*Original Research* *Highlight article*

# Atherosclerotic lesion-specific copper delivery suppresses atherosclerosis in high-cholesterol-fed rabbits

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### Impact statement

Copper reduction in the aortic wall is attributive to atherosclerosis induced by high-cholesterol feeding. A novel ultrasound contrast microbubble-assisted target-specific copper delivery procedure replenishes copper in the atherosclerotic lesion tissue, and blocks the progression of atherosclerosis without a disturbance of the stability of the lesion in the high-cholesterol-feeding rabbit model. This work demonstrates a high likelihood of an alternative approach to clinical treatment of atherosclerosis.

## Abstract

Dietary cholesterol supplements cause hypercholesterolemia and atherosclerosis along with a reduction of copper concentrations in the atherosclerotic wall in animal models. This study was to determine if target-specific copper delivery to the copper-deficient atherosclerotic wall can block the pathogenesis of atherosclerosis. Male New Zealand white rabbits, 10-weeks-old and averaged 2.0 kg, were fed a diet containing 1% (w/w) cholesterol or the same diet without cholesterol as control. Twelve weeks after the feeding, the animals were injected with copper-albumin microbubbles and subjected to ultrasound sonication specifically directed at the atherosclerotic lesions (Cu-MB-US) for target-specific copper delivery, twice a week for four weeks. This regiment was repeated 3 times with a gap of two weeks in between. Two weeks after the last treatment, the animals were harvested for analyses of serum and aortic pathological changes. Compared to controls, rabbits fed cholesterol-rich diet developed atherosclerotic lesion with a reduction in copper concentrations in the lesion tissue. Cu-MB-US treatment significantly increased copper concentrations in the lesion, and reduced the size of the lesion. Furthermore, copper repletion reduced the number of apoptotic cells as well as the content of cholesterol and phospholipids in the atherosclerotic lesion without a disturbance of the stability of the lesion. The results thus demonstrate that target-specific copper supplementation suppresses the progression of atherosclerosis at least in part through preventing endothelial cell death, thus reducing lipid infiltration in the atherosclerotic lesion.

**Keywords:** Atherosclerosis, copper, cholesterol, phospholipid, collagen, rabbits

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## Introduction

Dietary cholesterol supplements cause hypercholesterolemia and atherosclerosis in animal models.[1] Although the deposition of fatty substances and the hardening of the vascular wall in large-/medium-sized arteries are critical events in atherosclerosis,[2] changes in other vital compositions in the affected vessel wall are also noticed. Among these changes is a significant reduction of copper concentrations in the atherosclerotic wall compared to the normal aortic wall.[3,4] Therefore, copper deficiency has been postulated to be a trigger event of atherosclerosis in high-cholesterol-fed animals in addition to multiple other hypotheses on the etiology of atherosclerosis induced by high dietary cholesterol.[5–7]

Copper is an essential trace element in biological systems and is a constituent of superoxide dismutase and ceruloplasmin; both are importantly involved in preventing oxidative injury.[8,9] It was known that the oxidative modification of low-density lipoprotein (LDL) is a key event in human atherosclerosis.[10–12] Therefore, copper reduction in the aortic wall could weaken the antioxidant capacity leading to unchecked progression of atherosclerosis. In addition, it has been shown that copper is closely associated with cholesterol metabolism in human studies.

Two cross-sectional clinical studies with apparently healthy subjects showed that serum copper was associated negatively with low-density lipoprotein cholesterol (LDL-C), suggesting that a high copper level is linked to a better lipid metabolic state.[13] Importantly, dietary copper supplementation along with high-cholesterol feeding effectively prevented atherosclerosis in animal models, experimentally attesting the involvement of copper in cholesterol metabolism.[14]

Many studies from animal models to human clinical data found that plasma copper levels are significantly elevated along with hypercholesterolemia in atherosclerotic subjects.[15,16] Copper ions catalyze oxidative modification of LDL in vitro[17,18] and there are also reports on copper participation in the oxidation of LDL in vivo.[19,20] The increase in plasma copper from high-cholesterol feeding would promote oxidative modification of LDL, thus promoting atherosclerosis. Therefore, a simple dietary copper supplementation would augment the elevation of plasma copper concentrations, enhancing the process of copper-promotion of atherosclerosis.

Therefore, an organ specific copper delivery system would be a better solution for copper supplementation to the copper-deficient organ avoiding a further increase in serum copper concentrations. For this purpose, we developed a target-specific, ultrasound contrast microbubble-copper delivery procedure (Cu-MB-US) to increase copper content specifically in the atherosclerotic lesion in a high-cholesterol feeding rabbit model. We examined the effect of copper supplementation on the size, composition, and stability of the established atherosclerotic lesion.

## Materials and methods

### Experimental animals and treatments

Male New Zealand white rabbits (10-week-old), weighing 1.8–2.2 kg, were housed individually in metal cages in the experimental animal care and husbandry facility at the Sichuan University West China Hospital Research Center. Animals were free to access food and drinking water. All animal protocols were approved by the Institutional Animal Care and Use Committee at the Sichuan University West China Hospital, following the guidelines of the US National Institutes of Health (Approval no. 20211205A), and the research team signed terms of commitment.

Rabbits were randomly assigned to a group fed a laboratory rabbit HF (High Fiber) diet from LabDiet (Florida, USA) containing 1% (w/w) cholesterol purchased from GEN-VIEW (Florida, USA) for 12 weeks, and to the control group fed the same diet without cholesterol supplementation. After the 12-weeks high-cholesterol feeding, 10 rabbits were harvested as a prior-treated group; and 30 rabbits were switched to normal diet feeding: with a further division to three groups (10 in each group) for untreated controls, ultrasound-directed microbubble treatment (MB-US), or ultrasound-directed copper-microbubble treatment (Cu-MB-US).

### Cu-MB preparation and evaluation

The following solutions: $CuSO_4$ (1 mg $mL^{-1}$), human serum albumin (HSA, 5%, Chengdu Rongsheng Pharmaceuticals Co. Ltd) in saline, and 5% glucose solution were mixed at a ratio of 3:1:9 in a test tube, filled with perfluoropropane ($C_3F_8$) to a saturation point, and sonicated with a 400 W digital sonifier (450 D, Branson) using a 3/4″ diameter sonic horn, at an amplitude of 30% for 60 s, then 80% for 40 s. A series of tests were performed to determine the physical characteristics and copper content of Cu-MB, including measuring the concentration of Cu-MB using a Coulter Counter (Multisizer 4e; Beckman Coulter, Inc., USA), and analyzing the size variation using a Laser Particle Size Analyzer (LB-550, Horiba). Copper concentrations in Cu-MB were measured by graphite furnace atomic absorption spectrophotometer (AAS, ICE3500; Thermo). For vehicle control, HSA (5%) and 5% glucose solution were mixed at the ratio of 1:3 and filled with $C_3F_8$ and sonicated to generate albumin-coated microbubble (MB). The prepared Cu-MB and MB were sterilized and stored at 4 °C.

### Ultrasound-directed copper-microbubble treatment (Cu-MB-US)

Rabbits were kept in the supine position on a designated fixation plate. For the Cu-MB-US group, Cu-MB solution (5 mL) containing 0.05 mg/ml copper was infused via ear vein while a continued ultrasound impulse was applied directly pointing to the atherosclerotic lesions in the renal artery plane of the abdominal aorta. Ultrasound power was generated from L9-3 transducer (iU22, Philips). The entire treatment was completed in 5 min. In the MB-US group, the rabbits received the same treatment, except MB solution was administered instead of Cu-MB. This procedure was carried out twice a week for 4 weeks followed by a two-week rest, repeating 3 times for complete treatment (a total of 18 weeks treatment period).

### Blood sampling

Blood was drawn from the ear artery between 9:00 and 10:00 a.m. after overnight fasting. Serum was obtained by centrifugation at 4 °C, and stored at –80 °C for analysis.

### Aortic or atherosclerotic lesion tissues collection

At the end of the feeding and Cu-MB-US treatment, rabbits were sacrificed by an overdose of sodium pentobarbital solution (1.5 ml/kg intravenously) (Rhone Merieux, Harlow, Essex, UK). The ultrasound-irradiated abdominal aortas were isolated, cleaned free from adherent fat and fascia, and divided into several segments for late analyses: one half of the specimen was kept in liquid nitrogen for the measurement of tissue copper concentration and the measurement of lipid compositions and LOX activity, and the other was cut into small segments and embedded in paraffin wax or in an optimal cutting temperature compound gel (OCT, Leica, Germany) for histological examination.

### Measurement of copper concentrations

Copper concentrations were determined by graphite furnace atomic absorption spectrometry (AAS) (ICE3500, Thermo, USA), as described in our previous studies.[21]

Vessel tissue samples and serum samples were digested with nitric acid ($HNO_3$, Sigma, USA) at 60 °C overnight. Copper concentration was normalized by the dry weight of vessels or the volume of serum.

### Histological examination of atherosclerotic lesions

The renal artery was open longitudinally and stained with oil red O (G1260, Solarbio Life Sciences). A staining working solution was prepared by diluting the saturated oil red O stock solution with deionized water (3:2). The final solution was passed through a 0.2-μm filter paper before use. The polymethyl-fixed tissue samples were placed in the working solution in an oven at 37 °C for 1 h followed by a washing with 60% isopropanol to remove nonspecific staining. The area of atherosclerotic lesion was measured using an image analyzer Image J, and the ratio of atherosclerotic lesion area to the entire surface area of the artery was calculated.

### Measurement of lipids in the atherosclerotic lesion tissue

The lipid components in the atherosclerotic lesion including cholesterol (CHOL) and phospholipid (PL) were detected using a commercially available method (CHOL: A111-1, Nanjing Jiancheng Institute of Biological Engineering; PL: Mak122, Sigma Corporation) following the provided instruction. The frozen lesion tissue cleaned free from adherent fat and fascia was lyophilized and homogenized, and the homogenates were centrifuged and the cholesterol or phospholipid content in the supernatant was assayed. Lipid contents were normalized by the dry weight of lesion tissue.

### Detection of apoptosis

Apoptotic cell death was detected in situ using a TUNEL staining 50 kit (Roche, IN, USA) following the manufacturer's instruction. The tissue sections were fixed with 4% paraformaldehyde for 15 min, washed with PBS for 2 times, 3 min each time, and treated with a precooled permeabilization solution (0.1% Triton X-100) for 2 min. After washing (PBS for 2 times, 3 min each time), the labeling reaction was carried out in a solution containing terminal deoxynucleotidyl transferase and fluorescein-dUTP at 37°C for 1 hour. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma, D9542). TUNEL staining was examined by confocal microscope (ECLIPSE Ti A1, Nikon).

### Analyses of collagen deposition

Picrosirius red was used to stain collagen fibers as previously described.[22] Briefly, 6-μm-thick formaldehyde-fixed sections of abdominal aortas were stained in saturated picric acid containing 0.1% picrosirius red (Direct Red 80, SUI) for 0.5 h. Images were captured by a light microscope. The total collagen content of lesions was calculated as the ratio of red area to the lesion area using image analyzer Image J. The color in picrosirius red-stained sections varies under a polarized light microscope, which reflects the type of collagens: collagen I appears as yellow-red fibers, whereas collagen III appears as green fibers. We investigated whether copper repletion would change the stability of the atherosclerotic lesion by examining the effect of copper repletion on the variation of collagen contents and the ratio of collagen I/collagen III in the lesion.

### Immunohistochemical analysis of smooth muscle cells (SMCs)

The number of smooth muscle cells (SMCs) in the lesion also affects the stability of the atherosclerotic lesion, we determined the effect of copper repletion on SMCs. Tissue sections were incubated overnight with primary antibodies (Anti-HHF35, C34931, Enzo) at 4 °C, and were subsequently incubated with secondary anti-mouse IgG labelled with polymer and horseradish peroxidase (8125S, Cell Signaling Technology, USA) for 60 min at 37 °C according to the manufacturer's instruction. The immunoreactants were visualized using a diaminobenzidine substrate kit (Cell Signaling Technology, USA). Images were captured with a light microscope. The accumulation of SMCs was calculated as the ratio of brown positive area to the lesion area using image analyzer Image J.

### LOX activity

Collagen cross-linking catalyzed by lysyl oxidase (LOX) was another important factor influencing the stability of the atherosclerotic lesion. A fluorimetric LOX assay (AAT Bioquest, USA) was used for the detection of LOX enzyme activity. Frozen aortic and atherosclerotic lesion tissue samples were homogenized in PBS supplemented with a 1% complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Germany). Equal amount of proteins in each sample was ensured by prior quantitation using the Bio-Rad assay procedure (Bio-Rad Laboratories, USA). A 50 μl portion of the assay reaction mixture was added to each well of blank control (assay buffer) and test samples to obtain a total LOX assay volume of 100 μl per well. The wells were then incubated at 37 °C for 15 min. Fluorescence was excited at 560 nm and the emission was measured at 590 nm using a Bio-tek Synergy Multi-Mode reader (USA).

### RT-qPCR analysis

Matrix metalloproteinases (MMPs)-mediated degradation of collagens is critically responsible for the instability of atherosclerotic lesion. We analyzed the effect of copper repletion on the transcriptional levels for MMP2, MMP3, and MMP9. Total RNA was extracted from aortic and lesion tissues and purified in Trizol reagent (Invitrogen, USA) according to the manufacturer's instruction. The integrity of total RNA was verified by agarose gel electrophoresis, RNA concentration was quantified using a NanoDrop 2000

spectrophotometer (Thermo Fisher) and reverse transcribed to complementary DNA (cDNA) by using a Prime Script® RT reagent kit (TaKaRa, Japan) in the MJ Mini Personal Thermal Cycler (Bio-Rad, USA). The amount of cDNA corresponding to 1 ng of RNA was amplified using a SYBR green PCR kit (Bio-Rad Laboratories) with the primers for MMP2, MMP3, MMP9, and GAPDH. The primer sequences were listed as follow: MMP2 (forward: GAAGGTCAAGTGGTCCGTGT, reverse: CCGTACTTGCCATCCTTCTC); MMP9 (forward: CGCCGAGATA GGGAACAAGC, reverse: GGCAGTGCAGGATGTCAA AGC); MMP3 (forward: ACCCAGTCTACAACGCCTTC, reverse: GAGGGACAGGTTCCATAGGC); GAPDH (forward: AGGTCGGAGTGAACGGATT, reverse: ATGGC GACAACATCCACTTT).

### Statistical analysis

All data are expressed as mean ± SD. Normality of the data was determined via the Kolmogorov–Smirnov test. The analysis among untreated, MB-US and Cu-MB-US groups was carried out using one-way ANOVA followed by Tukey's multiple comparisons test (parametric test) or Kruskal-Wallis test (non-parametric test). Two-tailed bivariate correlations were estimated by the Pearson's coefficient. All analyses were conducted using standard statistical software (SPSS 21.0, USA). Differences are considered statistically significant at $P < 0.05$ level.

## Results

### Reversal of atherosclerosis by Cu-MB-US treatment

Atherosclerotic lesion area surface increased from an average 68.54 to 78.96% after 12 weeks of high-cholesterol feeding followed by 18 weeks of standard rabbit diet feeding. In the Cu-MB-US group, lesion surface decreased an average of 24.15% (average 78.96% without treatment to an average 54.81% after treatment) after treatment completion. In the MB-US group, lesion surface showed no signs of decrease (average 78.96% without treatment to an average 77.30% after treatment). Lesion surface decrease was statistically higher in the Cu-MB-US group ($P < 0.05$) (Figure 1(a)). Copper concentrations in the induced atherosclerotic lesion tissue were significantly decreased in comparison to that in normal aortic wall. It was found that copper concentrations increased in the Cu-MB-US group (Figure 1(b)). Further analysis revealed that there was an inverse correlation between copper concentrations and the size of the atherosclerotic lesion (Figure 1(c)). Serum copper concentrations increased significantly in rabbits with atherosclerotic lesions. This increase was unchanged after treatment both in both groups (Figure S1a). There were some slight increases in serum homocysteine levels by high-cholesterol feeding and the treatment with either MB-US or Cu-MB-US did not cause further changes (Figure S1b). Finally, both total SOD and Cu, Zn-SOD activities were decreased in the atherosclerotic lesion tissue and the treatment with MB-US or Cu-MB-US did not affect this change (Figure S1c).

### Reduction of lipid components in the atherosclerotic lesion by Cu-MB-US

The change of lipid components in the atherosclerotic lesion was determined to characterize the nature of the copper repletion-induced size reduction of the lesion. The results showed that copper repletion significantly reduced the cholesterol as well as phospholipid contents in the lesion tissue (Figure 2(a) and (d)). Further analysis revealed that the cholesterol (Figure 2(b)) and phospholipid (Figure 2(e)) level was positively correlated with the size of the atherosclerotic lesion. Consequently, copper levels were inversely correlated with the levels of cholesterol (Figure 2(c)) or phospholipid (Figure 2(f)) in the lesion. Since lipid infiltration is associated with impaired endothelial lining, as the result of endothelial cell apoptosis leading to exfoliation and dysfunction of the endothelial monolayer, we determined the effect of copper repletion on apoptosis in the atherosclerotic lesion. As shown in Figure 2(g), both Cu-MB-US and MB-US treatments significantly reduced apoptosis in the lesion, although the treatment with Cu-MB-US was more effective.

### Unchanged stability of the atherosclerotic lesion by Cu-MB-US

The results showed that copper repletion did not change collagen contents and the ratio of collagen I/collagen III in the lesion (Figure 3(a) and (b) and Figure S1d). The LOX activity and the SMCs contents in the lesion were unchanged after the treatment (Figure 3(c) and (d)).

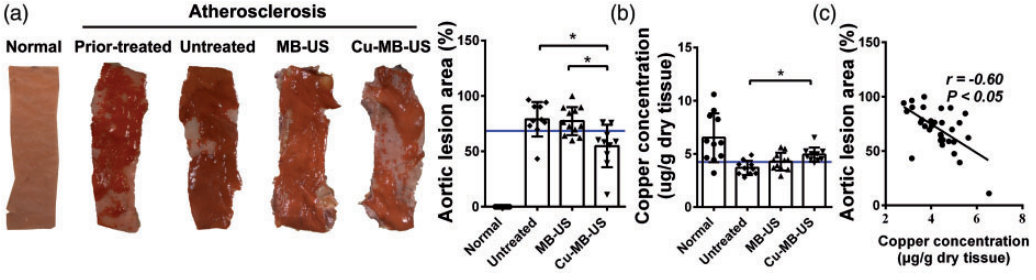


**Figure 1.** Cu-MB-US induced repression of atherosclerosis. (a) Representative ORO staining of abdominal aorta (left) and quantitative analysis of lesion aera (right). (b) The changes of copper concentrations in the aorta (intima-media membrane). (c) Linear correlation between copper concentrations and the severity of the atherosclerotic lesion, the regression coefficient $r = -0.60$. The blue line indicates the mean of prior-treated group. Data are presented as mean ± SD, $^*P < 0.05$. $n = 10–11$. (A color version of this figure is available in the online journal.)

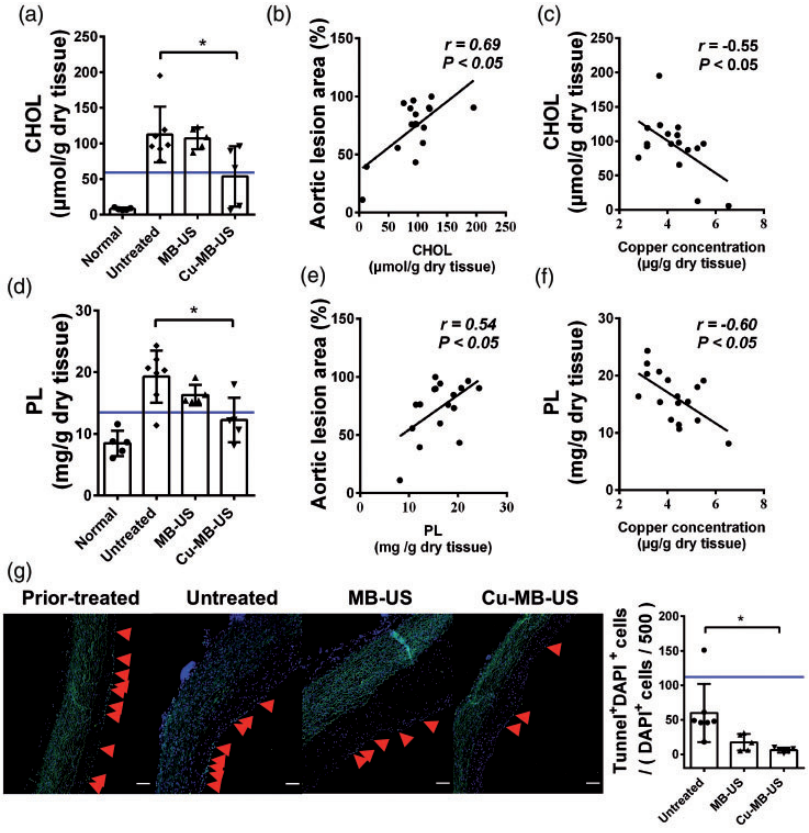


**Figure 2.** Effect of Cu-MB-US treatment on lipid content in the atherosclerotic lesion. (a) The changes of cholesterol (CHOL) content in the aorta (intima-media membrane). (b) Linear correlation between CHOL content and the severity of the atherosclerotic lesion, the regression coefficient $r = 0.69$. (c) Linear correlation between copper concentrations and CHOL content, the regression coefficient $r = -0.55$. (d) The changes of phospholipid (PL) content in the aorta (intima-media membrane). (e) Linear correlation between PL content and the severity of the atherosclerotic lesion, the regression coefficient $r = 0.54$. (f) Linear correlation between copper concentrations and PL content, the regression coefficient $r = -0.60$. (g) Representative images of apoptotic cells in the atherosclerotic lesion, identified by co-staining with TUNEL (green) and DAPI (blue) (left) and quantification of apoptotic cells in the atherosclerotic lesion (right). Scale bar = 100 μm. The blue line indicates the mean of the prior-treated group. Data are presented as mean ± SD, $^*P < 0.05$. $n = 5$–7. (A color version of this figure is available in the online journal.)

Importantly, it was found that Cu-MB-US actually decreased the transcriptional levels of the MMP2, MMP3, and MMP9 (Figure 3(e)).

## Discussion

There are several lines of evidence that support the copper deficiency hypothesis of cholesterol-induced atherosclerosis.[5,9,14,23] The present study was designed to specifically address the effect of atherosclerotic lesion-specific copper repletion on the progression of atherosclerosis. Using an ultrasound-directed copper-albumin microbubble copper delivery procedure, we showed here that atherosclerotic lesion-specific copper supplementation indeed increased copper concentrations in the lesion, along with a reduction of the atherosclerotic lesion size, and decreases in cholesterol and phospholipid contents in the lesion. However, these effects were not associated with any disturbance in the stability of the atherosclerotic lesion.

Although the reason for copper loss in the atherosclerotic wall is unknown, a recent study demonstrated an inverse correlation between copper concentrations and the severity of atherosclerotic lesion.[24] It was interesting to note that the reduction of copper in the atherosclerotic lesion was associated with an increase in the serum copper concentrations.[24] Thus, a simple dietary copper supplementation could not be a reasonable approach to replenish copper in the copper-deficient vessel wall due to the risk of adverse effects of further serum copper elevation.

In the present study, we used a copper-albumin microbubble in combination with ultrasound-assisted target-specific delivery procedure to specifically supplement copper to the atherosclerotic lesion site. This copper delivery is accomplished by the ultrasound impulse-induced collapse of copper-albumin microbubbles and an instant cell cavitation to promote the penetration of copper into lesion tissue.[25] This procedure indeed effectively delivered copper to the target tissue. We also noticed that the albumin microbubble alone with the same ultrasound treatment also increased copper concentrations in the lesion tissue. Since albumin is a major naturally-occurred copper-binding protein in the serum for copper target organ delivery, it is possible that the high concentration copper in the circulation of the atherosclerosis animals would interact with the instantly increased albumin to form the copper-albumin complex. This naturally-formed copper-albumin microbubble in vivo would act as the same as the premade Cu-MB in response to the ultrasonic impulse to deliver copper to the atherosclerotic lesion site. However, the amount of copper delivered by this mode was not as much as that delivered by the

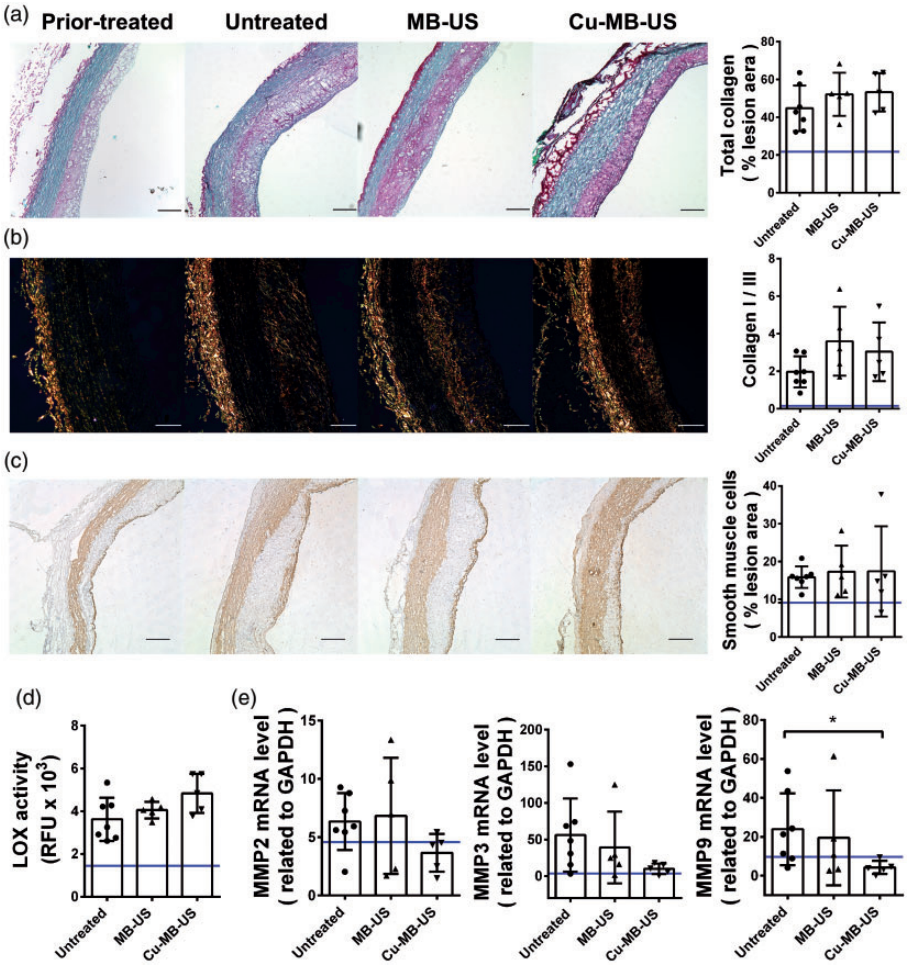


**Figure 3.** Changes of collagen compositions, LOX activity, and mRNAs for MMPs in the atherosclerotic lesion. (a) Collagen (red) deposition in atherosclerotic lesion identified by Sirius red (SR) staining (left) and semi-quantitative analysis of total collagen content in the atherosclerotic lesion (right). Scale bar = 200 $\mu$m. (b) Polarization microphotography of the SR staining sections, yellow–red collagen fibers (type I collagen) and green collagen fibers (type III collagen) (left) and semi-quantitative analysis of the ratio of type I/III collagen (right). Scale bar = 150 $\mu$m. (c) Immunohistochemical staining (marked by HHF35) of SMCs in the atherosclerotic lesion (left) and semi-quantitative analysis of SMCs in the atherosclerotic lesion (right). Scale bar = 200 $\mu$m. (d) The change of LOX activity in the aorta (intima-media membrane). (e) The changes of mRNA levels for MMP2 (left), MMP3 (middle) and MMP9 (right) in the aorta (intima-media membrane). The blue line indicates the mean of prior-treated group. Data are presented as mean ± SD, $^*P < 0.05$. $n = 5$–7. (A color version of this figure is available in the online journal.)

premade copper-albumin microbubble. Thus, the effects of copper repletion by this mode on varying measurements were not as effective as that of Cu-MB-US.

Copper replenishment significantly reduced the concentrations of cholesterol and phospholipid in the lesion tissue, and copper concentrations in the lesion tissue were inversely correlated with the size of the atherosclerotic lesion, as well as inversely with the concentrations of cholesterol and phospholipid in the lesion. The reduction of cholesterol and phospholipid would account for the reduced size of the atherosclerotic lesion. The endothelial cell dysfunction caused by endothelial cell apoptosis is the key factor leading to lipid infiltration in the vessel wall, a trigger for atherosclerosis.[26–28] In the atherosclerotic lesion induced by the cholesterol-rich diet feeding, there were abundant apoptotic cells in the endothelial layer. The treatment with Cu-MB-US effectively reduced the number of apoptotic cells. This would suggest that copper protection against apoptosis would result in a prevention of lipid infiltration in the lesion tissue. Since neither total SOD nor Cu, Zn-SOD activities were increased by the ultrasound-assisted copper delivery, the protective effect of copper may be related to the anti-apoptotic effect of copper per se, as demonstrated in previous studies.[29–31]

It was puzzling that copper repletion did not increase the activity of Cu, Zn-SOD in the lesion tissue. We have made an attempt to check any changes in copper transporters and chaperones in the lesion tissue, but failed to make any meaningful measurements due to the low expression of these molecules in the aortic wall. The activation of Cu, Zn-SOD requires copper transporters as well as copper chaperones to ensure copper intracellular trafficking for copper delivery to the binding site of the molecule, hence, the unchanged copper transporters and chaperones by this ultrasound-directed copper delivery would not activate Cu, Zn-SOD.

The instability of the atherosclerotic lesion poses a high risk for cerebrovascular and cardiovascular stroke. It is important to know if the lesion size reduction is associated with any changes in the stability of the lesion. Collagen

deposition and cross-linking catalyzed by LOX, as well as SMCs accumulation are major determinants for the stability of the lesion.[32,33] We found these parameters were not changed in association with the reduction of the lesion size, indicating the stability of the lesion was scurried in the Cu-MB-US reduced atherosclerotic lesion. Collagen degradation is critically involved in the instability of the atherosclerotic lesion.[34] MMPs are majorly responsible for the degradation process.[35,36] It was interesting to find that the treatment with Cu-MB-US reduced the mRNA levels for MMPs, further indicating the role of copper in securing the stability of the atherosclerotic lesion.

It is worth noting that the change of copper concentrations in human atherosclerotic tissues remains controversial.[37–41] There was study that showed in patients with coronary heart disease copper concentrations were decreased in the abdominal aorta.[41] Other study showed that patients with atherosclerotic occlusive disease had higher copper concentrations in calcified plaques than other types of lesions.[42] A difference in copper content between calcified plaques and fibrolipid plaques was also observed in patients with carotid atherosclerosis, with lower copper content in the fibrolipid plaques.[40] Therefore, the discrepancy in copper concentrations in human atherosclerotic tissues may reflect the stage of atherosclerotic lesions and various complications. However, in most cases of early stage of human atherosclerosis, copper concentrations were low in the affected aortic wall.[42,43]

In summary, the results obtained from the present study showed that atherosclerotic lesion-specific copper replenishment blocked the progression of atherosclerosis and reduced the lesion size without a disturbance of the stability of the lesion. This reduction was well correlated with the reduction of cholesterol and phospholipid in the lesion. This study further confirmed the observation published previously that copper reduction exaggerates the pathogenesis of atherosclerosis.[7,24] Importantly, copper suppression of atherosclerosis would provide an alternative approach to the treatment of the disease condition in humans.

## AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies and review of the manuscript; NW, XWX, HLL and HGW conducted the experiments; NW, XWX and HLL performed data analysis; QPF supplied critical reagents; YJK, NW and XWX wrote the manuscript, and YJK edited and approved the final version of the manuscript.

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The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## SUPPLEMENTAL MATERIAL

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