Biomedicine & Pharmacotherapy 92 (2017) 277–284

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www.sciencedirect.com

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EM|consulte
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Quantitative evaluation of atherosclerotic plaques and intraplaque neovascularization using contrast-enhanced ultrasound after treatment with atorvastatin in rabbits

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ARTICLE INFO

Article history:
Received 12 January 2017
Received in revised form 1 April 2017

Keywords:
Atorvastatin
Atherosclerotic plaque
Contrast-enhanced ultrasound
Neovascularization
Plaque formation
Quantitative evaluation

ABSTRACT

Introduction: This study is supposed to investigate the value of contrast-enhanced ultrasound (CEUS) in quantitative evaluation of atherosclerotic plaques and intraplaque neovascularization after treatment with atorvastatin (ATV) in rabbits.
Material and methods: Forty-five New Zealand white rabbits were enrolled to construct the rabbit model of AS. All rabbits were assigned into the control, AS group and ATV groups (n = 15 individually). The AS plaque formation and relative parameters were observed and calculated by CEUS respectively. Total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were measured. Serum levels of matrix metalloproteinase-3 and 9 (MMP-3/9) and high-sensitivity C-reactive protein (hs-CRP) were examined by ELISA. The histological changes plaques, maximal plaque thickness (MPT), plaque area (PA) and corrected plaque area (PA/CVA) were evaluated by HE staining. Immunohistochemistry (IHC) was used to measure the positive protein expressions of VEGF, FVIII-Rag, MMP-3, CD40L and F8. The correlation between CEUS parameters with ELISA and IHC was analyzed by the Spearman correlation analysis.
Results: At the 8th week, no plaque and new blood vessel were observed in the control group. The ATV group showed more plaques and new blood vessels, and lower IMT, plaque thickness and area than the AS group. The PI and RI were significantly increased in the AS and ATV groups compared to the control group. PI, RI. Plaque EI and its ratio in plaque and arterial lumen of the ATV group were lower than those in the AS group. Compared with the control group, the AS and ATV groups had higher serum levels of TC, TG, LDL, MMP-3, MMP-9 and hs-CRP, and higher AI. However, compared with AS group, serum levels of TC, TG, LDL and AI fell in ATV group. In comparison of the AS group, serum levels of MMP-3, MMP-9 and hs-CRP, MPT, PA and PA/CVA, and the positive expressions of VEGF, FVIII-Rag, MMP-3, CD40L and F8 were significantly reduced in the ATV group. The AS and ATV groups showed a positive correlation of EI in the plaque and its ratio in the plaque and arterial lumen with F8 protein expression, MMP-3 and MMP-9.
Conclusion: In conclusion, our results indicated that ATV stabilizes atherosclerotic plaques and reduces intraplaque neovascularization in a rabbit model with AS, which can be characterized using CEUS.

1. Introduction

Atherosclerosis (AS), a pathological development of chronic vascular inflammatory for a long time-course based on vascular lesions, could lead to serious complication such as apoplexy and myocardial infarction [1], and it is a killer of human beings health and is expected to become an important cause of mortality and morbidity in the world [2]. The primary potential causes of AS are coronary heart disease and the systemic degenerative inflammatory vascular disease [3]. It is well-known that the presence of AS in the carotid arteries can cause a substantial risk of ipsilateral cerebrovascular events [4]. Moreover, the presence of AS destroyed the normal protective mechanism which provided by the endothelium, and this mechanism was correlated with the pathophysiology of coronary artery disease and stroke [5]. Therefore, efforts are supposed to be put into the prevention of AS.

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http://dx.doi.org/10.1016/j.biopha.2017.04.068
0753-3322/

It is clear that the therapeutic strategies of AS included antiplatelet, lipid-lowering, antihypertensive drugs and hypoglycemic [6]. It is particularly worth mentioning that patients with AS are frequently treated with clopidogrel and atorvastatin (ATV) or other statins for the treatment of hypercholesterolemia [7]. Although all statins own the same main mechanism of action, their pharmacokinetic profile really does differ [8]. ATV, a reversible and competitive inhibitor of HMG-CoA reductase, could decrease the de novo cholesterol synthesis [9]. A meta-analysis demonstrated that the treatment with ATV particularly contributes to the regression or slowed progression in those patients with carotid AS or dyslipidemia [10]. Contrast-enhanced ultrasound (CEUS) is commonly used as medical diagnostic imaging modality because of its convenience, safety and portability on the basis of radiation exposure and low-cost [11]. Recently, the rapid development of CEUS technology has greatly enhanced the quality of ultrasound diagnoses and enriched the evaluation parameters of ultrasound images [12]. What's more, as an ultrasound technique, CEUS is used for patients by injecting a special intravenous contrast agent and it can be performed immediately in one session with non-contrast ultrasound once a lesion is found [13]. CEUS could detect neovascularization within AS plaques in vivo, and it has been reported that the enhancement within an AS plaque which is observed in CEUS can be attributed to plaque neovascularization [14].

Previous study indicated that intensive low-density lipoprotein cholesterol (LDL-C) lowering with statins could slow down AS plaque development and result in plaque regression [15]. However, less information is available yet with regard to the efficacy of ATV on AS by using CEUS. Therefore, in this study, we explored the value of CEUS in quantitative evaluation of atherosclerotic plaques and intraplaque neovascularization after treatment with ATV using a rat model of AS.

2. Materials and methods

2.1. Experimental animals and grouping

This animal experiment was performed with 45 healthy 2-month-old New Zealand white rabbits (weighted 1800–2500 g, purchased from the Experimental Center of Third Military Medical University, China). These rabbits were individually housed in cages with 24-h-free water available and 50% relative humidity at 20 °C. All rabbits were randomly divided into three groups (15 rabbits in each group): the control group, the AS group, and the ATV group. The present experiment was performed in accordance with the ethical principles as formulated in the Helsinki Declaration.

2.2. A rabbit model of atherosclerosis

After one week of basic diet feed, the rabbit model of AS was constructed. Rabbits in the control group were fed with a normal diet (100 g/d; purchased from the animal experimental center of Shandong University, China). Rabbits in the AS and ATV groups were fed with a high-fat diet containing 1% cholesterol, 5% lard and 94% basic diet (100 g/d; purchased from Beijing Ke'ao Xieli Feed Co. Ltd., China). After 2 weeks of high-fat diet fed, the hyperlipidemia was confirmed with blood lipid test in these rabbits. Balloon endothelial denudation of the abdominal aorta was performed in rabbits of the AS and ATV groups. Rabbits were anesthetized with 3% sodium pentobarbital (1 ml/kg, purchased from Sigma-Aldrich Chemical Company, St Louis MO, USA) via intravenous injection into the marginal ear vein of rabbit. The right femoral artery of rabbit was separated using blunt dissection, and inserted with a balloon catheter at an internal diameter of 3.0–3.5 mm. The balloon was selectively sent into the coeliac axis with a depth about 15–17 cm. The catheter was connected with a pressure pump. Normal saline was injected to inflate the balloon. Pressure was maintained at 10–15 kPa. Balloon catheter traction was repeated for 3 times. And then the balloon catheter was pushed out. Wound was stitched layer-by-layer. After operation, rabbits were intramuscularly injected with penicillin (80,000 U) to prevent postoperative infection. Rabbits in the control group were fed with a basic diet for 6 weeks, and rabbits in the AS group were fed with a high-fat diet for 6 weeks. Meanwhile, rabbits in the ATV group were fed with a high-fat diet and ATV calcium (5 mg/kg/d; Lipitor, 10 mg/tablet, batch number: D1420203390) for 6 weeks.

2.3. CEUS

The CEUS was performed on rabbits in the three groups using GE LOGIQ E9 ultrasound instrument (9 MHz, 16 l probe; GE Healthcare, Milwaukee, WI, USA) after 8 weeks of feed. After anesthesia, the rabbit was placed in the left tilt position on the table, and longitudinal and transverse conventional ultrasound was performed along the abdominal aorta from up to down. Plaque formation in the rabbit arteries was observed. Meanwhile, intima media thickness (IMT), plaque thickness, plaque area, peak systolic blood flow velocity (Vs), pulsatility index (PI), resistant index (RI) were measured. After injection with the contrast agent Sono Vue (2 ml; Bracco company, Italy), the intensity of contrast in the AS plaques was observed, and dynamic images were stored in the digital imaging and communication in medicine (DICOM) file format. Replaying the contrast dynamic images, the region of interest (ROI) in the plaque was quantitatively analyzed with QLAB software (PHILIPS, Andover, MA, USA), which is in accordance with the different plaque size and shape. Another ROI was placed in the center of abdominal aorta by using rectangular sampling box. After that, time-intensity curves (TICs) of the plaques and the lumen of the arteries were generated using QLAB software. And peak intensity (PI), basic intensity (BI) and enhanced intensity (EI) in plaque and arterial lumen were obtained (EI = PI − BI) [16]. The ratio of EI in the plaque and in the arterial lumen was calculated.

2.4. Sample collection

After 2 weeks and 8 weeks (i.e. after 6 weeks of drug intervention) of feed, blood samples (1.5 ml each rabbit) were collected from the marginal ear vein of rabbits, and then were centrifuged at 3000 r/min for 15 min. Then serum was collected and preserved in a −80 °C ultra-low temperature refrigerator for later measurement of blood biochemical indexes and Enzyme-linked immunosorbent assay (ELISA). After 8 weeks, rabbits were anesthetized with 3% pentobarbital (40 ml/kg, Sigma-Aldrich Chemical Company, St Louis MO, USA), and fixed in the operation table. Abdominal aortic tissue were isolated from the balloon-injured intima of rabbits and fixed in 10% formalin (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China), followed by paraffin embedding. Paraffin embedded tissue was sliced into sections with cryopreservation for haematoxilin-eosin (HE) staining and immunohistochemistry (IHC).

2.5. Blood biochemistry

After 2 weeks and 8 weeks of feed, serum level of total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were measured by an automatic biochemical analyzer (AU2700, Olympus company, Tokyo, Japan). And atherosclerosis index (AI) was calculated. Formula: AI = (TC − HDL-C)/HDL-C [17].