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Inhibiting Effects of Achyranthes Bidentata Polysaccharide and Lycium Barbarum Polysaccharide on Nonenzyme Glycation in D-galactose Induced Mouse Aging Model[1]

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Objective To investigate the inhibiting effects and mechanism of achyranthes bidentata polysaccharide (ABP) and lycium barbarum polysaccharide (LBP) on nonenzyme glycation in D-galactose induced mouse aging model. **Methods** Serum AGE levels were determined by AGE-ELISA, MTT method was used to determine lymphocyte proliferation, IL-2 activity was determined by a bioassay method. Spontaneous motor activity was used to detect mouse's neuromuscular movement, latency of step-through method was used to examine learning and memory abilities of mouse, colormetric assay was used to determine hydroxyproline concentration in mouse skin, pyrogallol autoxidation method was used to determine superoxide dismutase (SOD) activity of erythrocytes. **Results** Decreased levels of serum AGE, hydroxyproline concentration in mouse skin and spontaneous motor activity in D-galactose mouse aging model were detected after treated with ABP or LBP, while lymphocyte proliferation and IL-2 activity, learning and memory abilities, SOD activity of erythrocytes, were enhanced. **Conclusions** ABP and LBP could inhibit nonenzyme glycation in D-galactose induced mouse aging model *in vivo* and ABP has a better inhibiting effect than LBP.

Key words: Achyranthes bidentata polysaccharide(ABP); Lycium barbarum polysaccharide(LBP); D-galactose; Nonenzyme glycation; Aging

INTRODUCTION

Our previous works demonstrated that injection of D-galactose into mice could induce changes which resembled accelerated aging. The aging model shows neurological impairment decreased activity of anti-oxidant enzymes, and poor immune responses[1]. Achyranthes bidentata polysaccharide (ABP) is an active component isolated from the root of a Chinese medicinal herb Achyranthes bidentata Blume. Previous study showed that ABP could strengthen the ability of immune system, restrain metastasis of tumor, increase the number of leucocytes and protect liver cells[2]. Lycium barbarum polysaccharide (LBP) is a biological active substance from lycium barbarum. It can promote the function of T, B, CTL,

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NK and M_Φ immunocytes, formation of IL-2, IL-3 and TNF β cytokines, enhance immunological function of tumor-bearing mice and irradiation damaged mice and modulate NIM network[3]. The aim of this study was to investigate the effects of ABP and LBP on D-galactose induced mouse aging model, including neurological activities, serum AGE levels, skin hydroxyproline concentration and spontaneous motor activity, lymphocyte proliferation and IL-2 activity, learning and memory abilities, SOD activity of erythrocytes.

MATERIAL AND METHODS

Test Substances

Achyranthes bidentata polysaccharide (ABP) and lycium barbarum polysaccharide (LBP) were presented by professor Tian Gen-Yuan, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences. Drugs were resolved in phosphate-buffered saline (PBS) and filtered through 0.22 μm membranes.

Animals and Treatments

Three-month-old C57BL/6J female mice (a total of 32 mice, 18 g-22 g, provided by the Laboratory Animal Center, Chinese Academy of Medical Sciences, CAMS) were randomly divided into four groups with eight mice in each group. After one week adaptation period, the animals were given one of the following daily treatments for 8 weeks: (1) s.c 0.4 mL PBS as vehicle control, (2) s.c D-galactose at 50 mg/kg, (3) s.c D-galactose at 50 g/kg plus i.g ABP at 100 mg/kg, and (4) s.c D-galactose at 50 mg/kg plus i.g LBP at 100 mg/kg. After 8 weeks, four groups of mice were sacrificed and their sera, organs, and tissues were immediately collected for experiments or stored at -70℃ for later experiments.

AGE-enzyme-linked Immunosorbent Assay

Quantitative measurement of AGEs was performed by AGE-ELISA as described previously[4]. Briefly, 96-well plates were coated with 100 μL /well of 3 μg/mL AGE-BSA in coating buffer overnight at 4℃. Wells were washed three times with 150 μL washing buffer (PBS, 0.05%Tween-20, 1 mmol/L NaN_3), then blocked with 1% normal goat serum in 100 μL PBS for 2 h at 37℃. After washing, 50 μL of 1:10 diluted serum (sample) in dilution buffer (PBS, 0.02%Tween-20, 1 mmol/L NaN_3, 1% normal goat serum) and 50 μL anti-AGE polyclonal antiserum in dilution buffer (1:3 000) were added. Plates were incubated at room temperature for 2 h with gentle agitation on a horizontal rotary shaker. Wells were washed, 100 μL alkaline phosphate conjugated and antibody in dilution buffer (1:2 000) was added, and the plates were incubated at 37℃ for 1 h. They were then washed 6 times as above, 100 μL of *p*-nitrophenyl phosphate substrate was added to each well. After 30-60 min optical density (OD) at 405 nm was determined by an ELISA micro-plate reader (3550, Bio-Rad). The AGE-BSA was used as a competing antigen to generate an AGE standard curve. Sample AGE values were calculated from the standard curve.

CoA-induced Proliferation of Lymphocytes

Lymphocyte proliferation assay was performed as perviously described[5] with some modifications. The spleen was removed at sterilized condition after the animals were sacrificed. Cell suspensions were prepared by rubbing the tissue against sterile stainless steel wire mesh (100 μm) in 10 mL PBS. Contaminating erythrocytes were lysed by treating cell pellets with 5 mL of Tris-ammaonium chloride lysis buffer (0.18 mol/L NH_4Cl in 0.17

mol/L Tris, pH 7.2) for 2 min at room temperature. After washed with PBS, splenic lymphocytes were incubated in 100 μL RPMI 1640 medium at 10^6 cell/well with or without 7 μg/mL ConA. After incubation of the plates at 37℃ for 44 h, 20 μL MTT (5 mg/mL) was added to each well and incubated for additional 6 h at 37℃. The plates were read on a micro-plate reader (Model 3550, Bio-Read) at a wave-length of 570 nm. The proliferation of lymphocytes was expressed as stimulation index (OD at 570 of testing sample/ OD at 570 of non-stimulated control lymphocytes).

IL-2 Assay

IL-2 activity was determined using a bioassay as described previously[1,6]. Lymphocytes were incubated at 5×10^6 cells/mL for 24 h with 7 μg/mL ConA. Cell free supernatant was collected. The recombinant human IL-2 standard and testing supernatant were diluted with RPMI 1640 medium in 96-well micro-culture plates (100 μL/mL), and 100 μL of CTLL-2 cells (ATCC) at 4×10^5 cells/mL in RPMI 1640 medium were added to each well. The cells were incubated at 37℃ for 6 h to dissolve the dark blue crystal. OD at 570 nm was determined by a micro-plate reader and IL-2 activity was calculated from the standard curve. Data were expressed as U/mL.

Spontaneous Motor Activity Test

The photocell cage method was applied to test spontaneous neurological activity of mice[7]. The photocell apparatus (Model XZ-4, Institute of Materia Medica, CAMS) consisted of four dark boxes (19×10 cm). There were four light beams with corresponding photocell boxes in the periphery. The spontaneous motor activity of mice was detected using a photoelectric detector. At the end of treatment, mice were placed into the boxes, one per box. After 2-min adaptation, spontaneous activity was recorded for 10 min. The activity of PBS-treated young mice was regarded as a standard of 100% activity for data analysis.

Step-through Test for Acquisition of Memory

The step-through method[8] was used to evaluate the learning and memory ability of mice. The testing apparatus (36×12×12 cm, Model MX-5, Institute of Materia Medica, CAMS) consisted of two compartments: one dark box and one illuminated box with a light at a height of 25 cm from the top of the chamber. Between the two boxes, there was a hole of 3 cm in diameter. At 10 days prior to the end of treatment, each mouse was placed in the illuminated box, with its head against the hole. When the mouse entered the dark box, a scrambled footshock (30 V, 50 Hz) was delivered through the grid floor. The mouse could escape from the shock only by stepping back into the 'safe' illuminated side. Mice were trained for 5 min, 10 days prior to the memory test. At the test, mice were placed into the illuminated box and latency time (in seconds) and numbers of memory errors were recorded for 5 min. PBS-treated mice were used as controls.

Hydroxyproline Assay[9]

After sacrificing the mice, skin samples were excised from the back. For each mouse 25 mg (dried weight) of the defated sample was hydrolyzed with 2 mL 6 mmol/L HCl for 6 h at 140℃. The sample was neutralized with NaOH, and then dissolved in 15 mL distilled water. To a 1 mL hydrolysate solution the following were added sequentially: 1 mL chloramines T (1.41%), 1 mL 3.156 mol/L $HClO_4$, and 1 mL Ehrlichs reagent (20% *p*-dimethyl amino enzaldehyde). Formation of chromophore was measured at 560 nm. Standard hydroxyproline

solution was used to generate a standard curve ranging from 1-5 μg/mL.

Superoxide Dismutase (SOD) Assay

SOD activity of erythrocytes was determined by measuring the inhibition of pyrogallol autoxidation using a previously published method[10] with some modifications. Pyrogallol solution (0.1 mmol/L) was prepared with 1mM Tris-HCl, pH8.2. A stable autoxidation rate was reached and change of OD at 325 was ≈ 0.07/min at room temperature. A standard curve was produced with 0.1-0.5 μg/mL SOD. Sample OD was subsequently measured and activity was calculated based on the standard curve.

Data Analysis

Values were expressed as $\bar{x}\pm s$. The statistical significance of differences among experimental groups was evaluated by Student's *t*-test.

RESULTS

Serum AGE Levels

Mice treated with D-galactose showed an increased level of serum AGE (6.38±1.55) compared with the control mice (2.79±0.96) ($P<0.01$). Serum AGE levels of mice treated with D-galactose plus ABP or LBP, were 4.27±0.78 and 5.18±1.14 ($P<0.01$), respectively, indicating that ABP and LBP could prevent AGE increase in D-galactose treated mice (Fig.1).

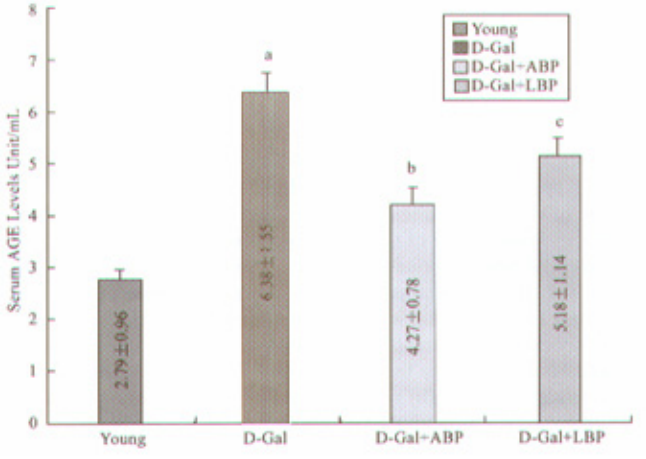

FIG. 1. Serum AGE levels of young and treated mice. Serum AGE levels were determined by a quantitative AGE-ELISA and data were expressed as relative AGE unit/mL ($\bar{x}\pm s$, n=8). [a] $P < 0.01$ vs young, [b] $P < 0.01$, [c] $P < 0.05$ vs D-galactose.

CoA-induced Proliferation of Lymphocytes and IL-2 Bioassay

Mice treated with D-galactose showed a significant decrease in lymphocyte proliferation

(2.08±0.25) and IL-2 production (9.32±1.54) *in vitro* compared with the 3-month-old controls ($P<0.01$). However, mice treated with D-galactose plus ABP or LBP had an increased lymphocyte proliferation and IL-2 production ($P<0.01$), indicating that the decline of immune response in D-galactose treated mice was prevented by ABP or LBP, and ABP had a better inhibiting effect than LBP (Fig. 2).

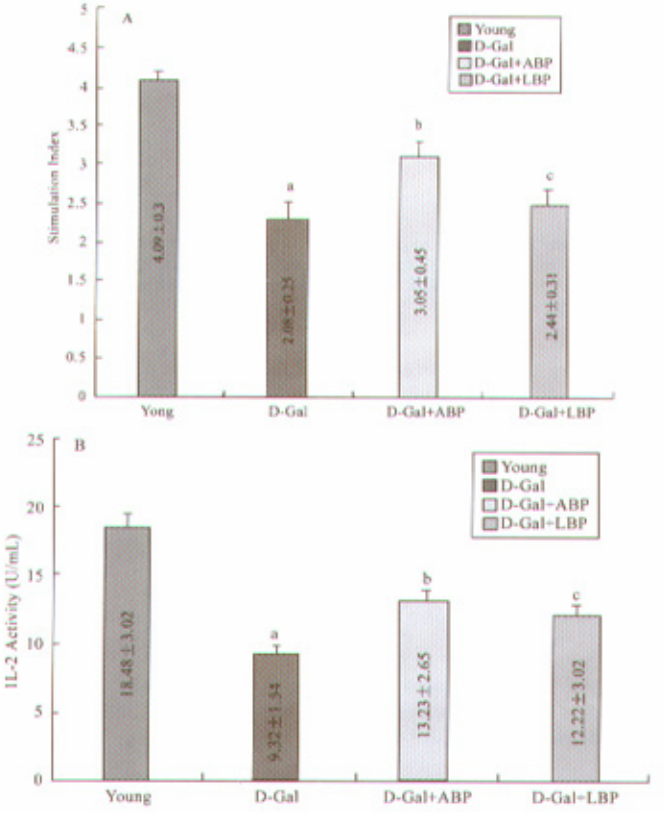

FIG. 2. Lymphocyte proliferation and IL-2 production of young and treated mice. (A) ConA-induced (7 μg/mL) proliferation of splenic lymphocytes was determined by MTT method. Stimulation index was calculated: OD at 570 of testing sample/OD at 570 of non-stimulated control. (B)IL-2 activity (U/mL) in ConA-treated splenic lymphocytes was determined by a bioassay. Data were expressed as $\bar{x}\pm s$. $n=8$, [a] $P<0.01$ vs young, [b] $P<0.01$, [c] $P<0.05$ vs D-galactose.

Spontaneous Motor Activity

Neuromuscular movement was determined by spontaneous motor activity. D-galactose treatment significantly lowered the spontaneous motor activity of young mice (155±45) compared with controls (282±66) ($P<0.01$). The effect of D-galactose was prevented by additional treatment of ABP or LBP ($P<0.05$) (Fig. 3). The inhibiting effect of ABP was 17.5% higher than that of LBP.

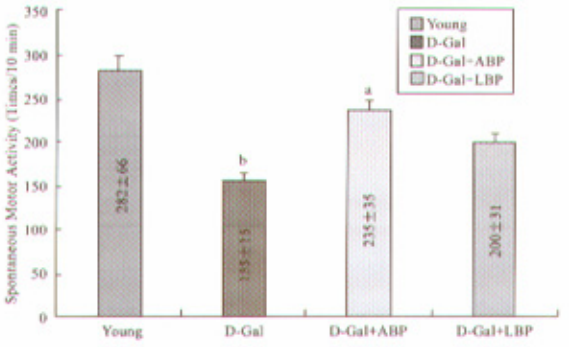

FIG. 3. Spontaneous motor activity of young and treated mice. Mice from different groups were placed in photocell cages and spontaneous motor activities were recorded electronically. Date were expressed as mean activity per 10 min ($\bar{x}\pm s$, n=8). Rate was calculated: activity of each group/activity of young. $^{b}P< 0.01$ vs young, $^{a}P< 0.05$ vs D-galactose.

Learning and Memory Abilities of Mouse

Learning and memory abilities were examined by latency of step-through method. D-galactose treated mice showed decreased latency of step-through (130±36) (P<0.01) and a greater number of errors (0.74±0.47) in comparison with the control. The shortened latency time and increased memory errors in D-galactose treated mice were prevented by ABP or LBP treatment (P<0.01) (Fig. 4). The latency time inhibiting effect of ABP was 28.7% higher than that of LBP, but the errors of ABP and LBP treated group were both lower than those of D-galactose group.

Hydroxyproline Concentration of Skin and SOD Activity of Erythrocytes

Treatment of young mice with D-galactose resulted in an increase of skin hydroxyproline content (63.44±8.00) and a decreased erythrocyte SOD activity (3.7±1.8) (P<0.01), while skin hydroxyproline content decreased and erythrocyte SOD activity increased after additionally treated with ABP or LBP, that is to say, the effect of D-galactose was prevented by ABP or LBP (Fig. 5).

DISCUSSION

Nonenzyme glycation has been widely accepted by modern medicine as a mechanism of diabetes mellitus and diabetic complication. Previous research from our laboratory also demonstrated that advanced glycation could account for the mechanism of D-galactose aging model[1]. It has become a hot spot to search for inhibitors of nonenzyme glycation in recent years. Traditional Chinese medicine with a history of two thousand years has been proved to be very effective in curing diabetes mellitus. Enlightened by this, many researchers in China have begun to study the inhibiting effect of traditional Chinese medicine on nonenzyme glycation. Some compounds, such as quercetin[11], silymarin[12], have been studied extensively and showed inhibition effect on nonenzyme glycation *in vitro* and *in vivo*. In this study, our results demonstrated that ABP or LBP treated mice had a significant

decrease in serum AGE levels, memory latency time and error rate, and skin hydroxyproline content. These mice also had a significant increase in motor activity, lymphocyte proliferation, interleukin-2 production, and superoxide dismutase (SOD) activity. All these results indicate that ABP and LBP are new traditional Chinese medicines which could prevent nonenzyme glycation reaction *in vivo*.

Both in human and animals, aging is associated with a decline of immune functions[13,14]. The decline of immune functions is linked to age-related increase in infectious diseases, malignancies, and autoimmune diseases. Mitogen-induced lymphocyte proliferation and IL-2 production are markers for aging[15].This investigation's finding that animals treated with ABP or LBP showed increased lymphocyte proliferation and IL-2 production supports that ABP and LBP could prevent the decline of immune functions by inhibiting the nonenzyme glycation.

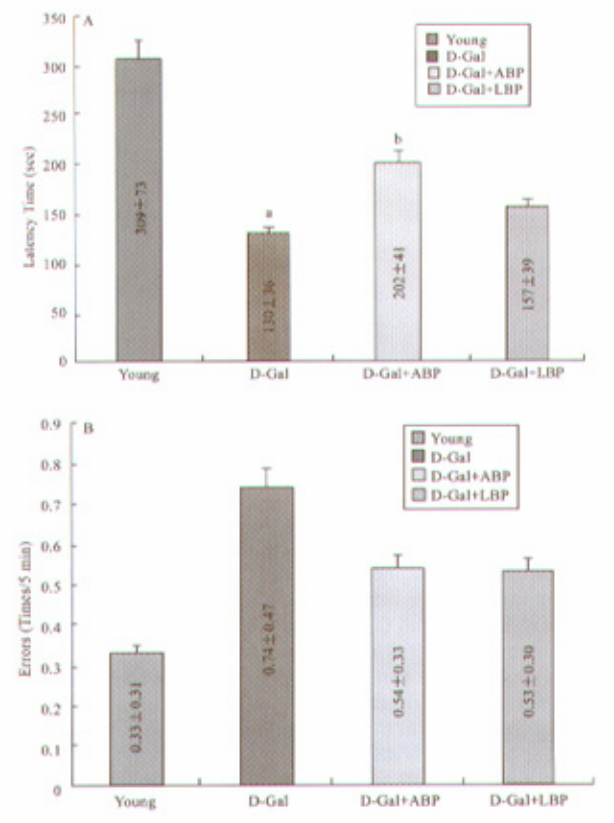

FIG. 4. Latency time and memory error rates of young and treated mice. Step-through method was used to determine the latency time (A) and memory error rates (B). Each mouse was trained for 5 min first to "remember" the system. Ten days later, mice were placed in the same cage and latency time (second) and number of errors were recorded electronically. n=8, ^{a}P<0.01vs young, ^{b}P<0.05 vs D-galactos.

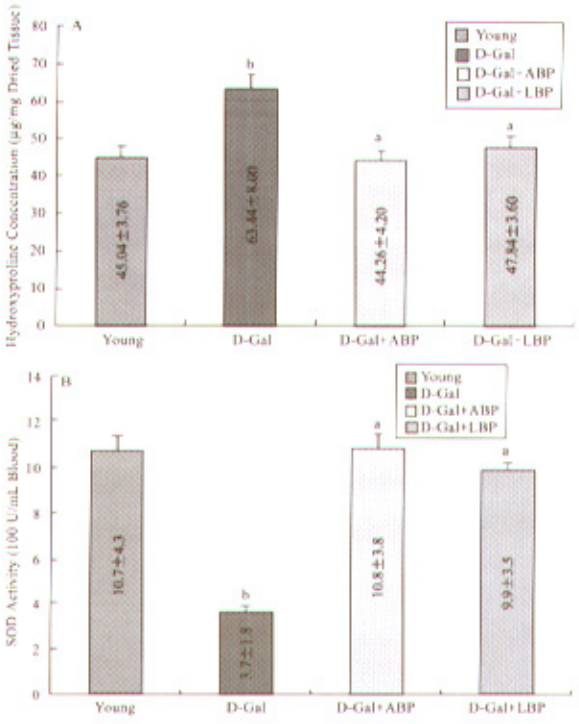

FIG. 5. Hydroxyproline and SOD levels in young and treated mice. (A)Hydroxyproline concentration in skin of mouse was determined by a colorimetric assay. Data were expressed as $\bar{x}\pm s$ (μg/mg dried tissue). (B)SOD activity in erythrocytes was measured and data were expressed as $\bar{x}\pm s$ (100 U/mL blood). n=8, $^{b}P< 0.01$ vs young, $^{a}P< 0.01$vs D-galactos.

Neurological aging is a complex process. Previous reports have shown that in D-galactose treated rats, there was an increase in lipofuscin, malondialdehyde, monoamine oxidase B, and superoxide dismutase in brain, heart, and liver, and a decreased level of superoxide dismutase(SOD) in serum[16]. These changes may directly or indirectly result in brain aging. Our results from this study indicated that ABP or LBP could prevent the decline of nervous system funcions by inhibiting nonenzyme glycation.

Free radicals play a very important role in aging process of body. SOD is a key enzyme in body which can get rid of the free radicals. When aging, body's ability to clean the free radicals decreases and the content of SOD is also reduced. Protein modified by AGE could produce a great deal of free radicals during oxidation. The activity of SOD will decrease or lose in body resulted from the SOD modified by NEG[17]. In our study, decrease of SOD activity in erythrocyte induced by D-galactose could be inhibited by ABP or LBP, which indicates that ABP or LBP restrains the decrease of SOD activity and enhances the ability to get rid of the free radicals by preventing nonenzyme glycation.

The aging theory of collagen cross-linking regards that increase of collagen cross-linking during aging could result in decrease of sensibility of collagen towards enzyme and

reduce dissolution, elasticity, flexibility of collagen[18]. Hydroxyproline content in skin is an indirect marker for collagen content. In this study, increased hydroxyproline content in skin induced by D-galactose could be inhibited by ABP or LBP, indicating that ABP or LBP delays the aging of collagen by inhibiting nonenzyme glycation.

From the results of our study we conclude that ABP has a better effect than LBP on inhibiting the nonenzyme glycation. Why would this happen? Is there a common mechanism of traditional Chinese herb of polysaccharide on inhibiting nonenzyme glycation? It is a problem awaits a further research.

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