

ICAM-1-Carrying Targeted Nano Contrast Agent for Evaluating Inflammatory Injury in Rabbits with Atherosclerosis

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Abstract

Purpose

To investigate the feasibility of using ICAM-1-carrying targeted nano ultrasonic contrast to evaluate the degree of inflammatory injury at different stages in the abdominal aorta of rabbits with atherosclerosis (AS).

Methods

Twenty-five experimental rabbits were assigned to five groups: the preoperative control group (A); the week-4 after modeling group(B); the week-8 after modeling group(C); the week-12 after modeling group(D); the week-16 after modeling group(E). All groups were given 2D ultrasonography, conventional ultrasonic contrast (SonoVue), and ICAM-1-carrying targeted nano ultrasonic contrast, respectively. Contrast parameters, including the peak intensity (PI), time to peak (TTP), and area under curve (AUC) of the region of interest, were used to evaluate the characteristics of vascular perfusion contrast.

Results

ICAM-1-carrying targeted nano ultrasonic contrast showed that the intensity of targeted micro-signals in the vascular wall of the abdominal bubble aorta gradually increased in B, C, D, and E groups (all $P < 0.05$). A positive linear correlation between PI and AUC and the expression of ICAM-1 ($r = 0.893, P < 0.001; r = 0.934, P < 0.001$). In ICAM-1-carrying targeted nano ultrasonic contrast, delayed imaging of the vascular wall of the abdominal aorta was observed, the outer membrane was thickened from week 4 to week 12, and both the intima-media membrane and outer membrane were thickened and with double-layer parallel echo at week 16, which was in line with the progression of atherosclerotic plaque vulnerability.

Conclusion

ICAM-1-carrying targeted nano contrast agent could evaluate the degree of inflammatory injury related to atherosclerotic progression and site high expression of specific adhesion molecules in early atherosclerotic lesions.

Introduction

Stroke has become the second leading cause of death from cardiovascular or cerebrovascular diseases worldwide, whereas the rupture of vulnerable plaques has been found to be closely related to the occurrence of acute cardiovascular diseases [1]. During the development of atherosclerosis (AS), the persistence of inflammation-related factors, the degree of vascular nourishment by the outer membrane, and the degree of neovascularization in plaques are closely related to plaque vulnerability [2]. Previous

studies have shown that the intercellular adhesion molecule 1 (ICAM-1) can be highly expressed on the surface of damaged vascular endothelial cells and that its upregulation has a key role in the inflammatory response [3].

The emergence of nanobubbles leads to revolutionary advances in ultrasonic molecular imaging technology and breaks the limitations of conventional ultrasonic contrast agents developing in the blood pool, making target tissue imaging outside the blood pool feasible [4–5]. The aim of this experimental study was to explore the feasibility and significance of ICAM-1-carrying targeted nano contrast agent in the evaluation of microvessels in atherosclerotic plaques.

Materials And Methods

Experimental animals

A total of 25 male New Zealand big-eared rabbits, 4 to 5 months old, weighing 2.0 to 2.6kg, purchased from Shanghai Jiagan Biotechnology Co., Ltd., with animal production license numbered SCXK (H.) 2015-0005, were used in this study. The rabbits were fed in cages with automatic washing that were automatically washed once every 4 hours. The temperature and humidity of the feeding room were recorded, and the room ventilation was checked every morning and afternoon.

This study was approved by the Medical Ethics Committee of Jiading District Central Hospital. (No.: 2018K15), and was also in accordance with ARRIVE guidelines.

AS rabbit models

The experimental rabbits were grouped into five following groups: group A, the preoperative control group; group B, the week-4 after modeling group; group C, the week-8 after modeling group; group D, the week-12 after modeling group; and group E, the week-16 after modeling group. AS rabbit models were built via high-fat cholesterol feed and surgical balloon injury[6]. Every day, all experimental rabbits were given 120g of high-fat cholesterol feed that contained 75.5% of basal rabbit feed, 10% of lard, 10% of yolk powder, 4% of cholesterol (of 95% purity), and 0.5% of cholate. After one week of adaptive feeding, the rabbits were subjected to balloon injury in the abdominal aorta. Before the surgery, the rabbits were anesthetized by intramuscular injection of 0.25/kg of suxinmian II (cetirizine hydrochloride injection, Jilin Shengda Animal Medicine Co., Ltd.); the hair from the left lower limb was removed, and the femoral artery was separated. During the surgery, a portable ultrasonic machine (M-Turbo, SonoSite, California) was used to guide the balloon catheter to 2cm above the level of the abdominal aortic, renal artery (of a feeding length of about 20cm). After that, the balloon was injected 7.5atm of air and dragged down to the bifurcation of the abdominal aorta and common iliac artery, which was repeated four times.

Preparation of ICAM-1-carrying targeted nanobubbles

ICAM-1-carrying targeted nanobubbles were prepared by the avidin-biotin binding method. An ultramix oscillator (Vortex-QL-861, at 2,000 oscillations/min and for 40 seconds) was used to activate ultrasonic

bare bubbles labeled the avidin USphere (800ul/vial). After 40ul of Biotin/FITC-ICAM-1 antibodies were added into the vial of the bare bubbles, and the vial was fully suspended for 10 seconds in a scroll oscillator and then placed at room temperature for 30 minutes for incubation. After that, it was placed in a centrifuge to remove unbound antibodies. A fluorescence microscope (LAICA DM2000, Germany, 400x) was used to observe the binding of nanobubbles to ICAM-1, and a flow cytometer was used to measure the binding rate of Biotin/FITC-ICAM-1 to nanobubbles.

2D ultrasonography

An ultrasonic diagnostic unit (Toshiba, Aplio400, Japan) was used at a probe transmission frequency of 11MHz, depth of 2cm, and frame rate of 45 frames/s. 2D ultrasonography was used to measure the thickness of the intima-media membrane at the site in the abdominal aorta that was the thickest. Color Doppler ultrasonography was used to observe blood filling in the lumen.

SonoVue ultrasonic contrast

The rabbits were anesthetized by intramuscular injection of 0.1ml/kg of suxinmian II (cetirizine hydrochloride injection, Jilin Shengda Animal Medicine Co., Ltd.), placed at the supine position on the operating bench; the hair was removed from the abdominal wall, and an indwelling needle (20G, Intima IITM, Suzhou Becton Dickinson Medical Devices Co., Ltd.) was placed in the left auricular vein. SonoVue (Bracco, Italy) was used as the ultrasonic contrast agent. An ultrasonic diagnostic unit (Toshiba, Aplio400, Japan) was used at a probe transmission frequency of 11MHz, depth of 2cm, a frame rate of 45 frames/s, and mechanical index of 0.08, with the focal depth below the posterior wall of the abdominal aorta. Before use, 5ml of 0.9% NaCl (China Otsuka Pharmaceutical Co., Ltd.) was injected into the bottle and shaken into microbubble suspension. After that, 0.1ml/kg of the contrast agent was extracted and quickly injected through the auricular vein, which was followed by an injection of 1ml of 0.9% NaCl.

ICAM-1-carrying targeted nano ultrasonic contrast

All experimental rabbits were given targeted nano ultrasonography under the same ultrasonic conditions as described above 1 hour after conventional ultrasonic contrast after being anesthetized in the same way as described above. Bolus injection of 0.3ml/kg of ICAM-1 targeted nanobubbles was quickly given to the rabbits at the site of the indwelling needle through the auricular vein. After that, the rabbits were immediately injected with 1ml of 0.9% NaCl to rinse the catheter, and continuous dynamic images were acquired in the contrast mode. Imaging of the target area was analyzed in the built-in software for the peak intensity (PI), time to peak (TTP), and area under curve (AUC) of contrast (Table 1).

Serum lipid test

After ultrasonic examinations, the rabbits' hair around the site of the strongest heart beating was removed, disinfected with iodophor, and extracted 5ml of blood through intercostal cardiac puncture. The whole blood was centrifuged at 4°C for 15 minutes for serum. Enzymes were used to measure the total

cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL), and low-density (LDL) in the serum samples.

HE staining

The extracted abdominal aorta was sealed in 5% formalin solution and embedded in paraffin, which was then cross-sectioned for a transverse section of tissues of about 3 μ m in thickness. The section was then HE stained to observe the degree of atherosclerotic lesions, and Image-ProPlus 6.0 software (Media Cybernetics, Maryland) was used to measure and calculate the maximum plaque thickness.

Western Blot

The BCA protein concentration quantitative kit (Thermo, USA) was used to measure the expression of ICAM-1 in the abdominal aorta, and the image analysis system (Quantity One, Bio-Rad, California) was used to analyze the optical density of the target.

Statistical analysis

SPSS 19.0 (IBM, Armonk, NY, USA) was used for data analysis. Continuous variables were expressed as means \pm standard deviation (SD). Independent samples t-test was performed for between-group comparisons, and one-way analysis of variance (ANOVA) followed by post-hoc LSD test for multiple-group comparisons. Pearson correlation analysis was used to analyze the correlation between CEUS parameters and pathological results. A P<0.05 was considered statistically significant.

Results

All rabbits were successfully given ultrasonic contrast and sampled for pathological tests.

Binding of ICAM-1 antibodies to ultrasonic nanobubbles

ICAM-1-carrying targeted ultrasonic nanobubbles were prepared by the avidin-biotin binding method. Nanobubbles bound to ICAM-1 antibodies showed green fluorescence rings on the surface compared to the control group, which proved that ICAM-1 antibodies could effectively bind to lipid microbubbles. Flow cytometry showed that the effective binding rate of ICAM-1 antibodies to bare bubbles was 72.6%.

Serum lipid

TC, TG, LDL, and HDL increased over time in all experimental rabbits, revealing statistically significant differences among the groups (P < 0.05, Table 1).

2D ultrasonography

A well-filled blood flow in the abdominal aortic lumen and a thin and smooth intima-media membrane were observed in the control group. B group showed enhanced intima-media echo in the vessel and no

obvious atherosclerotic plaque formation. C group had a slightly thickened intima-media membrane and no obvious atherosclerotic plaque formation. D group and E group showed gradual thickening of intima-media membrane in the abdominal aorta and different atherosclerotic plaque formation degrees. The differences in the intima-media membrane thickness were statistically significant among the groups ($P < 0.05$, Figure 1, Table 2).

SonoVue ultrasonic contrast

After the injection of SonoVue, the control group had well-filled contrast agents in the abdominal aortic lumen and smooth vascular wall; B group had no obvious abnormalities compared with the control group; C group had well-filled contrast agents in the lumen and slightly thickened intima-media membrane; group D and E had well-filled contrast agents in the lumen and different degrees of filling defects. The differences in the maximum thickness of the intima-media membrane were statistically significant among the groups ($P < 0.05$, Figure 2).

ICAM-1-carrying targeted nano ultrasonic contrast

Visual evaluation of ultrasonic contrast: after the injection of ICAM-1-carrying targeted nanobubbles, rabbits in all experiment groups had only a few microbubbles in the aortic lumen and no obvious filling of the contrast agent. The vascular wall of the control group was not obviously enhanced in the whole process. Group B showed no obvious abnormalities in the imaging of the vascular wall compared with the control group. C, D, and E groups had the contrast agent completely faded in the vascular lumen 2 minutes after the injection of contrast agent, as well as continuously enhanced imaging of the vascular wall. As the modeling time extended, the imaging intensity of the contrast agent gradually increased, and the fading time was delayed. E group showed obviously delayed enhancement of the intima-media membrane and the outer membrane, presenting double-layer parallel echo (Figure 2).

The built-in software in the machine was used to analyze the time-intensity curve of targeted ultrasonic contrast at the site in the abdominal aortic lumen wall that was the thickest, showing no significant difference in TTP among the groups as the modeling time extended, while there were statistically significant differences in PI and AUC (which increased gradually) among the groups ($P < 0.05$, Table 2).

HE staining

After HE staining, the control group had a complete arrangement of the vascular endothelium and orderly and long-fusiform arrangement of smooth muscle cells (SMCs) in the abdominal aorta and no vascular lipid deposition; group B had thickened smooth muscle layer of the medial membrane in the vascular wall, and disorderly arrangement of SMCs; C and D groups had obviously proliferated SMCs in the medial membrane, proliferated foam cells and disorderly arrangement of cells, and E group had a large number of foam cells accumulated into atherosclerotic plaques below the endothelial cells (Figure 3).

Western Blot

The expression of ICAM-1 in the abdominal aorta progressively increased over time, showing a statistically significant difference among the groups ($P < 0.05$) (Figure 4).

Correlation analysis

Correlation analysis showed that the expression of ICAM-1 was closely correlated with PI and AUC of targeted ultrasonic contrast ($r = 0.893, P < 0.001$; $r = 0.934, P < 0.001$).

Discussion

AS, one of the common diseases in clinical practice, is an inflammatory disease that runs through the disease's whole course, making plaques unstable and prone to rupture or erosion [7]. An essential feature in the early formation of plaques is the recruitment of white blood cells to the vascular wall. In general cases, endothelial cells increase the expression of leukocyte adhesion molecules, such as the expression of intercellular adhesion molecule-1 (ICAM-1), to resist the adhesion of white blood cells in the blood [8]. Therefore, ICAM-1 is one of the critical inflammatory markers related to AS. Previous studies have shown that up-regulated expression of ICAM-1 is involved in the occurrence and development of AS lesions and that to some extent reflects the degree of inflammatory endothelial injury. Hence, ICAM-1 can serve as a molecular probe reflecting the imaging diagnosis and treatment of targeted inflammation [9–10].

Nanoultrasonic contrast agent, for its small grain size, can freely pass through pathologically new vessels enabling the imaging of new vessels. It can also pass through the endothelial gap of new vessels, enabling extravascular tissue imaging with high sensitivity [11–12]. After binding to specific antibodies or ligands, ultrasonic contrast agents become targeted contrast agents that can actively bind to targeted areas and highly aggregated in such areas to enable specific imaging [13–14]. This experimental study was carried out in AS animal models. ICAM-1-carrying targeted ultrasonic nanobubbles were prepared for dynamic observation of the capability of ICAM-1-carrying targeted ultrasonic nanobubbles to recognize atherosclerotic inflammatory lesions at different stages. Our results revealed that ICAM-1-carrying targeted nano contrast agents had a longer time of imaging in the vascular intima-media membrane compared to the lumen, and conventional micron ultrasonic contrast agents in the lumen faded almost at the same time as lumen wall contrast agents, which was consistent with the study results of Fan et al [15]. The imaging intensity of ICAM-1-carrying nano contrast agents in the vascular wall significantly increased and the imaging time significantly extended with the progression of atherosclerotic lesions. Targeted nano contrast parameters PI and AUC in this experimental study revealed a positive linear correlation with the expression of ICAM-1 in tissues. It is possible that targeted nanobubbles were sucked into cells after adhering to activated neutrophils and monocytes but maintained the same acoustic properties. Also, after free microbubbles in the blood circulation were emptied, contrast agents in the lumen faded while intracellular targeted nanobubbles in the vascular intima-media membrane continued to enable ultrasonic imaging. Another possible reason might lie in the correlation between microbubble imaging's time and intensity with the site and severity of inflammation.

A previous study showed that new vessels in plaques gradually grew from the outer membrane to the atherosclerotic lesion site, providing a new pathway for monocytes and immune cells. Meanwhile, SMCs in the artery migrated into the medial membrane and proliferated, gradually forming fibrous caps. Also, the rupture was closely related to plaque vulnerability [16–17]. As shown in this experimental study, ICAM-1-carrying nano contrast agent had delayed imaging in the outer membrane of the vascular wall at week 4 after modeling, and enhanced imaging, with double-layer parallel echo, in both the intima-media membrane and outer membrane of the vascular wall at week 16 after modeling. Consequently, we speculated that ICAM-1-carrying targeted nanobubbles could specifically bind to ICAM-1 targets on the abdominal aortic wall. Also, their site of adherence in plaques was the same as the site vulnerable to atherosclerosis.

Conclusion

Compared with conventional ultrasonic contrast agents, ICAM-1-carrying targeted ultrasonic nanobubble contrast agent could reflect the degree of inflammatory injury at different stages of atherosclerotic lesions. Moreover, it could enable early qualitative diagnosis of plaque vulnerability, which provides a reference for early diagnosis of plaque vulnerability in future clinical practice.

Declarations

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

Author contributions:

Each author's contribution is as follows: Ping Li wrote the main manuscript. Lan Feng and Lin Jin made animal models. Yingchun Wang collected and analyzed of ultrasound images. Rong Yang was responsible for the pathological and immunohistochemical analysis. Lin Jin performed experimental design and overall planning. All the authors have reviewed the manuscript and agree with its content.

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Tables

Table 1. Comparison of serum lipid in experiment rabbits in different groups (mmol/L)

	TC	TG	LDL	HDL
Control group	1.67±0.14	1.11±0.09	1.27±0.25	0.59±0.09
Week-4 group	12.84±3.38*	1.85±0.25*	9.14±2.92*	0.75±0.09*
Week-8 group	22.73±3.97*#	3.14±0.57*#	15.03±3.86*#	1.24±0.30*#
Week-12 group	32.49±4.86*#△	4.71±1.12*#△	19.13±1.21*#△	1.66±0.14*#△
Week-16 group	38.54±4.30*#△□	8.49±1.43*#△□	22.46±1.88*#△□	1.96±0.17*#△□
F value	79.464	58.237	61.963	54.422
P	0.000	0.000	0.000	0.000

Note: * P < 0.05 when the value is compared with that of the control group; # P < 0.05 when the value is compared with that of the week-4 group; △ P < 0.05 when the value is compared with that of the week-8 group; □ P < 0.05 when the value is compared with that of the week-12 group.

Table 2. Ultrasonic examination results of experiment rabbits in different groups

	IMT (mm)	IMTCEUS (mm)	PI (dB)	TTP (s)	AUC
Control group	0.08±0.02	0.09±0.01	8.20±1.64	11.20±1.30	248.60±26.37
Week-4 group	0.17±0.02*	0.18±0.03*	11.80±0.84*	12.00±1.00	303.20±13.16*
Week-8 group	0.23±0.02*#	0.23±0.02*#	14.00±1.58*#	12.40±1.82	354.60±36.95*#
Week-12 group	0.30±0.03*#△	0.32±0.03*#△	18.60±1.14*#△	12.40±1.67	413.60±36.81*#△
Week-16 group	0.43±0.05*#△□	0.44±0.41*#△□	21.60±2.07*#△□	12.40±2.70	465.00±26.22*#△□
F value	85.285	105.964	61.887	0.422	43.139
P	0.000	0.000	0.000	0.791	0.000

Note: * P < 0.05 when the value is compared with that of the control group; # P < 0.05 when the value is compared with that of the week-4 group; △ P < 0.05 when the value is compared with that of the week-8 group; □ P < 0.05 when the value is compared with that of the week-12 group.

Figures

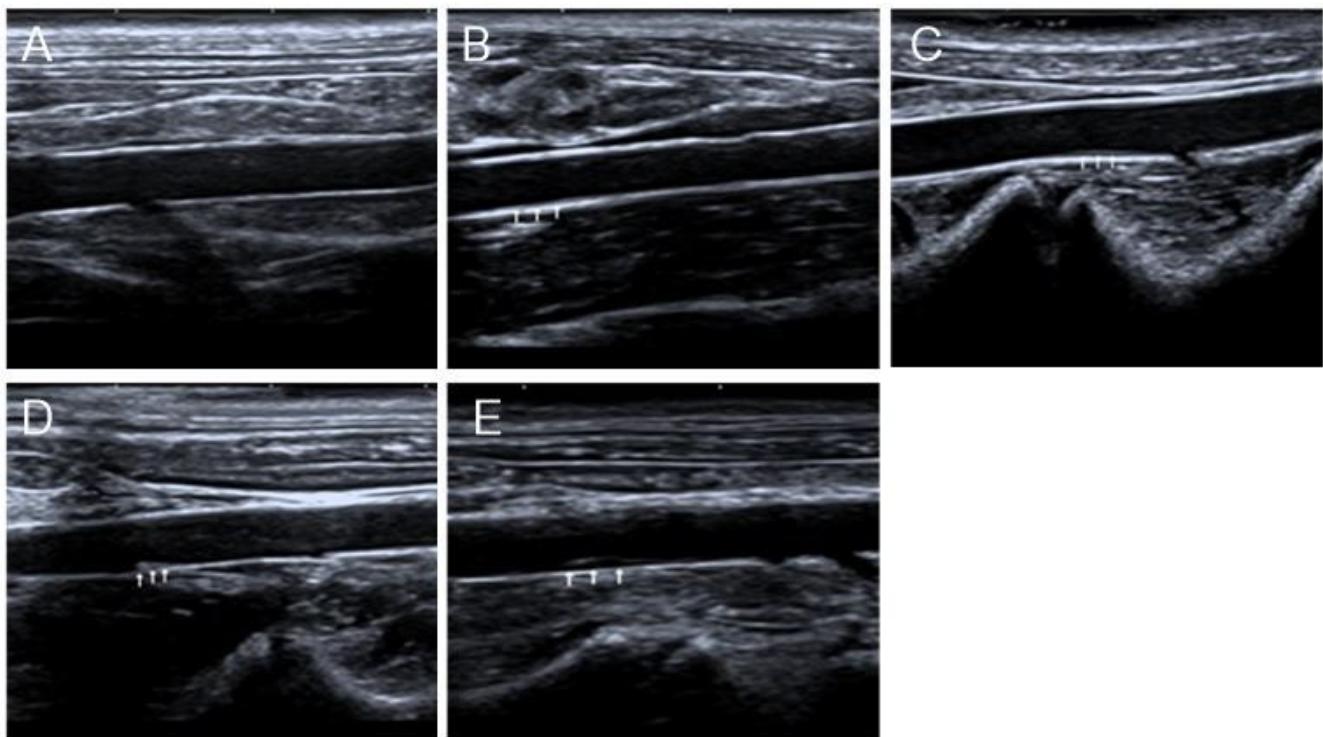


Figure 1

2D ultrasonography results of the abdominal aorta of experiment rabbits in different groups. A: Control group; B: Week-4 group; C: Week-8 group; D: Week-12 group; E: Week-16 group.

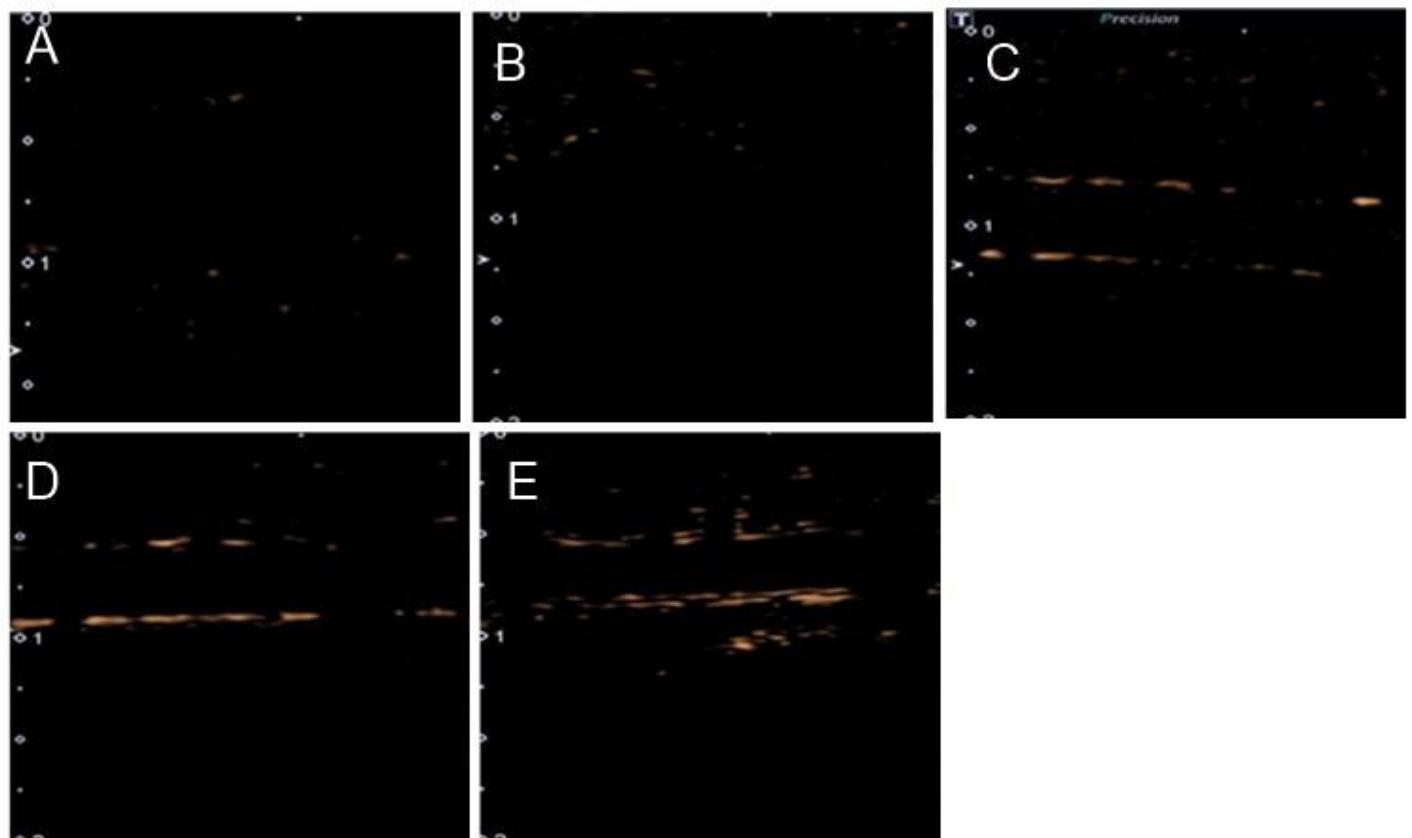


Figure 2

ICAM-1-carrying targeted nano ultrasonic contrast images of abdominal aorta of experiment rabbits in different groups. A: Control group; B: Week-4 group; C: Week-8 group; D: Week-12 group; E: Week-16 group.

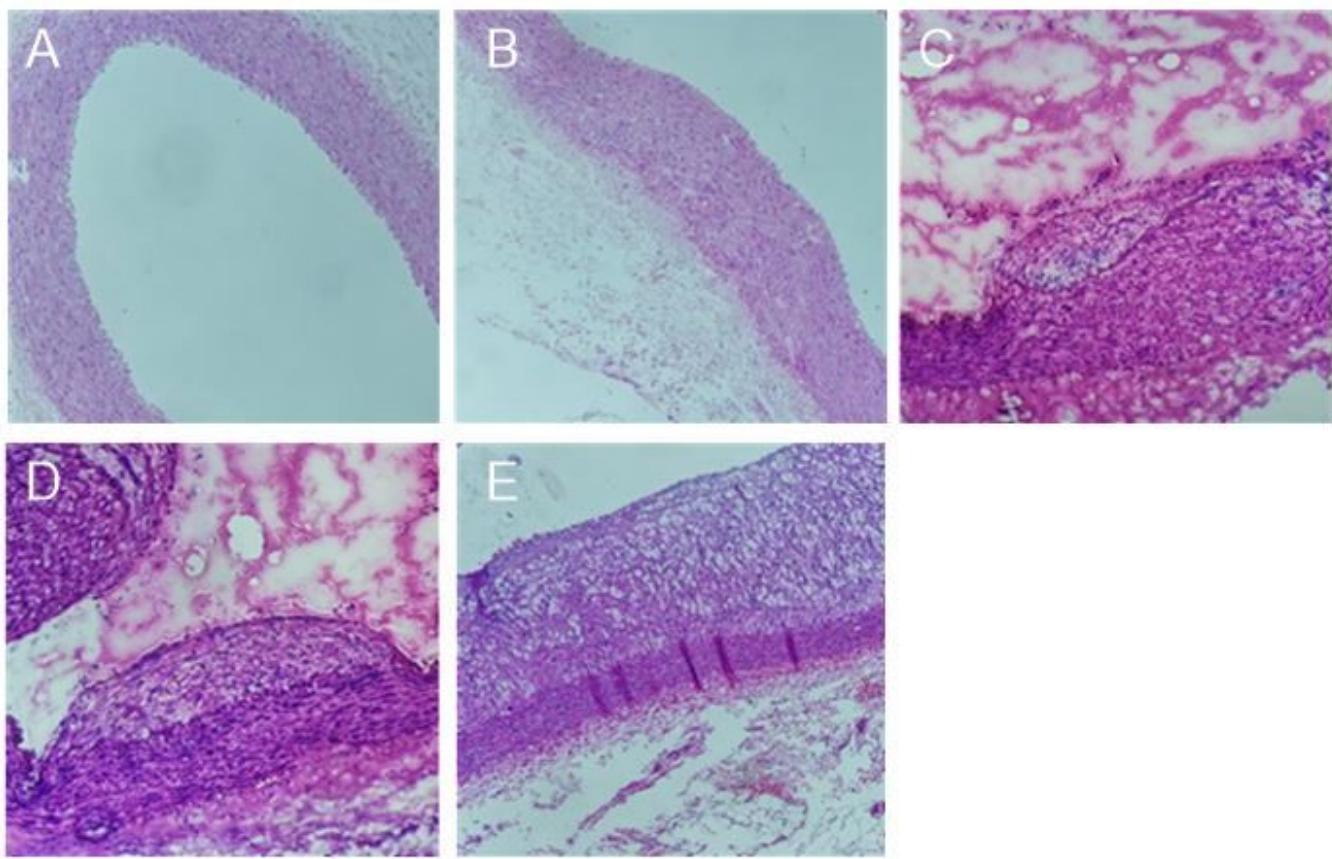


Figure 3

HE staining images of abdominal aorta of experiment rabbits in different groups ($\times 100$). A: Control group; B: Week-4 group; C: Week-8 group; D: Week-12 group; E: Week-16 group.

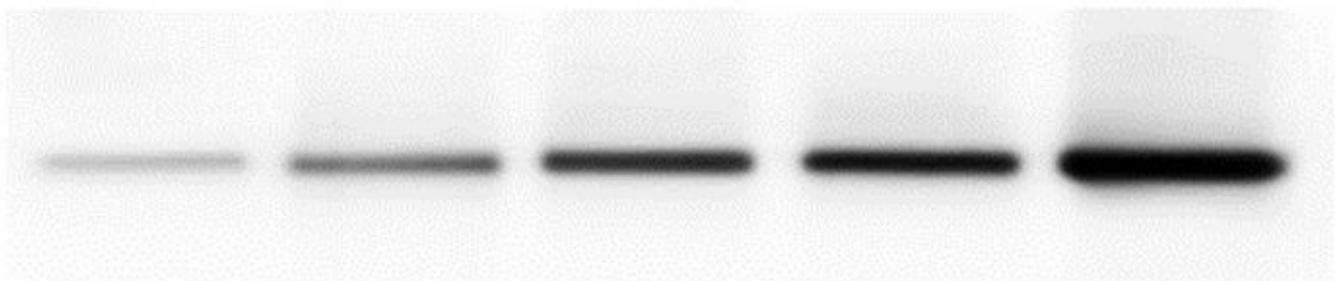


Figure 4

Western Blot images of abdominal aorta of experiment rabbits in different groups. A: Control group; B: Week-4 group; C: Week-8 group; D: Week-12 group; E: Week-16 group.