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Glycated Whey Proteins Protect NOD Mice against Type 1 Diabetes by Increasing Anti-Inflammatory Responses and Decreasing Autoreactivity to Self-Antigens

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Abstract

Our previous studies suggested that early glycation products (EGPs) generated in the first step of Maillard reaction/glycation were anti-inflammatory. The objectives of the present study were to determine the effects of EGPs derived from the whey protein isolate-glucose system on type 1 diabetes (T1D), and the underlying immunological mechanisms. In non-obese diabetic (NOD) mice, EGPs at the physiological dose of 600 mg/kg/day increased glucose metabolism, decreased non-fasting blood glucose levels and T1D incidence, decreased insulin resistance, and decreased the pancreatic immune infiltration. The protective effects were accompanied with decreases in CD4⁺CD8⁺ thymocytes, CD8⁺ T cells and serum insulin autoantibody levels, and increases in splenic CD4⁺CD25⁺ T cells, macrophage M2/M1 ratio and serum IL-10 level. However, similar treatment with EGPs produced minimal effect on the multiple low-dose streptozotocin-induced hyperglycemia. In conclusion, EGPs protected NOD mice against T1D via increasing anti-inflammatory immune responses and decreasing autoreactivity to self-antigens.

Graphical Abstract

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Author contributions

Y. C. and T. L. G. designed the studies; Y. C. performed the animal studies, T. L. G. conducted the flow cytometry experiments, and T. N. performed the histopathological assessment; Y. C. analyzed the data and performed the statistical analysis; Y. C. graphed the data and wrote the manuscript, and T. L. G. edited it.

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Conflict of interest

Declarations of interest: none.

Ethics Statements

The use of the animals was approved by UGA Institutional Animal Care and Use Committee (IACUC).

Keywords

glycation; whey proteins; non-obese diabetic (NOD) mouse; type 1 diabetes; anti-inflammation; insulin autoantibodies

1 Introduction

Type 1 diabetes (T1D) is a disease due to autoimmune destruction of insulin-producing pancreatic β -cells. In the non-obese diabetic (NOD) mouse, one of the murine models for human T1D, innate immune cells (e.g., dendritic cells, macrophages, neutrophils) infiltrate the pancreas as early as 3 weeks of age, and then attract CD4⁺ and CD8⁺ T cells into the pancreas at 4–6 weeks of age (Pearson, Wong, & Wen, 2016) to abruptly initiate insulinitis (intra-islet inflammatory infiltrate). The mechanisms of T cell induced β -cell death include: 1) direct cell-cell contact through recognition of MHC class I molecules by CD8⁺ T cells; 2) killing β -cells through surface receptor or secreted cytokines/chemokines by recognizing β -cell antigen presented by antigen-presenting cells (APCs; e.g., B cells, macrophages) (Mathis, Vence, & Benoist, 2001). T cells further recruit other immune cells to directly kill β -cells or facilitate the function of T cells. Other important pancreatic infiltrates are B cells and macrophages. B cell plays as APCs and costimulators for T cells, and boost the production of autoantibodies (Pearson et al., 2016). Macrophage is a vital APC and cytokine/chemokine-secreting cell.

Anti-inflammatory agents have the potential for T1D prevention. For example, the anti-inflammatory protein, alpha1-antitrypsin (AAT), prevented T1D in NOD mice (Song et al., 2004) and reduced %HbA1c and pancreatic autoantibodies in newly diagnosed T1D

children/adolescents (Rachmiel et al., 2016). Anti-inflammatory components from diet, such as polyherbal supplementation that decreased NF- κ B activity and chemotaxis chemokines (i.e., CCL2 and CXCL10) in 832/13 rat insulinoma cells, could stabilize blood glucose level (BGL) and prevent T1D in NOD mice with decreased CD3⁺ infiltrates in the pancreas (Burke et al., 2015). Early glycation products (EGPs) refers to the Amadori (or Henys) compounds generated at the first step of glycation (Beisswenger, 2012), and continuous reactions will lead to the production of notorious advanced glycation end-products (AGEs). AGEs are ubiquitous in foods (Uribarri et al., 2010), and about 10% of the orally consumed AGEs are absorbed in the gastrointestinal tract (Koschinsky et al., 1997) and contribute to body's AGE pool. When the parental NOD8.3 male and NOD/ShiLt female mice were exposed to a diet containing AGE during the periods of pregnancy and lactation, the AGE exposed female offspring had elevated circulating AGEs and exacerbated insulinitis (Borg et al., 2018), suggesting that dietary AGEs could contribute to the initiation and exacerbation of T1D. However, the consumption of infant formula, which had higher N^e- (carboxymethyl)lysine (CML) level (an AGE marker) compared to breast milk, did not change the BGL or plasma insulin level in old infants (Klenovics et al., 2013), suggesting other components in the infant formula might be anti-hyperglycemia. The quantitation of early and advanced glycation markers showed that EGPs considerably outweighed AGEs in infant formulas regardless of the product forms and processing methods (Birlouez-Aragon et al., 2004; Hegele, Buetler, & Delatour, 2008).

Our previous studies showed that dietary EGPs could regulate the inflammatory processes when applied to human macrophages (phorbol 12-myristate 13-acetate (PMA)-differentiated-U937 cells) (Chen, Filipov, & Guo, 2018), and polarize tumor-associated macrophages toward the anti-inflammatory phenotype M2 in C57BL/6 male mice (Chen & Guo, 2019), which implied a protective effect of EGPs against autoimmune dysregulation. Furthermore, the oral consumption of whey protein derived EGPs did not increase the serum CML level, excluding the possibility of biotransformation of EGPs into AGEs in physiological conditions (Chen & Guo, 2019). Therefore, it was hypothesized that EGPs could protect against T1D through the regulation of immune responses. To test this hypothesis, two T1D murine models were employed. EGPs delayed the T1D onset and reduced the T1D incidence in the autoimmune prone NOD mice. However, minimal effects were observed in the multiple low dose (MLD) streptozotocin (STZ)-induced hyperglycemia in C57BL/6 females, in which chemical destruction of pancreatic β -cells was the major mechanism. Because these EGPs are produced from food-grade raw materials involving only well-controlled heating, they are still considered food. Therefore, the beneficial effect observed on T1D prevention would highly qualify EGPs as nutraceuticals or medical food for patients with insulinitis and T1D.

2 Material and methods

2.1 EGP preparation and identification

Dietary EGPs were generated from whey protein isolate (WPI, Fonterra (USA) Inc, Rosemont, IL) and glucose (Sigma-Aldrich, St. Louis, MO). They were prepared as described before (Chen et al., 2018), and summarized in a simplified flow diagram (Fig.

1A). In brief, a solution containing WPI and glucose was freeze-dried, and the powders were further incubated in sealed desiccator maintained at a_w 0.53 and 55 °C for different durations. Glycation markers were quantitated and the 8 h samples were determined as EGPs (Chen et al., 2018). The reacted sample were stored at −20 °C prior to use, and thawing in a sealed desiccator containing silica gel at room temperature. The compositions of WPI and glycated products were analyzed by LC-MS (Bruker Daltonics, Billerica, MA) equipped with BioBasic-4 column (Thermo Fisher Scientific, Waltham, MA). The samples were dissolved in Milli-Q water at 1 mg/ml, and filtered through a 0.45 µm filter membrane. A 2 µl sample was injected at minute 11. Solvent A was aqueous solution containing 0.1% formic acid and solvent B was 100% acetonitrile. The elution was performed at a flow rate of 50 µl/min from 0 to 2%B within 11 min and to 100% in the following 50 min. A positive ionization mode was used, and the cone voltage was 45 V. The m/z range was 400–3000 with scan time of 5 s (0.2 s interval). The data were analyzed on MassLynx V4.1 and MassEnt1 (Waters Co., Milford, MA), and the average degree of substitution per protein (DSP) was calculated as the following formula (Chen, Liu, Labuza, & Zhou, 2013):

$$\text{Average DSP} = \frac{\sum_{i=0}^n i \times I_i}{\sum_{i=0}^n I_i}$$

Where i is the number of glucose attached; I_i is the peak intensity of proteins attached with i glucose.

2.2 Mice and treatment

C57BL/6 mice and NOD mice obtained from the Charles River Laboratories (Wilmington, MA) and Taconic Biosciences (Rensselaer, NY), respectively, were housed in Coverdell Vivarium at the University of Georgia (UGA). All mice were maintained on a 12-h dark/light cycle at 21–24 °C with 20–60% relative humidity, with access to food and filtered tap water *ad libitum*. Mice aged 0–4 week were kept on breeder chow (5058, PicoLab® Rodent Diet 20, LabDiet, MO), and switched to normal chow (5053) afterwards. All mice were euthanized by CO₂ inhalation followed by cervical dislocation. The use of the animals was approved by UGA Institutional Animal Care and Use Committee (IACUC).

Since there was no *in vivo* reference dose available for EGPs, the dose (600 mg/kg body weight (BW)/day) was determined following previous reports of *in vivo* dietary AGE-treatment (Cai et al., 2012), which would give the mouse an equivalent amount of AGEs to that they obtained from the diet (Supplementary Fig. 1). Toxicity (3 months duration) of both EGPs and AGEs was tested in female and male C57BL/6 mice prior to following experiments. No difference on BW was detected (**data not shown**), suggesting that these glycation products at this dose did not generate overt toxicity.

2.2.1 MLD STZ-induced hyperglycemia in C57BL/6 females—C57BL/6 female mice (6–8 weeks old) were randomly separated into 5 groups (5–6 per group), and gavaged with non-reacted samples (NR), EGP, AGEs, autoclaved purified water and glucose solution (14.1 mg/ml) in the same volume. After 8 weeks, the mice were rendered diabetic with STZ injection (50 mg/kg BW/day intraperitoneally (i.p.) in citrate buffer for 4 consecutive days). Dosing with glycation products were continued for another 4 weeks.

2.2.2 NOD mice—NOD mouse is a spontaneous T1D murine model in which the penetrance of diabetic genotype can be strongly modified by dietary factors (Leiter, 1993). Age-matched NOD females (6–8 weeks old) were randomly separated into 3 groups (4 in each group with one repeat), and gavaged with NR, EGP and autoclaved purified water in the same volume for 4 weeks. In the secondary experiment, two groups of age-matched NOD females (6–8 weeks old, 10–11 in each group) were treated in the same way for 8 weeks. Although a 2-week continuous exposure was sufficient to test the effect of a xenobiotic on the immune system, a 4-week exposure resulted in immunological alterations that were more consistent to humans (Guo & White, 2010). In our colonies, NOD females developed T1D starting at week 8–9. Therefore, the 4-week exposure was for the detection of the immunological changes prior to massive T1D initiation, while the 8-week exposure allowed a good comparison of T1D incidence upon different treatments. In the third experiment, age-matched non-diabetic NOD males were randomly separated into 2 groups (approximately 24 weeks old, 15–13 in each group) and gavaged with NR and EGPs for up to 6 months.

2.3 T1D relevant endpoints: BW, BGL, glucose tolerance test (GTT), insulin tolerance test (ITT), food and water consumptions

BW, food and water consumptions were measured weekly. BGL was measured weekly or bi-weekly in small sample of venous blood (tail nick) with Bayer's CONTOUR blood glucose meter (Bayer Health Care LLC, Mishawaka, IN). Diabetes was defined as BGL > 200 mg/dL in two consecutive weeks. For GTT, mice were fasted for 16 h, and i.p. injected with glucose water solution at the dose of 2 g/kg BW. For ITT, mice were fasted for 4 h, and i.p. injected with bovine insulin (Sigma-Aldrich) PBS solution at the dose of 6.4×10^{-3} mg/kg BW. To determine if the glucose in the glycation samples could elevate the non-fasting BGL, two groups of C57BL/6 females were used for orally challenged GTT: non-fasted mice were gavaged with water or 14.1 mg/ml glucose solution at 0.01 ml/g BW. The BGL was measured at 0, 15, 30, 60 and 120 min, and all mice were prevented from reaching food during the tests.

2.4 Histopathological scoring for the pancreatic β -islets

A section of tissue from the pancreas head were dissected and fixed in 10% buffered formalin. The hematoxylin and eosin (H&E) staining was performed by Clinical Pathology Laboratory (College of Veterinary Medicine, UGA), and the slides were analyzed blindly on an Olympus BX41 light microscope (Olympus Corporation, Tokyo, Japan) by a board-certified pathologist using the criteria described in Table 1. For STZ-destroyed pancreases, β -islet inflammation and vacuolation were scored. For NOD females, β -islet inflammation was graded.

2.5 Tissue processing and flow cytometry

Pancreatic immune infiltrates were purified following the protocol by Epshtein et al. (Epshtein, Sakhneny, & Landsman, 2017) with small modifications. A section of 0.1–0.15 g tissue from the pancreas head was dissected, cut into small pieces, and placed into 2 ml cold PBS. A 1 ml of digestive buffer was added to reach the final concentrations of collagenase

(Sigma-Aldrich) at 0.05 mg/ml and DNase (Sigma-Aldrich) at 5 U/ml. The tube was incubated at 37 °C for 30–45 min with the rotator speed at 20 rpm in a Robbins Scientific Model 1000 Hybridization Incubator. The digestion was stopped by adding 10 ml cold PBS, and the solution was centrifuged at 300 x g at 4 °C for 8 min. The supernatant was removed, and the pellet was resuspended in 6 ml PBS and passed through a 40 µm-cell strainer placed on a 50 ml conical polypropylene tube. The original tube was washed using 7 ml PBS twice by passing through the cell strainer into the collection tube. The PBS was removed by centrifugation, and the cells were resuspended in PBS to reach a proper concentration (10^6 /ml) for flow cytometric analysis.

Spleen was separated and placed into 3 ml cold PBS. Cell suspensions were prepared by mashing the spleen using the frosted ends of two microscope slides. Red blood cells were lysed using the ACK lysing buffer (Gibco by Life Technologies, Orand Island, NY) and the buffer was removed by centrifugation at 300 x g at 4 °C for 8 min. The cell pellet was washed once using cold PBS and then resuspended in PBS. Thymus was separated and placed into 2 ml cold PBS. The single-cell suspensions were prepared the same way as spleen but without lysis.

Splenic and pancreatic cells were stained for the following surface markers conjugated with fluorophores: CD3 (145–2C11, PerCP-Cy5.5, BD Pharmingen), CD 4 (RM4–5, V450, BD Horizon), CD8 (53–6.7, APC-H7, BD Pharmingen), CD25 (PC61, APC, BD Pharmingen), F4/80 (BM8, FITC, eBioscience), CD209 (LWC06, Alexa Fluor, Novus Biologicals), CD80 (16–10A1, PE-CD594, BD Horizon); B220 (RA3–6B2, FITC, eBioscience), CD40L (MR1, PE, eBioscience). Thymocytes were stained using CD4 (L3T4, PE, eBioscience) and CD8a (Ly-2, FITC, BD Pharmingen); CD44 (IM7, PE, BD Pharmingen) and CD25 (7D4, FITC, BD Pharmingen). Fluorescence Minus One (FMO) controls were performed for gating setup (Maecker & Trotter, 2006). 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 LSRII Flow Cytometer (BD Biosciences). The flow cytometric data were analyzed using FlowJo v10 (FlowJo, LLC, Ashland OR), and the gating strategies are shown in Supplementary Fig. 2. Leukocytes were characterized as CD4⁺ T cells (CD3⁺CD4⁺CD8[−]), CD4⁺CD25⁺ T cells (CD3⁺CD4⁺CD8[−]CD25⁺), CD8⁺ T cells (CD3⁺CD4[−]CD8⁺), macrophages (F4/80⁺), M1 (F4/80⁺CD80⁺CD209[−]), M2 (F4/80⁺CD80[−]CD209⁺), B (B220⁺), CD40 ligand positive B cells (B220⁺CD40L⁺), and thymocytes (CD8/CD4 or CD24/CD44).

2.6 Cytokine/chemokine quantitation

Blood was obtained from the orbital sinus after mouse being deeply anesthetized. The serum was collected and stored at −80 °C prior to use. Thirty-two cytokines/chemokines in the sera were detected by the bead-based multiplex ELISA technique using the MILLIPLEX MAP Mouse Cytokine/Chemokine Kit (Millipore, Billerica, MA) following the manufacturer's instructions. The beads were run on a Bio-Plex (Bio-Rad, Inc., Hercules, California), and the data was collected and analyzed using Bio-Plex Manager 6.1.1. The working range was 12.8–40,000 pg/ml for IL-13, and 3.2–10,000 pg/ml for the rest. IL-3 and IL-5 were not detected in the samples; IL-4 was below the lower limit of quantitation of the standard curve with some samples fell in the extrapolated range.

2.7 Measurement of immunoglobulin (Ig) isotypes and insulin autoantibodies (IAAs)

The serum levels of Ig isotypes (i.e., IgM, IgG₁, IgG_{2b}, IgG_{2c}) were detected using ELISA Quantitation Kits (Bethyl Laboratories Inc., Montgomery, TX). IgG_{2c} was a substitute for IgG_{2a} in NOD mice because IgG_{2a} was not expressed in inbred strains with *Igh1-b* allele (Martin, Brady, & Lew, 1998). The sera from NOD females were measured using two commercially available IgG_{2a} antibodies (i.e., Bethyl, SouthernBiotech), and the O.D. was consistently found to be ~0.2 at the dilution from 1/10 to 1/1000 (**data not shown**), which agreed with the previous study (Martin et al., 1998). Therefore, an O.D. < 0.2 was considered the background for all sera to eliminate the false positive caused by hemolysis. The 96 well flat-bottom high binding microplate was coated with 100 µl/well primary antibody (5 µg/ml) at 4 °C for overnight (16–18 h), and then blocked with 5% skim milk powder in 0.05% PBST at room temperature for 1 h. After washing with 0.05% PBST, a 100 µl standard or sample was added. The standard curve was prepared by diluting Mouse Reference Serum provided by the Manufacturer with the diluent (2% skim milk powder in 0.02% PBST). A series of dilutions was conducted for sera samples, and only the dilution with optimal detection was reported: IgM (1/5,000), IgG₁ (1/500,000), IgG_{2b} (1/20,000), IgG_{2c} (1/50,000). After 2 h incubation at room temperature, the plate was washed and a 100 µl HRP-conjugated secondary antibody was applied. The plate was incubated at room temperature at dark for 1 h, and then washed. A 100 µl Ultra TMB-ELISA Substrate Solution (Thermo Scientific, Rockford, IL) was added, and the reaction was stopped by an addition of 100 µl 1 M H₂SO₄ solution after 5–10 min. The absorbance was read at 450 nm in a Synergy 4 hybrid multi-mode microplate reader (BioTek Instrument, Inc., Winooski, VT).

For the IAA titers (Quintana & Cohen, 2001; Wan, Thomas, & Unanue, 2016), the plates were coated overnight with bovine insulin (10 µg/ml in carbonate solution (pH=9.6), 100 µl) at 4 °C. To check the specificity of the assay, diluted sera were divided into two aliquots and incubated in the presence or absence of 10 µg/ml insulin on ice for 1 h. HRP-conjugated goat anti-mouse IgM or anti-mouse IgG were used as the secondary antibodies (Bethyl). The O.D. threshold was 0.06, which was determined by a serial dilution from 1/5 to 1/500. The other steps were the same as described above.

2.8 Statistics

Data were presented as mean ± SE. Comparisons of means were tested by one-way ANOVA using JMP Pro 13 (SAS Inc., Cary, NC). Tukey-Kramer *post hoc* test was conducted when the omnibus test was significant for the comparison of three or more groups. The comparisons of means between EGPs and water/NR were tested using Student's *t* test, and the survival curves were compared using Gehan-Breslow-Wilcoxon test performed on GraphPad Prism 6 (GraphPad Software, San Diego, CA).

3 Results

3.1 The composition of EGPs

The composition of WPI powders was firstly verified by LC-MS. As shown in Supplementary Fig. 3, the powders were highly purified and contained mainly α-lactalbumin

(α -la, 14,178 Da) and two variants of β -lactoglobulin (β -lg, variant B: 18,277 Da; A: 18,363 Da). The two peaks with an increase in molecular weight of 324 Da of β -lg variants were probably resulted from 1 lactose attachment during powder manufacture, such as spray drying. When NR and EGPs were analyzed, the components in NR samples were highly consistent with WPI, suggesting that the freeze-drying steps did not generate glycation products (Fig. 1B upper panel). After 8 h reaction, no original forms of α -la or β -lg remained (Fig. 1B lower panel). All the α -la reacted with 3–11 glucoses, with the 6-glucose attached α -la being the most abundant and the average DSP of 7.2. Both variants of β -lg were attached by 5–15 glucoses. The 10-glucose attached form was the most abundant and the average DSP was 9.7 for both variants, which agreed with a previous study that the two variants showed similar reactivity during glycation (Chen, Liang, Liu, Labuza, & Zhou, 2012).

3.2 EGPs have minimal effect on MLD-STZ-induced hyperglycemia

C57BL/6 females were pretreated with EGPs for 8 weeks to stabilize the levels of circulating EGPs, and the effects of EGPs on BGL in these healthy animals were monitored at the same time. In addition to water control, two additional controls including glucose solutions and NR were included because NR and glycation product samples contained glucose, which might affect the BGL. Therefore, GTT was conducted with oral challenge of the glucose solution that was equivalent to the amount of glucose in NR sample. The results showed that orally intake of this amount glucose did not elevate the BGL immediately after dosing in healthy mice (Supplementary Fig. 4A). AGEs were used as a positive control since they were widely reported to be detrimental for both healthy and diabetic individuals (Cai et al., 2012). Supplementary Fig. 4B showed that no significant difference was detected for BW during treatment, suggesting none of the treatments generated overt toxicity. For the non-fasting BGL, it stayed between 110–125 mg/dL in EGP group, while it fluctuated between 110–160 mg/dL in the 4 control groups (Supplementary Fig. 4C). GTT was conducted after dosing for 8 weeks. The BGLs of EGP-treated group were significantly lower at 15 min after challenge when compared to those of NR and AGE-treated groups, although there were no differences compared to those of water- and glucose-treated groups (Supplementary Fig. 4D). During ITT, no significant difference was detected at any time points among the groups. These results suggested that EGPs could stabilize BGL. Therefore, the effect on T1D was further studied in two T1D models.

The first T1D model was the MLD-STZ-induced hyperglycemia in C57BL/6 females. After 8 weeks dosing, STZ was i.p. injected to specifically destroy pancreatic β -cells and thus induce hyperglycemia in these mice. After STZ injection, mice treated with glucose solution showed the least changes in BW, and water, NR and EGP-treated mice had moderate decreases in the BW while AGE-treated mice had the most decreases in BW (Fig. 2A). In addition, mice from the EGP group ate and drank the least (Fig. 2B–C). The glucose solution-treated mice maintained the non-fasting BGL around 150 mg/dL after STZ injection; the non-fasting BGLs in the water and EGP groups slightly increased; however, NR and AGE-treated mice showed a continuous increase in their non-fasting BGL (Fig. 2D). During GTT, NR and AGE-treated mice had increased BGL more than the mice from the other three groups within the first 30 min, and AGE group had a significantly increased BGL

at 120 min compared to water, glucose and EGP group (Fig. 2E left panel). During ITT, NR treated mice showed the highest fasting BGL, followed by AGEs, NR, EGPs, water and glucose solution. At 30 min after insulin injection, AGE group had the highest BGL, followed by NR/EGPs and water/glucose solution (Fig. 2E right panel). An overall evaluation of the diabetes severity suggested that AGEs > NR > EGPs, water glucose, based on the above various endpoints of T1D: BW, food and water consumption, non-fasting BGL, and BGL change during GTT and ITT. Histopathological assessment of β -islets was conducted from the aspects of inflammation and vacuolation, and the level of vacuolation but not inflammation correlated with the ranking of diabetes severity (Fig. 2F–G). Cytoplasmic vacuolation was due to the remarkable destruction of islets induced by STZ (Amniattalab, Malekinejad, Rezabakhsh, Rokhsartalab-Azar, & Alizade-Fanalou, 2016), and the results suggested that AGEs facilitated the destructive function of STZ while EGPs and NR control had similar effects.

3.3 Alleviation of T1D by EGPs in NOD mice is associated with reduced pancreatic immune infiltrates

3.3.1 EGPs delay T1D onset in NOD females—The other T1D murine model used was NOD mouse. NOD females were dosed with water, NR or EGPs for 4 weeks. There was no significant difference in BW between controls and EGPs (Fig. 3A) and organ weights including spleen, thymus, liver, kidney and heart (Supplementary Table 1) that were commonly used as toxicity indications of test compounds. These observations suggested that NR or EGPs at this dose did not generate overt toxicity to NOD females. Comparing to NR and water controls, EGPs significantly decreased non-fasting BGL of the non-diabetic mice after dosing for 2 weeks (Fig. 3B), but did not alter the BGLs during GTT or ITT, which were conducted after dosing for 4 weeks (Fig. 3C). Another set of NOD females were continuously dosed with NR and EGPs for up to 8 weeks to monitor the non-fasting BGL and the response to glucose and insulin challenges (Fig. 3D–E). EGPs significantly lowered the non-fasting BGL since week 1, increased the glucose metabolism during GTT, and decreased the insulin resistance during ITT, when compared to NR control. Additionally, the T1D incidence was recorded: NR treated mice started to show T1D incidence at week 3 (9–11 weeks old) and had a total incidence close to 60% at week 8 (14–16 weeks old); in comparison, EGPs treated mice did not show T1D incidence until week 7 (13–15 weeks old) and had an overall incidence at approximately 20% (Fig. 3F).

Since the mechanism of T1D in NOD is manifested as immune dysregulation and immune cells infiltrating into pancreas (Delovitch & Singh, 1997), the inflammation in β -islets was firstly evaluated by histopathology, but no significant differences were detected (Supplementary Fig. 5). The negative finding was probably because the treatment started between week 6–8 while insulinitis in NOD mouse started at week 4–6, and EGPs might have minimal effect on the already infiltrated immune cells. The subtle difference would be beyond the sensitivity of the semi-quantitative histopathology. Therefore, the pancreatic immune infiltrates of the non-diabetic NOD females after dosing for 4 weeks were dissociated and evaluated by flow cytometry. EGPs decreased the total immune infiltrates in the pancreas, and decreased the number of macrophages, CD8⁺ T cells and B cells by 50% when comparing to water and NR controls with significant changes observed in B cells

between NR and EGPs. However, there were no differences among the groups in the CD4⁺ T infiltrate number (Fig. 3G).

3.3.2 EGPs produce protective effects in NOD males—Non-diabetic NOD males were randomized into two groups (age matched) and dosed with NR or EGPs. NOD males have later T1D onset and lower incidence compared to females, so they were kept longer than 16 weeks. EGP-treated mice maintained the BW, whereas NR-treated mice started to show a decrease in BW starting at week 18 post treatment (Fig. 4A), suggesting that chronic oral intake of EGPs might have generated protective effects in the autoimmune male NOD mice. Indeed, EGPs decreased and maintained the non-fasting BGL at normal level (vs. NR), and significantly increased the glucose metabolism and decreased insulin resistance during GTT and ITT, respectively (Fig. 4B–D). Furthermore, fewer immune cells infiltrated into the pancreas, and fewer macrophages, CD8⁺ T and B infiltrates within pancreas were detected when compared to NR (Fig. 4E), and this was highly consistent with NOD females.

3.4 EGPs alter the immunity in NOD mice

By studying the effects of EGPs on T1D using two models (i.e., MLD-STZ-induced, NOD mouse), it was reasonable to hypothesize that EGPs exerted the protective effect against T1D through regulating the immunity. Therefore, the following section was to understand how the immunity, especially the aspects relevant to T1D progression, was affected by EGPs. After being treated for 4 weeks, non-diabetic NOD females were euthanized and the immune cell populations in primary and secondary lymphoid organs were studied. In the thymus, CD4CD8 and CD25CD44 thymocytes were studied (Fig. 5A). EGPs decreased the CD8 single positive cells but not the others. In the spleen, the percentage of M2 (anti-inflammatory) was slightly increased while %M1 (pro-inflammatory) was decreased by EGPs, leading to a significant increase of M2/M1 ratio (Fig. 5B upper panel). These results revealed the anti-inflammatory feature of EGPs. In addition, the percent CD4⁺CD25⁺ T cells was increased even though the total CD4⁺ T cells were unaffected, and %CD8⁺ T cells was decreased by EGPs (Fig. 5B lower panel). For the splenic B cells, EGPs decreased the CD40 ligand positive populations (B220⁺CD40L⁺, the $P \approx 0.05$, Fig. 5B lower panel).

The profiles of serum cytokines/chemokines were examined. Comparing to background (water), NR and EGPs consumptions had an enhancing effect on most cytokines/chemokines including G-CSF, GM-CSF, IL-1 β , IL-2, IL-6, IL-7, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, INF- γ , LIF, M-CSF, MCP-1, MIG, MIP-2, RANTES and VEGF (Supplementary Table 2). Key T1D regulators were separately graphed in Fig. 6. EGPs had little effects on the most important Th1 (INF- γ) and Th2 (IL-4) markers for pancreatic β -islets destruction and protection in NOD mice, respectively. For the other pro-inflammatory cytokines (i.e., IL-2, IL-12p40, IL-12p70, TNF- α), EGPs significantly increased IL-2 and IL-12p70, numerically increased IL-12p40, and had no effects on TNF- α when compared to water. EGPs significantly decreased IL-2 and IL-12p40, and had no effect on either IL-12p70 or TNF- α when compared to NR. All the other T1D relevant anti-inflammatory cytokines (i.e., IL-9, IL-10, IL-13) were either significantly or numerically increased by EGPs when compared to water and NR. Strikingly, IL-10, another vital Th2 marker other than IL-4, was increased 86- and 3-folds by EGPs compared to water and NR, respectively.

Taken together, EGPs shifted the cytokine/chemokine profile toward anti-inflammation despite the omnibus activation effect, which was highly in accordance with the M2 biased polarization in the spleen.

The serum levels of IgM, IgG₁, IgG_{2b} and IgG_{2c} were measured. The baseline levels of the 4 Ig isotypes in the water group was IgG₁ > IgG_{2c} > IgG_{2b} > IgM (Fig. 7A). EGPs significantly decreased IgM and IgG₁ when compared to water; and significantly decreased IgG₁ when compared to NR (**Supplementary Fig. 7A**). In the autoimmune prone NOD mice, autoantibodies were IgG predominant, and both IgG and IgM classes could recognize self-antigens known to induce diabetes, such as insulin (Quintana & Cohen, 2001). When IAA titers were measured, EGPs significantly decreased anti-insulin IgM and IgG when compared to water and NR (Fig. 7B).

4 Discussion

Overall, this study provides support for possible nutraceutical application of WPI-derived EGPs for patients with insulinitis and T1D. As data from the NOD females suggested, oral consumption of EGPs delayed the T1D onset and decreased the T1D incidence over 50%. Studies in both NOD males and females showed decreased and stabilized non-fasting BGLs, increased glucose metabolism during GTT and decreased insulin resistance during ITT, which were accompanied with reduced pancreatic immune infiltrates. However, in another T1D model, the STZ-induced β -cell destruction in C57BL/6 females that was much less immune-dependent, the protective effect of EGPs against T1D diminished. The seemingly contradictory results from two T1D models suggested that EGPs were likely to exert their effect through modulating the immunity.

When the immune changes in the non-diabetic NOD mice were evaluated to reveal the underlying mechanisms, EGPs were found to affect the immune system in the following three aspects. First, EGPs decreased the CD8⁺ T cells in NOD mice. Since CD8⁺ T cells were believed to be the main effector cells to attack β -islets (Mathis et al., 2001), a decrease of CD8 single positive cells in the thymus and CD8⁺ T cells in the periphery could be one reason how EGPs protected NOD mice from T1D. In the thymus, EGPs decreased the CD8 single positive thymocytes without affecting the other CD4CD8 and CD44CD25 subpopulations, which suggested that EGPs might affect the positive or negative thymic selection and required further studies.

Secondly, EGPs were anti-inflammatory for NOD mice. On the one hand, splenic M2/M1 ratio was increased by EGPs, suggesting a regulation of macrophage polarization towards anti-inflammatory M2. It was reported that injection of M2 macrophages to the prediabetic NOD recipients protected >80% of treated mice against T1D for at least 3 months (Parsa et al., 2012). On the other hand, serum IL-10 was up-regulated 86- and 3-folds by EGPs compared to water and NR, respectively. Importantly, IL-10-deficient mice experienced severe T1D (Tian et al., 2001). In our previous studies, IL-10 was also identified as one of the most up-regulated cytokines/chemokines when EGPs was applied to human macrophages (PMA-differentiated-U937 cells), peritoneal macrophages from C57BL/6 females (Chen et al., 2018), and C57BL/6 males (Chen & Guo, 2019).

The third aspect addressed the autoreactivity. It was reported that CD40 ligand expression on B cells could initiate insulinitis and antigen-specific T cell responses (Balasa et al., 1997). When B cells were activated by a combination of CD40 ligand and IL-4 *ex vivo*, an enhanced insulin-processing and presentation of B:12–20 epitope to 8F10 CD4⁺ T cells was observed. These T cells helped anti-insulin B cells to produce IAAs (Wan et al., 2016). Therefore, our observation that EGPs decreased the CD40L⁺ B cells might lead to a decreased autoreactivity. Indeed, when IAAs were quantitated, the level of anti-insulin IgG, the predominant anti-insulin Ig class in NOD mice, was dramatically decreased by EGPs.

The modes of action of EGPs, however, had not been investigated in this work. EGPs naturally existed in dairy products and could be synthesized in experimental conditions, but data regarding their absorption, distribution, metabolism and excretion were rare. It was reported that sugar moiety attachment through glycation protected the cleavage sites from trypsin hydrolysis (Chen et al., 2013). A chromatographic profiling of *in vitro* gastric and intestinal digested sodium caseinate showed that galactose glycation resulted in the reduction of peptide amount (Corzo-Martinez, Avila, Moreno, Requena, & Villamiel, 2012), which would possibly lead to a reduced intestinal absorption of the glycated proteins. Furthermore, a comprehensive review suggested that the absorption rate of protein-bound Amadori products was 3–10% (Pischetsrieder & Henle, 2012). In the small intestine, dietary protein antigens could induce periphery regulatory T cells (pTregs), which expressed high level of IL-10 and suppressed effector T cells (Kim et al., 2016). The consistently increased circulating IL-10 levels in both C57BL/6 males (Chen & Guo, 2019) and NOD females following EGP exposure suggested possible inductions of pTregs in the intestine, although other cells, such as M2 macrophages, CD4⁺CD25⁺Foxp3⁺ T cells, regulatory B cells including CD19^{hi}CD1d^{hi}CD5⁺ in mice (Yanaba et al., 2008) and CD24^{hi}CD27⁺ in humans (Iwata et al., 2011), could secrete IL-10 as well. In addition, the reduced intestinal absorption might increase the chances of gut fermentation. *Intestinimonas AF211* could convert Amadori compounds into butyrate in *in vitro* culture (Bui et al., 2015), and butyrate could attenuate 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis lesions in rats through affecting the Th17/pTreg lineage (Zhang et al., 2016). Further studies showed that protein bounded Amadori compounds derived from wheat gluten-glucose system could not inhibit the growth of *Escherichia coli* and *Staphylococcus aureus*, but their homologous advanced products could (del Castillo et al., 2007), suggesting that EGPs might not be as toxic as AGEs to gut microbes.

5 Conclusions

In summary, oral intake of WPI-derived EGPs delayed the T1D onset in NOD females through modulating the immunity. EGP consumption reduced CD8⁺ T cells and IAAs, and increased IL-10, CD4⁺CD25⁺ T cells and the anti-inflammatory responses by shifting M2/M1 balance toward M2. These data strongly suggested the applicable potential of EGPs as a nutraceutical for individuals with insulinitis and T1D. Using LC-MS, the active components had been narrowed down to mostly β -lg attached by 5–15 glucoses and small amount of α -la modified by 3–11 glucose. Additional studies are required to identify the active molecules. Besides WPI-derived EGPs, those produced from other proteins should also be studied regarding their effects on immunoregulation in the context of immune related

diseases such as T1D. Future studies would also focus on the molecular mechanisms underlying the anti-inflammatory effects produced by EGPs. Besides T1D, EGPs might be beneficial for other autoimmune diseases, and expanded studies with focuses on other autoimmune diseases might enlarge the application of EGPs as a nutraceutical.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AGEs	advanced glycation end-products
APCs	antigen presenting cells
BGL	blood glucose level
BW	body weight
CML	N ^ε -(carboxymethyl)lysine
DSP	degree of substitution per protein
EGPs	early glycation products
GTT	glucose tolerance test
IAA	insulin autoantibodies
ITT	insulin tolerance test
MLD	multiple low dose
NR	non-reacted
PMA	phorbol 12-myristate 13-acetate
pTregs	periphery regulatory T cells
STZ	streptozotocin
T1D	type 1 diabetes
WPI	whey protein isolate

α -la	α -lactalbumin
β -lg	β -lactoglobulin

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Highlights

Early glycation products protected non-obese diabetic mice from developing T1D.

Early glycation products decreased the pancreatic immune infiltration.

Early glycation products are anti-inflammatory.

Early glycation products decreased insulin autoantibodies.

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Fig. 1. EGPs contain mainly the glycated whey proteins.

(A) Scheme for EGP preparation. Whey protein isolate (WPI) and glucose were dissolved in Milli-Q water and the solution was freeze-dried. The resulted powders were incubated in a sealed desiccator maintained at a_w 0.53 and 55 °C. Samples were removed at different time points to quantitate the glycation markers for different glycation stages as described before. The 8 h samples were determined as EGPs, with 0 and 240 h samples as non-reacted (NR) and advanced glycation end-products (AGEs) controls, respectively. (B) Deconvoluted mass spectra (mass displayed: 14,000–21,500 Da) for NR and EGPs. NR samples were basically the same as WPI (Supplementary Fig. 3). In the EGP samples, all proteins were glycated: $\alpha\text{-la}$ had 3–11 glucoses attached; $\beta\text{-lg}$ had 5–15 glucoses attached.

Fig. 2. EGPs have minimal effects on multiple low dose (MLD)-streptozotocin (STZ)-induced hyperglycemia.

The hyperglycemia in C57BL/6 females were induced by STZ injection (50 mg/kg body weight (BW)/day, i.p., 4 doses). (A) BW, (B) food consumption, (C) water consumption and (D) non-fasting BGL were measured weekly or biweekly. (E) Glucose tolerance test (GTT) and insulin tolerance test (ITT) were measured at week 5 post STZ injection. Significance detected during ITT was shown in the table (food and water consumption were measured by cage; the other results were presented as mean $\pm$ SE, $n = 5-6$). (F) The histopathological changes of β -islets from each animal were evaluated from aspects of inflammation and vacuolation by scoring from 0 to 3. Representative images of β -islets of detected scores are shown in (G). Red arrows in the upper panels indicate inflammatory cells, and those in the lower panels indicate vacuoles. *a, b*, $P < 0.05$.

Fig. 3. EGPs delay type 1 diabetes (T1D) onset in NOD females.

NOD females (6–8 weeks old) were orally gavaged with water, NR or EGPs for 4 weeks. (A) BW and (B) non-fasting BGL were monitored weekly. The BGL change in the non-diabetic mice is summarized and graphed, the arrow indicates T1D incidence in water and NR groups. (C) GTT and ITT were conducted at the end of week 4 (mean $\pm$ SE, $n = 7-8$, the data was combined of two independent repeats). Another set of age matched NOD females were dosed with NR or EGPs. The non-fasting BGL change of the non-diabetic mice is shown in (D). (E) GTT and ITT were conducted after dosing for 8 weeks (mean $\pm$ SE, $n = 7-11$). (F) The diabetes incidence was recorded (diabetic: BGL > 200 mg/dL, $n_{NR} = 11$, $n_{EGPs} = 10$). (G) The pancreases of non-diabetic NOD females (being treated for 4 weeks) were harvested, and the number of immune cells (i.e., total immune infiltrates, macrophages, CD8⁺ T cells, CD4⁺ T cells, B cells) within pancreas were dissociated and analyzed by flow cytometry (mean $\pm$ SE, $n = 3-4$). +, $P < 0.05$, ++, $P < 0.01$ EGPs vs water; *, $P < 0.05$, **, $P < 0.01$ EGPs vs NR.

Fig. 4. EGPs have protective effects on NOD males.

Age matched diabetic free NOD males (> 16 weeks old) were dosed with NR or EGPs for 24 weeks. (A) BW and (B) non-fasting BGL were recorded. (C) GTT and ITT were performed at week 12 and 24 (mean $\pm$ SE, $n = 11-15$ for week 12, $n = 6$ for week 24). (D) After 24 weeks of treatment, the immune cells (i.e., total immune infiltrates, macrophages, CD8⁺ T cells, CD4⁺ T cells, B cells) within pancreas were dissociated and studied using flow cytometry (mean $\pm$ SE, $n = 6$). *, $P < 0.05$, **, $P < 0.01$.

Fig. 5. EGP decreases CD8⁺ T cells, and increases splenic M2/M1 ratio and CD4⁺CD25⁺ T cells. NOD females (6–8 weeks old) were orally gavaged with water, NR or EGPs for 4 weeks. (A) CD4/CD8 and CD44CD25 thymocytes were studied. (B) Splenic leukocytes, macrophages (M2 (F4/80⁺CD209⁺CD80⁻), M1 (F4/80⁺CD209⁻CD80⁺) and M2/M1 ratio), CD4⁺ T cells (CD3⁺CD4⁺CD8⁻), CD4⁺CD25⁺ T cells (CD3⁺CD4⁺CD8⁻CD25⁺), CD8⁺ T cells (CD3⁺CD4⁻CD8⁺), B cells (B220⁺) and CD40L⁺ B cells (B220⁺CD40L⁺) were studied by flow cytometry (mean ± SE, n = 7–8). +, P < 0.05, ++, P < 0.01 EGPs vs water; *, P < 0.05, **, P < 0.01 EGPs vs NR.

Fig. 6. EGPs increase serum IL-10.

The serum cytokines/chemokines from NOD females were quantitated, and the key regulators for T1D were graphed (mean $\pm$ SE, n = 7–8). The scale indicates the fold change. IL-10 was increased 86- and 3-folds by EGPs when compared to water and NR, respectively.

Fig 7. EGPs decrease IgG₁ and insulin autoantibodies (IAAs).

(A) The Ig isotypes (i.e., IgM, IgG₁, IgG_{2b}, IgG_{2c}) in the sera from NOD females were detected by ELISA (mean $\pm$ SE, n = 7–8). (B) Anti-insulin IgM and IgG were measured. To determine the specificity of IAAs, sera at the dilution of 1:5 were incubated with 10-fold excess of insulin (right column of both panels, mean $\pm$ SE, n = 4). +, P < 0.05, ++, P < 0.01 EGPs vs water; *, P < 0.05, **, P < 0.01 EGPs vs NR.

Table 1.Histopathological assessment of β -islets

Score	Pathological change of β -islets
Inflammation	
0	Unaffected.
1	Minimal affected. Only the periphery of the islet affected.
2	Inflammation present through the islet while the islet is still recognizable.
3	Islet destroyed.
Vacuolation	
0	No vacuole in the islet cells.
1	The islet cells have only small vacuoles, or one cell has many small vacuoles.
2	Mix of small vacuoles and large single vacuole per cell within the islets.
3	Predominantly large single vacuole in the islet cells.