Protective Role of Salidroside against Aging in A Mouse Model Induced by D-galactose[1]

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Objective To investigate the protective effects of putative AGEs (advanced glycation endproducts) inhibitor salidroside against aging in an accelerated mouse aging model induced by D-galactose. **Methods** A group of 5-month-old C57BL/6J mice were treated daily with D-galactose, D-galactose combined with salidroside, salidroside alone, and control buffer for 8 weeks. At the end of the treatment, serum AGEs levels, neurological activities, expression of glial fibrillary acidic protein (GFAP) and neurotrophin-3 (NT-3) in the cerebral cortex, as well as lymphocyte proliferation and IL-2 production were determined. **Results** D-galactose induced mouse aging model was developed as described before. As expected, salidroside blocked D-galactose induced increase of serum AGEs levels. It also reversed D-galactose induced aging effects in neural and immune system, as evidenced by improving motor activity, increasing memory latency time, and enhancing lymphocyte mitogenesis and interleukin-2 (IL-2) production. Furthermore, elevated expression of GFAP and NT-3 in the aged model mice was also reduced upon salidroside treatment. **Conclusion** Salidroside inhibits AGEs formation *in vivo*, which at least partially contributes to its anti-aging effect in D-galactose induced aging model.

Key words: Salidroside; Aging; D-galactose

INTRODUCTION

In this laboratory an aging model has been developed by injecting 50-500 mg/kg D-galactose intraperitonealy daily into C57BL/6J mice for 6-8 weeks, according to the protocol by Gong and Xu who first developed the model in 1991 in China[1]. This model resembles their aged control counterparts (16- to 24-month-old) both physiologically and pathologically[1-4]. Our previous study demonstrated that D-galactose injection led to an accelerated aging phenotypes manifested by an increased serum AGEs (advanced glycation endproducts) level, a significant decrease in motor activity, a decrease in memory latency time, and a decrease in lymphocyte mitogenesis and interleukin-2 (IL-2) production[5]. Treatment of AGEs formation inhibitor aminoguanidine (AG) in D-galactose induced aging mice reversed changes in the above parameters, indicating that AGEs may account at least partially for the mechanism of the accelerated aging[5]. Although the precise mechanisms involved remain obscured, our hypothesis is that AGEs are formed in the reaction of D-galactose with proteins and peptides *in vivo* and the increased AGEs accelerate the aging process.

AGEs are a heterogeneous group of reaction products that form between a protein's primary amino group and a carbohydrate-derived aldehyde group by reducing sugars, such as D-glucose and D-galactose, by nonenzymatic glycosation (NEG) *in vitro* and *in vivo*[6]. Many experimental evidences indicate that AGEs exacerbate and accelerate aging process and contribute to the early phases of age-related diseases, including atherosclerosis, cataract, neurodegenerative disease, renal failure, arthritis, and age-related macular degeneration[7].

Salidroside is a phenylpropanoid glycoside isolated from *Rhodiola rosea* L, a popular medicinal plant used in traditional Chinese medicine (see Fig. 1). It is reputable for improving depression, enhancing work performance, eliminating fatigue and treating symptoms of asthenia subsequent to intense physical and psychological stress. Due to these

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therapeutic properties, *Rhodiola rosea* L is considered to be one of the most active adaptogenic drugs. Our previous work has identified salidroside as one of most promising agents that are able to reduce the AGEs level *in vitro* in our NEG inhibitor screens (data not shown). Therefore, it is interesting to investigate further whether salidroside can inhibit AGEs formation *in vivo* and whether this would lead to any anti-aging effect in our D-galactose-induced aging model.

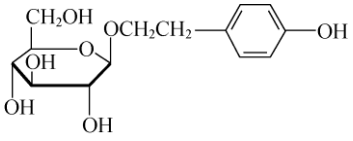

FIG. 1. Chemical structure of salidroside. Mr=300.3

In the present study, we treated a group of 5-month-old C57BL/6J mice daily with D-galactose, D-galactose combined with salidroside, salidroside alone, and control buffer for 8 weeks. At the end of the treatment, serum AGEs levels, neurological activities, expression of GFAP and NT-3 in the cerebral cortex as well as lymphocyte proliferation and IL-2 production were determined.

MATERIALS AND METHODS

Reagents

D-galactose, concanavalin A (ConA) and MTT were purchased from Sigma. RPMI 1640 medium and fetal calf serum (FCS) were from Gibco/BRL. Recombinant IL-2 was from Biotime. Interleukin-2 (IL-2)-dependent T lymphocytes (CTLL-2) were from ATCC. Salidroside was from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, China).

Animals and Treatment

C57 BL/6J female mice (Laboratory Animal Center, Chinese Academy of Medical Sciences, CAMS) were randomly divided into five groups of 10 each. Group two was 18-month-old aged controls and other groups were 5-month-old young mice. After one week adaptation period, the animals were given daily one of the following preparations subcutaneously for 8 weeks: (I) 0.4 mL PBS as vehicle control for the 5-month-old mice; (II) 18-month-old control mice with injection of PBS only; (III) D-galactose at 50 mg/kg for the 5-month-old mice; (IV) D-galactose at 50 mg/kg plus salidroside at 1 g/kg by intragastric injection for the 5-month-old mice; (V) salidroside at 1 g/kg by intragastric injection for the 5-month-old mice. Mice were sacrificed at the end of treatment and sera, organs, and tissues were immediately collected for experiments or stored at -70 ℃ for later experiments. All experimental procedures used in this study had been approved by the ethics committee in this institute and all animal experiments had been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The authors who performed experiments had given their informed consent prior to the study and had followed "principles of laboratory animal care" (NIH publication No. 86-23, revised 1985).

Immunohistochemistry

Immunohistochemical analysis was performed as previously described[8] with some modifications. Briefly, brains were removed, fixed for 4 h in formalin, equilibrated in PBS with 30% sucrose, and stored at 4 ℃. Each brain was cut in a coronal plane at a thickness of 40 μm with a freezing microtome. Brain sections were immerged into the PBS containing 0.2% Triton X-100 for 20 min and then blocked with 10% normal goat serum in PBS for 20 min at room temperature. Then, sections were incubated for 48 h at 4 ℃ with mouse monoclonal anti-GFAP IgG (Boehringer Mannheim, diluted 1:50) or rabbit polyclonal anti-NT-3 IgG (Santa Cruz, USA, diluted 1:50). After three washes with PBS, sections were incubated with biotinylated goat anti-mouse IgG (Santa Cruz, USA, diluted 1:100) or biotinylated goat anti-rabbit IgG (Santa Cruz, USA, diluted 1:100) for 1 h at room temperature prior to reaction with horseradish peroxidase-conjugated streptavidin (Santa Cruz, USA , diluted 1:100). The peroxidase reaction was developed for 15 min at room temperature in a DAB (diamino benzidine) solution (0.05% DAB+0.03% H_2O_2, PBS, pH7.2).

Protocols for Other Assays

AGEs ELISA assay, spontaneous motor activity test and step-through test as well as lymphocyte proliferation assay and IL-2 bioassay were carried out as described in our previous publication[5].

Data Analysis

Data from the various groups were compared by Student's *t*-test. In each case, $P<0.05$ was considered statistically significant. All data listed in the figures or the tables were expressed as $(\bar{x} \pm s)$.

RESULTS

Effects of Salidroside on Serum AGEs in vivo

All groups of mice gained weight normally throughout the study. Old mice treated with PBS had a higher level of serum AGEs than young mice (Table 1). Young mice treated with D-galactose showed a remarkably increased level of serum AGEs compared with young control mice (P<0.01, group III *versus* group I). Salidroside treatment significantly blocked the increase of AGEs in D-galactose treated mice (P<0.01, group IV *versus* group III), though it remains unclear whether salidroside inhibited AGEs formation directly or indirectly *in vivo*. It should be noticed that salidroside had little effect on the serum AGEs levels in young mice (Table 1, Group V). Next, we asked whether the decreased serum AGEs caused by the drug would lead to reversal of any aging effects, such as neurological activities in D-galactosed-induced aging model.

TABLE 1

Body Weight and Serum AGE Levels

Group	Treatments	Age (Month)	Body Weight (g)		AGE (U/mL)
			Pre-treatment	Post-treatment	
I	Young Control	5	22.0±0.3	23.0±0.2	2.95±1.10
II	Old Control	18	27.0±0.3	28.0±0.3	6.10±1.54[a]
III	D-galactose	5	21.9±0.2	23.0±0.1	6.18±2.14[b]
IV	D-galactose + Salidroside	5	21.8±0.2	23.2±0.2	3.85±1.32[c]
V	Salidroside	5	22.0±0.2	23.0±0.3	2.49±1.29

Note. C57BL/6J female mice (5-month-old or 18-month-old, n=10 in each group) were treated daily with PBS (s.c, young control and old control), D-galactose (50 mg/kg, s.c), D-galactose (50 mg/kg, s.c) with salidroside (1 g/kg, ig) and salidroside alone (1g/kg, ig). Body weights of pre- and post-treatment are shown in grams ($\bar{x} \pm s$) Serum AGE levels at the end of the treatment were determined by a quantitative AGE-ELISA and data are expressed as relative AGE U/mL ($\bar{x} \pm s$) . Statistically significant difference: [a] P<0.01, *vs* I; [b] P<0.01, *vs* I; [c] P<0.01, *vs* III.

Effects of Salidroside on Neurological Activities in Mice Model

Neuromuscular movement was determined by spontaneous motor activity as described[5]. Old mice showed significant lower activity per 10 min than young mice as a common change associated with aging. D-galactose treatment significantly lowered the spontaneous motor activity of young mice compared with controls, and this lowered activity can be reversed by the additional treatment of salidroside (Table 2). Learning and memory abilities were examined by latency of step-through method as described[5]. In old mice the passive avoidance time was shortened compared with young mice. D-galactose treated young mice also showed decreased latency of step-through. However, the shortened latency time in D-galactose treated mice was prevented by salidroside treatment (Fig. 2A). Similarly, salidroside decreased the number of memory errors in D-galactose treated mice (Fig. 2B).

TABLE 2.

Spontaneous Motor Activity of Salidroside

Group	Treatments	Age (Months)	Motor Activity (Times/10 min)
I	PBS	5	252±60
II	PBS	18	189±42[a]
III	D-galactose	5	160±29[b]
IV	D-galactose + Salidroside	5	249±36[c]

Note. Mice from different groups (n=10) were placed in photocell cages and spontaneous motor activities were recorded electronically. Data were expressed as mean activity per 10 minutes ($\bar{x} \pm s$) . [a] P<0.05, *vs* I; [b] P< 0.01, *vs* I ; [c] P<0.01 *vs* III.

Influence of Salidroside on Expression of GFAP and NT-3 in Cerebral Cortex

One of the most obvious phenotypes of neurological aging is the physiological and pathological changes of astrocytes, the major glial cells showing enlargement and accumulation of middle-fiber

containing GFAP[9-12]. Therefore, the elevated GFAP and NT-3 expression in the cerebral cortex is often an indicator of brain aging. We performed immunohistochemical study to detect the expression of GFAP and NT-3 in the cerebral cortex in D-galactose-induced aging mice. As expected and shown in Fig. 3A, the number of GFAP positive cells in the cerebral cortex was higher in aged mice

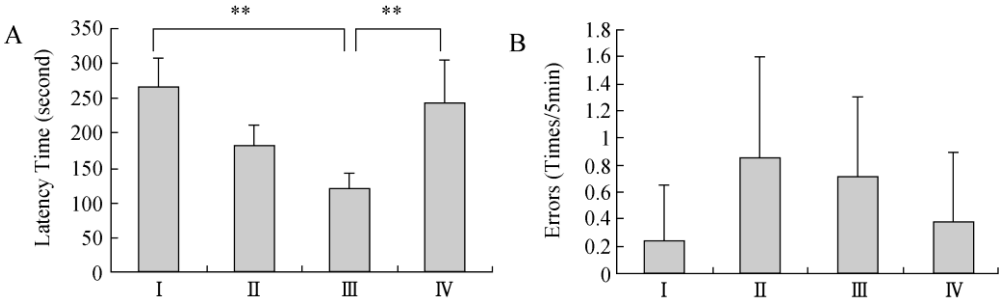

FIG. 2. Latency time and memory error rates of young (I), old (II), D-galactose treated (III), and D-galactose combined with salidroside treated (IV) mice. The step-through method was used to determine the latency time (A) and memory error rates (B). Each mouse was trained for 5 min first to "remember" the system. After 10 days, mice were placed in the same cage and latency time (second) and the number of errors was recorded electronically. Data were results of three experiments and expressed as $(\bar{x} \pm s)$. $^{**}P<0.01$.

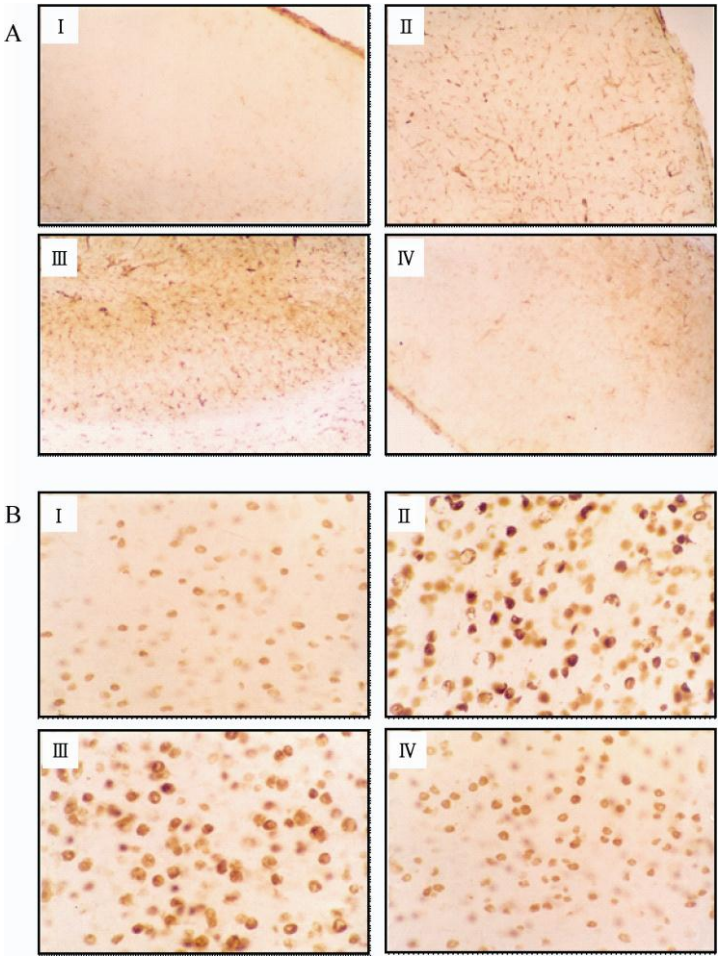

FIG. 3. Expression of GFAP (A) and NT-3 (B) in the cerebral cortex of young (I), old (II), D-galactose treated (III), and D-galactose combined with salidroside treated (IV) mice. Immunostaining with anti-GFAP IgG or anti-NT-3 IgG was performed as indicated in MATERIALS AND METHODS, and representative images were acquired from three different experiments.

compared with that in young mice (Fig. 3A II *versus* I). The D-galactose treated mice (III) showed similar GFAP expression pattern as the aged mice. However, the number of GFAP positive cells was lower in D-galactose combined with salidroside treated mice, which was similar to that of the young mice (Fig. 3A IV). Similar results were found for NT-3 expression (Fig. 3B).

Effects of Salidroside on Splenic T Lymphocyte Proliferation and IL-2 Activity

It is well documented that aging is associated with the decline of immune responses such as mitogen-induced T lymphocyte proliferation and IL-2 production[13]. As anticipated, 18-month-old mice showed a significant decrease in lymphocyte proliferation and IL-2 production compared with 5-month-old controls (see Fig. 4). Young mice treated with D-galactose also showed similar decreased splenic lymphocyte proliferation and IL-2 production *in vitro*. However, both lymphocyte proliferation and IL-2 production can be increased about 2 fold to the similar level of young mice upon salidroside treatment. Notably, salidroside alone did not demonstrate any significant effect in young mice (Fig. 4).

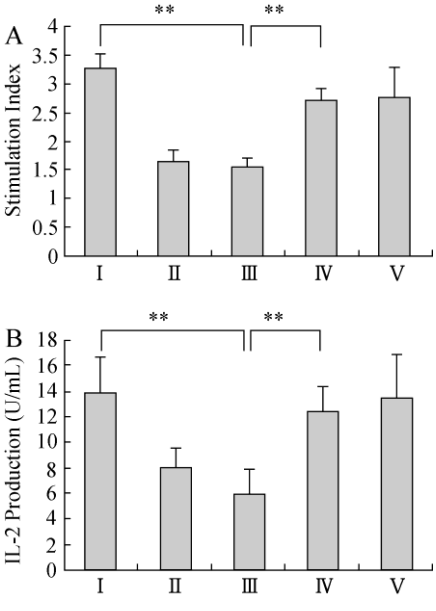

FIG. 4. Lymphocyte proliferation and IL-2 production of young (I), old (II), D-galactose treated (III), D-galactose combined with salidroside treated (IV), and salidroside alone treated (V) mice. (A) ConA-induced (7 mg/mL) proliferation of splenic lymphocytes was determined by MTT method. The stimulation index was calculated as: 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 results of three experiments and expressed as $(\bar{x} \pm s)$. $^{**}P<0.01$.

DISCUSSION

Normally accumulated with aging process, AGEs can induce both pathological and physiological changes in aging process[14-16]. In the D-galactose induced aging animals models developed by this lab oratory before, an increased level of AGE is thought to account at least partially for the underlying mechanism as the AGEs inhibitor aminoguanidine (AG) could block most of the aging phenotypes in the D-galactose induced mouse model[5]. That is why we selected AGEs as the target for anti-aging drugs screening. Salidroside is identified as a putative AGEs inhibitor from an ELISA-based AGEs inhibitor screening system. The naturally occurring drug is well-known for its adaptogenic effects and plays multiple roles such as anti-fatigue, anti-depression, and anti-infection *etc*. Although it is well proposed that salidroside may have anti-aging effect as well due to its antioxidant and neuroprotective role[17], the direct supportive experimental evidences linking the drug with aging have rarely been reported so far. Our work has for the first time direct proved the anti-aging efficacy of the small molecule agent *in vivo* with our unique aging mouse model. The underlying mechanisms could be complicated. One possibility is the inhibition of AGEs. Structurally different from AG, salidroside may function differently in inhibiting AGEs when compared with AG. AG not only prevents proteins from cross-linking by binding to sugars, thus preventing them from binding to the lysine group of proteins, but also decreases the AGEs-induced cross-linking of the extracellular matrix. However, salidroside is a beta-D-glucoside of a phenol and could bind to many receptors or enzymes that bind D-glucose or oligosaccharides, although it is unclear whether it could serve as a substrate or inhibitor *in vivo*. Moreover, it is anticipated that salidroside's phenol group contributes to some antioxidant properties. In fact, one study has suggested that salidroside may play a protective role against hydrogen peroxide (H_2O_2)-induced apoptosis in human neuroblastoma cells[18] and that AGEs can induce the activation of free radicals including reactive oxygen species (ROS)[15,19-21]. We also performed an *in vitro* experiment using human fetal lung diploid firbroblast 2BS cell line treated with H_2O_2 and found that salidroside could reverse H_2O_2-induced increase of ROS (data not shown). This further confirms the anti-oxidative effect of salidroside. Interestingly, AG can prevent oxidative modification of LDL (low-density lipoproteins) as well, and inhibits the formation of atherosclerotic plaques. Meanwhile, the increased free radicals were also observed in the

D-galactose model[22-23].

Taken together, it is possible that salidroside exerts its anti-aging effects at least partially by its NEG-inhibiting effect. Although several natural flavonoids showing potent inhibitory activity on AGEs formation are potent antioxidants *per se*[24], further work need to be done regarding the relationship between the NEG inhibitory activity and antioxidant property of this drug.

In conclusion, salidroside, as a putative AGEs inhibitor, is proved to block D-galactose induced increase of serum AGEs levels in D-galactose induced aging mouse model. Additionally, it can also reverse D-galactose induced aging effects in both neural and immune system. Our work is the first one to direct prove the anti-aging efficacy of the small molecule agent *in vivo* with our unique aging mouse model, thus inspiring the new application of this drug in gerontological area.

REFERENCES

1. Gong G Q, Xu F B (1991). Study of aging model in mice. *J China Pharm Univ* **22**, 101-103.
2. Li W B, Wei F, Fan M, *et al.* (1995). Mimetic brain aging effect induced by D-galactose in mice. *Chin J Pharm Toxicol* **9**, 93-95.
3. Cui X, Zuo P, Zhang Q, *et al*. (2006). Chronic systemic D-galactose exposure induces memory loss, neurodegeneration, and oxidative damage in mice: protective effects of R-alpha-lipoic acid. *J Neurosci Res* **84**(3), 647-654.
4. Wei H, Li L, Song Q, *et al*. (2005). Behavioral study of the D-galactose induced aging model in C57BL/6J mice. *Behav Brain Res* **157** (2), 245-251.
5. Song X, Bao M, Li D, *et al.* (1999). Advanced glycation in D-galactose induced mouse aging model. *Mech Ageing Dev* **108** (3), 239-251.
6. Bassi AM, Ledda S, Valentini S, *et al.* (2002). Damaging effects of advanced glycation end-products in the murine macrophage cell line J774A.1. *Toxicol In Vitro* **16** (4), 339-347.
7. Tian J, Ishibashi K, Ishibashi K, *et al*. (2005). Advanced glycation endproduct-induced aging of the retinal pigment epithelium and choroid: a comprehensive transcriptional response. *Proc Natl Acad Sci U S A* **102** (33), 11846-11851.
8. Satoh S, Matsumura H, Kanbayashi T, *et al*. (2006). Expression pattern of FOS in orexin neurons during sleep induced by an adenosine A2A receptor agonist. *Behav Brain Res* **170**(2), 277-286.
9. Smith JR, Pereira-Smith OM(1996). Replicative senescence: implications for *in vivo* aging and tumor suppression. *Science* **273**(5271), 63-67.
10. Wu Y, Zhang A Q, Yew D T (2005). Age related changes of various markers of astrocytes in senescence-accelerated mice hippocampus. *Neurochem Int* **46**(7), 565-574.
11. O'Callaghan J P, Miller D B (1991). The concentration of glial fibrillary acidic protein increases with age in the mouse and rat brain. *Neurobiol Aging* **12**(2), 171-174.
12. Nichols N R, Day J R, Laping N J, *et al*. (1993). GFAP mRNA increases with age in rat and human brain. *Neurobiol Aging* **14**(5), 421-429.
13. Franklin R A, Arkins S, Li Y M, *et al*. (1993). Macrophages suppress lectin-induced proliferation of lymphocytes from aged rats. *Mech Ageing Dev* **67** (1-2), 33-46.
14. Ramasamy R, Vannucci S J, Yan S S, *et al*. (2005). Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation. *Glycobiology* **15** (7), 16R-28R.
15. Alikhani M, Maclellan C M, Raptis M, *et al*. (2007). Advanced glycation end products induce apoptosis in fibroblasts through activation of ROS, MAP kinases, and the FOXO1 transcription factor. *Am J Physiol Cell Physiol* **292** (2), C850-856.
16. Bucala R, Cerami A (1992). Advanced glycosylation: chemistry, biology, and implications for diabetes and aging. *Adv Pharmacol* **23**, 1-34.
17. Kucinskaite A, Briedis V, Savickas A, (2004). Experimental analysis of therapeutic properties of *Rhodiola rosea* L. and its possible application in medicine. *Medicina (Kaunas)* **40**(7), 614-619.
18. Zhang L, Yu H, Sun Y, *et al*. (2007). Protective effects of salidroside on hydrogen peroxide-induced apoptosis in SH-SY5Y human neuroblastoma cells. *Eur J Pharmacol* **564** (1-3), 18-25.
19. Wang Z, Li D D, Liang Y Y, *et al*. (2002). Activation of astrocytes by advanced glycation end products: cytokines induction and nitric oxide release. *Acta Pharmacol Sin* **23**(11), 974-980.
20. Wong A, Luth H J, Deuther-Conrad W, *et al*. (2001). Advanced glycation endproducts co-localize with inducible nitric oxide synthase in Alzheimer's disease. *Brain Res* **920**(1-2), 32-40.
21. Li S Y, Sigmon V K, Babcock S A, *et al*. (2007). Advanced glycation endproduct induces ROS accumulation, apoptosis, MAP kinase activation and nuclear O-GlcNAcylation in human cardiac myocytes. *Life Sci* **80**(11), 1051-1056.
22. Ho S C, Liu J H, Wu R Y (2003). Establishment of the mimetic aging effect in mice caused by D-galactose. *Biogerontology* **4**(1), 15-18.
23. Lei M, Hua X, Xiao M, *et al*. (2008). Impairments of astrocytes are involved in the d-galactose-induced brain aging. *Biochem Biophys Res Commun* **369**(4), 1082-1087.
24. Urios P, Grigorova-Borsos A M, Sternberg M (2007). Flavonoids inhibit the formation of the cross-linking AGE pentosidine in collagen incubated with glucose, according to their structure. *Eur J Nutr* **46**(3), 139-146.

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