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Structural and Functional Changes of Immune System in Aging Mouse Induced by *D*-Galactose[1]

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Objective To investigate the role of *D*-galactose, especially in the structural and functional changes of the immune system in aging. **Methods** Serum levels of advanced glycation end-products (AGE) were determined by ELISA method. Ultra-structures of thymus and spleen were detected by transmission electron microscopy. MTT method was used to determine the lymphocyte proliferation. IL-2 activity was determined by bioassay. Northern blot was used to detect the IL-2 mRNA levels. **Results** Serum AGE levels of *D*-galactose- ($P<0.01$) and AGE-treated ($P<0.05$) mice ($n=8$) were increased significantly. The ultra-structures of thymus and spleen in *D*-galactose- and AGE-treated mice showed regressive changes similar to those in the aged control group. The lymphocyte mitogenesis and IL-2 activity of spleen were also decreased significantly ($P<0.01$, $n=8$). The change of IL-2 activity shown by Northern blot resulted from the change of mRNA expression. The AGE plus aminoguanidine group, however, showed no significant change in these parameters in comparison with the young control group ($P<0.01$ or $P<0.05$, $n=8$). **Conclusion** *D*-galactose and AGE lead to a mimic regression change of aging in the immune system *in vivo*.

Key words: *D*-galactose; Aging-mimetic model; Advanced glycation end-products; Ultra-structure; Nonenzyme glycation; Aminoguanidine

INTRODUCTION

A mimetic aging model developed by injecting *D*-galactose subcutaneously into rats or mice for over a month has been used widely for research and drug screening in China[1-2]. The changes include neurological impairments, increased free radicals, decreased activity of anti-oxidant enzyme, and diminished immune responses[3]. Free radicals account for the mechanism of aging[4-5]. However, the mechanism responsible for the aging changes induced by *D*-galactose remains largely unknown, especially the knowledge on the structure and functional changes of tissues or organs is limited.

AGE can be induced by reducing sugars, such as *D*-glucose, *D*-galactose in a non-enzymatic glycation with amino groups of proteins, lipids and nucleic acids *in vitro* and *in vivo*[6]. It is clear that the advanced glycation end-products (AGE) can produce a range of chemical, cellular and tissue effects, which are linked not only to aging but also to the development of several metabolic diseases, such as diabetic and uremic complications, atherosclerosis, amyloidosis and Alzheimer's disease[7-8]. Many reports have shown that aminoguanidine, the AGE formation inhibitor, prevents certain age-related changes in the aging rat model[9]. The hypothesis here is that AGE is formed in the reaction of *D*-galactose with proteins and peptides *in vivo* and the increased AGE accelerates the aging process.

In the present study, mice were injected with *D*-galactose, AGE, aminoguanidine, or PBS buffer for 8 weeks. Serum AGE levels, ultrastructure of thymus and spleen, lymphocyte proliferation, IL-2 activity, and IL-2 mRNA levels were determined to investigate the structure and function changes of immune system and the role of *D*-galactose in aging.

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MATERIALS AND METHODS

Reagents

D-galactose, L-lysine, aminoguanidine hydrochloride (AG), and MTT were purchased from Sigma. Glutaral, hydrogen osmate, Epon812 epoxy resin, dimethyl formamide, and dimethyl sulfoxide were analytical pure reagents made in China. AGE standards and rabbit anti-AGE-RNAase antibody were provided by Picower Institute, USA. α-32p-dATP was purchased from Yahui Biomedical Technology Inc. *Eco*R I, *Bam*H I and nylon membrane were purchased from Sino-American Biotechnology Co. Recombinant human IL-2 standard was purchased from Chinese Academy of Military Medical Sciences. AGE was prepared as previously described[8].

Animals and Treatment

C57BL/6J female mice (Laboratory Animal Center, Chinese Academy of Medical Sciences, CAMS) were randomly divided into 5 groups of 8 each. After one-week adaptation, the animals in each group were given daily one of the following preparations subcutaneously for 60 days: 0.4 mL PBS as vehicle control for the 2-month-old control mice, 0.5 mL PBS for the 22-month-old control mice, and *D*-galactose at 50 mg/kg for the 2-month-old mice, AGE at 50 mg/kg (tail vein injection) for the 2-month-old mice, *D*-galactose at 50 mg/kg plus 0.1% AG in drinking water for the 2-month-old mice. Mice were sacrificed at the end of treatment and sera, organs, and tissues were collected immediately for experiment or stored at -70℃ for later use.

Enzyme-linked Immunosorbant Assay of AGE

Quantitative AGE was measured by ELISA as described previously[8,10]. Briefly, 96-well plates were coated with AGE-BSA in coating buffer overnight at 4℃, and then blocked with normal goat serum. After washing, diluted serum (sample) and anti-AGE polyclonal antiserum in buffer (1:3000) were added. Plates were incubated at room temperature for 2 h. Wells were washed, and then alkaline phosphate conjugated secondary antibody in dilution buffer (1:2000) was added, and the plates were incubated at 37℃ for 1 h. *p*-Nitrophenyl phosphate substrate was then added and optical density (OD) at 405 nm was determined with an ELISA microplate reader (3550, Bio-Rad). The standard curve was generated using AGE-BSA as a competing antigen. AGE values in samples were calculated from the standard curve.

Ultrastructure of Thymus and Spleen[11]

After the animals were sacrificed, thymus and spleen were removed under sterile condition, fixed immediately in glutaral and hydrogen osmate, dehydrated for 10 min in 50%, 70%, 80%, 90%, 95% alcohol, and 95% alcohol: 95% acetone (V/V=1:1) successively, then dehydrated for 40 min with 100% acetone, infiltrated for one hour with epoxypropane and epoxy resin (V/V=1:1), embeded with Epon812 epoxy resin and aggregated for 12 h at 35℃, 45℃, and 55℃, successively. Then they were cut into 70 nm slices with LKB-Ⅲ microtome (Sweden), stained with uranyl acetate and lead citromalic acid, observed and photographed under JEM-1200EX transmission electron microscope (Japan).

ConA-induced Proliferation of Splenic Lymphocytes

Lymphocyte proliferation assay was performed as perviously described[8,12] with some modifications. Briefly, the prepared splenic lymphocytes were incubated in RPMI 1640 medium with or without 7 mg/L ConA. After incubation at 37℃ for 44 h, MTT (5 g/L) was added to each well and the plates were incubated again. The plates were read on a microplate reader (Model 3550, Bio-Rad) at a wavelength of 570nm (A_{570}). The proliferation of lymphocytes was expressed as stimulation index (the ratio of OD for testing sample: non-stimulated control lymphocytes at A_{570}).

IL-2 Bioassay

IL-2 activity was determined by bioassay[8,13]. Briefly, lymphocytes were incubated with 7 mg/L ConA and then the cell-free supernatant was collected. The recombinant human IL-2 standard and the testing supernatants were diluted with RPMI 1640 medium, and CTLL-2 cells (ATCC) were added. After the cells were incubated at 37℃ for 6 h to dissolve the dark blue crystal, OD_{570} was determined with the microplate reader and IL-2 activity was calculated from the standard curve. Data were expressed as $U \cdot mL^{-1}$.

Determination of IL-2 mRNA Levels by Northern Blot

Total RNA was extracted from splenic lymphocytes by a single step method using acid guanidinium thiocyanate-phenol-chloroform[14]. RNA was quantified with a spectrophotometer, and the ratio of OD_{260} to OD_{280} exceeding 1.8 indicated a high quality of RNA. Equal amounts of RNA (50 μg) were filtered onto nylon membranes as described in the protocol of molecular clone Ⅱ. The IL-2 cDNA probe was obtained from a pJ1 plasmid. After

amplification, extraction (alkali method), and purification (phenol:chloroform) of plasmid DNA containing target cDNA, then restriction digestion (*Eco*R I and *Bam*H I), 1% agarose gel electrophoresis, recovery, and purification of target cDNA, the radio-labelled cDNA probes were labelled with α-32p-dATP, using random primes kits (Promega) as described in the protocol provided by the manufacturer. Following such steps as prehybridization, hybridization and washing, membranes were autoradiographed at -70℃.

Data Analysis

All data in the text and figure are presented as $\bar{x} + s$. Statistical analysis was performed by Student's *t*-test.

RESULTS

Serum AGE

During the period of treatment, neither young nor aged mice showed significant abnormality detected by naked eye. All mice gained weight normally throughout the study (Table 1). As anticipated, the serum AGE level in Group Ⅱ treated with PBS was higher than that in Group Ⅰ ($P<0.01$). Also, mice in Group Ⅲ and Group Ⅳ treated with *D*-galactose and AGE, respectively, showed an increased level of serum AGE, compared with that in Group Ⅰ ($P<0.01$ or $P<0.05$, vs Group Ⅰ). In group Ⅴ treated with *D*-galactose, however, AG prevented the increase of AGE ($P<0.05$, vs Group Ⅲ).

TABLE 1

Body Weight and Serum AGE Levels (n=8)

Group	Age	Body Weight (g)		AGE
		Pre-treatment	Post-treatment	(U/mL)
Young Control	2 months	21.2±0.2	22.3±0.4	2.99±1.07
Old Control	22 months	30.1±0.5	30.9±0.6	7.12±0.01[a]
D-Galactose	2 months	21.1±0.3	22.1±0.3	6.55±0.57[a]
AGE	2 months	21.1±0.3	22.2±0.3	5.49±1.60[b]
D-Galactose+AG	2 months	21.0±0.2	22.1±0.2	4.24±0.89[c]

Note. [a]vs Young control, $P<0.01$; [b]vs Young control, $P<0.05$; [c]vs *D*-galactose, $P<0.01$.

Ultrastructure of Thymus and Spleen

The ultrastructure of thymus in each group is shown in Fig. 1. The lymphocytes in thymus of young mice were round with big nuclei, and the mass of heterochromatin was found in the cells. Mitochondria and numerous free ribosomes were found in cytoplasm, and the structure of mitochondria was clear. The nuclei of epithelial-reticular cells were elliptical and had mass of euchromata, with clear nucleolus. The cytoplasm abounded in structurally normal mitochondria, with distributed rough endoplasmic reticula and free ribosomes (Fig. 1-A). The lymphocytes in thymus of old mice were irregular, with visible pyknosis. Mitochondria in epithelial-reticular cells were swollen with vacuoles, the cristae of mitochondria were broken, and tonofilaments and lysosomes appeared in cytoplasm (Fig. 1-B). Moreover, the thymic cortex in *D*-galactose and AGE-treated mice had aging-mimetic ultrastructure. The irregular lymphocytes appeared as a pyknotic phenomenon, with condensed intranuclear chromatin (Fig. 1-C, D). However, the structure of lymphocytes in thymus of *D*-galactose plus AG-treated mice was almost normal and its ultrastructure was similar to that of the young control mice (Fig. 1-E).

The ultrastructures of spleen in different groups are shown in Fig. 2. The lymphocytes in spleen of young mice were closely arranged, with round nuclei, clear nucleoli, and numours free ribosomes in the cytoplasm. Structurally normal mitochondria, rough endoplasmic reticula and free ribosomes were distributed in the cytoplasm (Fig. 2-A). In contrast, in the spleen of old mice, some lymphocytes were irregular, the nuclei were also irregular and pyknotic, the karyotheca was partly broken, and the nuclear matrix had cavitation. Free ribosome decreased in cytoplasm, mitochondria were swollen, and the cristae of mitochondria were broken (Fig. 2-B). In addition, spleen in *D*-galactose- and AGE-treated mice showed aging-mimic structures (Fig. 2-C, and Fig. 2-D), which were obviously changed, compared with those in young mice. The size of some lymphocytes in spleen was unequal, many nuclei were irregular and pyknotic. Mitochondria and ribosomes in cytoplasm were decreased and swollen. Few lysosomes were shown in the cytoplasm. The ultrastructure of lymphocytes in the spleen of mice treated with *D*-galactose plus AG was similar to that of young mice (Fig. 2-E).

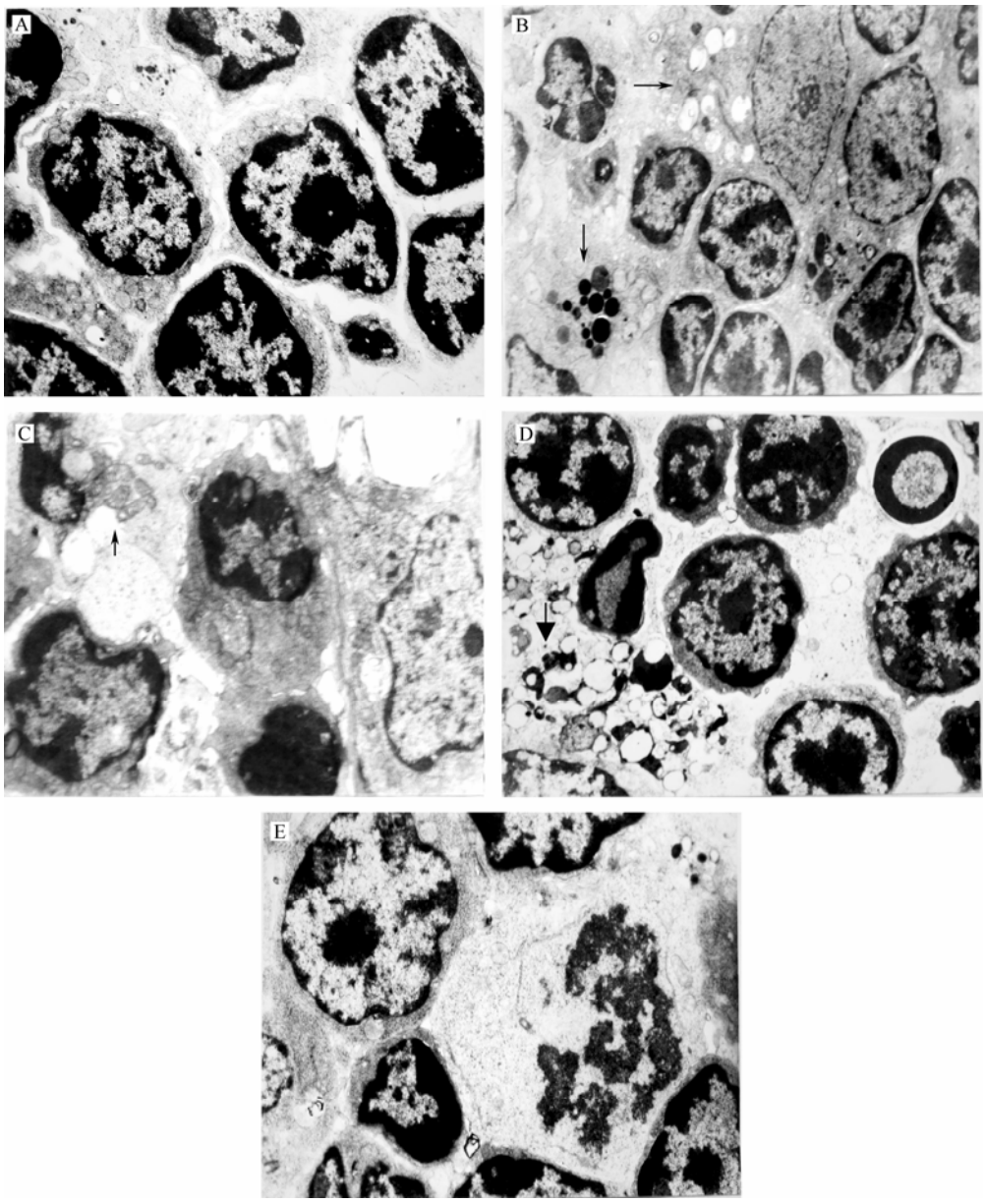

FIG. 1. Ultrastructure of thymus gland of young mice (A), old mice (B), mice treated with *D*-galactose (C), AGE (D), *D*-galactose and aminoguadinine (E). Bar=1μm.

ConA-induced Proliferation of Splenic Lymphocytes and Activity of IL-2

As anticipated, mice in group Ⅱ showed a significant decrease in lymphocyte proliferation and IL-2 activity, compared with those in group Ⅰ ($P<0.01$, *vs* group Ⅲ). In addition, mice in groups Ⅲ and Ⅳ treated with *D*-galactose and AGE showed decreased splenic lymphocyte proliferation and IL-2 production *in vitro* ($P<0.01$, *vs* group Ⅲ). However mice in group Ⅴ showed that AG prevented the decline of immune response in *D*-galactose-treated mice ($P<0.01$, *vs* group Ⅲ) (Table 2).

IL-2 mRNA Expression

The Northern blot assay showed that the change of IL-2 activity resulted from the change of mRNA expression. The IL-2 mRNA expression in *D*-galactose- and AGE-treated old mice was lower than that in young mice. AG inhibited the decreased IL-2 mRNA expression induced by *D*-galactose (Fig. 3).

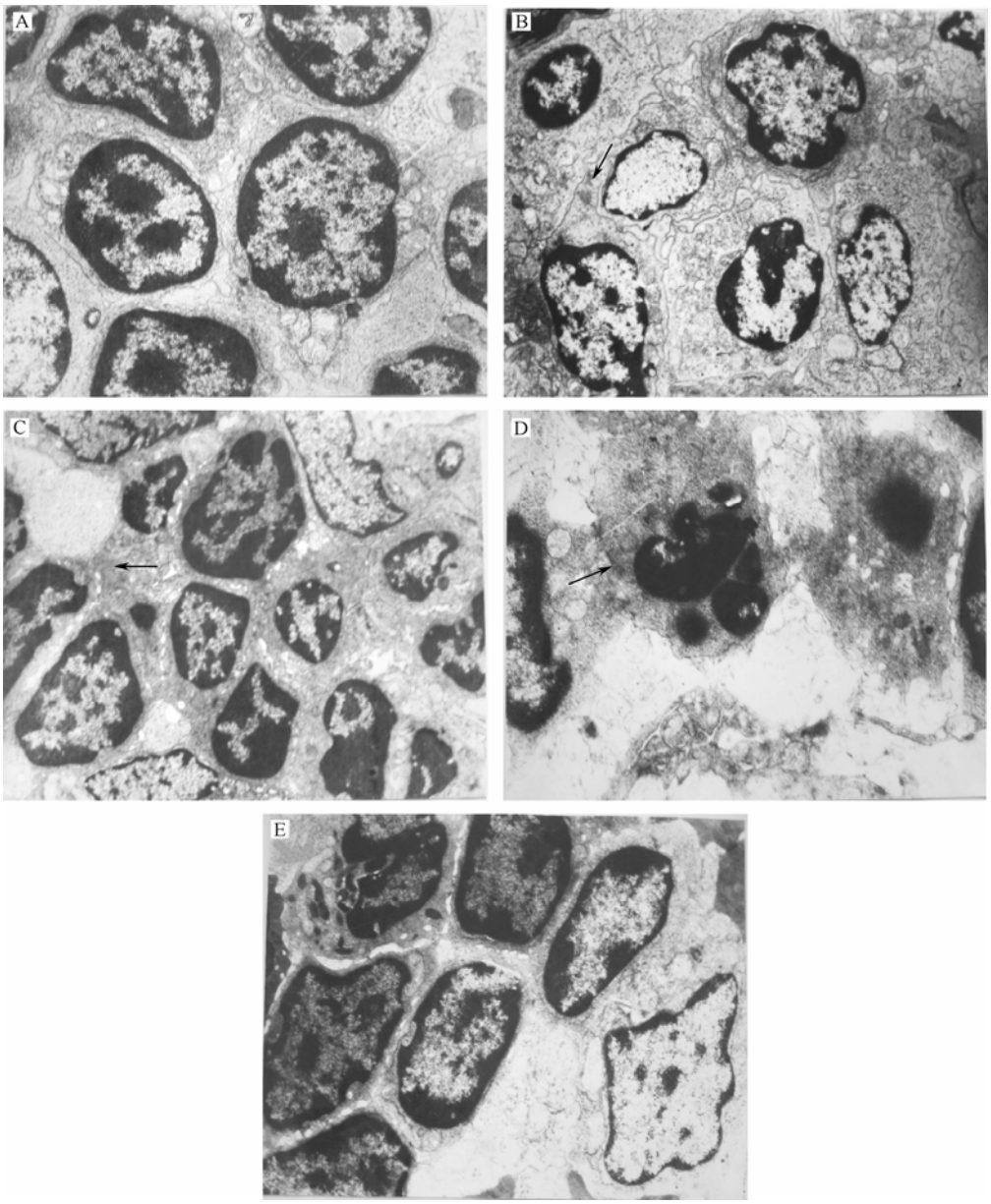

FIG. 2. Ultrastructure of spleen of young mice (A), old mice (B), mice treated with *D*-galactose (C), AGE (D), *D*-galactose and aminoguadinine (E). Bar=1μm.

DISCUSSION

Our study demonstrated that the serum AGE levels in mice treated with *D*-galactose and AGE increased significantly, and the ultrastructure of thymus gland and spleen showed retrogressive morphologic changes. The lymphocyte proliferation and IL-2 activity in these mice also decreased significantly. Such changes are in accord with the aging process. Furthermore, aminoguanidine, the AGE inhibitor, could prevent the accelerated aging process in *D*-galactose-injected mice. All these results strongly suggest that the aging effect induced by *D*-galactose is related to the nonenzyme glycation (NEG)[15]. The decline of the function of thymus and spleen in *D*-galactose-treated and AGE-treated mice could be induced by NEG, and AG could inhibit the NEG reaction. This would throw a new light on the anti-aging drug research.

Aging involves morphological alterations of the thymus, spleen and deregulation of various immune

TABLE 2

Proliferation of Splenic Lymphocytes and IL-2 Activity (n=8)

Groups	Age	Proliferation Stimulation Index	IL-2 Activity ($U \cdot mL^{-1}$)
Young Control	2 months	3.83±0.43	16.67±3.33
Old Control	22 months	1.74±0.38**	7.35±1.66 [a]
D-Galactose	2 months	2.04±0.22**	8.24±1.27 [a]
AGE	2 months	2.08±0.24**	8.59±1.12 [a]
D-Galactose +AG	2 months	3.06±0.40$^{\Delta\Delta}$	11.65±2.32 [b]

Note. [a]vs Young control, $P<0.01$; [b]vs *D*-galactose, $P<0.01$.

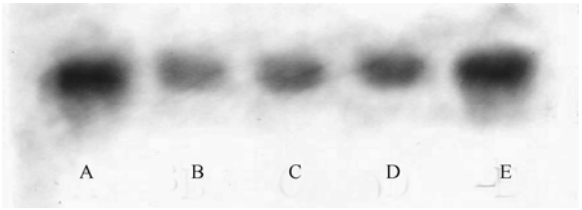

FIG. 3. Expression of IL-2 mRNA in mice (Northern blot). A: Young mice. B: Old mice. C: *D*-galactose-treated mice. D: AGE-treated mice. E: *D*-Galactose+AG-treated mice.

response parameters[10]. Thymus plays an important role as the central immune organ secreting thymosin, in regulating the function of immune system and the maturity of T cells. Many studies have shown that thymus undergoes great retrogressive morphologic changes with aging, including weight loss and structural changes, such as atrophy of thymic cortex and medulla, decreased number of lymphocytes and epithelial-reticular cells, destroyed cell membrane integrity, swollen mitochondria, disrupted crystallin and increased lysosomes. Spleen is the main peripheral immune organ undergoing immune reaction. It was reported that the structure of spleen changes in the process of aging, during which lymphocytes decrease in number, epithelial-reticular cells and macrophages increase, mitochondria become swollen, and crystallin and lipofuscin are disrapted in macrophages[16].

Until now there are few reports on the structural and functional changes of thymus and spleen in *D*-galactose-induced aging. In our study, the ultrastructure of thymus and spleen in mice treated with *D*-galactose and AGE showed a regressive change and AG could inhibit the effects induced by *D*-galactose, suggesting that the structural change of immune system caused by *D*-galactose results from nonenzyme glycation.

Aging is associated with a decline in immune responses including mitogen-induced lymphocyte proliferation and IL-2 production[17-18]. A decrease of lymphocyte proliferation and IL-2 production in animals treated with *D*-galactose and AGE further suggests that *D*-galactose imitates the natural process of biological aging. The Northern blot assay confirmed that the change of IL-2 activity is consistent with the IL-2 mRNA expression. *D*-galactose also causes a functional change of immune system resulting from the nonenzyme glycation (NEG) as confirmed in the previous studies[8].

In conclusion, the nonenzyme glycation accelerates the aging process of immune system. The mimetic aging effects in the immune system induced by *D*-galactose may relate to the nonenzyme glycation. The nonenzyme glycation in the functional deterioration of immune system may be one of the mechanisms involving *D*-galactose in the process of aging.

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