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RESEARCH ARTICLES

Pomegranate extract and exercise provide additive benefits on improvement of immune function by inhibiting inflammation and oxidative stress in high-fat-diet-induced obesity in rats☆

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Abstract

Background: Obesity is reported to be associated with immune dysfunction and a state of low-grade, chronic inflammation. Either pomegranate extract (PomE) or exercise (Ex) has been shown to have antiobesity, anti-inflammatory and antioxidant effects. Nevertheless, no study has addressed the additive benefits of PomE and Ex on the restoration of obesity-induced immune defects.
Objective: The present work aims to study the effect of PomE and Ex as a combined intervention on immune function and the underlying mechanism involved in inflammation and oxidative stress in rats with high-fat-diet (HFD)-induced obesity.
Results: Our results demonstrate that the combination of PomE and Ex showed additive benefits on inhibition of HFD-induced body weight increase and improvement of HFD-induced immune dysfunction, including (a) attenuating the abnormality of histomorphology of the spleen, (b) increasing the ratio of the CD4+:CD8+ T cell subpopulations in splenocytes and peripheral blood mononuclear cells (PBMC), (c) inhibition of apoptosis in splenocytes and PBMC, (d) normalizing peritoneal macrophage phenotypes and (e) restoring immunomodulating factors in serum. We also find that immune dysfunction in HFD-fed rats was associated with increased inflammatory cytokine secretion and oxidative stress biomarkers, and that the combination of PomE and Ex effectively inhibited the inflammatory response and decreased oxidative damage.
Conclusions: The effect of PomE and Ex as a combined intervention is greater than the effect of either PomE or Ex alone, showing that PomE and Ex may be additively effective in improving immune function in HFD-fed rats by inhibiting inflammation and decreasing oxidative stress.

Keywords: Pomegranate extract; Exercise; Immune function; Inflammation; Oxidative stress; Obesity

1. Introduction

Obesity is often associated with an increased risk of dyslipidemia, hypertension, atherosclerosis, diabetes, nonalcoholic fatty liver disease and certain cancers [1]. Obesity has recently been reported to be associated with immune dysfunction. Changes in immune function have been proposed to underlie the pathogenesis of all these diseases. In obese individuals, reduced lymphocyte numbers and reduced responsiveness to mitogen have been observed [2]. Obesity induces immune defects that lead to attenuated host responses to bacterial infection [3,4]. Defects in specific immunity such as reduced lymphocyte numbers in the spleen, thymus and peripheral blood have been reported in Ob/Ob mice, db/db mice and Zucker rats [5], indicating that obesity may suppress the immune response. Conversely, innate immune dysfunction, associated with the absence of interleukin (IL)-6, granulocyte-macrophage colony-stimulating factor, IL-1R1 and IL-18, could be induced by obesity [6–8]. And

Abbreviations: Con, control rats fed a standard chow; HFD, rats fed an high-fat-diet (HFD); HFD + PomE, rats fed an HFD and administered a daily oral gavage of pomegranate extract (PomE); HFD + Ex, rats fed an HFD and exercised; HFD + PomE + Ex, rats fed an HFD and administered a daily oral gavage of PomE and exercised.

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substantial evidence has indicated that the obesity-induced immune dysfunction may contribute to the progression of several diseases, such as type II diabetes and cardiovascular disease [9]. Given the involvement of adipose tissue in the inflammatory process, one of the clinical objectives is to identify lifestyle factors that may affect the obesity–immune system dynamic. For instance, exercise and nutrition have shown to be significant lifestyle factors influencing the inflammatory cytokines associated with the state of obesity [10]. Particularly, it is well documented that obesity with chronic inflammation is also highly correlated to nutritional factors such as the type and amount of carbohydrates, proteins and fats that are consumed in the diet [11–12]. Thus, to evaluate the impact of physical activity and nutrition on obesity-related immune function is of significance.

Pomegranate extract (PomE) contains an array of compounds that have been attributed antiobesity effects. We have previously reported that pomegranate-extract-enriched punicalagin may be a useful nutrient for the treatment of obesity-associated nonalcoholic fatty liver disease that promotes mitochondrial function and eliminates oxidative stress and inflammation [13]. Dietary pomegranate-seed-oil-enriched punicic acid ameliorates high-fat-diet (HFD)-induced obesity and insulin resistance in mice [14]. Diet, in particular, combined with exercise (Ex) has been proven to be more effective than diet alone on improving obesity-related vascular dysfunction in obese children [15]. Nevertheless, no study has addressed the additive benefits of PomE and Ex on the restoration of obesity-induced immune defects. In our current work, we present the beneficial effects of PomE and Ex on improving immune function in HFD-induced obesity rats.

2. Materials and methods

2.1. Animals and experimental design

Sprague–Dawley male rats (180–220 g) were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). Animal rooms were maintained on a 12-h light–dark cycle at 20°C ± 3°C and 60% relative humidity. After 1 week of acclimatization, the rats were randomly assigned into the following five groups: (a) control rats fed a standard chow (Con, 12% kcal fat content); (b) rats fed an HFD (HFD, 45% kcal fat content); (c) rats fed an HFD and administered a daily oral gavage of PomE (HFD + PomE, 150 mg/kg/day); (d) rats fed an HFD and exercised (HFD + Ex, 20 m/min for 60 min at five times per week); (e) rats fed an HFD and administered a daily oral gavage of PomE and exercised (HFD + PomE + Ex, 150 mg/kg/day, 20 m/min for 60 min at five times per week). Punicalagin-enriched PomE was purchased from Tianjin Jianfeng Natural Products Research and Development Co., Ltd., China. The growing area of pomegranate is in Shaanxi province of China. The main composition is polyphenol, and punicalagin accounts for 40%. The extract preparation method is mainly column chromatography. The standard chow for control group contained 27.5% protein, 12% fat and 60.5% carbohydrate. The high-fat diet contained 19% protein, 45% fat and 36% carbohydrate and a premix to confirm the essential nutrients. Both diets have the same content of antioxidant (tertiary butylhydroquinone).

The exercise training was according to Bedford's protocol [16]. The training protocol we used is as follows: Rats in Ex groups commit 8 weeks of treadmill running (20 m/min, 60 min/day for 5 days per week). The exercise intensity was about 45% maximum oxygen uptake. Before these 8 weeks, there was 1 week of adaptive training for rats; the running time was increased day by day from 15 min to 60 min. Each group contained 10 rats. Body weight and food intake were measured twice weekly. After 8 weeks of PomE feeding and/or training, all rats were fasted overnight (about 12–16 h) and then sacrificed before afternoon. There was more than 48 h between the last time exercise or PomE feeding and sacrifice. This study involving animals was conducted according to the guidelines in the Declaration of Helsinki, and all procedures were approved by the Institutional Animal Care Committee of Xi'an Jiaotong University.

2.2. Body weight and immune organ weight

Body weight was measured and recorded every week. The weights of thymus and spleen were measured when the rats were sacrificed, and the relative weights of spleen and thymus for each rat were calculated by the formula organ weight (mg)/body weight (g).

2.3. Blood sample and peripheral blood mononuclear cells (PBMC) preparation

After the rats were sacrificed, blood samples were obtained by cardiac puncture, and the serum was separated by centrifugation (3000 rpm, 10 min). Serum samples were stored at −80°C.

PBMC were isolated from the peripheral blood of rats by density centrifugation followed by dextran sedimentation and lysis of contaminating red blood cells. Briefly, blood was collected in EDTA-treated tubes, diluted 1/2 with RPMI 1640 medium and carefully layered onto a density gradient Ficoll-Paque (Tianjin Haoyang, Tianjin, China). After centrifugation, the bands of PBMC were aspirated; PBMC were washed three times with RPMI 1640 medium and then preserved with frozen solution containing 90% fetal calf serum and 10% dimethyl sulfoxide (DMSO). Frozen PBMC were used for flow cytometry analysis.

Serum levels of C-reactive protein (CRP), leptin, adiponectin, immunoglobulin (Ig)A, IgG, IgM, tumor necrosis factor (TNF)-α, IL-1β, IL-6 and IL-4 were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's standards and protocols (R&D Systems, Minneapolis, MN, USA). The levels of triglyceride (TG) and total cholesterol (CHO) were analyzed using an automated biochemistry analyzer (Hitachi Ltd., Tokyo, Japan).

2.4. Splenocyte preparation

Spleens were removed aseptically at the time of sacrifice, and single-cell suspensions were prepared by forcing spleens through 200-mesh stainless steel. Splenocytes were washed with phosphate-buffered saline (PBS), and then erythrocytes in spleen were completely lysed using lyse/fix buffer (BD Bioscience, San Jose, CA, USA). This red blood cell (RBC) lysis buffer is formulated for optimal lysis of erythrocytes in single-cell suspensions of spleen and peripheral blood. After washing with PBS, the cells were resuspended in RPMI 1640 medium and then preserved with frozen solution containing 90% fetal calf serum and 10% DMSO. Frozen splenocytes were used for flow cytometry analysis.

2.5. Peritoneal macrophage preparation

Resident peritoneal macrophages were prepared by washing the peritoneal cavity with ice-cold PBS. After washing twice, the peritoneal cells were suspended in RPMI 1640 medium, and 2×10^6 cells were plated. Peritoneal macrophages were purified by 2-h adherence to dishes. More than 95% of the adherent cells were consistently found to be peritoneal macrophages, as assessed by their morphology after staining with Giemsa. Resident preparations were 95% macrophages. The cells were frozen for subsequent experiments.

2.6. Histopathological analysis of thymuses and spleens

At the time of sacrifice, thymi and spleens were collected, trimmed of fat and weighed before being stored in buffered 10% formalin. After fixation, one middle cross section from the spleen and one lobe of the thymus were embedded in paraffin, and five 5–6-μm sections were prepared and stained with hematoxylin and eosin for histopathological evaluation by light microscopy.

2.7. Analysis of T lymphocyte subpopulations in PBMC and splenocytes

The PBMC and splenocytes were suspended in RPMI 1640 medium at a concentration of 5×10^6 cells/ml, then recentrifuged and suspended in 100 μl PBS. The suspensions were incubated with 5 μl PerCP-conjugated anti-CD8 monoclonal antibody, 2 μl PE-conjugated anti-CD4 monoclonal antibody and 2 μl FITC-conjugated anti-CD3 monoclonal antibody (BioLegend, San Diego, CA, USA) for 30 min and then examined by flow cytometry. Data analysis was performed using EXPO32 ADC analysis software.

2.8. Analysis of PBMC and splenocyte apoptosis

To determine the extent of early apoptosis and necrosis in PBMC and splenocytes, cells were stained with annexin V and propidium iodide (PI). PBMC and splenocytes were collected as described above when the rats were sacrificed, adjusted to 2×10^6/ml, washed with cold PBS three times, centrifuged and incubated with 5 μl annexin V–fluorescein isothiocyanate (FITC) and 5 μl PI for 15 min in the dark at room temperature, and 400 μl of binding buffer was added to each sample. Samples were collected using a flow cytometer (Beckman Coulter), and data were analyzed using EXPO32 ADC analysis software. At least three independent experiments were performed.

2.9. Macrophage phagocytosis assay

For phagocytosis assays, macrophages were washed with PBS. After washing, FITC-labeled zymosan particles (100 g/ml) (Molecular Probes, Life Technologies) were added to the cells, and the cells were incubated at 37°C for the indicated times. The cells were then put on ice and washed thoroughly with PBS to remove unbound particles. The macrophages were detached and analyzed by flow cytometry immediately.

2.10. Assessment of antioxidant status

Rat serum was used for the measurement of biomarkers of oxidative stress. The total antioxidant capacities (T-AOC), lipid peroxidation malonaldehyde (MDA), superoxide dismutase (SOD), glutathione (GSH) and oxidized glutathione (GSSG) kits (Nanjing Jiancheng, Nanjing, China) were used according to the instructions of the manufacturer.