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Protective effects of ginsenoside Rg1 on splenocytes and thymocytes in an aging rat model induced by D-galactose

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ABSTRACT

Physiological aging is associated with a range of medical problems. However, the treatment of aging-associated diseases and prolonging human life are vital to our current aging societies. *Panax ginseng*, a traditional Chinese medicine, has been shown to have anti-oxidative and anti-aging effects. In the current study, aging rats induced by D-galactose were administered ginsenoside Rg1, then splenocytes and thymocytes were extracted and changes in activity were detected. The results demonstrated that compared with the D-gal group, the level of advanced glycation end products (AGE), the ratio of splenocytes and thymocytes in G0 phase (%), and apoptosis (%) of splenocytes and thymocytes, the ratio (%) of SA-gal positive splenocytes and thymocytes, the content of reactive oxygen species (ROS) and malondialdehyde (MDA), the ratio of glutathione (GSH) to oxidized glutathione (GSSG) and senescence-associated protein expression were significantly decreased and the index of the spleen and thymus, the proportion of white pulp in the spleen, the proportion of cortex in the thymus, the content of interleukin-2 (IL-2), interleukin-6 (IL-6), granulocyte-macrophage colony stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), the activities of superoxide dismutase (SOD), and the proliferative capacity of splenocytes and thymocytes were increased in the Rg1 + D-gal group. These findings demonstrated that ginsenoside Rg1 may antagonize spleen and thymus damage in D-galactose-induced aging rats by alleviating oxidative stress injury and down-regulating the expression of senescence-associated protein.

1. Introduction

The spleen and thymus are crucial organs in the human immune system, which have irreplaceable effects in the defense of pathogen invasion. The structure and function of the spleen and thymus decline with age. Currently, there are no effective methods with which to delay the aging of immune cells in the spleen and thymus. Ginsenoside Rg1 is the main active pharmaceutical ingredient in *Panax ginseng*, a traditional Chinese medicine, which exerts anti-oxidative and anti-aging effects [1,2]. Our previous studies suggested that oxidant stress can accelerate hematopoietic stem aging [2], and ginsenoside Rg1 can exert anti-aging effects by delaying hematopoietic stem cell aging [3]. Previous studies provided evidence that almost all of the immune cells come from hematopoietic stem cells. Thus, we deduced that Rg1 may be able to protect immune cells in the spleen and thymus against oxidative injury.

Oxidative stress and mitochondrial dysfunction have been reported to play a significant role in the aging process [4]. In addition, past studies have reported that mitochondria respiratory chains form the main intracellular source of reactive oxygen species in most tissues [5]. Hence, damaged mitochondria progressively lead to the generation of reactive oxygen species (ROS) [6]. Mitochondria are crucial intracellular organelles in which adenosine triphosphate (ATP) is generated. Dysfunction of mitochondria has been detected to generate ROS, impair intracellular calcium levels, reduce mitochondrial ATP production, and increase mitochondrial DNA mutations [7]. Increased production of ROS and damage to mitochondrial function influences the progress of senescence.

D-Galactose is a nutrient mainly obtained from lactose in milk. In animals, galactose is mainly metabolized by D-galactokinase and galactose-1-phosphate uridyltransferase and then absorbed by the body, but over-supply of D-galactose results in abnormal metabolism [8].

Abbreviations: ROS, reactive oxygen species; DNA, deoxyribonucleic acid; AGE, advanced glycation end products; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; GSSG, oxidized glutathione; SA-β-gal, senescence-associated β-galactosidase; IL-2, interleukin-2; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; GM-CSF, granulocyte-macrophage colony stimulating factor; HSC, hematopoietic stem cell

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Galactitol is converted from D-galactose and accumulates in the cell, which leads to the generation of ROS [9]. D-Galactose reacts with peptides and free amines of amino acids in proteins to form advanced glycation end products (AGE), and these products in turn activate the AGE receptor. These reactions induce cellular damage and oxidation [10,11]. Compared with other aging models including SAMP aging model, aging model induced by O_3, aging model induced by removal of thymus and natural aging model, the advantages of D-galactose model were significant, it was efficient, simple, cheap and stable. Therefore, we established an aging rat model induced by D-galactose to investigate the mechanism responsible for the protective effects of ginsenoside Rg1 on spleen and thymus cells.

2. Materials and methods

2.1. Animals

Male SD rats, 6–8 weeks old, 180–200 g weight, purchased from Chongqing Medical and Laboratory Animal Center (qualified number: SCXK yu. 2012–0001), were housed at 20–25 °C in a light-controlled room with free access to water and food. All experiments were performed according to the regulations of experimental animal administration issued by the State Committee of Science and Technology of the People's Republic of China and were ratified by the Ethics Committee of Chongqing University (CQU-2009011).

2.2. Reagents

Ginsenoside Rg1 (purity > 98%) was purchased from Hong Jiu Biotech Co., Ltd. (Tonghua, China). D-Galactose (purity > 99%) was acquired from Shanghai Bioengineering Co., Ltd. (Shanghai, China). SA-β-gal staining kit was purchased from Cell Signaling (Boston, MA, USA), and the ROS, SOD, GSH, GSSG, and MDA kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The AGE kit was purchased from Shanghai Yuanye Biological Technology Co. Ltd. (Shanghai, China). P53, P21, and Rb polyclonal antibodies were obtained from Prointech. TNF-α, GM-CSF, IL-2, and IL-6 kits were purchased from Boster (Wuhan, China).

2.3. Rat model [12]

A total of 40 rats were used and these were randomly divided into four groups (n = 10). In the D-gal-administration group (D-gal group), the rats were subcutaneously and persistently injected with D-galactose ($120\ mg{\cdot}kg^{-1}{\cdot}d^{-1}$) for 42 days [13,14]. In the Rg1 plus D-gal-administration group (Rg1 + D-gal group), rats were injected with D-galactose for two weeks, followed by a combined administration of Rg1 ($20\ mg{\cdot}kg^{-1}{\cdot}d^{-1}$) and D-galactose for 4 weeks [15,16]. In the normal control group, the rats were administered saline at the same dose and time. In the Rg1 control group, rats were injected with saline ($120\ mg{\cdot}kg^{-1}{\cdot}d^{-1}$) for two weeks and then administered both Rg1 ($20\ mg{\cdot}kg^{-1}{\cdot}d^{-1}$) and saline ($120\ mg{\cdot}kg^{-1}{\cdot}d^{-1}$) by subcutaneous injection for 4 weeks. On the 44th day, the rats were euthanized by cervical dislocation, and rats' body weight and spleen weight and thymus weight were measured to calculate the spleen and thymus index. Blood samples from the rats were also collected for white blood cell differential counts. Finally, the spleens and thymuses were halved, with one half being used for tissue sectioning and the other for disaggregation into single cells.

2.4. Preparation of spleen and thymus single cell suspensions

Spleen and thymus tissue was thoroughly mixed using a syringe piston, filtered through a 75-μm cell strainer, and centrifuged at 300 ×*g* for 5 min at room temperature (RT). Then, single cells were collected by high speed centrifugation. Following centrifugation, the precipitate was rinsed in 2 ml of PBS buffer, centrifuged at 300 ×*g* for 5 min at RT, and then resuspended in 4 ml RPMI 1640 medium.

2.5. Assessment of lymphocytes in peripheral blood and AGE in serum

Peripheral blood harvested from the rat tail vein was stored temporarily in a Heparinized Biomedical Polymer Anticoagulative tube (EDTA 1.5–2.2 mg/ml), then mixed sufficiently and put under the blood lancet of an automatic blood cell analyzer (Sysmex XE-2100, Kobe, Japan), next, pressed start key and the result will be read in 1 min. The accumulation of AGEs can speed up multisystem functional decline during the aging process. The levels of AGE in the serum were detected by an ELISA kit according to the manufacturer's instructions.

2.6. Calculating the indexes and structural analysis of the spleen and thymus

The indexes of the spleen and thymus were calculated using the equation: the organ index = organ weight (mg) / body weight (kg). The spleen and thymus were excised and fixed in 4% paraformaldehyde (pH 7.4), dehydrated in graded alcohol, and embedded in paraffin. Then 5–8-μm sections were sliced and stained with hematoxylin and eosin to observe the structure of the spleen and thymus. For every individual, there were at least 3 sections sliced in different position of the spleen and thymus. Then, the proportion of white pulp in the spleen and the proportion in the cortex of the thymus were measured, respectively, using Image Pro Plus software (Media Cybernetics Inc., Atlanta, GA).

2.7. Senescence-associated β-galactosidase cytochemical staining

Senescence-associated β-galactosidase (SA-β-gal) stain is a useful tool in the evaluation of organ senescence. SA-β-gal was exerted in accordance with the manufacturer's instructions. Senescent cells were subjected to blue staining under light microscopy. The frozen tissues slices were prepared and fixed by infusing fixatives at 20 °C for 20 min, and were then washed with phosphate-buffered saline (PBS) for 5 min, three times. Then staining solution (including β-galactosidase staining solution A 10 μl, β-galactosidase staining solution B 10 μl, β-galactosidase staining solution C 930 μl and X-Gal 50 μl) was added at 37 °C for 12 h. Senescent cells were observed and counted under light microscopy and the percentages of SA-gal-positive cells were determined. For every individual, there were at least 3 sections sliced in different position of the spleen and thymus.

2.8. Assessment of spleen and thymus cell proliferation

Single cell suspensions were collected and centrifuged at 1000 ×*g* for 5 min at 25 °C. The cells were seeded at 5×10^3 cells/well in a 96-well culture plate, then ConA with final concentration of 5 mg/l was added to each well and cultured, and then 10 μl of CCK-8 working solution was added to each well on days 1–4 and the absorbance was finally detected at 450 mm by microspectrophotometry.

2.9. Detection of cell cycle phase distribution and cell apoptosis

Splenocytes and thymocytes were harvested and adjusted to a concentration of 5×10^5 cells/ml, and then fixed with 70% cold ethanol at 4 °C overnight. Cells were cultured with propidium iodide (PI) at 4 °C for 30 min in the dark. Then cells were subjected to flow cytometry using a FAC Scan Flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) to detect the percentage of cells in different cell cycle phases. Flow cytometry was also used to measure the percentage of active cells during the apoptotic process with an Annexin V/PI Apoptosis Detection Kit (Roche, Shanghai, China). After culturing in a 5% CO_2 incubator at 37 °C for 48 h with reagents, cells were resuspended in binding buffer, then Annexin V (1:20) was added and