Silencing ferritin alleviates atherosclerosis in mice *via* regulating the expression levels of matrix metalloproteinases and interleukins

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This study was conducted to investigate the roles of ferritin in atherosclerosis. The mouse model of atherosclerosis was established by feeding ApoE knockout mice with a high-fat diet. The mice were then treated with ferritin-overexpressing and -silencing constructs, and assessed for interleukins (ILs) and matrix metalloproteinases (MMPs) levels using ELISA and Western blot analysis. After being fed with a high-fat diet, the ApoE knockout mice developed pro-atherogenic lipid profiles with elevated total cholesterol (TC), triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C). They also showed increased atherosclerotic lesions including narrowed lumen diameter, reduced lumen area, and increased plaque size. Following injection of the overexpression and silencing constructs, mRNA levels of ferritin were increased and decreased, respectively, and at the same time the atherosclerotic lesions were aggravated and alleviated, respectively. Further analysis indicated that silencing of ferritin gene reduced IL-1β and IL-10 levels while overexpressing ferritin increased them. On other hand, the TNF-α levels showed an opposite trend. MMP8, MMP12 and MMP13 levels were increased or decreased significantly after the mice were injected with ferritin over-expression or silencing vectors, respectively. Western blot analysis showed that compared to the control, overexpressing ferritin resulted in increased expression of p-JNK while silencing ferritin decreased the expression. Meanwhile, the levels of pc-Jun remained unchanged. Our work demonstrates that ferritin can regulate the progress of atherosclerosis *via* regulating the expression levels of MMPs and interleukins. Silencing ferritin inhibits the development of atherosclerosis and is, therefore, worth being further investigated as a potential therapeutic approach for this disease.

Keywords: atherosclerosis, interleukin, ferritin, tumor necrosis factor, matrix metalloproteinases, inflammatory reaction

INTRODUCTION

Cardiovascular and cerebrovascular diseases caused by atherosclerosis are common and leading causes of disability and death (Barquera *et al.*, 2015). Atherosclerosis is a chronic inflammatory disease affecting large and medium arteries (Fatkhullina *et al.*, 2016; Moss *et al.*, 2018). A better understanding of cellular and molecular mechanisms underlying the pathogenesis of atherosclerosis is important for developing new prevention and treatment strategies as well as therapeutics for the disease. Studies have shown that inflammatory response plays an important role in the pathogenesis of atherosclerosis. For example, inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6 and lipid mediators could promote inflammatory reactions in atherosclerotic plaque (Auguet *et al.*, 2016; Gostner & Fuchs, 2016). The abnormally elevated expression of matrix metalloproteinases (MMPs) can lead to atherosclerotic plaque rupture, acute myocardial infarction and other cardiovascular events (Newby, 2016). On the other hand, other cytokines such as IL-10, transforming growth factor β (TGF-β), and extracellular matrix (ECM) proteins can reduce atherosclerotic inflammation, leading to plaque regression (Hassan *et al.*, 2018; Rahman *et al.*, 2017).

Ferritin is an iron storage protein whose level reflects the iron reserve and iron load in the body (Golan *et al.*, 2021). It is elevated during infectious diseases, tumors and inflammation (Jaksch-Bogensperger *et al.*, 2020; Kim *et al.*, 2013). Studies have shown that iron at high concentration could accelerate the formation of free radicals and oxidation of atherogenic lipoproteins such as cholesterol (Cozzi *et al.*, 1990; Ikeda *et al.*, 2006; Tuomainen *et al.*, 2003). Therefore, increased ferritin and iron stores may be a risk factor for cardiovascular disease and have a causal role in the pathogenesis of atherosclerosis (Lauffer, 1991; MacDonald, 1993). Since pro-inflammatory and anti-inflammatory macrophages within arterial plaques have a different amount of intracellular iron (Kraml, 2017), it is still largely unclear how ferritin participates and regulates the atherosclerotic process, although iron level appeared to increase in the atherosclerotic plaques in deceased patients with coronary heart disease (Vlad *et al.*, 1994). In addition, high ferritin was shown to predict poor prognosis in patients with coronary artery disease (Zhu *et al.*, 2006).

To better understand the role of ferritin in pathogenesis of atherosclerosis, we examined the effect of ferritin

(FTH1 gene) on atherosclerosis and related inflammatory reactions using the mouse model of atherosclerosis. The findings would provide new insights on the relationship between ferritin and atherosclerosis and clues for the treatment of atherosclerosis.

MATERIALS AND METHODS

Animals

Specific pathogen-free male apolipoprotein E-knockout (ApoE$^{-/-}$) mice, aged 6 to 8 weeks, weighing 21.2 to 25.5 g, were purchased from Tsinghua Animals, Beijing, China. All experimental protocols for the use of animals were approved by the Animal Care and Use Committee of Hebei Medical University. All animal experiments complied with the ARRIVE guidelines. Mice were housed under pathogen-free conditions, had access to standard mice feed and water ad libitum and were maintained at a 12/12 hour day/night cycle in climate-controlled conditions (22±1°C, 40–70% humidity). Animals were sacrificed by CO_2 asphyxiation after completion of the experiments and tissues were collected. CO_2 was supplied at a flow rate of 20% of the cage volume per minute (5 L/min). The death after exposure to carbon dioxide was confirmed based on a careful assessment of the mice for cardiac arrest.

Reagents and instruments

Multi-shRNA vector pLKO.1-TRC was obtained from Addgene, USA; 293A cells were purchased from ThermoFisher Scientific, USA; automatic biochemical analyzer was purchased from Sigma-Aldrich, USA; blood lipid test reagents and ELISA kits were purchased from Wallysong Biotechnology, Wuhan, China; Trizol reagent (CW0580S) and Ultrapure RNA Extraction Kit (CW0581M) were products of CWbiotech, Beijing, China; cDNA Synthesis Kit (CW2141S) was obtained from Cwbiotech, Beijing, China; 2x SYBR Green PCR Master Mix (A4004M) was obtained from Lifeint, Beijing, China; mouse anti-human monoclonal antibodies against phospho-Ser63 c-Jun (pc-Jun) (1,50, ab195924) and phospho-Thr183/185 JNK (p-JNK) (1,50, ab131499) were obtained from Abcam, Cambridge, UK; HiScript II SuperMix for qPCR (R223-01) was from Vazyme, USA; PVDF membrane (IPVH00010) was a product of Millipore, USA; real-time fluorescent PCR system (CFX Connect) was obtained from Biorad, Shanghai; ultrasensitive luminescent solution (RJ239676) and pENTR Directional TOPO Cloning Kit were purchased from ThermoFisher Scientific, USA.

Construction of silencing and overexpression vectors

Using ThermoFisher's online RNAi Designer (https//www.thermofisher.com/us/en/home/life-science/rnai/vector-based-rnai.html), short hairpin RNA sequences for ferritin (shRNA, 5'-ATTTTTGGCAACTGC-CTCTG) were designed and inserted into pLKO.1-TRC. The lentivirus vector was amplified in 293A cells, tittered according to the manufacturer's instructions and stored at -80°C before use. To construct overexpression vector RNA was extracted from mice using Trizol RNA extraction kit and reversely transcribed into cDNA using cDNA synthesis kit according to the manufacturers' protocols. The cDNA was used as a template to amplify ferritin coding sequence using a pair of primers (forward 5'-CTGCAGATGCTTCTCAAGGCCTCCGC-

CGCTCTC, reverse 5'-GGTACCCTATGCCTGATG-CAACTTTCCTTCATCCAGCAGCA). The amplified DNA fragment was recovered from the gel and cloned into pENTR™ Directional TOPO to generate pENTR-ferritin according to the manufacturer's instructions.

Atherosclerosis model and treatments

ApoE$^{-/-}$ mice were fed a high-fat diet (containing 21.0% fat and 1.5% cholesterol, Research Diets, New Brunswick, NJ, USA) to generate an atherosclerosis model or regular diet (10% fat and 0% cholesterol, Research Diets, USA) for use as a control. After 24 weeks, three mice were randomly selected from each group to examine blood lipid profiles to determine whether atherosclerosis modeling was successful. The model animals were then randomly divided into three groups (n=10) to receive an intravenous injection of silencing construct (10 µl at 10^8 UT/mL), overexpression construct (5 µg at 1 µg/µL), or empty vector (5 µg at 1 µg/µL) via tail vein as previously reported (Gorgens et al., 2019). After injection, the animals were reared at the same conditions as described above for 20 days and sacrificed for analysis.

Blood lipid measurements

Venous blood was collected from three randomly selected mice per group and analyzed for total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) using COD-PAP and GPO-PAP methods according to the supplier's instructions.

Hematoxylin and eosin (HE) staining

HE staining was carried out to examine the plaque as described previously (Fischer et al., 2008). Briefly, the thoracic aortas were isolated and fixed in 4% paraformaldehyde, dehydrated in 70%, 80%, 90% and 100% alcohol and cleaned with xylene. Dehydrated tissue was embedded in paraffin, sectioned, dewaxed, and hydrated. The sections were stained with an aqueous hematoxylin solution for 3 min, differentiated with hydrochloric acid for 15 s, briefly washed, and counter-stained with eosin for 3 min. After being washed in distilled water, dehydrated and cleared, the sections were sealed and examined under a microscope for aortic morphological changes.

Real-time fluorescent quantitative PCR (qRT-PCR)

Total RNA was isolated from blood 5 days after injection of constructs using the Trizol reagent according to the manufacturer's instructions and was reversely transcribed to cDNA for mRNA expression analysis using cDNA Synthesis kit according to the manufacturer's protocols. HiScript II qRT SuperMix for qPCR was used for quantification of cDNA according to the manufacturer's protocol using primers for ferritin (forward, GCCGAGAAACTGATGAAGCTGC, reverse, GCACACTCCATTGCATTCAGCC). Normalization was performed with GAPDH. The PCR was carried out in a total volume of 10 µl containing 1.5 µl of diluted and pre-amplified cDNA, 10 µl of 2× SYBR Green PCR Master Mix and 1 µl of each fluorescence probe. The cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 45 cycles, each one consisting of 10 s at 95°C and 30 s at 58°C. Samples were run in triplicate and the mean value was calculated for each treatment. The data were managed according to the previously described protocol (Livak & Schmittgen, 2001).

Table 1. Levels of serum lipids and atherosclerotic lesions parameters in mice after feeding with the high-fat diet (model) and the normal diet (ND) for 24 weeks.

Lipids and atherosclerotic lesions	ND, mmol/L (n=6)	Model, mmol/L (n=6)	t	P
TG	0.88±0.23	4.89±0.63	2.811	0.022
TC	10.06±1.23	25.06±2.23	4.221	0.011
LDL-C	8.06±1.11	15.76±2.23	2.183	0.031
HDL-C	2.06±0.23	2.09±0.33	2.875	0.082
Minimal lumen diameter (mm)	0.82±0.08	0.55±0.03	7.815	0.012
Lumen area (mm^2)	0.44±0.02	0.35±0.03	6.215	0.017
Atherosclerotic plaque size (mm^2)	0.04±0.01	0.13±0.02	5.236	0.011

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected from mice 20 days after injection. Serum concentrations of IL-1β, TNF-α, IL-10, MMP8, MMP12, and MMP13 were measured using ELISA kits according to the manufacturer's protocols. The absorbance (OD value) was read with a plate reader at a wavelength of 450 nm within 15 min after adding the stop solution.

Western blot

Thoracic aorta tissues were lysed with RIPA buffer containing protease inhibitors to extract proteins. Total protein was quantitated using BCA kit according to the manufacturer's instructions. After denaturation, 50 μg of protein was separated using polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Non-specific binding was blocked by incubating in 5% non-fat milk in 1× Tris-buffered saline containing 0.1% Tween for 4 hours at room temperature and then the membranes were incubated with primary antibodies (mouse anti-human phospho-Thr183/185 JNK (p-JNK) (1.50) and mouse anti-human phospho-Ser63 c-Jun (pc-Jun) (1.50)) overnight. The blots were then incubated with goat anti- mouse horseradish peroxidase-conjugated secondary antibodies (1 2000) and the immunoreactive bands were visualized with a chemiluminescence kit. The gray values of bands were analyzed using Quantity One software.

Statistical analysis

All data were expressed as means ± standard error of the mean (S.E.M.) obtained from at least three independent experiments. Statistical comparisons between groups were performed using one-way ANOVA followed by the Tukey post-hoc test. A value of $P<0.05$ was considered statistically significant.

RESULTS

High fat-diet generated pro-atherogenic lipid profiles and atherogenic lesions in ApoE$^{-/-}$ mice

After being fed with a high-fat diet for 24 weeks, the animals were tested for serum lipid levels. The results showed that compared to the mice fed with normal diet (ND), high-fat diet feeding resulted in significantly elevated levels of pro-atherogenic lipids in ApoE$^{-/-}$ mice. The levels of TG, TC, LDL-C and HDLC increased from 0.88, 10.06 , 8.06 and 2.06 to 4.89, 25.06, 15.76 and 2.09 mmol/L, respectively ($P<0.05$, Table 1), while HDL-C remained unchanged ($P>0.05$, Table 1). The size

of atherosclerotic plaques was significantly greater in mice fed with the high-fat diet than fed with the normal diet. In addition, the vessel lumen was significantly narrowed ($P<0.05$, Table 1).

Ferritin silencing reduced atherosclerotic lesions

The atherosclerotic model mice were then intervened with ferritin silencing and over-expression constructs and assessed for atherosclerosis-related changes. PCR assay showed that five days after injection of silencing and overexpression vectors, the serum ferritin mRNA levels were significantly down and up-regulated (Fig. 1), indicating that the vectors were effective in reducing and increasing ferritin expression, respectively. 20 days after the intervention, compared to mice treated with an empty vector, the area of atherosclerosis plaques was significantly increased in the mice treated with ferritin over-expression vector and significantly decreased in the mice treated with the silencing vector ($P<0.05$, Table 2). The diameter and area of arterial lumen were significantly decreased in the mice treated with ferritin over-expression vector and significantly increased in the mice treated with ferritin silencing vector ($P<0.05$, Table 2).

Ferritin silencing changed IL-1β, IL-10, and TNF-α levels

We then measured IL-1β, IL-10, and TNF-α levels in the mice 20 days after injection. Results showed that compared to mice treated with an empty vector the levels of IL-1β and IL-10 levels were significantly increased or decreased in the mice treated with ferritin over-expression or silencing vector ($P<0.05$, Table 3), respectively. On the other hand, the levels of TNF-α levels were significantly decreased when the mice were treated with ferritin over-expression vector and significantly increased when the mice were treated with ferritin silencing vector ($P<0.05$, Table 3).
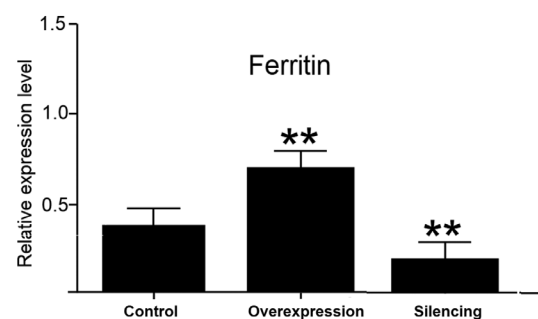

Figure 1. Serum ferritin mRNA levels following delivery of over-expression and silencing vectors in mice.
**denotes $P<0.01$ vs control.

Table 2. Atherosclerotic plaque size and minimal lumen diameter and area 20 days after atherosclerotic mice were treated with ferritin silencing and overexpression constructs.

Group	No. mice	Minimal lumen diameter (mm)	Lumen area (mm²)	Atherosclerotic plaque size (mm²)
Control (empty vector)	10	0.51±0.08	0.29±0.05	0.14±0.01
Overexpression	10	0.42±0.11[a]	0.25±0.04[a]	0.28±0.02[a]
Silencing	10	0.55±0.03[b]	0.39±0.06[b]	0.08±0.01[b]

[a]and [b]denote $P<0.05$ vs control and overexpression vector, respectively.

Table 3. Levels of IL-1β, IL-10 and TNF-α in mice 20 days after atherosclerotic mice were treated with ferritin silencing and overexpression constructs

Group	No. mice	IL-1β (pg/mL)	IL-10 (pg/mL)	TNF-α (pg/mL)
Control (empty vector)	10	20.51±3.08	10.04±1.11	16.29±3.25
Overexpression	10	24.21±2.88[a]	16.04±1.41[a]	12.19±3.15[a]
Silencing	10	16.51±1.08[b]	10.04±1.11[b]	33.04±5.15[b]

[a] and [b]$P<0.05$ vs control and overexpression vector, respectively.

Table 4. Levels of MMP8, MMP12, and MMP13 in mice 20 days after atherosclerotic mice were treated with ferritin silencing and overexpression constructs.

Group	No. mice	MMP8 (pg/µL)	MMP12 (pg/ µL)	MMP13 (pg/ µL)
Control (empty vector)	10	20.21±1.08	26.69±1.12	20.04±1.11
Overexpression	10	23.61±1.38[a]	29.33±1.12[a]	26.14±1.71[a]
Silencing	10	18.21±1.01[b]	22.49±1.02[b]	16.04±0.91[b]

[a] and [b] denote $P<0.05$ vs control and overexpression vector, respectively.

Ferritin silencing changed the expression of MMP8, MMP12, and MMP13

In addition, MMP8, MMP12, and MMP13 levels were measured and results showed that they were increased significantly following treatment with ferritin over-expression vector and significantly decreased after treatment with ferritin silencing vector ($P<0.05$, Table 4).

Ferritin silencing activated p-JNK/c-Jun signaling pathways

The expression of proteins in the p-JNK / c-Jun signaling pathways was assessed and results are shown in Figure 2. Compared to the control ferritin over-expression resulted in increased expression of p-JNK, while ferritin silencing decreased the expression of the protein ($P<0.05$, Fig. 2A). On the other hand, the levels of pc-Jun remained unchanged ($P>0.05$, Fig. 2B).

DISCUSSION

Ferritin is an important storage protein for iron supply and its role in atherosclerosis has not been fully elucidated at the molecular level, although clinical studies showed that patients with carotid atherosclerosis displayed elevated serum ferritin levels (Ma et al., 2015; Xu et al., 2017) and a population-based study in northeast Germany showed that there is a relationship between serum ferritin level and carotid atherosclerosis that was potentiated by LDL cholesterol (Wolff et al., 2004). The present study found that silencing FTH1 gene expression could alleviate the development of atherosclerotic lesions in atherosclerotic mice. FTH1 gene codes ferritin which is a major intracellular iron storage protein in prokaryotes and eukaryotes (Muhoberac & Vidal, 2019). Silenc-

ing this gene also reduced the levels of inflammatory cytokines and MMPs and down-regulated p-JNK signaling pathways. On the other hand, overexpressing the gene promoted the development of atherosclerotic lesions, increased the levels of inflammatory cytokines and MMPs, and up-regulated p-JNK signaling pathways. Since overexpression of ferritin would result in increased body iron
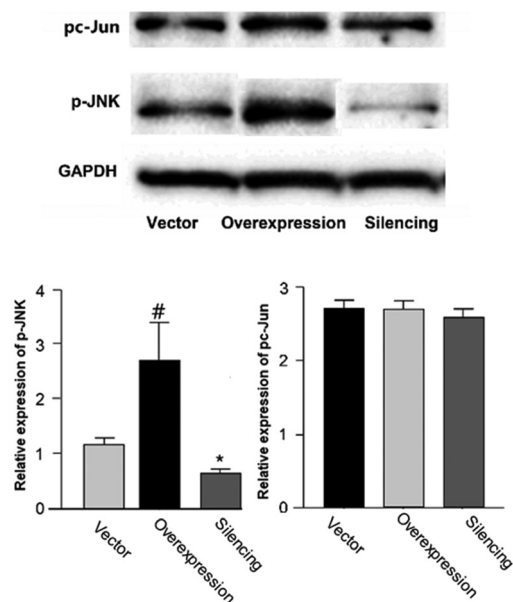

Figure 2. Expression of p-JNK and pc-Jun following overexpression and silencing of ferritin gene in mice.
Upper panel: representative Western Blots, lower panel: relative protein levels. * and #denote $P<0.05$ vs control.

load, this may lead to enhanced progression of atherosclerosis as previously reported (Araujo *et al.*, 1995; Lee *et al.*, 1999). Furthermore, previous studies showed that the expression of the ferritin gene is up-regulated in atherosclerotic vessels (Pang *et al.*, 1996), which is consistent with our observations that over-expression of ferritin has atherogenic activity, leading to increased plaque size and reduced aorta lumen diameter.

Inflammatory response plays an important role in the development of atherosclerosis which is regarded as a form of chronic vascular inflammation lesions (Soeki & Sata, 2016; Taleb, 2016). Measurements showed that the levels of inflammatory cytokines such as IL-1β and IL-10 were significantly increased following treatment with ferritin overexpression construct, and significantly reduced once ferritin silencing construct was used, suggesting that ferritin may regulate atherosclerosis progression via inflammatory pathways. These findings are consistent with the previous observation that the secretion of serum ferritin is regulated by inflammatory hormones (Tran *et al.*, 1997). For instance, it was found that IL-1β may increase the expression of both the heavy (H) and light (L) ferritin subunit (Rogers *et al.*, 1994) and ferritin could stimulate secretion of IL-10 and TNF-α in mice (Wang *et al.*, 2017). Since ferritin level is statistically correlated with the levels of inflammatory biomarkers such as TNF-α, IL-10, and high-sensitivity C reactive protein (hs-CRP) and mortality of patients with the peripheral arterial disease (PAD), including atherosclerosis (Depalma *et al.*, 2010), it is likely that ferritin has pro-atherogenic activity and further study is needed to investigate the mechanisms underlying ferritin-induced elevation of inflammatory hormones.

In atherosclerotic lesions, monocyte chemotaxis is induced to transform monocytes into macrophages. An important component of the inflammatory response is the secretion of MMPs to promote plaque rupture and to produce inflammatory cytokines such as TNF-α, IL-1β as well as lipid mediators to promote the inflammation in the plaque (Moore *et al.*, 2013; Ruytinx *et al.*, 2018). On the other hand, some reparative macrophages, such as alternatively activated M2 macrophages, could phagocytize dead cells or damaged tissue and release IL-10, TGF-β and ECM proteins to resolve inflammation and stabilize or even reverse atherosclerosis (Barrett, 2020). Our study showed that ferritin increases the levels of IL-1β and IL-10 as well as the levels of MMPs, suggesting that ferritin may influence the polarization of macrophages, leading to an increased inflammatory response. Previously, it was found that iron overload could result in M2-like polarization of macrophages (Kao *et al.*, 2020) and expression of the heavy subunit of ferritin is a key factor determining the macrophage polarization in isolated bone marrow-derived mouse monocytes (Bolisetty *et al.*, 2015).

Our study showed that silencing of ferritin down-regulates the expression of MMP8, MMP12, and MMP13. MMPs and their endogenous tissue inhibitors (TIMPs) play complex dual role during late-stage progression and rupture of atherosclerotic plaques. MMPs can degrade the fibrous cap of the lesion, resulting in the rupture of the lesion, subsequent thrombus formation (Zhang *et al.*, 2017), and acute myocardial infarction (Hong-Brown *et al.*, 2015). Therefore, selective MMP inhibition would help limit cardiovascular morbidity and mortality.

p-JNK/c-Jun signaling pathway is a major signaling cassette of the mitogen-activated protein kinase (MAPK) signaling pathway. It is involved in a number of cellular processes, including proliferation, embryonic development and apoptosis. Our data showed that JNK was down-regulated when the expression of ferritin was suppressed, suggesting that ferritin may regulate the progression of atherosclerosis *via* the p-JNK/c-Jun signaling pathway. Early works also showed that when berberine was used to suppress atherosclerosis (Wan *et al.*, 2018) or the chemerin gene was knocked down to alleviate atherosclerotic lesion (Liu *et al.*, 2019), p-JNK expression was down-regulated, suggesting that p-JNK expression is associated with atherosclerosis.

CONCLUSION

By overexpressing and silencing the ferritin gene, our work demonstrated that ferritin regulates atherogenesis in ApoE$^{-/-}$ mice. Silencing ferritin alleviated atherosclerotic lesions, reduced the levels of inflammatory cytokines and MMPs and deactivated the p-JNK/c-Jun signaling pathway, while ferritin overexpression resulted in opposite outcomes. Further study is needed to investigate the mechanism underlying the ferritin-mediated regulation of atherosclerosis.

DECLARATIONS

Ethics approval and consent to participate

This study was approved by the Animal Care and Use Committee of Hebei Medical University, Hebei, China.

Consent for publication

Not applicable.

Availability of data and material

The datasets used during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MZ, LL and XQ designed the study. MZ, LL, YL and YL conducted the experiments, collected the data and performed analysis. YL, YL and XQ drafted the manuscript. All authors read and approved the final version of the manuscript.

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