Pakistan Veterinary Journal

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

RESEARCH ARTICLE

Kinetin Protects D-Galactose-Induced Aging Rats from Splenic Oxidative Damage

Mengyun Li[1,2], Wuqing Ouyang[1]*, Xiaoli Wu[1], Yin Zheng[1], Rui Gao[1], Xu Ouyang[1] and Lei An[1]

[1]College of Veterinary Medicine, Northwest A&F University, Yangling 712100, Shaanxi, P. R. China; [2]College of Animal Science and Technology, Henan University of Science and Technology, Luoyang 471003, Henan, China
*Corresponding author: oywq2014@163.com

ARTICLE HISTORY (14-005)

Received:
Revised:
Accepted:

Key words:
Aging
D-galactose
Kinetin
Oxidative damage

ABSTRACT

The molecular mechanisms specifying anti-oxidative and anti-aging action of kinetin (Kn) on the splenic oxidative damage of aging rats are poorly understood. Here, the Sprague Dawley (SD) rats (*Rattus norregicus*) aging model was proposed by napes subcutaneous injection of D-galactose, and anti-aging group were subsequently dealt with doses of 5, 10, 20 mg/kg·BW of Kn. Superoxide dismutase (SOD), malondialdehyde (MDA), glutathione peroxidase (GSH-PX), catalase (CAT), lipid peroxide (LPO), spleen indexes and histopathology were evaluated. Our results showed that Kinetin significantly decreased the MDA and LPO activity, while spleen indexes, SOD, GSH-PX and CAT were higher than aging model group; histopathological studies for spleen confirmed the rescue from the aging rats. These results demonstrate that kinetin recovers the splenic cell structure and could effectively protect the spleen cells from oxidative damage and aging.

To Cite This Article: Li M, W Ouyang, X Wu, Y Zheng, R Gao, X Ouyang and L An, 2014. Kinetin protects D-galactose-induced aging rats from splenic oxidative damage. Pak Vet J, 34(3): 386-390.

INTRODUCTION

Animal aging is a spontaneous and inevitable process. Identifications of tissues and organs exhibit age-related decline in functions and atrophy such as flabby skin, brain atrophy, senile dementia and lowered immunity (Aw *et al.*, 2009; Hosek *et al.*, 2013). According to the free radical theory of aging (Grune, 2000) and anti-senescence agents, activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), catalase (CAT) become low with aging, which causes excessive accumulation and clearing defect of the free radicals *in vivo* that are produced in the process of metabolism, resulting to damage of the cellular structure with a large number of malondialdehyde (MDA), and lipid peroxide (LPO) (Park *et al.*, 2006; Sharaf *et al*., 2013; Zhang *et al*., 2013). Meanwhile, immunologic function is associated with the organismic age, in other words, immune organs first exhibit the aging atrophy, lowered indexes and decline of immune functions.

Kinetin (Kn), a type of cytokinin, is adenine derivative that promotes cell division, differentiation and growth (Won *et al.*, 2008). It has been involved in anti-oxidative damage and the powerful anti-aging effects. Kn can improve the activity and increase the content of SOD in plants and can be used as a direct free radical antagonist. In addition, the previous study proved that Kinetin had positive lethal effect on tetrachloride-induced hepatic fibroblasts and inhibited formation of collagenous fiber in the livers of rats. Consistent with this, kinetin could delay maturity (Cronin and Draelos, 2010), and Kn has also been showed the obvious protective effect on D-gal-induced oxidative damage of ovary and uterus tissues (Sun *et al.*, 2013).

In this study, we firstly characterized immunohisto-chemistry of D-galactose-induced aging and rescue of kinetin in rat spleen, which is effectively protect the spleen cells from aging and splenic atrophy, and is helpful for mechanism of the antioxidative and anti-aging in the immunological aspect.

MATERIALS AND METHODS

Animals: SD rats (100 rats with 2-month-old, 200±20 g, distributed with random composition of males and females) were obtained from laboratory animal center of Xian Jiaotong University (the certification of qualification number 2012-003, Xi'an, China) and housed in our groups and maintained on a clean environment with food and water available. All procedures were performed according to the institutional guidelines for animal experiments (The Measures for the Administration of Experimental Animals in Shaanxi Province, China. 2011). All of the animal experiments were performed according

to the Guidelines for the Care and Use of Laboratory Animals of Northwest A&F University.

Reagents: Kinetin (K3378, USA) and D-galactose were obtained from sigma. Assay kits of SOD, MDA, GSH-PX, CAT, LPO were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Electronic balance was purchased from Shanghai Precision instruments Co. Ltd. (JA2003, Shanghai, China). Spectrophotometer was purchased from Shimadzu Co. Ltd (UV2450, Japan). KD-T Water bath-slide drier, KDM Embedding center, LEICA RM2016 microtome, OLYMPUS-CX21 Microscope, JEOL-1230 Transmission electron microscopy and LETCA EMUC7 Ultra microtome were stored in my lab.

Groups and treatments: The rats were random divided into 5 groups of 20 rats and raised for 1 week to reduce stress. The control group was injected subcutaneously with normal saline continuously for 45 days. Rats in other groups (aging rats, low Kn group, mid Kn group, high Kn group) were injected subcutaneously with D-galactose (125 mg/kg·BW) to make aging rat models continuously for 45 days. In the process of experiment day 11, rats in control group and aging model group were given normal saline while rats in other groups were given different doses (5 mg/kg·BW, 10 mg/kg·BW and 20 mg/kg·BW) of kinetin continuously to day 45.

Biochemical assay: All rats were euthanized at day 45. The spleens were collected and weighted separately. The spleen index was calculated according to the formula as followed: Spleen Index = the weight of spleen (mg) × weight of body $(g)^{-1}$×100%.

The fresh spleen of each rat was collected and grinded into 10% tissue homogenate (1g spleen and 9g saline water) instantly. And then the homogenate were centrifuged (3000r/min, 10 min). The supernatant were collected and the activity levels of SOD, GSH-PX, CAT, MDA and LPO were measured according to the manuscripts, individually.

Histopathology and ultrastructure observation: Part of spleen of rat was collected and fixed in 4% formaldehyde solution for 24h. The histological structures of spleen were observed through optical microscope and the images were collected using paraffin sections and HE staining.

Part of spleen of rat was collected and fixed in 2.5% glutaraldehyde solution for 24h. After washed with phosphate buffer solution, tissues were fixed in 1% osmic acid solution, and then tissues were rinsed, gradient dehydrated and permeated. At last, the ultrathin sections of spleens were prepared and stained by lead citrate and uranyl acetate. The ultrastructure of spleen was observed by transmission electromicroscope.

Statistical analysis: Statistical analysis was conducted by the SPSS (version 20.0). One-way ANOVA was used to process inter-group comparisons and post hoc Duncan's was used to operate multiple comparisons. All data were expressed as Means±SD.

RESULTS

Changes of the spleen indexes: According to Fig. 1A, spleen indexes of aging model group, low dose group, middle dose group are 1.15±0.10, 1.47±0.04 and 1.72±0.04, respectively, which showed significant difference ($P<0.01$) with that of control group (1.97±0.32) and there is no significant difference between high group (1.91±0.08) and control group ($P>0.05$).

Effect of Kn on oxidative indexes of rats in different groups: As Fig. 1 showed, the activity level of SOD (Fig.1B), CAT (Fig.1E) and GSH-PX (Fig.1D) of rats spleen in aging group were obviously lower than that in control group ($P<0.01$) while the MDA (Fig.1C) content of rats spleen in aging group was higher than that in the control group ($P<0.05$). Additionally, the oxidase activity of the spleens in different treatment groups had reduced fairly. Our results showed dose-depended effect of the Kn on oxidase activity.

The Kn rescue from the aging spleen to normal event: In the control group, the distribution of white pulps in spleen showed much more than red pulps with a clear and obvious demarcation between them, and a large number of lymphocytes could be found in splenic nodules and lymphoid tissue sheaths surrounding the arteries were clear. There were few apoptotic cells in white pulps and marginal zone, and splenic cord and sinus in red pulps were identified clear as shown in Fig. 2A and Fig. 2B. In comparison, in the rats of aging model group, there was unclear boundary between white pulps and red pulps with shrunken white pulps, reduced splenic nodules, sparse lymphocytes, unobvious germinal center and hyperplasia of connective tissue in trabecula. In addition, as shown in Fig. 2C/2D, thickly dyed karyopyknosis and many apoptotic cells were found as well as congested splenic blood sinus and increased macrophages. After treatment with Kn, in the rats of low dose group and mid dose group, there were gradually generating splenic nodules and nodule lymphocytes compared with that of the aging group, but there were numerous hemosiderins and macrophages (Fig. 2E and Fig. 2F). Our results also showed that the different between high dose group and control group was little obvious, with similar splenic tissue structure and less hemosiderins.

Identification and changes of splenic ultrastructure dealt with Kn: From the Fig. 3G (the control group), we could see that compact cell arrangement with complete and clear karyotheca. And even distributed chromatins, obvious nucleolus and abundant organelles were found as well. But in the aging group, sparse and dissolved splenocyte were found as well as enlarged intercellular space, and some cytomembranes happened to rupture and dissolve, which caused obscure boundary, chromatin pyknosis, swinging edges and degenerate organelles (Fig. 3H). Some karyothecas began to sunken and dissolve, and cell nucleus showed popcorn shape (Fig. 3I). Meanwhile, hyperplasia and proliferation of endoplasmic reticulum were identified as well as diffuse cytoplasm around the nucleus and free ribosome (Fig. 3J). Obviously, there were increased lipofuscins but mitochondria reduced with

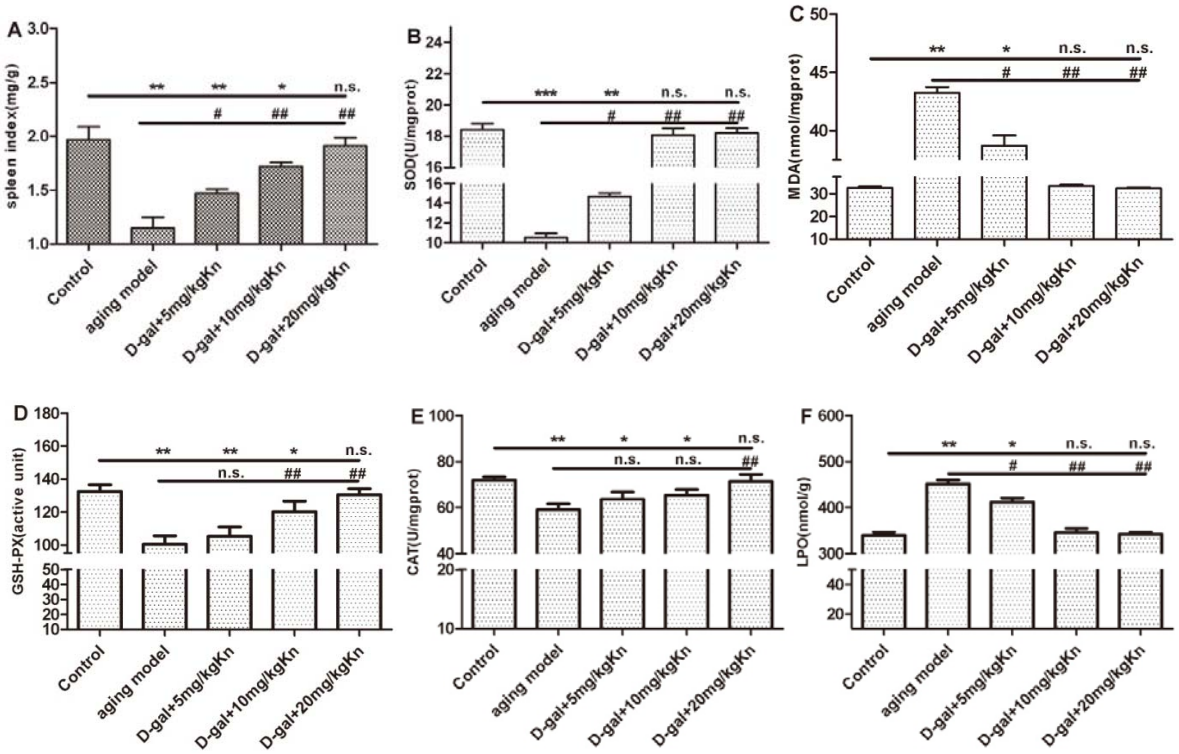

Fig. 1: A spleen indexes (mg/g); **B** SOD (U/mgprot); **C** MDA (nmol/mgprot); **D** GSH-PX (active unit); **E** CAT (U/mgprot); **F** LPO (nmol/g). P-values represent mean±SEM determined from independent experiments. ***P<0.0001, **P<0.01, *P<0.05, ##P<0.01, #P<0.05, n.s., no significant. Note: *compared with control group and #compraed with aging group.

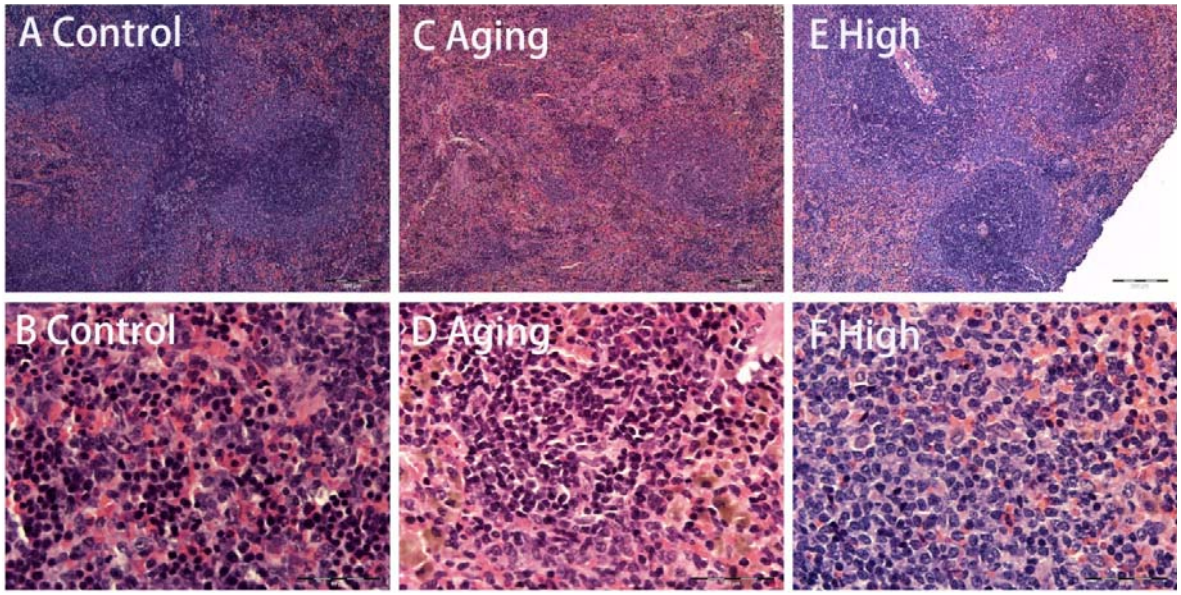

Fig. 2: A and B The control group. The tissue with splenic nodules, the lymphocyte density and clear boundary between red and white pulp. **C and D** The aging model group. Less Splenic nodules with fuzzy boundary and more hemosiderin. **E and F** The high dose group. The activity of lymphocytes was dense and large number of blood red element and macrophages appeared in red pulp area. Bars: A, C, E, 500μm; B, D, F, 50μm.

swelling, crest fracture, partial dissolution of mitochondrial membrane and cavitation. Apoptotic bodies wrapped by invaginated cytomembrane and formed by fragments were noticed. Especially, after treated with Kn, the cell arrangement began gradually to become compact, and the basic cell structures of spleen in high dose group had been rescued to normal (Fig. 3K and Fig. 3L).

DISCUSSION

The application of D-gal can cause disturbance of carbohydrate metabolism in organism and produced a large amount of free radicals and H_2O_2, which lead to injury and defect of mitochondria and endoplasmic reticulum, and last, the enzymes activity changed and the

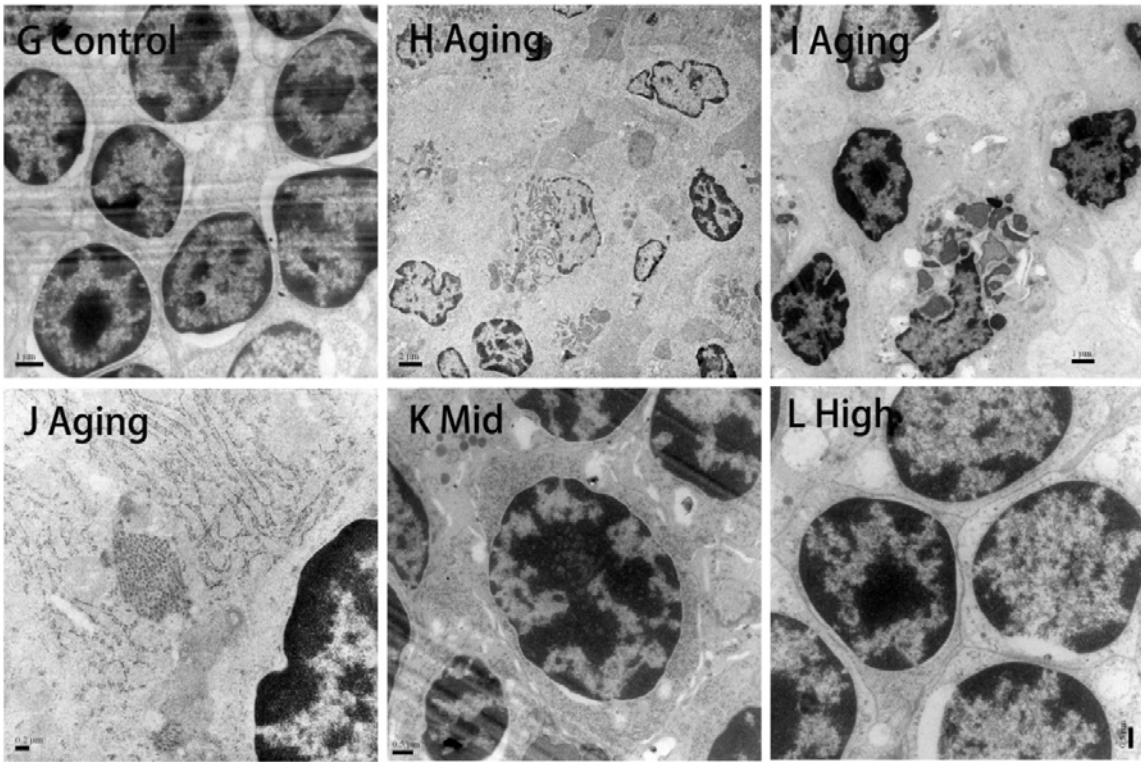

Fig. 3: G The control group. Closely spleen cells, clear completed nuclear membrane, equably chromatin, obvious nucleolus and abundant organelles. Bars,1 μm; **H** The aging group. Sparse spleen cells, rupture cell membrane, dissolved cell, degenerated organelles, and Chromatin pyknosis. Bars, 2 μm; **I** The aging group. Some spleen cells dissolved and some nuclear membrane showed sag like popcorn. Bars, 1 μm; **J** The aging group. Some spleen cells showed hyperplasia of endoplasmic reticulum, expansion, increased ribosome, scattered nuclear cytoplasm and swelling mitochondria with dissolved cristae and membrane. Bars, 0.2 μm; **K** The mid dose group. Some spleen cells were still sparse but closely rescued from the aging group. Bars, 0.5 μm; **L** The high dose group. Identification of spleen cell structure showed the rescue from aging model. Bars, 0.5 μm.

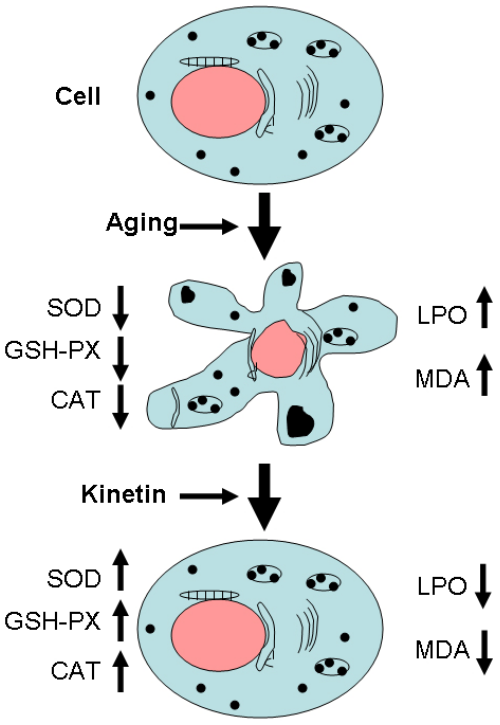

Fig. 4: Schematic diagram of rescue of Kinetin from aging rats. The aging cell shows the changes of morphology and activation of LPO and MDA and inhibition of SOD, GSH-PX, and CAT, but the treatment of Kinetin induces reversed event, which involves adjusting of the aging delay.

aging appeared. Since Kn and its derivative were recent characterized (Novotna *et al.*, 2012) and a recent study showed that mice received subcutaneous injection of D-gal accelerated aging degree like the natural aging mice (Liu *et al.*, 2010). Consistent with this, our results showed that rats in aging model group had lowered organ coefficients and we rescued from aging model deal with Kn. The results of lower activity of SOD, CAT, GSH-PX and higher MDA and LPO contents suggested that the aging model was successful created for study further. Kinetin (Kn) was not only used for studies of anti-oxidation and anti-aging, but kinetin could inhibit the free radicals accumulation, which proved that Kn had the potential capacity of anti-oxidant and anti-aging (Zhong *et al.*, 2009). Moreover, kinetin could increase antiproliferative and apoptogenic activity against various human cancer cell lines (Rajabi *et al.*, 2012).

Our studies showed that, compared with the control group, spleen index of the aging model decreased significantly. After treatment with different concentration of Kn, the spleen indexes of the groups have rebounded, suggesting that Kn had the positive effect of anti-aging and delaying aging atrophy of immune organs. As a free-radical scavenger, Kn can be used to activate activity of SOD and other enzymes further, it has also obvious anti-oxidative and anti-aging effect (Brink *et al.*, 2009). The results showed that enzymatic activities of SOD, CAT and GSH-PX in aging model group decreased while MDA and LPO contents were increased significantly. Interestingly, in treatment groups, oxidation-related splenic indexes

proved a well character compared to aging model, and the degree of rescue was positively correlated with the Kn dosage. The results support further the effect of Kn on anti-oxidative and anti-aging.

With the development of aging, there is a substantial reduction in lymphocytes of splenic white pulp and huge increasing macrophages (Kumar and Burns, 2008). This experiment showed consistent results, compared with control group; volume of white pulp in aging group was relatively small with fewer splenic nodules. In addition, sparse lymphocyte, thickly dyed karyopyknosis and a large amount of apoptotic cells were identified, and splenic blood sinus congestion and numerous hemosiderin were found. Rats in Kn treatment groups had been rescued model characterized with well spleen tissue structures compared with the aging group, and rats of high dose group had similar splenic tissue structure with the Control Group, indicating that Kn had obvious anti-aging effect to confront spleen of subacute aging rats.

To investigate the role of Kn in the splenic ultrastructure, we introduced effect of Kn on spleen cell using transmission electron microscopy. The analytical results were in agreement with previous researches results that Kn most likely stimulates apoptosis mechanism of cancer cells and prove a approach against human leukemias (McDermott *et al*., 2012, Dudzik *et al*., 2011). According to the experiment, aging group had sparse splenocytes, most organelles degenerated and disappeared with chromatin pyknosis and swinging edge as well as apoptotic bodies formed by cytomembrane invagination. Kinetin has been identified to increase chromium absorption and change the activity of catalase and ascorbate peroxidase (Zhao *et al*., 2011). Our results also demonstrate that Kn was applied to strengthen cell compact and retreat organelles damage, showing that Kn could protect the spleen cells from aging and improve the activity of spleen cell.

In summary, we have presented evidence that Kinetin improve the antioxidant activity of SOD, GSH-PX and CAT in aging spleen and reduced the content of MDA and LPO. Furthermore, our current study suggests that Kn can not only protect spleen cells and recover the splenic tissue structure, but also inhibit effectively splenic aging atrophy and delay the aging of spleen. Understanding effect of Kn contributing to aging may lead to the mechanism of aging and we can search powerful drugs to enhance immune function. However, the detailed molecular mechanisms underlying this regulation await further to refine.

Authors' contributions: WO conceived and designed the study. ML performed all the experiments and wrote the manuscript. XW and YZ helped to perform the biochemical assay. RG and XO helped to perform the histopathology and ultrastructure observation. LA helped to analyze data. All authors have read and approved the manuscript for publication.

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