Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Effects of Kinetin on Thymus and Immune Function of Aging Rats

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ARTICLE HISTORY (15-474)

Received: November 25, 2015
Revised: March 24, 2016
Accepted: April 01, 2016
Published online: June 29, 2016

Key words:
Aging
Immune
Kinetin
Thymus

ABSTRACT

To investigate the effects of kinetin (Kn) on thymus and immune function in a rat model of D-galactose (D-gal)-induced aging, fifty SD rats were randomly divided into five groups: young control group, aging model group, low-dose Kn-treated group, middle-dose Kn-treated group and a high-dose Kn-treated group. Rats in the aging model group were subcutaneously injected with D-gal (125 mg/kg BW/day) in the back of the neck. Rats in the Kn-treated groups were intraperitoneally injected with 5, 10 or 20 mg/kg BW/day Kn solution. Changes in the thymus index, activity of antioxidant enzymes such as superoxide dismutase (SOD), the content of metabolic products such as malondialdehyde (MDA), the content of serum immunoglobulins and Interleukin–2 (IL-2), as well as histological changes in the thymus were recorded and analyzed. Results showed that the thymus index, antioxidant enzyme activity and the immunoglobulin contents of experimental animals in the aging model group decreased significantly compared with the young control group. The contents of metabolic products increased significantly compared with the young control group. The thymus index, content of immunoglobulin and IL-2, as well as antioxidant enzyme activity of the rats in the Kn-treatment groups significantly increased compared with the aging model control group, while the contents of Interleukin–6 (IL-6) and MDA decreased significantly. The structure and substructure of thymus cells were protected by Kn treatment. Therefore, we conclude that Kn can protect the tissue structure of the thymus and effectively antagonize oxidative damage in the thymus of aging rats induced by D-gal, and thereby improve the body's immune ability and delay aging of the thymus.

To Cite This Article: Li M, Ouyang W, Li J, Si L, Li X, Guo J and Li H, 2016. Effects of kinetin on thymus and immune function of aging rats. Pak Vet J, 36(3): 356-362.

INTRODUCTION

Immunology theory supposes that the decline of immune function is an important factor contributing to aging of the body (Smith *et al*., 2015, Filiano and Kipnis, 2015). The thymus is the hub of the immune system and the place where T lymphocytes differentiate and mature. It plays an import role in the maintenance of cellular immunity and regulation of the immune response (Takaba *et al*., 2015). With increasing age, the thymus atrophies, the thymus index decreases, and levels of immune cells and subcellular factors also decrease, all of which cause a decline in the body's immune function (Hosek *et al.*, 2013).

Kn is a type of cytokinin. The molecule can not only promote cell division, differentiation and growth, but also has protective effects against oxidative damage and delays the aging (Alemi *et al.*, 2013). Natural Kn is found in almost all animal and plant tissues. There have been a large number of Kn studies and it is widely used as an anti-aging cosmetic ingredient (Sun *et al.*, 2013; Goindi *et al.*, 2015). However, the mechanism of Kn in anti-immune aging needs further elucidation. In this study, we investigated the mechanism of Kn in protecting against the thymus aging by analyzing the changes of the thymus and the immune function of the aging rats induced by D-gal.

MATERIALS AND METHODS

Materials: Fifty two-month-old SD rats (25 males and 25 females) were provided by the Experimental Animal Center of the Medical College of Xi'an Jiao Tong

University (Qualification certificate number: 2012-003). The weight of the animals was 200±20 g.

Main reagents and instruments: Main reagents and instruments included: D-galactose (Sigma, St Louis, MO, USA), Kinetin (sigma, K3378), superoxide dismutase (SOD), malondialdehyde (MDA), Glutathion Peroxidase (GSH-PX), Catalse (CAT), Lipid Peroxide (LPO), Coomassie brilliant blue staining kit (Nanjing Jiancheng Biological Institute, Nanjing, Jiangsu, China), JA2003 Electronic Precision Balance (Shanghai Precision Instrument Co. Ltd., precise to 0.001g), Shimadzu-UV2450 ultraviolet spectrophotometer (Shimadzu, Corporation, Kyoto, Japan), KD-T Water bath-slide drier, KD-BM biological tissue embedding machine (Leica microsystems, Wetzlar, Germany), Leica RM2016 paraffin slicing machine (Leica microsystems, Wetzlar, Germany), Olympus-BX53 microscope (Olympus, Tokyo, Japan), Jeol-1230 transmission electron microscope (Jeol, Tokyo, Japan), and Leica EMUC7 ultramicrotome.

Experimental animals: The SD rats were randomly divided into 5 groups: a young control group, an aging model group, a low-dose Kn-treated group, a middle-dose Kn-treated group and a high-dose Kn-treated group. Each group comprised 10 rats.

The rats were acclimatized for one week after purchase to reduce the stress response. The experiment began when the rats had adapted to their new environment. Throughout the experiment, the rats were supplied with a normal diet and clean water. The room temperature was controlled at 20°C, and the humidity between 65-75%. The animals in the young control group were subcutaneously injected with physiological saline in the dorsal neck daily for 45 days. The animals in the other four groups received subcutaneous injection of D-gal (125 mg/kg BW) daily for 45 days to establish the aging model. From day 11 to day 45, the animals in the Kn-treated groups received daily intraperitoneal injections of Kn (5, 10 or 20 mg/kg BW, respectively). Animals in the young and aging control groups received an equal volume of physiological saline.

Calculation of thymus organ index: Morphological changes of the experimental animals were observed and recorded. Blood was collected on day 45 and serum was isolated. After the rats were humanely killed, the thymus tissues were harvested under sterile conditions. The connective tissues were dissected out and the blood on the surface was absorbed using filter paper. Each thymus was weighed using an analytical balance and the changes of the thymus index were then analyzed. A portion of the thymus tissue was then grinded and homogenized for the test of the activities of antioxidant enzymes. The remainder of the thymus tissue was divided into two and fixed using 4% formaldehyde or 2.5% glutaraldehyde solution before use for paraffin sections and ultrathin sections, respectively.
Calculation formula: Thymus organ index = thymus weight (mg)/body weight (g) ×100.

Biochemical indicator examination: Tests for serum immunoglobulins (IgA, IgG and IgM) and immune factors (IL-2 and IL-6), the SOD, GSH-PX and CAT activity, and LPO and MDA contents of thymus were carried out strictly following the manufacturers' instructions.

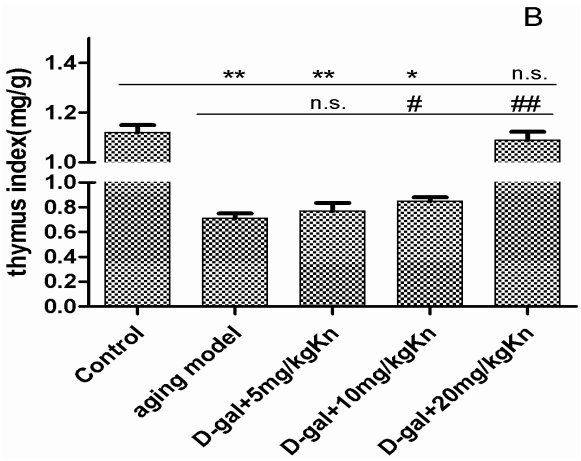

Fig. 1 Thymus indexs of the rats in the different experimental groups (mg/g); *compared with control group, #compared with aging group, ** or ## P<0.01, * or # 0.01<P<0.05, n.s. P>0.05.

Pathological examination: Regular paraffin sections were prepared using the formaldehyde-fixed thymus tissue. After HE staining, the sections were observed and photographed under an optical microscope. The thymus tissue fixed in glutaraldehyde was rehydrated through a series of washes in PBS (0.1 M/L pH 7.2) and then fixed using 1% osmic acid. Ultrathin sections were prepared through the processes of rinsing, gradient dehydration, infiltration, and embedding of the specimen in resin. Sections were stained with lead citrate/uranyl acetate and observed and photographed under a transmission electron microscope.

Statistical analysis: SPSS software was used to perform single factor analysis of variance (one-way ANOVA). Duncan's test was used to test the statistical significance of differences between experimental groups and the control group. Experimental data are represented as mean±SE. P value was used to judge significance (P>0.05: difference not statistically significant; P<0.05: difference is statistically significant; P<0.01: difference is extremely significant).

RESULTS

Changes of the thymus organ index: As shown in Fig.1, the thymus index of the rats in the aging model groups decreased significantly over the course of the experiment. In the Kn-treatment groups, atrophy of the thymus was inhibited. The thymus index of the high-dose Kn group showed no significant difference compared with the control group, but did show an significant difference compared with the aging model group (P<0.01).

Serum Immunoglobulins: As shown in Fig.2, the contents of serum IgG (Fig.2 A), IgA (Fig.2 B) and IgM (Fig.2 C) of the aging model group decreased significantly as compared with the young control group(P<0.01). In contrast the contents of IgG, IgA and IgM of the Kn-treated groups significantly increased compared with the aging model group. The difference between the high-dose Kn group and the aging model group was significant (P<0.01), but there was no significant difference between the high-dose Kn group and the control group.

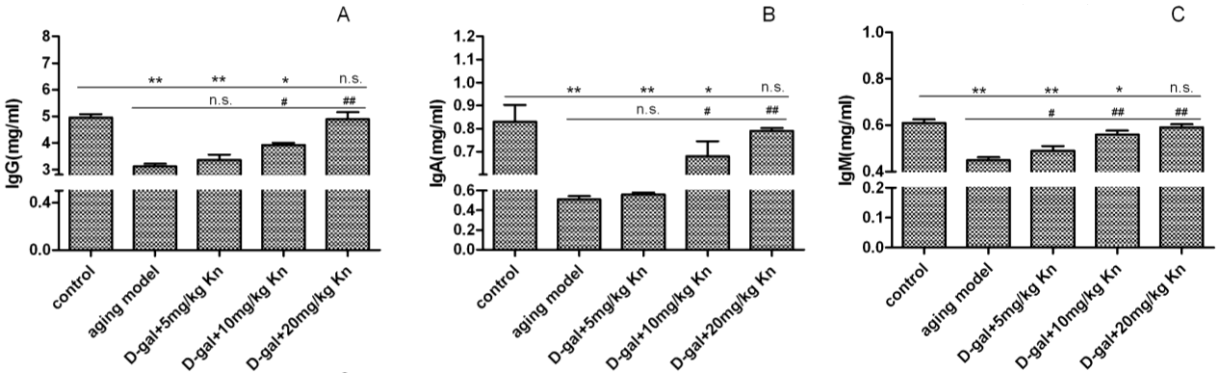

Fig. 2 Serum immunoglobulin content; In the aging model group, the contents of IgG, IgA and IgM in serum were all lower compared with the control group.In contrast, the contents of IgG, IgA and IgM in the serum of Kn-treated rats were all higher than in the untreated aging model group. **A** IgG content (mg/mL)**. B** IgA content (mg/mL)**. C** IgM content (mg/mL).

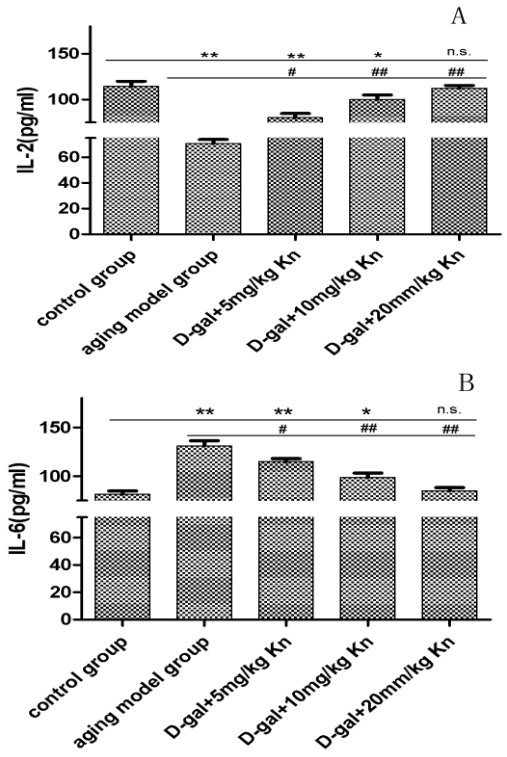

Fig. 3 Changes in IL-2 and IL-6 in the serum of individual groups; The levels of IL-2 and IL-6 in the high-dose Kn-treated group were significantly different from the aging model group but had no significant difference compared with the young control group. **A** IL-2 content (pg/mL). **B** IL-6 content (pg/mL)

Tests of serum immune factors: As shown in Fig.3, the content of serum IL-2 (Fig.3 A) of the rats in the aging model group decreased significantly and the contents of serum IL-6 (Fig.3 B) increased significantly. The differences in the two indices between the aging model group and the young control group were significant (P<0.01). After treatment with various concentrations of Kn, the content of IL-2 increased and IL-6 decreased and the degree of the change was positively proportional to the dose of Kn.

Activity of thymus antioxidant enzymes and their metabolites: As shown in Fig.4, compared with the control group, the activity of SOD (Fig.4 A), CAT (Fig.4 B) and GSH-PX (Fig.4 C) in the thymus of rats in the aging model group decreased, and the difference was significant (P<0.01). Kn treatment increased the activity of all three enzymes, but the increase in the high-dose Kn group was significant, and there was no significant difference between the high-dose Kn group and the young control group. The contents of MDA (Fig.4 D) and LPO (Fig.4 E) of the rats in the aging model group increased compared with the young control group and the difference was significant (P<0.01). After Kn treatment, the contents of MDA and LPO were significantly reduced in the high-dose Kn group (P<0.01) restoring the levels to those of the young control group.

Observation of paraffin sections of thymus: The boundary of the cortex and medulla of the thymus lobe of the rats in the young control group were clear; the cortex was relatively thick and the medulla was relatively thin; lymphocytes were arranged in close proximity (Fig.5 A, B). The lobe structure of the rats in the aging model group was maintained but the gap between the lobes widened; the cortex thinned and the medulla thickened; the boundary between the cortex and medulla was indistinct; the lymphocytes were sparse and the number of macrophages increased; a large number of apoptotic cells could also be seen (Fig.5 C, D). The cortex of the rats in the Kn-treatment groups thickened and the medulla became thin, and the number of lymphocytes was greater than in the aging model group. Apoptotic cells were occasionally observed in the high-dose Kn group (Fig.5 E, F).

Observation of the ultrastructure of the thymus: The thymus lymphocytes of the rats in the young control group were arranged closely, the distribution of chromatin was uniform, and the cell organelles were abundant (Fig.6 A); The thymus cells of the rats in the aging model group were sparse, the cell membrane had dissolved, the outline of the cells was indistinct, nuclei appeared shrunken and condensed, chromatin moved to the edge of the cell, mitochondria were swollen and cristae were broken and even dissolved, lipid peroxides were increased, and the mitochondrial membrane was partially dissolved and showed vacuolar degeneration (Fig.6 B, C, D). The number of thymus lymphocytes of the rats in the Kn-treatment groups increased compared with the aging model group, and the number of injured mitochondria were reduced. In the high-dose Kn group the thymus was basically recovered to normality although a few apoptotic cells, as well as swollen mitochondria accompanied by broken cristae and vacuolar degeneration were still observed (Fig.6 E, F).

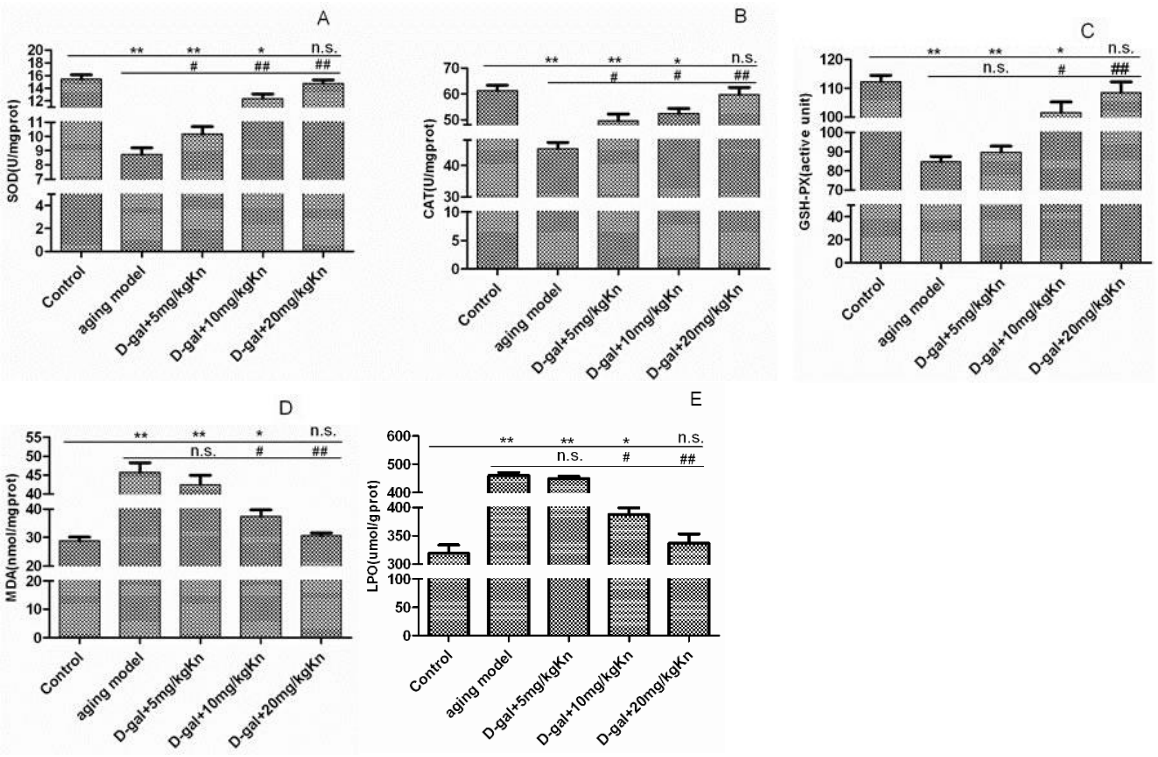

Fig. 4 Antioxidant enzyme and metabolite levels in the thymuses of rats induced by D-gal; **A** SOD (U/mg prot); **B** MDA (nmol/mg prot); **C** GSH-PX (active unit); **D** CAT (U/mg prot); **E** LPO (nmol/g prot)

DISCUSSION

With increasing age, the thymus, where T lymphocytes develop and mature, first undergoes aging and atrophy which is detected as a decrease in the thymus index (Khalyavkin and Krut`kov, 2015). Observation under an optical microscope reveals a reduction in the number of functional thymus cells; cells are few and scattered; lobule gaps increase and the organ atrophies. Transmission electron microscopy shows that nuclei shrink and are dark-stained. Chromatin moves toward the periphery of the nuclei and a large number of apoptotic bodies can be observed (Ka *et al*., 2014; Li *et al*., 2014). Since structure is the basis of function, changes in the structure lead to impaired function. Thus the ability of the thymus to produce and secrete IL-2 and immunoglobulins decreases, immune function declines and infection risk increases (Ferrando-Martinez *et al*., 2011; Rajabi *et al*., 2012).

Kn has the effect of promoting cell division and proliferation. In addition to its functions of promoting cell division, differentiation and growth, Kn also acts as an anti-oxidant, playing roles in protecting against damage, cell protection and delaying aging (Li *et al*., 2014). It has been shown that Kn can affect cell morphology and internal cytoskeleton structure, as well as resisting the aging of human fibroblasts in vitro (Mik *et al*., 2011).

When the human body ages, organs cease development, even atrophy, and the organ index decreases (Tang *et al*., 2013). In this study, anatomic examination revealed that the thymus of rats in the aging model group shrank significantly compared with the control group. However, the condition of the thymus of the rats in the Kn-treated groups improved significantly. Atrophy of the thymus was inhibited and the organ index also increased. This suggests that Kn has the function of delaying thymus aging and atrophy.

IL-2 and IL-6 are produced by lymphocytes and play a key role in immunomodulation. The decrease of IL-2 is a fundamental factor causing the immune deficiency of elderly people, while increasing IL-6 is also associated with the infection and death of the elderly. With aging, the content of IL-2 decreases and the content of IL-6 increases (Meneguello-Coutinho *et al*., 2014; Essa *et al*., 2015). As our study indicates, the serum content of IL-2 in the aging model group rats decreased significantly compared with the control group while the content of IL-6 increased significantly compared with the control group. This result was in accordance with the result obtained by Ciaramella *et al*. (2012). Treatment with different doses of Kn increased the expression of IL-2 and decreased the expression of IL-6, suggesting that Kn to a certain extent improves immune function and inhibits thymus atrophy.

T and B lymphocytes are the material foundation of normal immune function. The level, structure and function of lymphocytes are all closely correlated with age (Ewald *et al*., 2014). As the thymus atrophies with increasing age, the generation, differentiation and maturation of T and B lymphocytes becomes disordered. The relative numbers of functional cells reduce, the types of functional cells change, and the activity of receptors on the cell surface declines. The immunoglobulins produced by B cells reduce and lead to a further decrease in the immune response (He *et al*., 2013; Zhou *et al*., 2014). Our results show that the contents of IgA, IgG and IgM of the aging model group decreased significantly compared with the young control group. Treatment with Kn restored the levels of immunoglobulins to a certain extent compared with the aging model group and showed an apparent dose-effect relationship. Our study suggests that Kn can improve the content of immunoglobulins and increase the immune function of the human body.

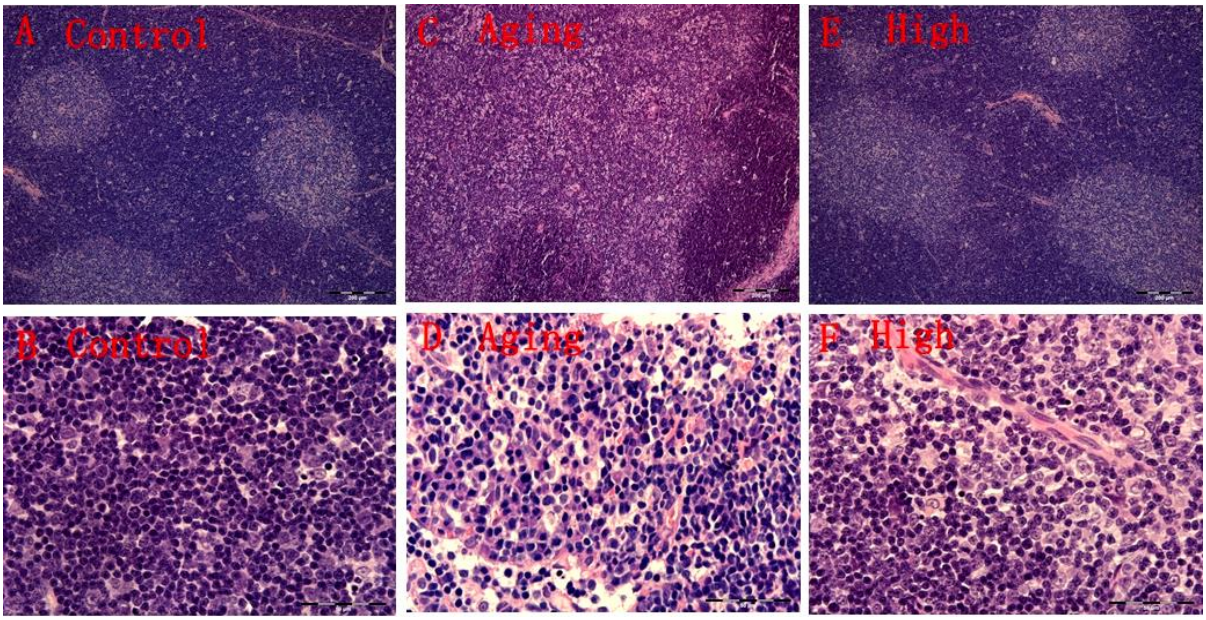

Fig. 5 Paraffin sections of rat thymus: **A** In the thymuses of rats in the control group, the thymic lobule cortex was thicker, and the medulla was thinner compared with other groups, bar 200 μm. **B** The thymuses of rats in the control group. the lymphocyte density, bar 50 μm. **C** The thymuses of rats in the aging model group; the thymic lobule cortex was thinner, and the medulla thicker compared with the control group, bar 200 μm. **D** The thymuses of the rats in the aging model group.Karyopyknosis and dark-stained nuclei could also be observed.Lymphocytes were sparsely distributed, Bar 50 μm. **E** The thymuses of the rats in the high-dose Kn group.The thymic lobules of the cortex were thicker, and the medulla thinner, bar 200 μm. **F** The thymuses of rats in the high-dose Kn group. The arrangement of lymphocyte was more dense compared with the aging model group, and a very small number of apoptotic cells were visible, bar 50 μm.

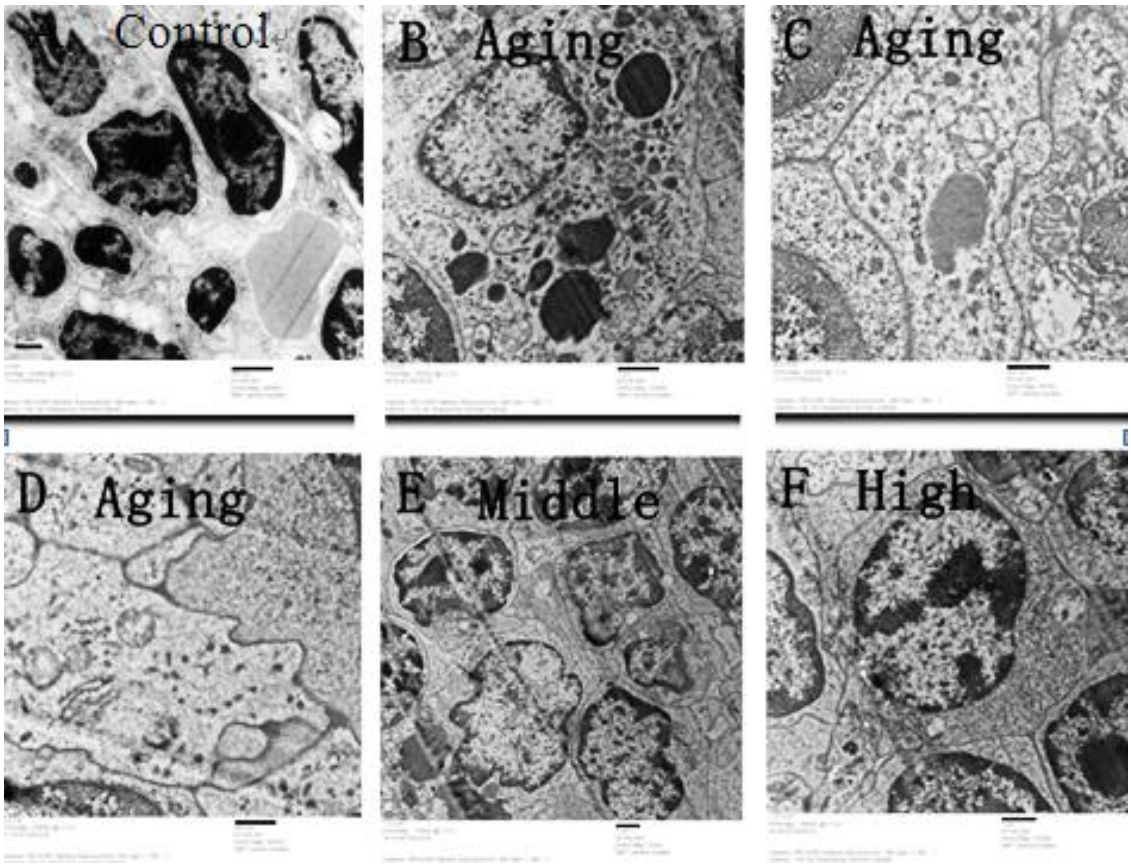

Fig. 6 Ultra-fine sections of rat thymus: **A** Young control group. Closely arranged thymic cells, clearly complete nuclear membrane,evenly-distribute chromatin, obvious nucleolus and abundant organelles, bar 1 μm. **B** Aging group. Sparsely-distributed thymic cells, ruptured cell membrane, pyknotic chromatin localized close to the nuclear membrane, lipofuscin increased, bar 1 μm. **C** Aging group. lipofuscin increased of some thymic cells increased.mitochondrial swelling with dissolved cristae,degenerated vacuoles, bar 500 nm. **D** Aging group. Sparse thymic cells,lipofuscin increased of some thymic cells increased,mitochondrial swelling with dissolved cristae, degenerated vacuoles, bar 600 nm. **E** Middle-dose Kn group. Some thymic lymphocytosis, clear complete nuclear membrane, a few thymic cells with mitochondrial swelling with dissolved cristae, degenerated vacuoles, bar 1 μm. **F** High-dose Kn group. Closely-arranged thymic cells, clear complete nuclear membrane, evenly-distributed chromatin, decrease in the number of damaged mitochondria, the structure of the thymic cells was returned to normal, bar 1 μm.

The activity of antioxidant enzymes such as SOD decreases with aging, causing the free radicals generated during metabolic reactions to accumulate excessively in the body. The accumulated free radicals cause damage to DNA and further induce mutation and cancer. The free radicals can also oxidize a variety of substances in cells and injure the cell membrane. During this process, large amounts of MDA and LPO are generated, so that cells are further damaged, thus accelerating the body's aging process (Avramovic *et al*., 2012; Ahn *et al*., 2015). Our results show that the activity of antioxidant enzymes including SOD in the aging model group of rats decreased significantly compared with the control group, while the contents of MDA and LPO increased significantly. After Kn treatment, the activity of antioxidant enzymes improved and the contents of MDA and LPO significantly decreased. This suggests that Kn can restore the activity of antioxidant enzymes and facilitate the clearance of toxic metabolic products such as MDA and LPO so as to effectively inhibit the damage to cell structure caused by free radicals.

The results of this study show that the spaces in the thymus lobules of the rats in the aging model group widened, the cortex became thinner, the medulla thickened, and the border of the cortex and medulla became blurred. The distribution of lymphocytes was scattered, macrophages increased, and a large number of apoptotic cells could be observed. These results are consistent with previous studies (Li *et al*., 2014; Solti *et al*., 2015). The cortex of the Kn-treated rats thickened and the medulla thinned, the number of lymphocytes increased and the structure of the thymus improved. The thymus structure of the rats in the high-dose Kn group was similar to that of the control group, suggesting that Kn has a significant protective effect on the thymus tissue structure of subacute aging rats and delays aging of the thymus.

Under the electron microscope, the thymus cells of rats in the aging model group were sparse, the arrangement of the cells was disordered and the spaces between cells increased. In some cells, the cell membrane was broken and dissolved, and the cell boundary was indistinct. Most cell organelles appeared degenerated and dead, or even disappeared. Chromatin was dark-stained and accumulated around the edge of cells. Part of the nuclear membrane was sunken and dissolved. Lipid peroxides increased significantly. Mitochondria swelled and cristae were broken. Part of the mitochondrial membrane dissolved and showed vacuolar degeneration. In addition, apoptotic bodies were evident. Our results were consistent with other studies (Dudzik *et al*., 2011; Zhao *et al*., 2011; Mcdermott *et al*., 2012). Following treatment with Kn, the cells were closely arranged, cell organelles were complete, and the structure was clear and visible. This indicates that Kn can effectively protect the substructure of thymus cells.

Conclusions: Our results suggest that Kn can protect the structure and substructure of thymus cells and effectively protect against oxidative damage in aging rats induced by D-gal. Consequently, Kn has the effect of increasing immune function and delaying thymus aging.

Acknowledgements: This work is sponsored by the Henan University of Science and Technology Student Research Training Plan Foundation (SRTP:2015144), the Henan University of Science and Technology Education Teaching Reform Foundation(2015YB-017), the Henan University of Science and Technology experimental technology development fund (SY1516040), the Henan University of Science and Technology Dr. research start-up fund (13480072).

Authors' contributions: WO conceived and designed the study. ML performed all the experiments and wrote the manuscript. JL and LS helped to perform the biochemical assay. XL and JG helped to perform the histopathology and ultrastructure observation. HL helped to analyze data. All authors have read and approved the manuscript for publication.

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