B-E) with significant changes observed for total splenocytes, M1, CD4⁺ T cells, CD8⁺ T cells and B cells. However, the M2/M1 ratio was not significantly altered by EGP treatment (Fig 4C).

To further determine if EGPs generated anti-inflammatory effects, the cytokines/chemokines in sera were measured (Fig 5). The up-regulated ones among the 31 cytokines/chemokines examined were IL-10, G-CSF, IL-3, IL-5, IL-6 and IL-17 with IL-10 showing statistically significant increases (5678.85 ± 979.60 (NR) versus 9644.43 ± 764.86 (EGPs), $p = 0.0096$, Supplementary table 2). IL-17 was increased 1.664 folds by EGPs, and the mean $\pm$ SE was 72.34 ± 15.24 (NR) versus 192.68 ± 114.38 (EGPs, Supplementary table 2).

EGPs modulate gut microbiome

Correlations has been found between symptom scores and disease severity of CP/CPPS and the degree of dysbiosis in gut microbiomes¹⁷. 16S rRNA sequencing was used to characterize the changes in the microbiome community composition. Principal coordinate analysis of the β diversity metrics showed that the gut bacterial communities in the NR and EGP groups were well separated when taking difference in abundance into account using

weighted UniFrac (Fig. 6A). The weighted UniFrac result was supported by ANOSIM ($p < 0.05$ with 999 permutation), which suggested the difference in the gut microbiome induced by EGP exposure was readily observable and well differentiated. However, EGP treatment did not show a clear pattern on the unweighted Unifrac, an index of β diversity indicating presence/absence (data not shown). Both the PD whole tree and chao1 , indexes of α diversity that reflected the genetic diversity of the communities under study¹⁸, were not significantly altered by EGP treatment (data not shown).

The taxonomic profiles of the EGP treated versus the NR mice were then compared by assigning taxonomy to OTUs and subsequently splitting the OTU table into phylogenetic level. The relative abundances of taxonomy for each treatment at the phylum level are shown in Fig. 6B, with each color representing an individual bacterial phylum. Phylum *Firmicutes* represented 60.0% of the total bacteria in the NR group; while in EGP group, it represented 50.3% of the total taxa, which was significantly different from the NR group. *Firmicutes/Bacteroides* (F/B) ratio was decreased following EGP treatment (2.19 ± 0.41 vs. 1.30 ± 0.10); however, it did not reach the level of statistical significance. When the microbial taxa at the class level were compared, EGP treatment increased Erysipelotrichia, and decreased Coriobacteriia in terms of relative abundance. When the microbial taxa at the order level were compared, EGP treatment increased Erysipelotrichales, and decreased Coriobacteriales in terms of relative abundance. When the microbial taxa at the family level were compared, EGP treatment increased *Porphyromonadaceae*, *Prevotellaceae*, *Erysipelotrichaceae* and *Bacteroidaceae*, and decreased *Coriobacteriaceae* in terms of relative abundance (data not shown). When the microbial taxa at the genus level were compared, EGP treatment increased *Anaerostipes*, *Parabacteroides*, *Prevotella*, *Allobaculum* and *Bacteroides*, and decreased *Adlercreutzia* and *Roseburia* in terms of relative abundance (Fig. 6C). When the microbial taxa at the species level were compared, EGP treatment increased *Allobaculum s*, *Anaerostipes s*, *Prevotella s*, *uniformis*, *acidifaciens* and *Bacteroides s*, and decreased *Adlercreutzia s* and *Roseburia s* in terms of relative abundance (Fig. 6D).

Correlation between immunity and gut microbiome

Fig. 7 illustrates the relationships between pairs of the variables that were significantly altered following EGP treatment. Spearman's correlation analysis revealed that the number of prostatic macrophages positively correlated with total splenocytes, splenic M1 macrophages, CD4^+ T cells, CD8^+ T cells and B cells, and negatively correlated with blood eosinophils, serum IL-10 and *Bacteroides* at the genus level (Fig. 7A). When *Bacteroides* at species levels were further analyzed, the number of prostatic macrophages negatively correlated with *Bacteroides acidifaciens* and another unidentified *Bacteroides* species (Fig. 7B). Blood eosinophils positively correlated with serum IL-10, *Prevotella* and *Anaerostipes* at the genus level and *Bacteroides uniformis* at species level, and negatively correlated with total splenocytes, splenic M1 macrophages, CD4^+ T cells, CD8^+ T cells and B cells (Fig. 7A&B). The number of total splenocytes positively correlated with splenic M1 macrophages, CD4^+ T cells, CD8^+ T cells and B cells, and negatively correlated with serum IL-10 and *Bacteroides*, *Parabacteroides*, *Prevotella* and *Anaerostipes* at the genus level and *Bacteroides uniformis* and *acidifaciens* at species level (Fig. 7A&B). Splenic M1

macrophages positively correlated with splenic CD4⁺ T cells, CD8⁺ T cells and B cells, and negatively correlated with serum IL-10, *Bacteroides* and *Parabacteroides* at the genus level and *Bacteroides uniformis* and *acidifaciens* at species level (Fig. 7A&B). Splenic CD4⁺ T cells positively correlated with splenic CD8⁺ T cells and B cells, and negatively correlated with serum IL-10 and *Bacteroides* at the genus level and *Bacteroides acidifaciens* at species level (Fig. 7A&B). *Bacteroides* positively correlated with *Prevotella* at the genus level (Fig. 7A). *Parabacteroides* positively correlated with *Prevotella* and *Allobaculum* at the genus level (Fig. 7A). *Prevotella* positively correlated with *Anaerostipes* at the genus level (Fig. 7A). Taken together, the correlational analysis showed that all the significantly altered immune parameters were correlated to each other, and their further correlation with gut microbes suggested an overall anti-inflammatory feature.

Discussion

In this work, the chronic effects of EGPs were studied in aged non-diabetic NOD males; the survival rate of these NOD mice was significantly increased by EGP treatment (Fig 1), which might be related to a systemic anti-inflammatory effect generated by EGPs. EGPs decreased total splenocytes, splenic M1 macrophages, CD4⁺ T cells, CD8⁺ T cells and B cells (Fig 4), while increasing the serum IL-10 level (Fig 5). Upregulation of anti-inflammatory cytokine IL-10 by EGPs was previously observed in human macrophage culture ⁷, as well as in the NOD females ⁹ and C57BL/6 males subcutaneously transplanted with TRAMP-C2 prostate cancer cells ⁸. Interestingly, IL-10 has been shown to prevent aging-associated inflammation and insulin resistance ¹⁹, and aging-induced endothelial dysfunction ²⁰. Moreover, IL-10 might mediate its anti-inflammatory effect by metabolically reprogramming macrophages ²¹, and decreased IL-10 could result in enhanced mortality from Gram-negative sepsis ²². However, the Th2 response is also characterized by the production of IL-10, which may contribute to the immediate hypersensitivity response and eosinophil influx ²³. It has been shown that certain *Prevotella* species promotes the differentiation of Th17 cells, which synergizes with eosinophils to accelerate multiple myeloma progression ²⁴. Further studies should be conducted to determine the effects of EGP-induced increases in blood eosinophils and IL-17.

Aged male NOD mice develop a wide spectrum of organ specific autoimmune diseases ¹⁰, such as autoimmune prostatitis, that was characterized by leukocyte infiltration in the prostate gland ¹¹. We have found that EGP treatment moderately decreased prostatic inflammation (Fig 2). In addition, flow cytometric analysis suggested that the infiltrating immune cells, especially macrophages, in the anterior prostate were decreased by EGPs. The number of prostatic macrophages positively correlated with total splenocytes, splenic M1 macrophages, CD4⁺ T cells, CD8⁺ T cells and B cells, and negatively correlated with levels of serum IL-10 and gut *Bacteroides* (Genus). Specifically, the number of prostatic macrophages negatively correlated with *Bacteroides acidifaciens* and another unidentified *Bacteroides* species. *Bacteroides uniformis*, which was also increased by EGPs, could induce higher IL-10 production from Raw264.7 macrophages than other *Bacteroides* strains ²⁵. However, the function of IL-10 in prostatitis is still being debated. Although several cytokines and chemokines including IL-10 were significantly increased in seminal plasmas from patients with CP/CPPS ^{26, 27}, peripheral IL-10 was not significantly changed in these

patients²⁸. Further studies by measuring cytokines in the seminal fluid following EGP treatment are warranted.

Only 3–10% protein-bound Amadori products were absorbed in the intestine after ingestion²⁹. After eating a single meal containing fructoselysine, the human fecal secretion of fructoselysine was 2.6–5.6%³⁰. Thus, it could be assumed that about 90% of orally consumed EGPs were decomposed by the gut microbes. In this study, EGP treatment altered the microbiome profile (β diversity) without decreasing the genetic diversity of the communities (α diversity). In contrast, advanced glycation end-products (AGEs), the homologous products of EGPs, were inflammatory and decreased the species richness and α diversity of gut microbiota in Sprague-Dawley rats³¹ and C57BL/6 mice³². For CP/CPPS patients, significantly decreased α diversity was observed comparing to controls who were asymptomatic or just had urinary tract symptoms³³. However, gut microorganism alteration in clustering but not diversity was reported for T1D patients³⁴.

Sequencing of 16S rRNA-encoding gene has identified Bacteroidetes and Firmicutes as the most abundant phyla in human gut microbiota³⁵, and in fecal samples from NOD mice³⁶. The F/B ratio has been extensively examined and correlated with various diseases. It has been reported that F/B ratio was higher in obese subjects and overweight subjects³⁷. In this study, F/B ratio was numerically decreased by EGPs, and these NOD mice had fewer prostatic immune infiltrates and higher survival rate. Similar phenomena were observed in our previous genistein studies, in which genistein prevented the hyperglycemia and numerically decreased F/B ratio in NOD mice³⁶. However, a significant lower F/B ratio was also observed in patients with systemic lupus erythematosus³⁸, and these patients have higher IL-10 levels³⁹. In contrast, it is also reported that aging-dependent decline of IL-10-producing B cells coincides with production of antinuclear antibodies⁴⁰. Therefore, it is important to determine if EGP consumption has any adverse effects in lupus patients.

EGP treatment increased *Erysipelotrichia* at the class level, *Erysipelotrichales* at the order level and *Erysipelotrichaceae* at the family level. The presence of a core microbial ecology including *Erysipelotrichaceae* is essential for a successful therapeutic fecal microbiota transplantation, which controls intestinal inflammation through inducing IL-10 secretion by immune cells⁴¹. *Porphyromonadaceae* at the family level and *Parabacteroides* at the genus level, which were also upregulated by EGPs, were positively correlated with IL-10 in healthy human stools⁴² and in mice⁴³, respectively. In addition, EGPs significantly upregulated *Allobaculum*, *Bacteroides*, and *Prevotella*, and downregulated *Roseburia* at genus level. Other significantly regulated genera included an increased *Anaerostipes* and a decreased *Adlercreutzia*. The SCFA-producing bacteria *Anaerostipes* can improve insulin sensitivity by generating butyrate⁴⁴. On the other hand, genus *Adlercreutzia* was elevated in old vs. young wild type mice⁴⁵. Overall, EGP-treated NOD males exhibited a healthier gut microbiome than the NR controls.

Among these significantly regulated genera, *Bacteroides*, which was predominantly contributed by *Bacteroides acidifaciens* (Fig 7B), correlated with all the immune parameters except for the eosinophils (Fig 7A). The expansion of gut *Bacteroides acidifaciens* was identified in mice with Atg7 conditional knockout in dendritic cells, which showed a lean

phenotype with improved insulin resistance, lower body weight and fat mass ⁴⁶. In our previous study, decreased *Prevotella* and *Anaerostipes* were detected in hyperglycemic male CD-1 mice (multiple low dose streptozotocin-induced) following chronic 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure, and correlated with liver weight, one of the indexes for liver toxicity ¹⁶. In CP/CPPS patients, underrepresented *Prevotella* was detected comparing to controls ³³. In this study, EGP-mediated increases in *Prevotella* and *Anaerostipes* correlated with changes in eosinophils and IL-10 positively, and splenocytes negatively (Fig 7A). It should be noted that eosinophils can contribute to the inhibition of prostate cancer cell growth ⁴⁷ and resolution of lung-allergic responses following repeated allergen challenge by producing IL-10 ⁴⁸.

Conclusions

In summary, this work demonstrated that chronic exposure to glycated whey protein alleviated autoimmune prostatitis and increased the survival rate in aged NOD male mice, which extended the potential application of EGPs from T1D ⁹ to a broader spectrum of autoimmune diseases. Anti-inflammation and modulated gut microbiome profiles were suggested to be the underlying mechanisms how EGPs produced their protective effects. To our knowledge, this is the first animal study exploring the effects of EGPs on gut microbiome. Direct evidence has been presented that EGPs could modulate gut microbes in mice, and the data suggest that microbiome alterations correlate with immunity changes. Admittedly, the cause-effect relationship between immune system and gut microbiome following EGP exposure has not been heavily focused on within this work. Future approaches, including fecal transfer and the application of immunodeficient mice model, should be carried out to address the knowledge gap.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig 1. Survival rate of male NOD mice.

The mice were orally gavaged with NR or EGPs at the dose of 600 mg/kg BW/day for 6 months. Moribund events were recorded when the mice reached the humanity endpoints (n = 15–13). *, $p < 0.05$; **, $p < 0.01$.

Fig 2. Chronic exposure to EGPs reduces the prostatic inflammation.

After dosing for 3 or 6 months, the mice were euthanized and the urogenital organs were harvested. Longitudinal sections were H&E stained, and the inflammations associated with the 4 lobes were scored. Representative images for each score are shown: black arrows indicate scattered or aggregated leukocyte infiltrates. The inflammatory statuses of NR and EGP groups at month 3 and 6 are summarized in the last panels. Immune cell infiltration was only detected for (A) dorsal and (B) lateral prostates. In addition to histological assessment, (C) the inflammatory infiltrates (i.e., macrophages, CD4⁺ T cells, CD8⁺ T cells, B cells) in the anterior prostate (AP) after 6-month treatment were quantitated by flow cytometry (mean $\pm$ SEM, $n = 5-6$). *, $p < 0.05$.

Fig 3. EGPs alter the white blood cells (WBC) in the circulation.

The hematology analysis with differentials was conducted, and the numbers of WBC, neutrophils, lymphocytes, monocytes and eosinophils were shown (mean ± SEM, n = 5–6).

**, $p < 0.01$. One blood sample in the EGP group had clots, so the analysis of this sample was not done.

Fig 4. EGPs decrease the numbers of splenic leukocytes without altering the spleen weight.

(A) The spleens were harvested and weighted. (B) The splenic leukocyte number was significantly decreased by EGPs. The analysis of macrophages, CD4⁺ T cells, CD8⁺ T cells and B cells are summarized in (C-E) (mean $\pm$ SEM, n = 6). *, $p < 0.05$; **, $p < 0.01$.

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Fig 5. Heatmap of cytokines/chemokines regulated by NR and EGPs in male NOD mice treated for 6 months.
The average concentrations of cytokines/chemokines of NR groups are shown (pg/ml), and those of EGP groups were expressed as % change of NR. All averages and SEM were shown in Supplement Table 2.

Fig 6. The composition of gut microbiome based on 16S rRNA sequencing in NOD males treated with EGPs or NR for 6 months.

(A) Weighted UniFrac beta diversity difference between NR (blue) and EGPs (red). (B)

Taxonomy of gut microbiome shown at the phylum level. Linear Discriminant Analysis

Effective Size (LEfse) results for genus level (C) and species level (D) are shown. N = 6.

Fig 7. Correlational analysis using Spearman's correlation test.

(A) Significantly regulated immune endpoints and microbes at genus level were correlated.

(B) Significantly regulated *Bacteroides* sp. were correlated with immune endpoints. Positive correlation, + ($p < 0.05$), + ($p < 0.01$); negative correlation, - ($p < 0.05$), - ($p < 0.01$).