Alleviation of renal injury in rabbits by allisartan

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ABSTRACT

The objective of this study was to determine the relationship between renal injury and inflammatory response induced by high-fat diet in rabbits and the interventional effect of allisartan. Fifteen 6-week-old healthy male rabbits were randomly divided into three groups: normal control (NC) group, high-lipid diet (HLD) group, high-lipid diet and allisartan (HLD+ALST) group. After allisartan treatment for 12 weeks, changes in total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), serum creatinine (Scr) and blood urea nitrogen (BUN) were measured enzymatically in the three groups. The left side of the kidney tissue was kept for paraffin section, and HE staining, periodic acid-Schiff (PAS) staining and Masson staining were used to observe the renal pathologic changes. TC, TG, LDL-C, Scr and BUN levels were all higher and HDL-C levels were lower in the HLD group compared with the NC group. Compared with the HLD group, Scr and BUN levels were significantly decreased in the HLD+ALST group. The results of HE staining showed that allisartan improved the changes of renal tissue morphology in rabbits on high-fat diet, reduced glomerular mesangial cell proliferation and improved glomerulosclerosis; PAS staining showed that glomerular glycogen deposition was reduced and glomerular red staining was significantly lighter; Masson staining showed that renal tubular blue-stained collagen fibers were reduced. In conclusion, hyperlipidemia can lead to aberrant expression of multiple cellular proteins and kidney tissue morphological damage in rabbits. On the other hand, allisartan attenuated renal injury and the mechanism may be related to the downregulation of the inflammatory response.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Hyperlipidemia is a risk factor for the progression of glomerulosclerosis with structural changes in renal tissue cells.
⇒ The kidney is another important target organ that suffers from hyperlipidemia besides cardiovascular and cerebrovascular.

WHAT THIS STUDY ADDS

⇒ We found that high-fat diet caused renal tissue damage and upregulated inflammatory factors in rabbits, and the intervention of allisartan improved renal histologic morphology, alleviated damage and downregulated inflammatory factor levels in rabbits, indicating that allisartan may alleviate hyperlipidemia-induced renal injury by reducing inflammatory response.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The protective effect of allisartan on hyperlipidemia-induced renal injury may be dependent on the control of inflammation.
⇒ Our study provides clue for specific mechanism of action of allisartan in the inflammatory response pathway and determine if the same effect is present in clinical cases.

INTRODUCTION

Hyperlipidemia is a clinical disease that manifests itself when the body's serum total cholesterol (TC) or triglycerides (TG) are too high or the high-density lipoprotein cholesterol (HDL-C) is too low.[1 2] In recent years, the incidence of hyperlipidemia caused by high-sugar and high-fat diet in China has been increasing year by year, and the overall prevalence of dyslipidemia among adults in China has reached 40.4%.[3] Due to the insidious, progressive and systemic characteristics of hyperlipidemia, it is often overlooked, which in turn leads to disorders of lipid metabolism and eventually induces the occurrence of diseases.[4 5] Previous studies reported about the relationship between kidney disease and lipid deposition.[6 7] The 'lipid nephrotoxicity hypothesis' proposed by Moorhead *et al* demonstrated that hyperlipidemia is a risk factor for the progression of glomerulosclerosis with structural changes in renal tissue cells.[8] Since then, scholars have found that glomerulosclerosis and atherosclerosis share similar pathologic changes and pathophysiologic mechanisms.[9] The kidney is another important target organ that suffers from hyperlipidemia besides cardiovascular and cerebrovascular.

Although most animal and clinical studies support the 'lipid nephrotoxicity hypothesis', there also exists some evidence that hyperlipidemia does not have a significant effect on kidney function. For example, the Watanabe hereditary hyperlipidemic rabbit model has hyperlipidemia but no kidney damage.[10] Renal damage rarely

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occurs in patients with primary familial hyperlipidemia, except for obese patients.[11] The current view in the mainstream has expanded on the doctrine of lipid nephrotoxicity as the joint involvement of inflammatory factors and lipids in the progression of kidney disease.[12 13] Therefore, treatments that can reduce the inflammatory response to nephrotoxicity may become available.

Allisartan is a new domestic angiotensin II receptor blocker (ARB) that has been widely used in renal disease and has been shown to have renoprotective effects independent of antihypertensive properties, but the specific mechanism is unclear.[14] In addition, recent studies have found that ARB drugs have anti-atherosclerotic effects by attenuating the inflammatory response.[15 16] Owing to the multiple pharmacologic effects and targets of ARB drugs, attention has been paid to their protective effects on a variety of organs. However, it is still unclear how ARB drugs alleviate lipid kidney injury.

In this study, we found that high-fat diet caused renal tissue damage and upregulated inflammatory factors in rabbits, and the intervention of allisartan improved renal histologic morphology, alleviated damage and downregulated inflammatory factor levels in rabbits, indicating that allisartan may alleviate hyperlipidemia-induced renal injury by reducing inflammatory response.

MATERIALS AND METHODS

Animals

Fifteen normal grade male New Zealand white rabbits (4 months old, weighing 2~3 kg) were purchased from Hebei Medical University. The animals were housed in the Animal Experiment Center of Hebei Medical University (ambient temperature 24°C±2°C, humidity 50%–70%, alternating light and dark for 12 hours each. The rabbits have free access to water and feed during feeding. The environmental facilities of the feeding environment conformed to the Grade II standard of the Standard for Laboratory Animals of the Ministry of Health of the People's Republic of China). Feed: Common rabbit pellet feed was purchased from Hebei Medical University; high-fat feed was purchased from Beijing Keao Cooperative Feed. Formula: 1% cholesterol+6% peanut oil+93% basal feed. After allisartan treatment for 12 weeks, the rabbits were sacrificed for testing.

Allisartan treatment

Allisartan was ground into powder with a grinding rod, then 0.9% saline was added to dissolve it and diluted into 8 mg/mL allisartan solution. The animals were randomly divided into three groups after 1 week of adaptive feeding: normal control group (NC group), high-fat diet group (HLD group), high-fat diet and allisartan intervention

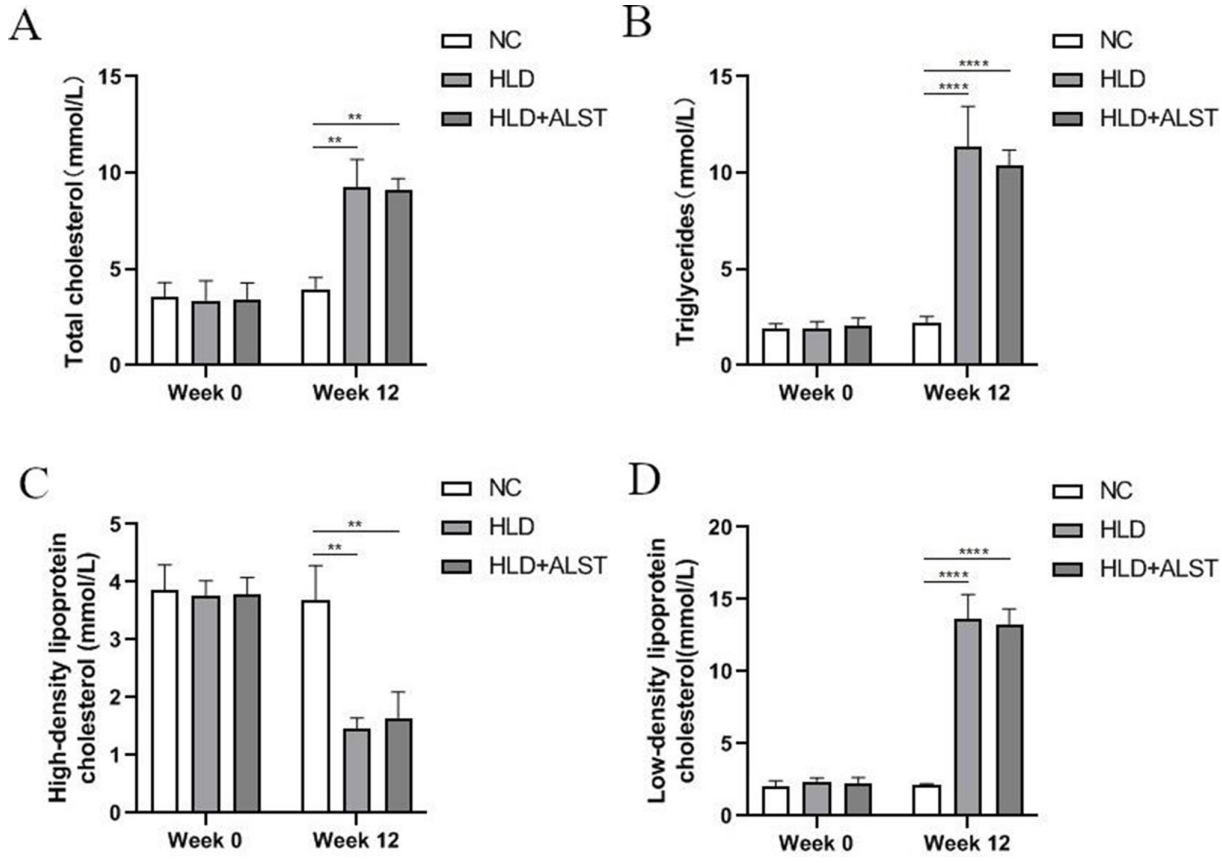

Figure 1 Comparison of serum lipids in the three groups. (A) Total cholesterol (TC) in the three groups. (B) Triglycerides (TG) in the three groups. (C) High-density lipoprotein cholesterol (HDL-C) in the three groups. (D) Low-density lipoprotein cholesterol (LDL-C) in the three groups. Week 0 indicates before treatment with allisartan, while week 12 indicates allisartan treatment for 12 weeks. HLD, high-fat diet group; HLD+ALST, high-fat diet and allisartan intervention group; NC, normal control group. **p<0.01; ****p<0.0001.

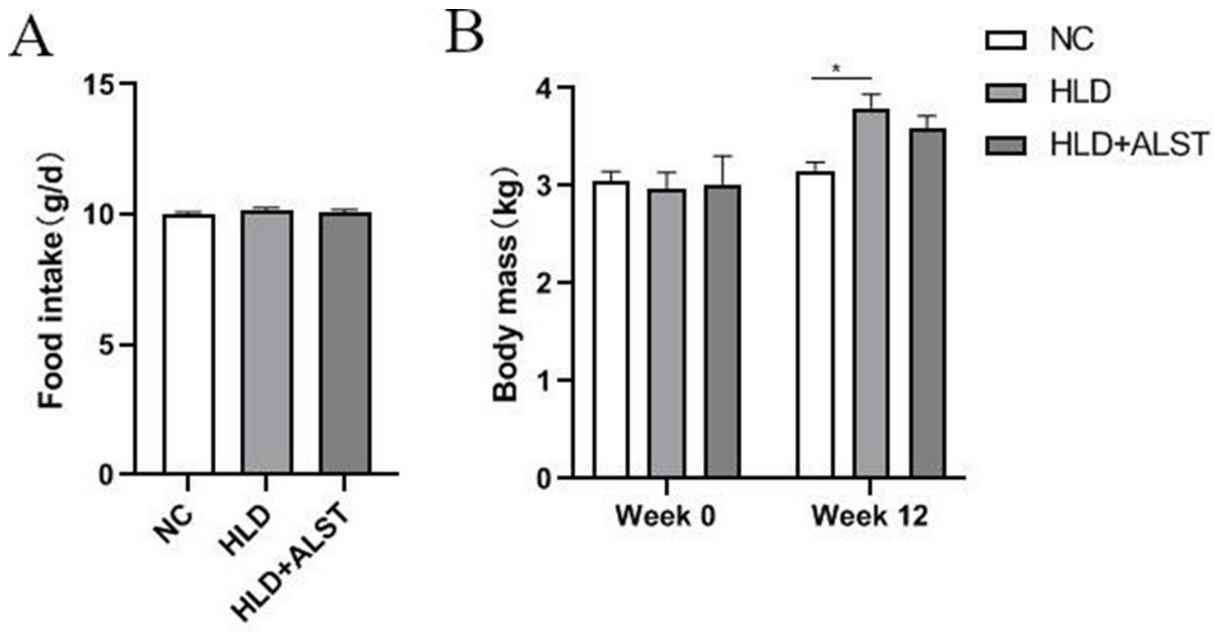

Figure 2 Changes in food intake and body mass in the three groups. (A) Food intake before allisartan treatment. (B) Body mass in the three groups. Week 0 indicates before treatment with allisartan, week 12 indicates allisartan treatment for 12 weeks. HLD, high-fat diet group, HLD+ALST, high-fat diet and allisartan intervention group; NC, normal control group. *p<0.05.

group (HLD+ALST group). The NC group was fed with normal rabbit pellet diet; the HLD and HLD+ALST groups were fed with high-fat diet (150 g of high-fat diet per rabbit per day, supplemented with regular feed). The HLD+ALST group was given allisartan 40 mg/kg by gavage once daily in the morning on an empty stomach for 12 weeks, while the NC and HLD groups were given the same volume of saline by gavage. Week 0 indicates pretreatment, while week 12 indicates completion of treatment.

Blood tests

At week 0 and week 12, 2 mL of specimen of rabbit's blood was collected for enzymatic determination of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C). The blood specimens were rested at room temperature for 1 hour, and the serum was separated by centrifugation at 3000 rpm for 10 min. Serum creatinine (Cr) level and blood urea nitrogen (BUN) level were measured. The serum interleukin-6 (IL-6) and interleukin-1β (IL-1β) levels were detected by ELISA.

Renal pathologic examination

The left kidney was taken from the rabbits after being sacrificed, fixed in 4% formalin, rinsed, dehydrated, routinely paraffin embedded, sectioned (5 μm), and stained by HE staining (HE),[17] periodic acid-Schiff stain (PAS)[18] and Masson.[19] Pathologic changes were then observed under light microscopy.

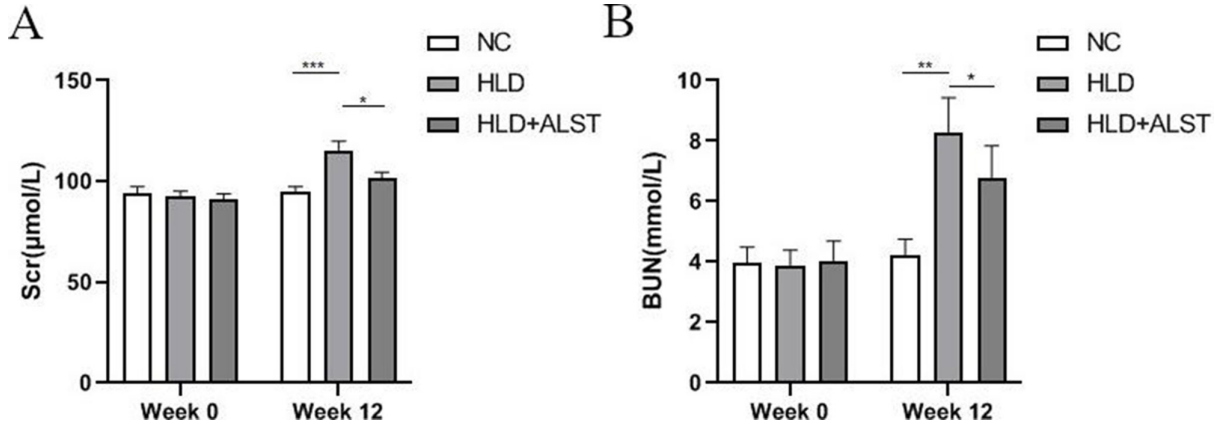

Figure 3 Comparison of (A) Scr and (B) BUN levels in the three groups. Week 0 indicates before treatment with allisartan, while week 12 indicates allisartan treatment for 12 weeks. BUN, blood urea nitrogen; HLD, high-fat diet group, HLD+ALST, high-fat diet and allisartan intervention group; NC, normal control group; Scr, serum creatinine. *p<0.05; **p<0.01; *** p<0.001.

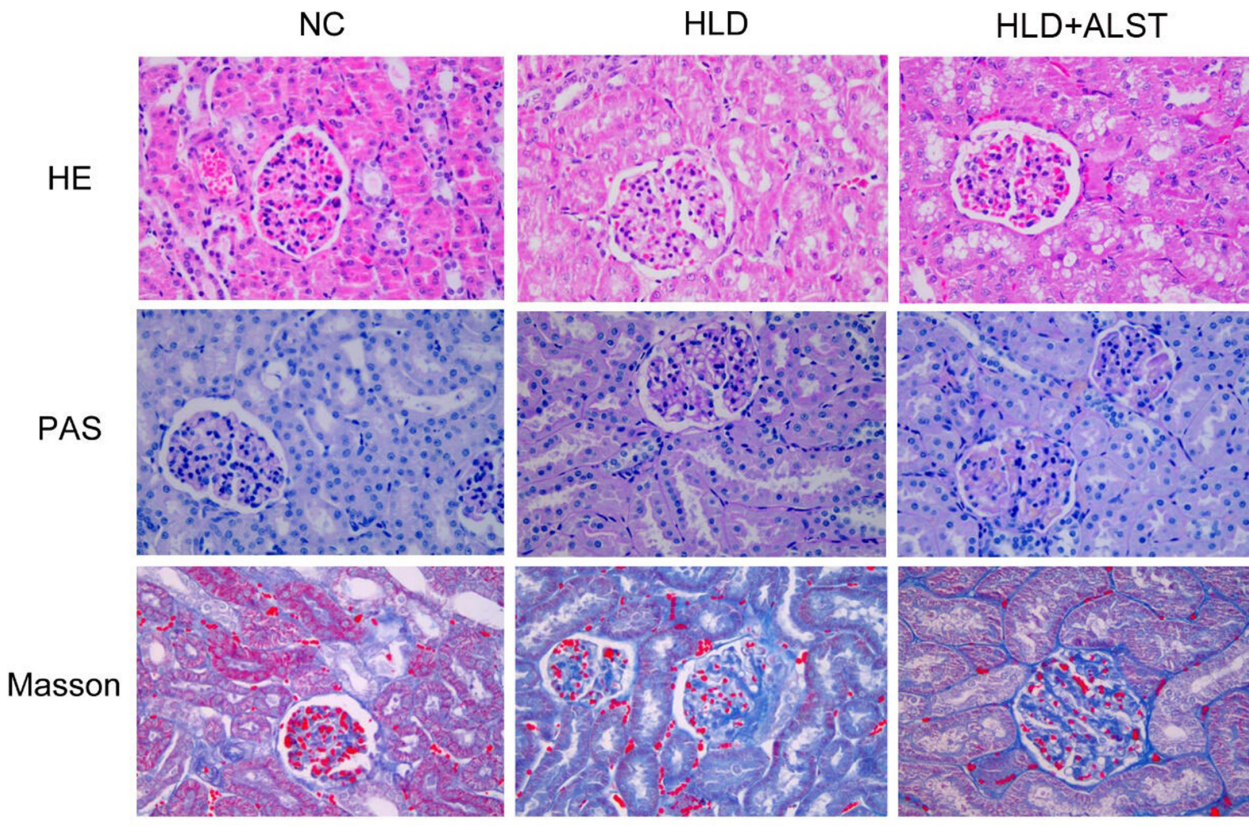

Figure 4 HE, periodic acid-Schiff (PAS) and Masson staining of the rabbit kidney tissues from the three groups (×400). HLD, high-fat diet group; HLD+ALST, high-fat diet and allisartan intervention group; NC, normal control group.

Immunohistochemistry

Briefly,[20] kidney tissue sections were dewaxed for antigen repair, then primary antibodies of α-smooth muscle actin (α-SMA) and desmin were incubated overnight for 4 days, secondary antibodies were incubated after phosphate buffer saline washing, diaminobenzine color, hematoxylin re-staining and then dehydrated, transparent and sealed for observation.

Statistics

All data were statistically processed with the SPSS V.21.0 software. Measurement data were expressed as mean±SD if normally distributed, and one-way analysis of variance was used for comparison between groups; χ^2 test was used for comparison between groups. $p<0.05$ was statistically significant.

RESULTS

Rabbit model of hyperlipidemia

To examine whether we have constructed rabbit model of hyperlipidemia successfully, we tested serum lipids in the NC, HLD and HLD+ALST groups. We observed a significant increase of TC, TG and LDL-C in the HLD group, while HDL-C, which promotes cholesterol metabolism, was decreased in the HLD group (figure 1A–D).

Moreover, no significant change was observed in food intake and body mass (figure 2A,B). These results suggest that we constructed the hyperlipidemia model successfully.

Allisartan improved the renal function of the rabbit

We therefore tested Scr and BUN, which may reflect the renal function. Compared with the NC group, both Scr and BUN in the HLD group increased, indicating that hyperlipidemia impaired the renal function of the rabbit. By treatment with allisartan, Scr and BUN were decreased in the HLD+ALST group compared with the HLD group, indicating that allisartan protects the renal system from being damaged (figure 3A,B).

Allisartan ameliorates the histopathologic morphology of rabbit's kidney

To perform the morphologic changes in the kidney, we employed HE staining. We found that the structure and morphology of the kidney tissue in the NC group were basically normal, and no obvious pathologic changes were observed, while in the HLD group, the glomerular mesangial cells were significantly proliferated, stromal cells were increased, and with apparent glomerulosclerosis. Meanwhile, the nuclei size were reduced and with inflammatory cell infiltration. Compared with the HLD group, the pathologic changes of the renal tissue in the HLD+ALST group were significantly reduced.

PAS staining showed a small amount of glycogen deposition in the glomeruli of the NC group, and a large amount of glycogen deposition in the glomerular mesangial cells was observed in the HLD group. Compared with the HLD

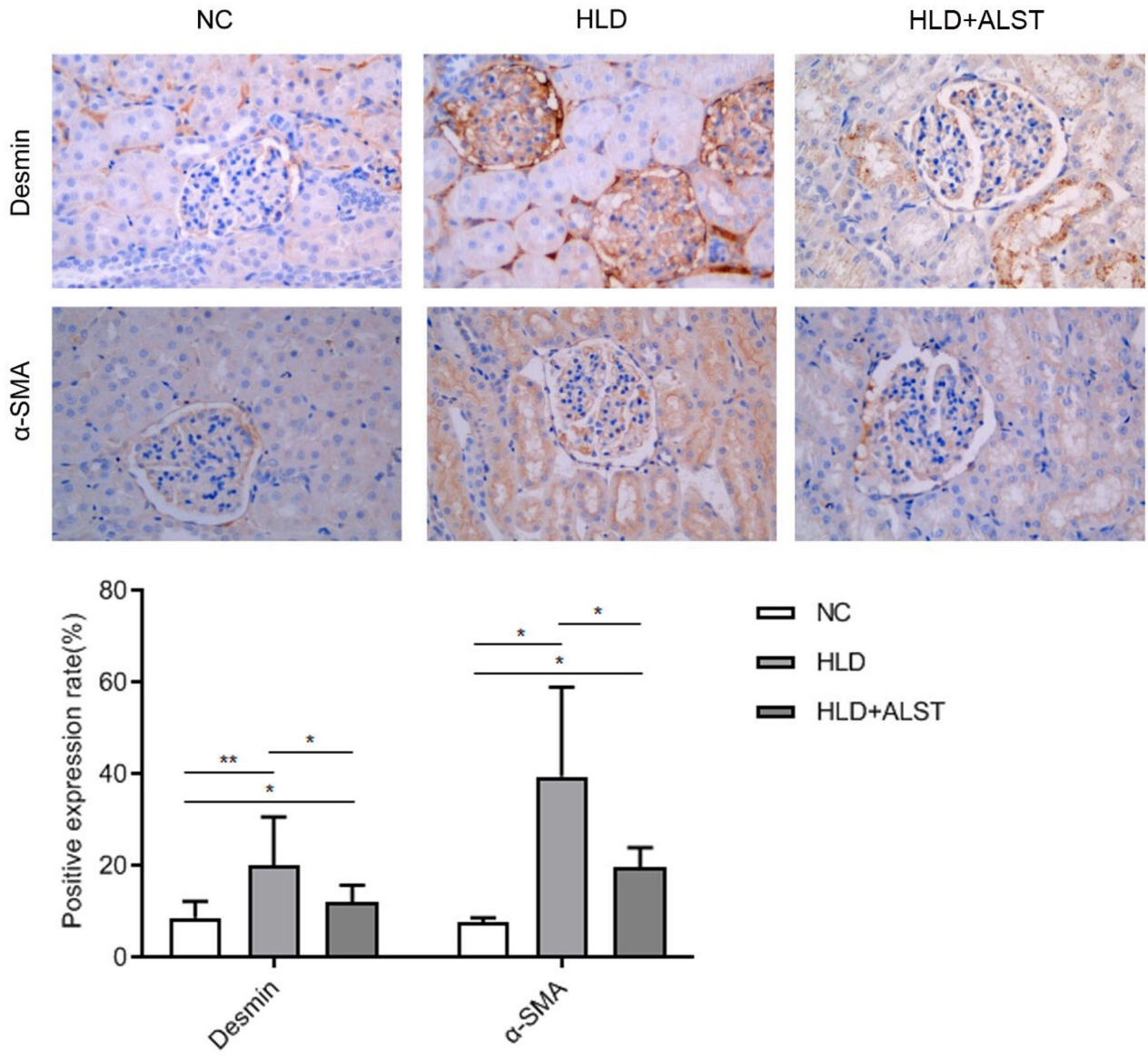

Figure 5 Immunohistochemical staining of desmin and α-smooth muscle actin expression in the kidney tissues of each group (×400). HLD, high-fat diet group; HLD+ALST, high-fat diet and allisartan intervention group; NC, normal control group. *p<0.05; **p<0.01.

group, the renal histopathologic changes were significantly reduced in the HLD+ALST group (figure 4).

Masson staining showed that a small amount of blue-stained collagen fibers were visible in the renal tubules of the NC group, while diffuse blue staining was visible in the renal tubules of the HLD group, and the content of collagen fibers was significantly elevated. In comparison with the HLD group, the blue-stained collagen fibers in the tubules of the HLD+ALST group were clearly decreased (figure 4).

Desmin is a typical cytoskeletal protein, which is normally expressed negatively or in small amounts in renal podocytes, and is one of the early markers of podocyte injury. Desmin was mainly expressed in the glomerular cytosol and plasma, with a brownish-yellow granular expression, and a small amount was seen in the NC group. A large number of desmin-staining positive cells were observed in the glomeruli of the HLD group, and desmin-staining positive cells were reduced in the HLD+ALST group compared with the HLD group. Quantitative data analysis showed that the rate of desmin was obviously decreased in the HLD+ALST group compared with the HLD group (figure 5).

α-SMA is an important marker of the degree of interstitial fibrosis and the progression of renal disease, and a significant increase in its expression level indicates significant impairment of the renal function. α-SMA was mainly expressed in the plasma, with brownish-yellow granular expression. A small amount of α-SMA expression was seen in the NC group, and a large number of α-SMA-staining positive cells were seen in the glomeruli of the HLD group. α-SMA staining positive cells were reduced in the HLD+ALST group compared with the HLD group. Quantitative data revealed a significant decrease in the rate of α-SMA positivity in the HLD+ALST group compared with the HLD group (figure 5).

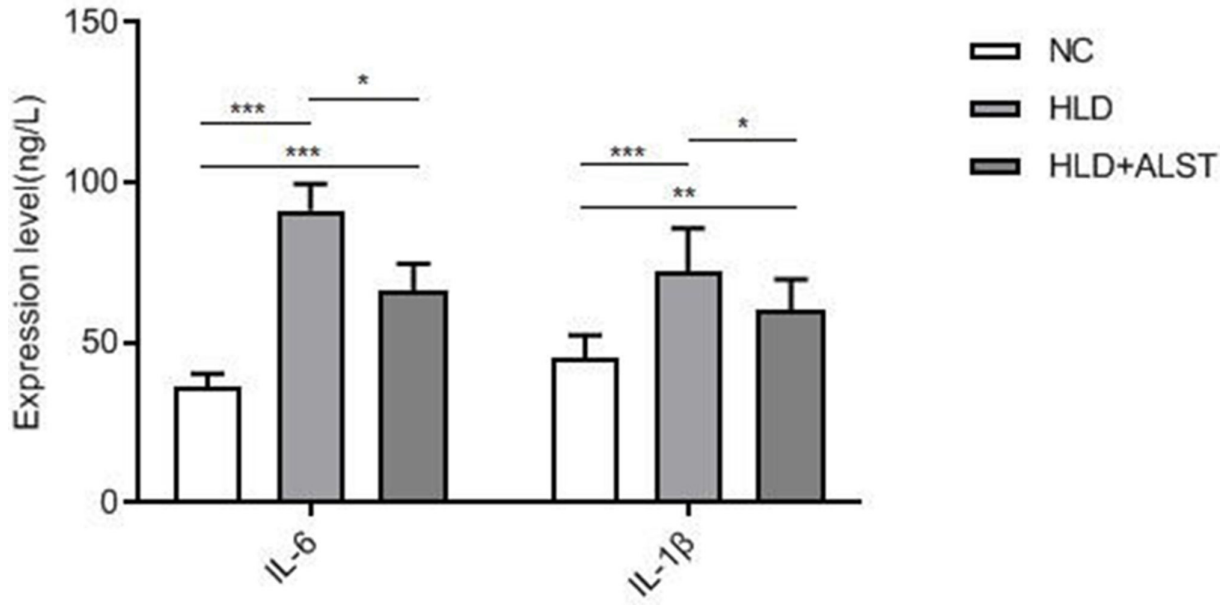

Figure 6 Comparison of IL-6 and IL-1β in the three groups after allisartan treatment for 12 weeks. HLD, high-fat diet group; HLD+ALST, high-fat diet and allisartan intervention group; IL-1β, interleukin-1β; IL-6, interleukin-6; NC, normal control group. *p<0.05; **p<0.01; ***p<0.001.

Allisartan alleviates inflammatory response

To determine the inflammation in rabbit, we tested serum IL-6 and IL-1β of the NC, HLD and HLD+ALST groups. The results demonstrated that IL-6 and IL-1β were significantly higher in the HLD group compared with the NC group, while in the HLD +ALST group treated with allisartan, IL-6 and IL-1β were both significantly decreased in comparison with the HLD group (figure 6).

DISCUSSION

Hyperlipidemia is a series of clinical syndromes caused by high blood lipid levels in patients.[21] Many studies have now shown that hyperlipidemia can lead to changes in glomerular structure and function and is an independent risk factor for progression to end-stage renal disease.[22] Clinical studies have found that serum levels of free fatty acids such as palmitic acid are elevated in patients with hyperlipidemia, which in turn causes inflammation and oxidative stress, leading to renal fibrosis and renal cell apoptosis.[23] In addition, the glomerular filtration rate of patients with early hyperlipidemia decreases gradually with renal injury and loss of renal units.[24] In this study, we found that TC, TG, LDL-C, Scr and BUN levels were higher in the HLD group compared with the NC group after high-fat diet. HE staining results showed that glomerular mesangial cells and endothelial cells in the HLD group were significantly proliferated, and stromal cells were increased, showing obvious glomerulosclerosis. Meanwhile, the nuclei were reduced to varying degrees and inflammatory cells infiltrated. PAS staining and Masson staining showed massive glycogen deposition and collagen fibers in glomerular mesangial cells. All the results suggest that we successfully constructed a rabbit model of lipid kidney injury by high-fat diet.

The renin-angiotensin system plays an important role in maintaining blood pressure, water-electrolyte balance, and other physiologic processes.[25 26] Allisartan is a new generation of Ang II type 1 receptor (AT1R) antagonist independently developed in China, which can effectively inhibit the vasoconstrictive effect of Ang II by binding to AT1R on vascular smooth muscle and reduce peripheral vascular resistance. Several studies have demonstrated its efficacy in the treatment of essential hypertension,[27] cardiac left ventricular hypertrophy,[28] and angina pectoris,[14] without aggravating the burden of the liver. In addition, allisartan also acts on the kidney to dilate the small efferent arteries, reduce glomerular pressure, and improve the degree of basement membrane lesions, thus achieving renal protection and delaying renal function damage.[14 28 29] The results of our study showed that Scr and BUN levels were significantly decreased and glomerular mesangial cell proliferation was reduced in the HLD+ALST group compared with the HLD group. Immunohistochemical staining showed that the positive staining for α-SMA and desmin protein was diminished in the HLD+ALST group compared with the HLD group, and the protein positivity rate decreased. Furthermore, both inflammatory factors IL-6 and IL-1β showed a significant decrease. This suggests that the protective effect of allisartan on hyperlipidemia-induced renal injury may be dependent on the control of inflammation. Next, we will focus on the specific mechanism of action of allisartan in the inflammatory response pathway and determine if the same effect is present in clinical cases.

There are some limitations in this study. The sample size of this study was small and the experimental time was short. More samples and longer experimental time are needed to study the intervention effect of allisartan on renal injury caused by long-term hyperlipidemia. Allisartan is an Ang II receptor antagonist with antihypertensive effects, but the blood pressure and blood glucose levels of the rabbits were not measured during the experiment, and all the results of

the study cannot yet exclude the influence of blood pressure and blood glucose levels on lipid kidney injury, while the current experimental data are in support of the conclusion that allisartan alleviates kidney injury without blood pressure and blood glucose levels. Moreover, the present experiment illustrates the protective effect of allisartan on lipid kidney injury, but the exact mechanism is not clear and needs further study.

In summary, our study concluded that renal injury was obvious and inflammation levels were elevated in the high-fat diet group of rabbits, and that allisartan may have a protective effect on the renal function by downregulating inflammatory factors and reducing the expression of α-SMA and desmin to attenuate the renal injury caused by hyperlipidemia.

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Contributors LRB and LL have made substantial contributions to the conception and design of the study. ZJD and WD were involved in acquisition of data, data entry and data cleaning. CXR and BL were involved in analysis and interpretation of data. LRB and ZL have been involved in drafting the manuscript. YXH was involved in revising manuscript critically for important intellectual content. All authors contributed substantially to its revision. All authors read and approved the final manuscript. YXH is the guarantor of this study.

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Competing interests None declared.

Patient consent for publication Not applicable.

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REFERENCES

1 Ou T-H, Tung Y-T, Yang T-H, *et al*. Melatonin improves fatty liver syndrome by inhibiting the lipogenesis pathway in hamsters with high-fat diet-induced hyperlipidemia. *Nutrients* 2019;11:748.
2 Karr S. Epidemiology and management of hyperlipidemia. *Am J Manag Care* 2017;23:S139–48.
3 Pan L, Yang Z, Wu Y, *et al*. The prevalence, awareness, treatment and control of dyslipidemia among adults in China. *Atherosclerosis* 2016;248:2–9.
4 Kling MA, Trojanowski JQ, Wolk DA, *et al*. Vascular disease and dementias: paradigm shifts to drive research in new directions. *Alzheimers Dement* 2013;9:76–92.
5 Ogungbe O, Akil L, Ahmad HA. Exploring unconventional risk-factors for cardiovascular diseases: has opioid therapy been overlooked? *Int J Environ Res Public Health* 2019;16:2564.
6 Su H, Wan C, Lei C-T, *et al*. Lipid deposition in kidney diseases: interplay among redox, lipid mediators, and renal impairment. *Antioxid Redox Signal* 2018;28:1027–43.
7 Mitrofanova A, Fontanella AM, Merscher S, *et al*. Lipid deposition and metaflammation in diabetic kidney disease. *Curr Opin Pharmacol* 2020;55:60–72.
8 Ruan XZ, Varghese Z, Moorhead JF. An update on the lipid nephrotoxicity hypothesis. *Nat Rev Nephrol* 2009;5:713–21.
9 Langley-Evans S, Swali A, McMullen S. *Lessons from animal models: mechanisms of nutritional programming*. NY, USA: Nova Science Publishers, 2013: 253–80.
10 Watanabe Y. Serial inbreeding of rabbits with hereditary hyperlipidemia (WHHL-rabbit): incidence and development of atherosclerosis and xanthoma. *Atherosclerosis* 1980;36:261–8.
11 Ruan XZ, Varghese Z, Moorhead JF. Inflammation modifies lipid-mediated renal injury. *Nephrology Dialysis Transplantation* 2003;18:27–32.
12 Gai Z, Wang T, Visentin M, *et al*. Lipid accumulation and chronic kidney disease. *Nutrients* 2019;11:722.
13 Mihai S, Codrici E, Popescu ID, *et al*. Inflammation-related mechanisms in chronic kidney disease prediction, progression, and outcome. *J Immunol Res* 2018;2018:1–16.
14 Zhang J-Q, Yang G-H, Zhou X, *et al*. Effects of allisartan isoproxil on blood pressure and target organ injury in patients with mild to moderate essential hypertension. *Medicine* 2019;98:e14907.
15 Yang G, Tan Z, Zhou L, *et al*. Effects of angiotensin II receptor blockers and ACE (angiotensin-converting enzyme) inhibitors on virus infection, inflammatory status, and clinical outcomes in patients with COVID-19 and hypertension: a single-center retrospective study. *Hypertension* 2020;76:51–8.
16 Dandona P, Dhindsa S, Ghanim H, *et al*. Angiotensin II and inflammation: the effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockade. *J Hum Hypertens* 2007;21:20–7.
17 Wu J, Pan X, Fu H, *et al*. Effect of curcumin on glycerol-induced acute kidney injury in rats. *Sci Rep* 2017;7:1–11.
18 Lin L, Lin H, Wang D, *et al*. Bone marrow mesenchymal stem cells ameliorated kidney fibrosis by attenuating TLR4/NF-κB in diabetic rats. *Life Sci* 2020;262:118385.
19 Wang Z, Han Z, Tao J, *et al*. Role of endothelial-to-mesenchymal transition induced by TGF-β1 in transplant kidney interstitial fibrosis. *J Cell Mol Med* 2017;21:2359–69.
20 Meier Bürgisser G, Heuberger DM, Giovanoli P, *et al*. Delineation of the healthy rabbit kidney by immunohistochemistry - A technical note. *Acta Histochem* 2021;123:151701.
21 Langlois MR, Nordestgaard BG. Which lipids should be analyzed for diagnostic workup and follow-up of patients with hyperlipidemias? *Curr Cardiol Rep* 2018;20:1–14.
22 Lupattelli G, Pisciotta L, De Vuono S, *et al*. A silent mutation of Niemann-Pick C1-like 1 and apolipoprotein E4 modulate cholesterol absorption in primary hyperlipidemias. *J Clin Lipidol* 2013;7:147–52.
23 Li J, Yang M, Yu Z, *et al*. Kidney-secreted erythropoietin lowers lipidemia via activating JAK2-STAT5 signaling in adipose tissue. *EBioMedicine* 2019;50:317–28.
24 Chapman DP, Wheaton AG, Anda RF, *et al*. Adverse childhood experiences and sleep disturbances in adults. *Sleep Med* 2011;12:773–9.
25 Tomson C, Tomlinson LA. Stopping RAS inhibitors to minimize AKI: more harm than good? *Clin J Am Soc Nephrol* 2019;14:617–9.
26 Szczepanska-Sadowska E, Czarzasta K, Cudnoch-Jedrzejewska A. Dysregulation of the renin-angiotensin system and the vasopressinergic system interactions in cardiovascular disorders. *Curr Hypertens Rep* 2018;20:1–24.
27 Zhang G, Fan Y, Qiu Y, *et al*. Allisartan Isoproxil improves endothelial function and vascular damage in patients with essential hypertension: a single-center, open-label, randomized controlled trial. *Adv Ther* 2020;37:3551–61.
28 Liu Y, Wang H, Cheng Y, *et al*. A 26-week repeated-dose toxicity study of allisartan isoproxil in Sprague-Dawley rats. *Drug Chem Toxicol* 2013;36:443–50.
29 Li Y, Li X-hui, Huang Z-jun, *et al*. A randomized, double blind, placebo-controlled, multicenter phase II trial of Allisartan Isoproxil in essential hypertensive population at low-medium risk. *PLoS One* 2015;10:e0117560.