

Exercise reduces hyperlipidemia-induced kidney damage in apolipoprotein E-deficient mice

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Abstract. Hyperlipidemia is a risk factor of kidney damage that can lead to chronic kidney disease. Studies have shown that exercise reduces kidney damage; however, the specific mechanisms underlying the protective effects of exercise remain unclear. For 12 weeks, 8-week-old male apolipoprotein E-deficient (ApoE^{-/-}) mice were randomly divided into four treatment groups (n=7/group) as follows: Mice fed a normal diet (ND group); mice fed a ND and exercised (ND + E group); mice fed a high-fat diet (HD group); and mice fed a HD and exercised (HD + E group). Exercise training consisted of swimming for 40 min, 5 days/week. Metabolic parameters, such as low-density lipoprotein-cholesterol, total cholesterol and creatinine levels were higher in the ApoE^{-/-} HD mice compared with those in the ApoE^{-/-} HD + E mice. Serum levels of glutathione peroxidase and superoxide dismutase were significantly decreased in the HD group compared with those in the HD + E group. Significant pathological changes were observed in the HD + E group compared with in the HD group. Immunohistochemistry and immunoblotting revealed increased levels of oxidative stress

(nuclear factor erythroid-2-related factor 2) and fibrosis (Smad3 and TGF-β) markers in the ApoE^{-/-} HD group; however, the expression levels of these markers were significantly decreased in the ApoE^{-/-} HD + E group. Furthermore, NF-κB expression in the HD + E group was significantly lower compared with that in the HD group. These results suggested that exercise may exert protective effects against kidney damage caused by hyperlipidemia.

Introduction

Hyperlipidemia is a disorder of lipid metabolism that causes blood lipid levels to increase to abnormally high levels, including increases in the levels of low-density lipoprotein-cholesterol (LDL-c) and total cholesterol (TC). In addition, hyperlipidemia can cause kidney damage (1). Clinical studies have suggested a causative role of dyslipidemia in the development and progression of CKD and its complications (2,3). Increasing evidence has shown that lipid deposition, oxidative stress, fibrosis and inflammation are major pathophysiological mechanisms of hyperlipidemia-induced kidney damage, which may progress to chronic kidney disease (CKD) (4,5). Exercise has been shown to reduce kidney damage in several studies. Zeynali *et al* (6) suggested that aerobic exercise may reduce cisplatin-induced nephrotoxicity, and a favorable effect on renal function was induced by increasing the activation of the antioxidant system (6). In an animal model of high consumption of cola soft drinks, it has been observed that moderately intense exercise reduced the kidney damage caused by cola (7). In addition, Braun *et al* (8) showed that free-running wheel exercise may attenuate the long-term effects of high blood pressure in spontaneously hypertensive rats with kidney damage (8). Apolipoprotein E-deficient (ApoE^{-/-}) mice, a well-established animal model of hyperlipidemia, have been extensively used to study the effects of kidney damage on atherosclerosis and renal injury (9-11). A hyperlipidemia-induced kidney damage animal model using ApoE^{-/-} mice was established in the present study by administering a high-fat diet (HD) and subjecting the mice to exercise in the form of swimming for 40 min/day, 5 days/week for 12 weeks. Whether exercise reduced renal damage caused by hyperlipidemia, and the underlying mechanisms, was assessed.

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Abbreviations: ApoE^{-/-}, apolipoprotein E-deficient; LDL-c, low-density lipoprotein-cholesterol; TC, total cholesterol; CRE, creatinine; GSH-PX, glutathione peroxidase; SOD, superoxide dismutase; CKD, chronic kidney disease; PAS, Periodic acid-Schiff; IHC, immunohistochemistry; NRF2, nuclear factor erythroid-2-related factor 2

Key words: hyperlipidemia, ApoE^{-/-} mice, kidney damage, exercise, TC

Materials and methods

Animals. For establishment of an animal model of hyperlipidemia-induced kidney damage, 8-week-old male ApoE^{-/-} mice (n=28; weight, 24.30±1.04 g) were obtained from Beijing Vital River Laboratories Animal Technology Co., Ltd. Mice were provided with *ad libitum* access to food and water, and were maintained under constant conditions, including 40-60% humidity, 24-26°C and a 12-h light/dark cycle. ApoE^{-/-} mice were randomly divided into four groups as follows: Mice fed a normal diet (ND group); mice fed a ND and exercised (ND + E group); mice fed a HD (HD group); and mice fed a HD and exercised (HD + E group). Based on a previous study, the number of mice in each group was seven (12). The HD mouse food consisted of 1.25% (w/w) cholesterol, 22.5% (w/w) protein, 20.0% (w/w) cocoa fat and 45.0% carbohydrate (cat. no. MD12017; Jiangsu Medicience Co., Ltd.; https://www.medicience.com/proshow_13.html). Exercise training was initiated in an experimental swimming pool (temperature, 30°C; water depth, 44 cm; radius, 120 cm) 1 week prior to administration of the experimental diets. The progressive exercise program initially involved swimming for 5-10 min and was gradually extended to 30 min/day. When the experimental diets were administered, mice were subjected to swimming exercise for 40 min/day, 5 days/week for 12 weeks. After 12 weeks, the mice were euthanized with a high dose of pentobarbital (100 mg/kg, intraperitoneally), and lack of respiration and heartbeat was used as an indicator of mouse death. All animal experiments were approved by the Ethics Committee of the Zhejiang Rongjun Hospital (Jiaxing, China).

Biochemical measurements. Blood samples were obtained from the inferior vena cava and preserved in tubes. The blood samples were immediately centrifuged at 1,006 x g for 10 min at 4°C after collection, and the serum were subsequently stored at -80°C. Total cholesterol (TC; cat. no. A111-1-1), triglyceride (TG; cat. no. A110-1-1), LDL-c (cat. no. A113-1-1), creatinine (CRE; cat. no. C011-2-1), superoxide dismutase (SOD; cat. no. A011-3-2) and glutathione peroxidase (GSH-PX; cat. no. A005-1-2) levels were measured using ELISA kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocols. The concentrations of TC, TG, LDL-c, CRE, SOD and GSH-PX were calculated based on measurement of the optical density at the respective wavelengths for each compound according to the manufacturer's protocol.

Morphological analysis. Kidney tissues were fixed in 10% buffered formalin solution for 30 min at room temperature and dehydrated in 75% ethanol overnight, followed by paraffin embedding. Serial sections (4 µm, n=3/group randomly selected) were stained with hematoxylin for 15 min and eosin for 5 min at temperature to assess pathological changes using a BX40 upright light microscope (Olympus Corporation). Kidney damage scores were determined according to the extent of kidney injury, as previously described (13,14), by two blinded researchers. Scoring was primarily based on the presence or absence of hemorrhaging, tubular cell necrosis, tubular dilatation and cytoplasmic vacuole formation. The grading system was scored as follows: 0, 0% damage (normal kidney); 1, 0-5% damage (minimal damage); 2, 5-25% damage

(mild damage); 3, 25-75% damage (moderate damage); and 4, 75-100% damage (severe damage).

Masson's trichrome and Periodic acid-Schiff (PAS) staining. Kidney-tissue sections (n=3/group) were deparaffinized via immersion in xylene (three times, 5 min each) and rehydrated in a descending alcohol series (100, 90, 80 and 70% alcohol; 5 min each). Slides were stained using Masson's trichrome and PAS staining to investigate changes in kidney tissues. In Masson's trichrome staining, after deparaffinization and rehydration the procedure was as follows: i) Fixation in Bouin liquor overnight at 4°C, followed by washing in running water until the yellow color disappeared and rinsing in two changes of distilled water; ii) staining with Mayer's Hematoxylin for 5 min at room temperature, followed by immersion in 0.5% hydrochloric acid in 70% alcohol for 5 sec at room temperature, washing in running tap water for 30 sec and rinsing in two changes of distilled water; iii) staining with acid ponceau for 5 to 10 min at room temperature, followed by rinsing in three changes of distilled water; iv) immersion in 1% phosphomolybdic acid aqueous solution; and v) staining with aniline blue for 5 min at room temperature, followed by immersion in 1% glacial acetic acid for 5 min at room temperature, dehydration of the stained sections and sealing using resin glue. In PAS staining, after deparaffinization and rehydration, the sections were placed in 1% periodic acid for 15 min at room temperature followed by washing with water, incubation with Schiff's reagent (MilliporeSigma) for 20 min at room temperature and staining with Gill's Hematoxylin (Thermo Fisher Scientific, Inc.) for 3 min at room temperature. All sections were examined using a BX40 upright light microscope (Olympus BX43; Olympus Corporation). Blue staining indicated collagen accumulation in Masson's trichrome staining and red staining indicated lipid deposition in PAS staining.

Immunohistochemistry (IHC). Paraffin-embedded sections (n=3/group) were deparaffinized with xylene and rehydrated through a graded series of ethanol, as described above. Sections were treated with 3% H₂O₂ in methanol for 15 min to inactivate endogenous peroxidases and were then incubated with rabbit anti-NF-κB (cat. no. 10745-1-AP), anti-nuclear factor erythroid-2-related factor 2 (NRF2, cat. no. 16396-1-AP), anti-Smad3 (cat. no. 25494-1-AP) and anti-TGF-β (cat. no. 18978-1-AP) antibody (all at 1:200 dilution; ProteinTech Group, Inc.) overnight at 4°C. The tissues were incubated with the secondary antibody (HRP-labeled goat anti-rabbit IgG) from N-Histofine Simple stain kit (cat. no. 414341F Nichirei Biosciences Inc.) for 30 min at room temperature. Subsequently, the signal was visualized using 3,3'-diaminobenzidine (Metal Enhanced DAB Substrate Kit; cat. no. DA1015; Beijing Solarbio Science & Technology Co., Ltd.) for 10 min and the development was stopped by adding distilled water, according to the manufacturer's instructions. Finally, the stained sections were dehydrated in an ascending series of ethanol concentrations (70, 80, 90 and 100% ethanol; 5 min each) and sealed using resin glue. All sections were examined using a BX40 upright light microscope (Olympus BX43; Olympus Corporation). Three sections were randomly selected from each group. Semi-quantitative assessment of the NF-κB positive cells was performed using ImageJ software version 1.8.0 (National Institutes of Health).

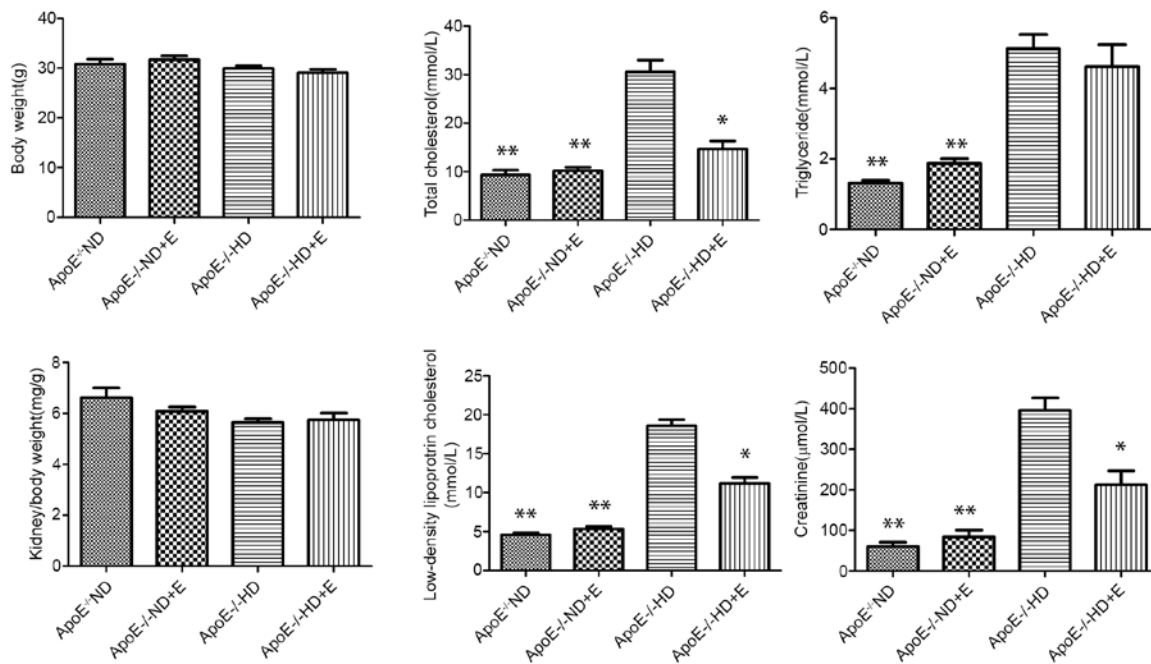


Figure 1. Metabolic data from the mice in the different groups after 12 weeks of feeding with different diets. Kidney/body weights, and total cholesterol, triglyceride, low-density lipoprotein and creatinine levels in the mice after 12 weeks of different treatments. Data are presented as the mean \pm standard error of the mean. $n=7$ /group. * $P<0.05$, ** $P<0.01$ vs. ApoE^{-/-} HD group. ApoE^{-/-}, apolipoprotein E-deficient; HD, high-fat diet; ND, normal diet; E, exercise.

Western blotting. Proteins were extracted from kidney tissues ($n=3$ /group) using RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Protein concentrations were determined using a BCA protein assay kit (cat. no. DQ111-01; Beijing Transgen Biotech Co., Ltd.). Protein samples (20 μ g per lane) were separated by SDS-PAGE on 10-15% gels and were transferred to PVDF membranes. Subsequently, membranes were blocked in Tris-buffered saline with 0.1% Tween-20 (TBS-T) containing 5% skimmed milk and then incubated at room temperature for 2 h. The membranes were then incubated with primary antibodies diluted in primary antibody diluent (cat. no. P0023A; Beyotime Institute of Biotechnology) and lightly shaken overnight at 4°C. Primary rabbit anti-Smad3 (1:1,000 dilution; cat. no. 25494-1-AP), anti-TGF- β (1:1,000 dilution; cat. no. 18978-1-AP), anti-NF- κ B (1:1,000 dilution; cat. no. 10745-1-AP) and anti- β -actin antibodies (1:1,000 dilution; cat. no. 20536-1-AP; all from ProteinTech Group, Inc.) were used. After washing with TBS-T three times (15 min each), membranes were incubated with a secondary antibody (HRP-conjugated goat anti-rabbit IgG; 1:2,000; cat. no. SA00001-2; ProteinTech Group, Inc.) for 1 h at 37°C. Enhanced chemiluminescence reagent (cat. no. 32106; Thermo Fisher Scientific, Inc.) was used to visualize bands. Signals were imaged using a Bio-Rad imaging system (Bio-Rad Laboratories, Inc.) with a Chemi 410 HR camera (Analytik Jena AG) and analyzed using Gel-Pro Analyzer version 4.0 (Media Cybernetics, Inc.). The analysis was performed independently three times. Densitometry analysis was performed using ImageJ software version 1.8.0 (National Institutes of Health) as described previously (15).

Statistical analysis. All data are presented as mean \pm standard error of the mean. SPSS software version 23.0 (IBM Corp.) was used to analyze the data. Differences between multiple

groups were measured using a one-way ANOVA with a post-hoc Tukey's test. Kidney damage scores are expressed as median values and were analyzed using a Kruskal-Wallis test with post hoc Dunn's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Metabolic characterization. The metabolic characteristics of the animals are shown in Fig. 1. Body weight did not differ significantly amongst the four groups. The ApoE^{-/-} HD and HD + E groups showed significantly increased LDL-c, TC, TG and CRE levels compared with those in the ND and ND + E groups. In addition, the levels of TC, LDL-c and CRE were significantly higher in the HD group compared with those in the HD + E group, whereas TG levels did not differ significantly. These results suggested that exercise was effective in reducing TC, LDL-c and CRE levels in mice fed a HD, but was less effective in terms of TG status.

Exercise induces histopathological changes in the kidney tissues of ApoE^{-/-} mice fed a HD. H&E, Masson's trichrome and PAS staining were used to evaluate histopathological changes in renal tissues (Fig. 2). Kidney samples from ApoE^{-/-} ND mice appeared normal. ApoE^{-/-} HD mice exhibited obvious renal lipid deposition and pro-inflammatory cell infiltration compared with in the ND mice. H&E staining results showed that the kidneys of the HD group mice displayed hemorrhaging, inflammatory infiltration, detachment and swelling of tubular epithelial cells, interstitial edema, tubular cell casts, dilatation and necrosis (Fig. 2A). Kidney damage scoring showed that exercise treatment significantly decreased renal injury in the HD group, consistent with H&E results (Fig. 2B). Collagen deposition was determined using Masson's staining. Heavy

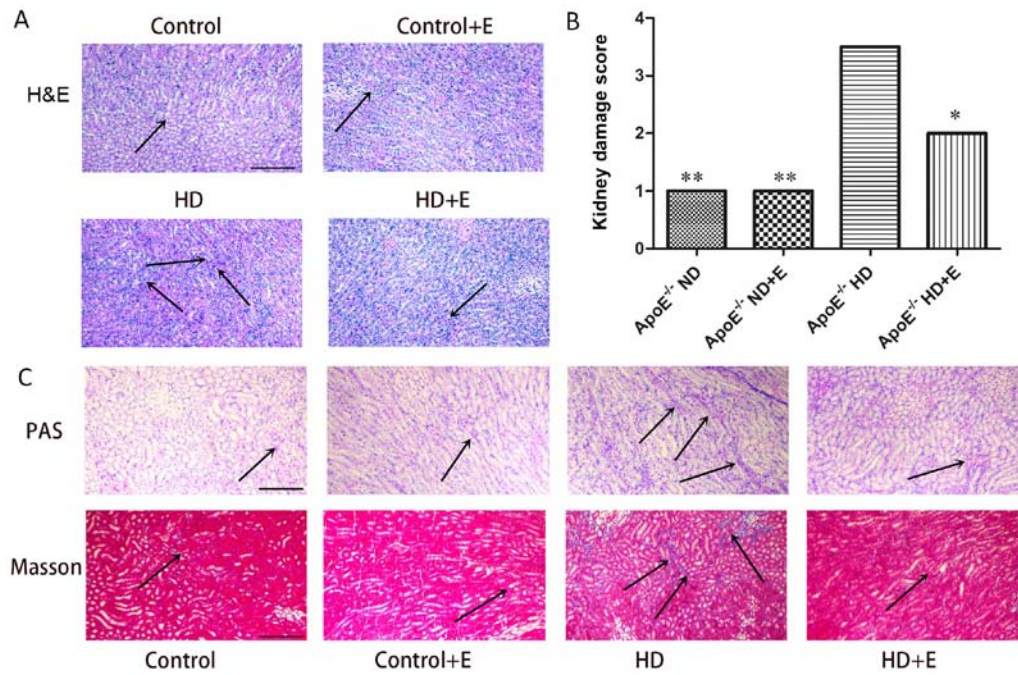


Figure 2. Histological analysis of kidney samples. (A) Notably reduced inflammatory infiltration was observed in the kidney tissue of the mice in the HD+E group compared with the ND group of mice, as determined by H&E staining. Arrows indicate inflammatory infiltration. (B) Kidney damage scores are expressed as the median and were analyzed using the Kruskal-Wallis test and Dunn's post hoc test. Semi-quantitative injury scores ranged from 0 to 4 [0, normal kidney; 1, minimal damage (0-5% injury); 2, mild damage (5-25% injury); 3, moderate damage (25-75% injury); and 4, severe damage (75-100% injury)]. * $P < 0.05$, ** $P < 0.01$ vs. ApoE^{-/-} HD group. (C) Masson and PAS staining in the kidney tissues with different treatments. Exercise reduced lipid deposition, collagen deposition and fibrosis in ApoE^{-/-} HD group mice. Scale bar, 100 μ m. $n = 3$ /group. Masson staining: Collagen fibers, mucus and cartilage are blue; muscle fibers, cellulose and red blood cells are red; and the nucleus is blue and black. The arrows indicate damage. Data are presented as the mean \pm standard error of the mean. ApoE^{-/-}, apolipoprotein E-deficient; HD, high-fat diet; ND, normal diet; E, exercise; PAS, Periodic acid-Schiff.

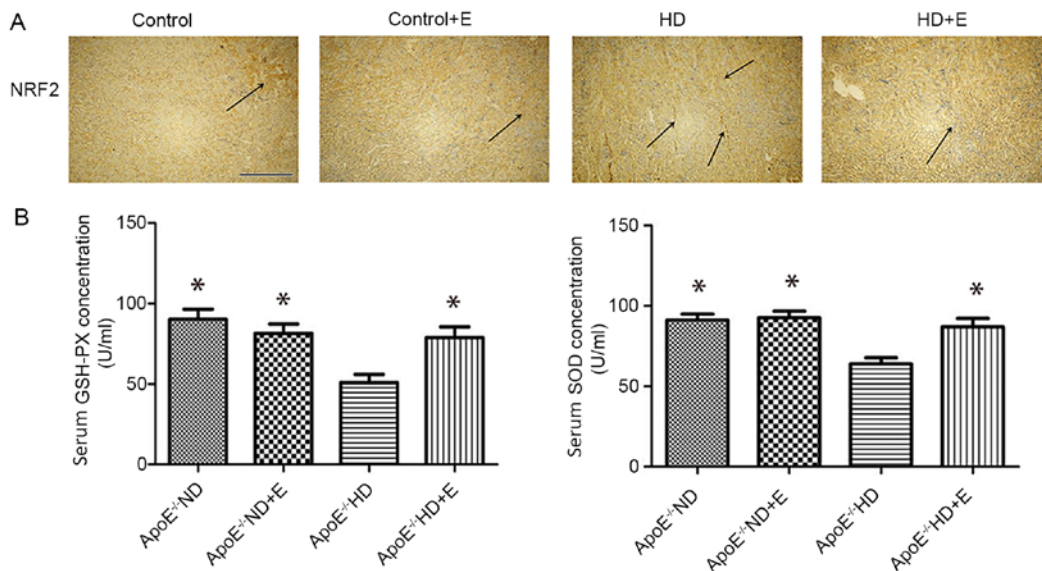


Figure 3. Oxidative stress characteristics. (A) NRF2 expression in the kidney tissues of mice in the different groups after 12 weeks of feeding with different diets. Representative images of immunohistochemistry staining for NRF2 in kidney tissues of mice with the different treatments. Scale bar, 100 μ m. Arrows indicate positively stained cells. (B) GSH-PX and SOD expression in the serum of the four groups after 12 weeks with different treatments. Data are presented as the mean \pm standard error of the mean. $n = 3$ /group. * $P < 0.05$ vs. ApoE^{-/-} HD group. ApoE^{-/-}, apolipoprotein E-deficient; HD, high-fat diet; ND, normal diet; E, exercise; NRF2, nuclear factor erythroid-2-related factor 2; GSH-PX, glutathione-peroxidase; SOD, superoxide dismutase.

collagen deposition was observed in the HD group. Notably, this damage was suppressed in the ApoE^{-/-} HD + E mice (Fig. 2C).

Oxidative stress characteristics. Images of NRF2 expression in the IHC-stained kidney samples are presented in Fig. 3A.

Compared with the ND and ND + E groups, the expression of NRF2 in the ApoE^{-/-} HD group was visibly increased. Exercise reduced NRF2 expression in the ApoE^{-/-} HD + E group compared with that in the ApoE^{-/-} HD group. Serum levels of GSH and SOD are shown in Fig. 3B and C. GSH and SOD

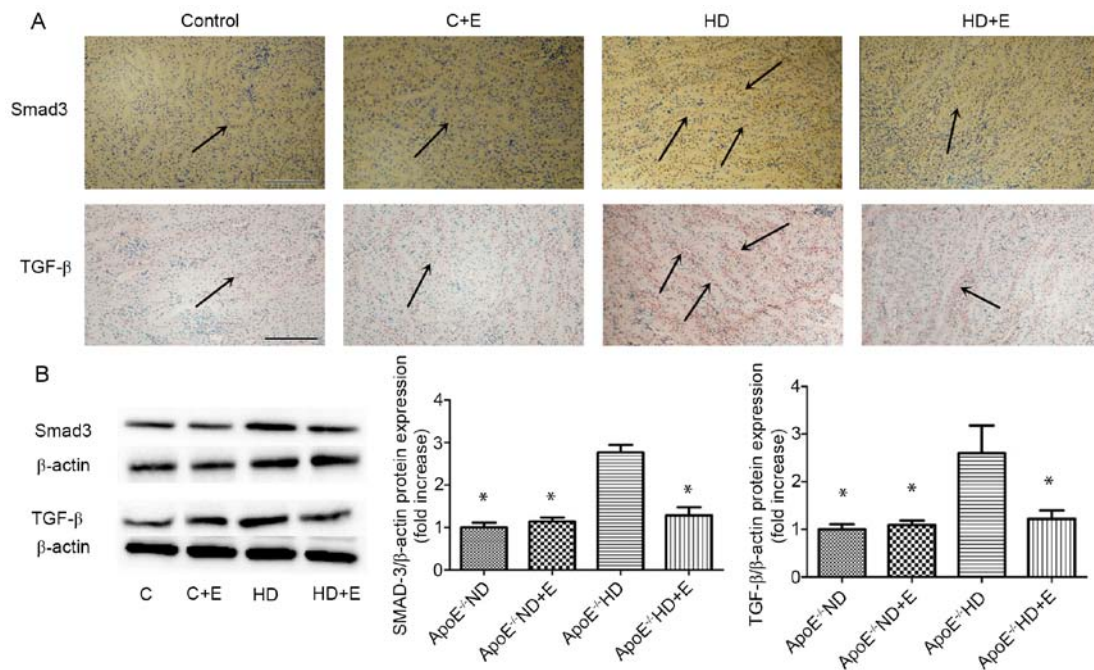


Figure 4. Characteristics of fibrosis. (A) Representative images of immunohistochemistry staining for TGF- β and Smad3 in kidney tissues of mice with different treatments. Scale bar, 100 μ m. Arrows indicate positively stained cells. (B) Western blot analysis of TGF- β and Smad3 protein expression levels in renal tissues. Data are presented as the mean \pm standard error of the mean. $n=3$ /group. * $P<0.05$ vs. ApoE^{-/-} HD group. ApoE^{-/-}, apolipoprotein E-deficient; HD, high-fat diet; ND, normal diet; E, exercise.

levels were significantly higher in the ApoE^{-/-} HD + E mice compared with those in the ApoE^{-/-} HD mice.

Characteristics of fibrosis. To investigate the mechanism of fibrosis in kidney damage, TGF- β and Smad3 expression was visualized using IHC (Fig. 4A) and immunoblotting (Fig. 4B). IHC staining showed that the numbers of TGF- β - and Smad3-positive cells in the ApoE^{-/-} HD mice were markedly increased compared with the ND group. However, exercise markedly reduced this increase. Compared with in the ApoE^{-/-} HD mice, HD + E mice exhibited significantly reduced TGF- β and Smad3 expression levels, as determined by immunoblotting (Fig. 4B). These results indicated that exercise reduced TGF- β and Smad3 expression in ApoE^{-/-} HD mice.

NF- κ B signaling pathway. IHC analysis of NF- κ B was used to investigate kidney damage caused by hyperlipidemia (Fig. 5). NF- κ B protein expression in kidney tissues was lower in the ApoE^{-/-} HD + E mice compared with that in the ApoE^{-/-} HD mice.

Discussion

The present study demonstrated that exercise may exert a protective effect against kidney damage induced by hyperlipidemia, including limiting the progression of lipid deposition, oxidative stress and fibrosis. Compared with in ApoE^{-/-} mice that consumed a ND, higher LDL-c, TG and TC levels were observed in ApoE^{-/-} mice that consumed a HD, in agreement with Faran *et al* (16). Furthermore, CRE levels were higher in the HD group compared with those in the ND group. These results suggested that a hyperlipidemia-induced kidney damage model was established in the ApoE^{-/-} mice. Notably, LDL-c, TC and CRE levels were significantly lower in the HD + E mice

compared with those in the HD mice, suggesting that exercise exerted a protective effect in reducing LDL-c, TC and CRE levels in mice with kidney damage by progressive lipid deposition, but was less effective in altering TG levels. Several studies have indicated that exercise can attenuate kidney damage caused by other factors, such as cisplatin (6), cola (7) and hypertension (8).

Hyperlipidemia is a major independent risk factor for the development of kidney disease (17). In the present study, using H&E, PAS and Masson staining, it was shown that kidney tissue disorders, lipid deposition, inflammatory cell infiltration, collagen accumulation and increased fibrosis resulted in kidney damage in the HD group. However, kidney damage was significantly reduced in the HD + E group. These results suggested that exercise may reduce kidney tissue damage in ApoE^{-/-} mice fed a HD.

Oxidative stress and inflammation are important characteristics of CKD, and can induce its progression (18,19). The balance between oxidative stress and antioxidant defenses maintains stability in living organisms (20). Moreover, hypercholesterolemia has been reported to cause exacerbation of inflammation and increase oxidative stress in kidney tissues (21,22). Napoli and Lerman (23) showed that increased free radical production was associated with increased accumulation of cholesterol in serum and tissue (23). Increased lipid peroxidation is frequently observed concurrent with a reduction in endogenous antioxidants, such as SOD, catalase and GSH-PX (24). In the present study, SOD and GSH-PX levels were decreased in the HD group compared with those in the ND group, suggesting that the HD-induced lipid deposition and disrupted the oxidative stress/antioxidant defense balance. However, SOD and GSH levels were increased in the HD + E group compared with those in the HD group, suggesting that exercise reduced lipid peroxidation and enhanced antioxidant

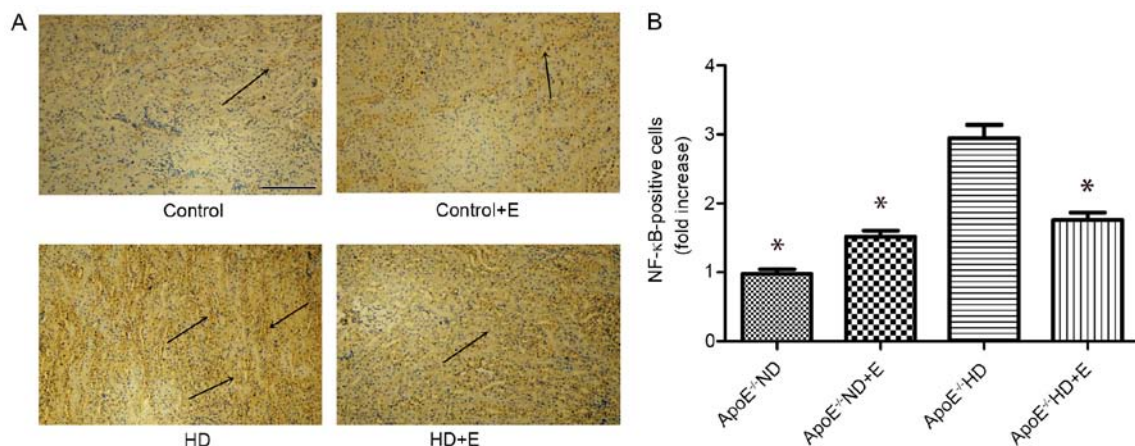


Figure 5. NF- κ B protein expression in the kidney tissue of mice in the different groups after 12 weeks of feeding with different diets. (A) Representative immunohistochemistry staining for NF- κ B in kidney tissues of mice with different treatments. Arrows indicate positively stained cells. (B) Bar graph showing the fold increase in NF- κ B-positive cells compared with in the ApoE^{-/-} ND group. Scale bar, 100 μ m. Data are presented as the mean \pm standard error of the mean. $n=3$ /group. * $P<0.05$ vs. ApoE^{-/-} HD group. ApoE^{-/-}, apolipoprotein E-deficient; HD, high-fat diet; ND, normal diet; E, exercise.

activity. NRF2 is a key regulator of the cellular response to oxidative stress (25). Physiologically, NRF2 is maintained in an inactive state through binding with Keap1 in the cytoplasm (26). Oxidative stress can induce nuclear accumulation of NRF2, upregulate downstream antioxidant gene transcription and promote the expression of antioxidant enzymes (27,28). In the present study, it was shown that the protein expression levels of NRF2 were higher in the HD group compared with those in the ND group, suggesting that oxidative stress was activated. Additionally, less oxidative stress was observed in the HD + E group compared with that in the HD group, as estimated by the lower expression levels of NRF2. Thus, exercise resulted in a protective effect against kidney damage caused by hyperlipidemia through attenuation of oxidative stress. Ishikawa *et al* (29) demonstrated that exercise can alleviate diabetic renal injury through reduced renal oxidative stress and inflammation, in agreement with the results of the present study.

Fibrosis is a characteristic of CKD and has been recognized as an independent predictor of the progression of kidney disease (30). In diabetic glomerular injury, oxidative stress has been shown to induce mRNA expression of TGF- β (31). TGF- β acts through a canonical signaling pathway that involves phosphorylation and activation of Smad3 by the TGF- β receptor, and then induces renal fibrosis (32). Renal fibrosis can thus be accelerated by increasing the levels of TGF- β and Smad3 (31,32). In the present study, collagen deposition was determined using Masson's staining. Heavy collagen deposition was prominently observed in the HD group, whereas exercise reduced collagen deposition in the HD + E group. In addition, the expression of TGF- β and Smad3 was examined using IHC and immunoblotting. Compared with in the ApoE^{-/-} HD group, TGF- β and Smad3 were significantly suppressed in mice in the ApoE^{-/-} HD + E group. This result indicated that exercise training reduced renal fibrosis caused by hyperlipidemia.

Several studies have shown that the NF- κ B signaling pathway is associated with fibrosis (33,34). NF- κ B is constitutively expressed in various types of tissues during inflammation and fibrosis (35-38). Inhibiting the activation of activated hepatic stellate cells to alleviate NF- κ B signaling has been shown to contribute to the treatment of hepatic fibrosis (39).

Furthermore, Zhang *et al* (40) showed that all-trans retinoic acid suppressed epidural fibrosis by regulating the NF- κ B signaling pathway (40). To investigate the inflammatory responses in kidney damage caused by hyperlipidemia, IHC analysis of NF- κ B was performed. NF- κ B protein expression in kidney tissues was lower in the ApoE^{-/-} HD + E mice compared with in the ApoE^{-/-} HD mice, thus suggesting that exercise may regulate the NF- κ B pathway, and improve inflammation and oxidative stress status in hyperlipidemia-induced kidney damage.

In conclusion, the results of the present study showed that exercise exhibited a protective effect against kidney damage caused by hyperlipidemia. Thus, exercise may be an additional means of clinical management of CKD.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YS and HL designed the present study. CQ and QY performed the experiments. HZ, XY and LG analyzed and interpreted the results of experiments. CQ prepared figures. QY drafted the manuscript. LG revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of Zhejiang Rongjun Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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