Effects of Raloxifene on the Aortic Valve Function of Mice Fed with High-sugar and High-fat Diets

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The current study aimed to explore the effects of raloxifene on the aortic valve function of mice fed with high-sugar and high-fat diets. C57 mice were randomly divided into five groups, 10 mice per group, normal diet group or control group, high-sugar, high-fat group, raloxifene 4.5 mg/kg+high-sugar, high-fat group, raloxifene 9 mg/kg+high-sugar, high-fat group, and raloxifene 18 mg/kg+high-sugar, high-fat group. Raloxifene was intragastrically administered every day for a total of three months. Steatosis and calcification of aortic valve was detected through hematoxylin and eosin staining and alizarin red staining and relative expression levels of caspase-3 and caspase-8 using immunohistochemical staining and real-time quantitative polymerase chain reaction. Compared with the high-sugar, high-fat group, both the hematoxylin and eosin staining and alizarin red staining showed that aortic valve steatosis and calcification in mice significantly improved after treatment with raloxifene, especially in the group administered with 18 mg/kg. In addition, quantitative immunohistochemical staining and real-time quantitative polymerase chain reaction showed that raloxifene treatment sharply decreased mRNA expression levels of caspase-3 and caspase-8 compared to the high-sugar, high-fat group. Raloxifene at certain concentrations induced inhibitory effects on calcification and apoptosis in the aortic valves of mice after high-sugar, high-fat diet, thereby laying a foundation for therapeutic benefits raloxifene.

Key words: RAL, aortic valves, high-sugar, high-fat diet, apoptosis

With increasing aging and higher incidence of atherosclerosis, the incidence of valvular heart disease (VHD) increases annually[1,2]. Aortic valvular interstitial cells are the most abundant cells in the aortic valve of the heart and these are important in maintaining the normal structure and function of valves[3]. Valvular interstitial cells and calcium nodules are involved in the valvular degeneration and calcification. Changes in the biological functions of these cells have important effects on tissues[4].

Raloxifene (RAL) is a second-generation selective oestrogen receptor modulator (SERM) that is initially used mainly for treating postmenopausal osteoporosis and breast cancer and later for treating cardiovascular disease. Studies have shown that, RAL and other SERMs could improve the functions of vascular endothelial cells, expand coronary arteries, regulate blood lipids, and exert protective effects on atherosclerosis, venous thrombosis, and others[5,6]. RAL regulates the functions of vascular endothelial cells through oestrogen-response elements or by other means, thereby protecting endothelial functions. RAL is expected to play a potential role in treating cardiovascular disease[7,8].

RAL and other SERMs have been studied in basic and clinical cardiovascular diseases. These drugs were shown to improve endothelial dysfunction, increase coronary dilatation, and improve lipid profiles[9]. Aortic valve disease can be triggered by high-sugar and high-fat has been confirmed[10]. It is not clear whether RAL has a dose-dependent effect on the improvement of cardiovascular disease. In the present study, a high-sugar and high-fat diet mouse model was designed and different concentrations of RAL were administered for three months after high-sugar and high-fat diet with the aim of detecting the effects of RAL on aortic valve

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steatosis, calcification, and apoptosis-related genes, laying foundation for further treating tissues with RAL.

A total of 50 male C57 mice (10 w old) were bought from Beijing Vital River. The animals were housed in a non-specific-pathogen free animal house in Shenzhen People's Hospital. The Experimental Animal Ethics Committee of Shenzhen People's Hospital approved all experimental protocols in the present study (LL-KT-201801027).

Fifty male C57BL/6 mice were randomly divided into the 5 groups of 10 mice each, normal diet group (control group), high-sugar, high-fat group, RAL 4.5 mg/kg+high-sugar, high-fat group, RAL 9 mg/kg+ high-sugar, high-fat group and RAL 18 mg/kg+high-sugar, high-fat group. On the day after grouping, the groups to be fed with high-sugar and high-fat diets were marked and the normal diets of these groups were replaced with high-sugar, high-fat diets. The next day, treatment group mice were intragastrically administered RAL daily for 3 mo.

Animals were sacrificed with an overdose of pentobarbital sodium after the 3 mo of RAL administration. Using RT-qPCR, the relative expression levels of caspase-3 and caspase-8 were detected. Aortic tissues were dissected for extracting total RNAs using a TRIzol kit (Invitrogen, USA). The concentration and purity of RNA were detected by using an ultraviolet spectrophotometer with the absorbance of 260/280 nm. Reverse transcription reaction was completed using PrimeScript RT reagent kit (TaKaRa Biotechnology Co., Ltd., Shanghai, China), with every 10 µl of the total system. RT-qPCR was completed using SYBR Premix Ex Taq II kit (TaKaRa Biotechnology Co., Ltd., Shanghai, China), with every 20 µl system. PCR amplification was further completed using a quantitative PCR system (Applied Biosystems, Foster City, CA, USA). For the relative expression levels of caspase-3 and caspase-8, the standard glyceraldehyde 3-phosphate dehydrogenase (GDPDH) was used as a reference. The data was expressed by $2^{-\Delta\Delta Ct}$. Differences between the samples were assessed. Primers used for caspase-3/caspase-8 and GDPDH were listed in Table 1. Aortic valve tissues were taken and fixed in 4 % formaldehyde buffer for 12 h, fully dehydrated, cleared using ethanol and xylene stepwise, and embedded in paraffin at 56-58°. Tissue slices of 5 µm thickness were cut, deparaffined and placed in water for staining using hematoxylin and eosin (H and E) staining kit (Nanjing Jancheng Technology Co., Ltd.). Alizarin red staining was used to observe calcium deposition in the aortic tissue. For alizarin red staining, the deparaffined section was rinsed in water for 2 min, stained with 0.1 % alizarin red S for 30 min, and mounted in glycerinated gelatin.

TABLE 1: SEQUENCES OF PRIMERS FOR RT-QPCR

Gene	Primer sequences
Caspase-3	Forward Primers 5'-TGTCATCTCGCTCTGGTACG-3' Reverse primers 5'-CCCTTTCTGCCTGTCTTCTG-3'
Caspase- 8	Forward primers 5'-ACAATGCCCAGATTTCTCCCTAC-3'
GDPDH	Reverse primers 5'-CAGACAGTATCCCCGAGGTTTG-3'
	Forward primers 5'-ACCTCAACTACATGGTCTAC-3' Reverse primers 5'-TTGTCATTGAGAGCAATGCC-3'

After the deparaffined section was placed in water, endogenous peroxidase was inactivated in 3 % H_2O_2 solution. Antigen retrieval was completed under high pressure or in the form of enzymatic digestion. After blocking with 5 % bovine serum albumin for 30 min. Caspase-3 (GeneTex, Inc. GTX24051, USA) and caspase-8 (GeneTex, Inc. GTX60510, USA) antibodies (1:200) were added and cultured for 2 h at room temperature. Phosphate-buffered saline was used as a replacement for primary antibody as the negative control. A 50 µl volume of ready-to-use MaxVisionTM reagent was added dropwise, and incubated at room temperature for 10 min. 3,3′-diaminobenzidine tetrahydrochloride substrate solution was dropped into the mixture. The section was stained with hematoxylin, conventionally dehydrated, cleared, and mounted, and brown particles showed a positive signal. Image Proplus 6.0 (IPP6.0) was adopted for quantitative image analysis and determining the relative integral optical density value of different groups.

All data were processed using SPSS 17.0. The test data were represented as means±SD. The group treated with different concentration of RAL was compared with the control group by one-way analysis of variance, which showed statistical differences ($p<0.05$).

Caspase-3 and caspase-8 are major apoptosis-related genes, and the expression levels of the two genes were closely related to cell apoptosis. In the present study, after 3-mo administration of RAL that, the mRNA expression levels of caspase-3 and caspase-8 were reduced in the aortic valves of the high-sugar,

high-fat mice group, in particular, those in the RAL 9 mg/kg+high-sugar, high-fat group and RAL 18 mg/kg+high-sugar, high-fat group decreased sharply (fig. 1A and B).

In observing the effects of RAL on the aortic valves of the high-sugar high-fat mice group by H and E staining, it was found that in the aortic valves of the high-sugar, high-fat mice group showed abundant dotted steatosis areas, and the aortic valve steatosis areas after the administration of different concentrations of RAL were improved to different extents, especially in the RAL18 mg/kg+high-sugar, high-fat group, in which few of such areas were found (fig. 2A-E).

Calcium deposition in aortic tissues was observed by alizarin red staining. In the aortic valve tissue of the high-sugar, high-fat mice group, numerous areas with red-dotted calcium deposition were found; however, in the group treated with different concentrations of RAL, these dotted areas are considerably diminished,

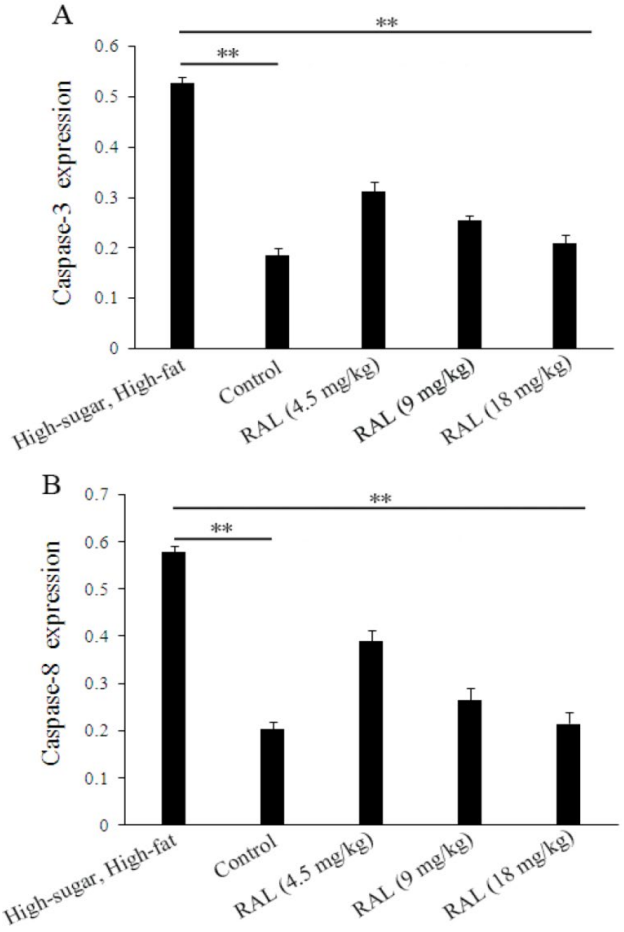

Fig.1: Effects of RAL on mRNA expression of caspase-3 and caspase-8 in the high-sugar, high-fat mice group
Effects of different concentrations of raloxifene on caspase-3 and caspase-8 mRNA expression in cardiac valves of the mice fed with high-sugar and high-fat, (A) caspase-3; (B) caspase-8, the results are expressed as mean±SD, **p<0.01

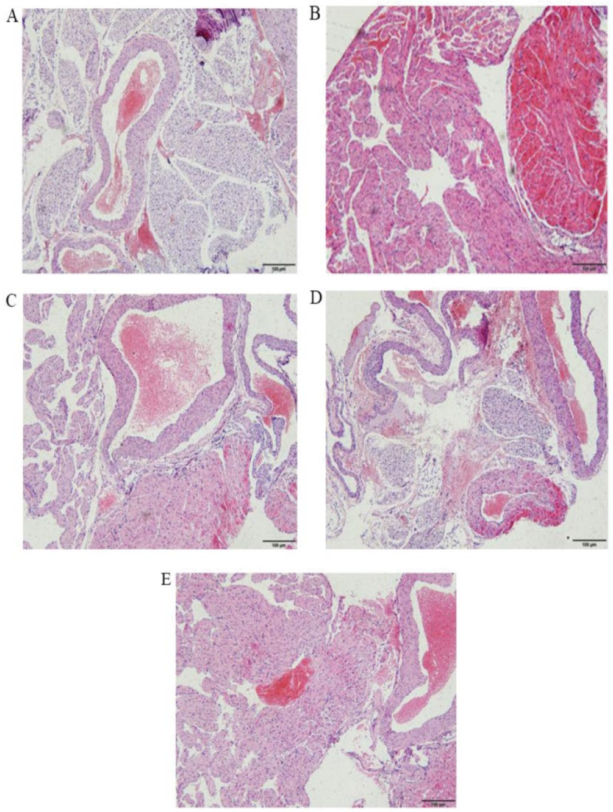

Fig. 2: Effects of RAL on the aortic valves stained with H and E stain
Effects of different concentration of raloxifene on the H and E stained aortic valves from the high-sugar, high-fat fed mice. (A) high-sugar, high-fat group; (B) control group; (C) RAL 4.5 mg/kg+high-sugar, high-fat group; (D) RAL 9 mg/kg+high-sugar, high-fat group; and (E) RAL 18 mg/kg+high-sugar, high-fat group

particularly in the high-concentration RAL18 mg/kg +high-sugar, high-fat group, in which no such calcification was found (fig. 3A-E).

While analysing the expressions of caspase-3 and caspase-8 by immunohistochemistry, we found that the expression levels of proteins regulating the apoptosis of valvular interstitial cells after administration of RAL were inhibited to a certain extent compared with the high-sugar, high-fat group. In the sliced aortic valve tissue specimens from the high-sugar high-fat group mice, abundant positively expressed brown caspase-3 (fig. 4A-F) and caspase-8 (fig. 5A-F) were observed; in addition, through quantitative analysis of areas with positive expression, the expression levels in the high-sugar, high-fat group were significantly higher than those of the group treated with RAL.

VHD is an important disease that threatens human health and advancement of age. Issues involving degenerative diseases occupy a dominant position in VHD lesions, which constitute the most common

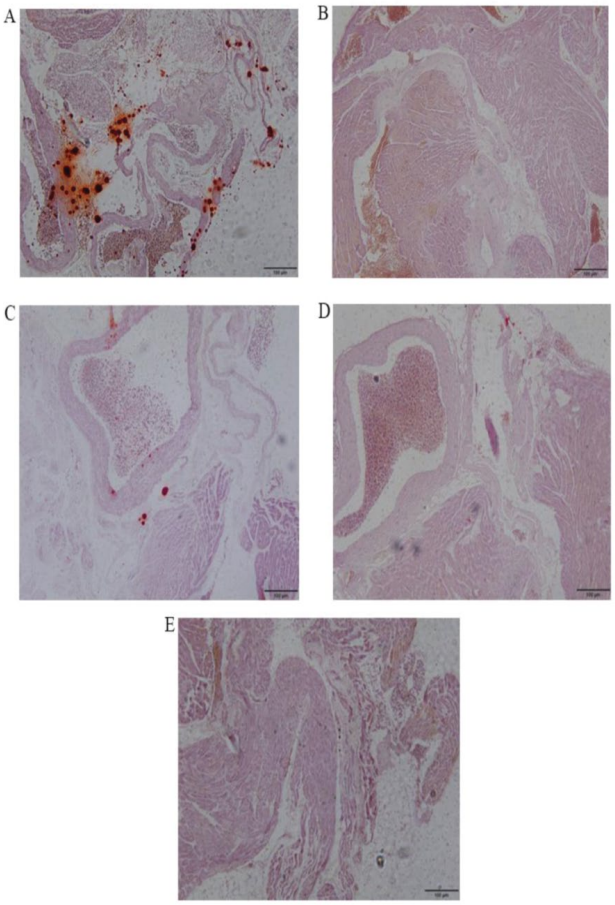

Fig. 3: Effects of RAL on the aortic valves stained with alizarin red

Effects of different concentrations of raloxifene on the alizarin red stained aortic valves from the high-sugar, high-fat fed mice group. (A) High-sugar, high-fat group; (B) control group; (C) RAL 4.5 mg/kg+high-sugar, high-fat group; (D) RAL 9 mg/kg+high-sugar, high-fat group; and (E) RAL 18 mg/kg+high-sugar, high-fat group

cause of aortic valvular disease and the most important reason for aortic valve replacement surgery[11,12]. Aortic valvular disease is a complex pathologic process. In the past years, the disease is generally considered as a passive and non-regulated process under long-term mechanical stress. At present, owing to the lack of understanding of the occurrence and development of these lesions, extremely few effective interventions are available for early valve lesions. Valvular calcification is a main pathological symptom of the disease and is an active biological process subject to active regulation[13]. In this complex regulatory process, the proliferation and apoptosis of valvular interstitial cells involve the formation of valve lesions and calcification[14,15]. Studies have shown that a high-fat diet may induce valvular lesions in New Zealand white rabbits, with significantly increased apoptosis in the lesion of the valves[16].

RAL belongs to the second-generation SERM group, and is mainly used initially for the treatment of post-menopausal osteoporosis and breast cancer[17]. Subsequently, the medicine was found to exert

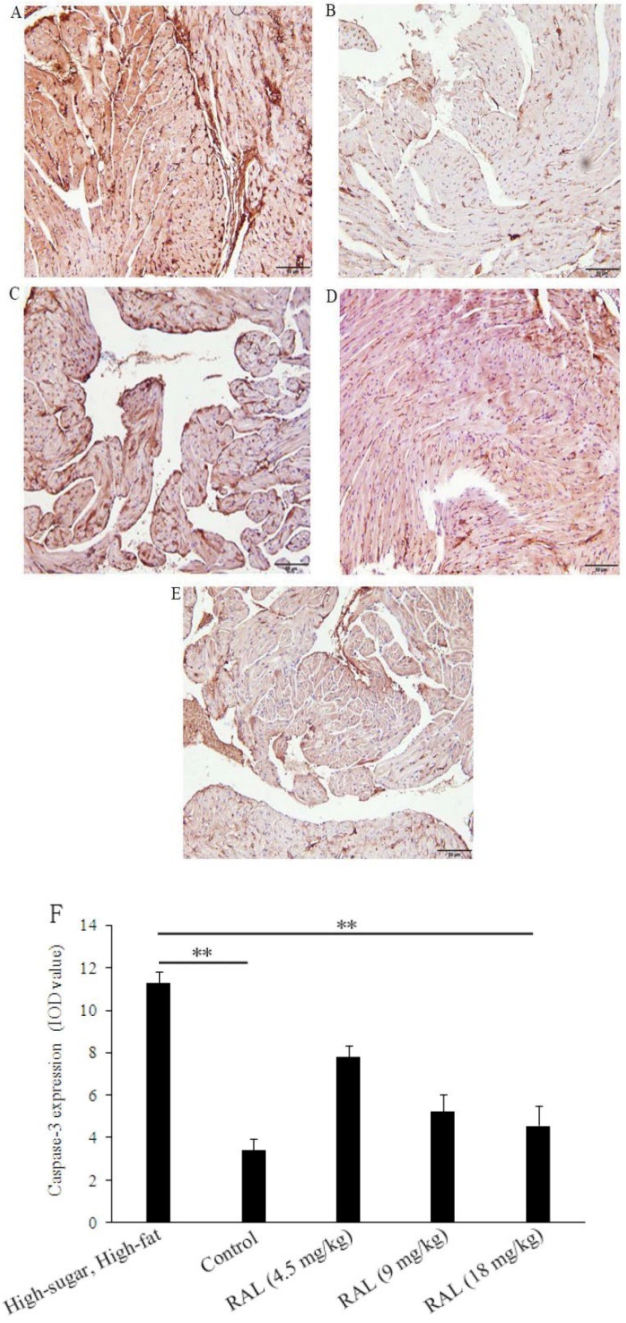

Fig. 4: Effects of RAL on caspase-3 expression levels in the aortic valves

Effects of different concentrations of raloxifene on caspase-3 expression levels in the aortic valves from the high-sugar, high-fat fed mice studied using immunohistochemistry. (A) high-sugar, high-fat group; (B) control group; (C) RAL 4.5 mg/kg+high-sugar, high-fat group; (D) RAL 9 mg/kg+high-sugar, high-fat group; and (E) RAL 18 mg/kg+high-sugar, high-fat group and (F) results of the quantitative analysis on the caspase-3 expression levels are expressed by means±SD, **p<0.01

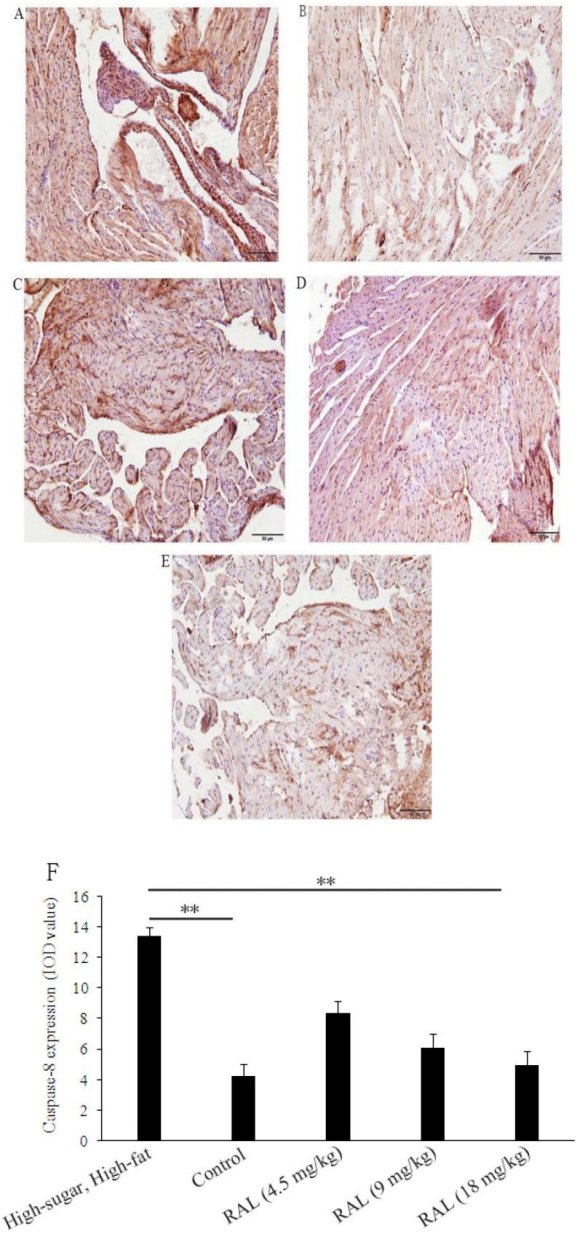

Fig. 5: Effects of RAL on the caspase-8 expression in the aortic valves

Effects of different concentrations of raloxifene on caspase-8 expression levels in the aortic valves from the high-sugar, high-fat fed mice studied using immunohistochemistry. (A) High-sugar, high-fat group; (B) control group; (C) RAL 4.5 mg/kg+high-sugar, high-fat group; (D) RAL 9 mg/kg+high-sugar, high-fat group; and (E) RAL 18 mg/kg+high-sugar, high-fat group; (F) The results of the quantitative analysis on caspase-3 expressions are expressed as means±SD, **p<0.01

therapeutic effects on cardiovascular disease. RAL and other SERMs exhibit certain effects, such as improving the functions of vascular endothelial cells[18,19], dilating the coronary arteries[20], regulating the lipids, and significantly decrease soluble human vascular endothelial cadherin and increase serum total matrix metalloproteinase-2 in healthy postmenopausal women[21], and protecting from atherosclerosis and venous thrombosis, and others[22,23]. RAL can improve the renal failure-induced valvular calcification by inhibiting the apoptosis of aortic valvular interstitial cells; however, the effects of RAL in animals with VHD and related clinical studies have been rarely reported[24].

In the present study, the effects of different concentrations of RAL on the biological functions of aortic valves in the high-sugar, high-fat group of mice were discussed, and reviewed the literature and previous study summaries and designed the corresponding concentrations and length of treatment duration. After three-month administration of RAL, the mRNA expression levels in caspase-3 and caspase-8 in the aortic valves of the high-sugar, high-fat mice group were found to be decreased compared with those of the high-sugar, high-fat group. In particular, those of the RAL9 mg/kg+high-sugar, high-fat group and the RAL 18 mg/kg+high-sugar, high-fat group decreased sharply. A long-term high-sugar, high-fat diet is the main cause of aortic valve steatosis. Numerous dotted steatosis areas were found in the aortic valves of the high-sugar, high-fat mice group; then, after the administration of different concentrations of RAL, the aortic valve steatosis areas were improved to varying degrees. Analysis results of the degree of calcification show numerous areas with red-dotted calcium deposition in the aortic valves of the high-sugar, high-fat mice group. In comparison, these dotted areas are markedly diminished in the group treated with different concentrations of RAL; particularly, the high-concentration RAL 18 mg/kg+high-sugar, high-fat group virtually showed no such calcification. As observed by quantitative analysis on regulating the protein expressions related to the apoptosis of valvular interstitial cells, the expression levels after RAL administration were also inhibited to a certain extent compared with those of the high-sugar, high-fat group.

In the present study, an aortic valve degeneration model for high-sugar, high-fat mice was established, and the effects of different concentrations of RAL on the biological functions of the aortic valves were observed. An appropriate concentration of RAL is found to exert certain inhibitory actions on tissues, thereby providing experimental reference for preventing and treating aortic valve disease with RAL.

Conflicts of interest:

The authors declare that there is no conflict of interests.

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