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# Protective effect of atorvastatin meditated by HMGCR gene on diabetic rats with atherosclerosis: An *in vivo* and *in vitro* study

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ABSTRACT

*Background:* Accelerated atherosclerosis in patients suffering from diabetes represents a major cause of morbidity and mortality. The aim of present study was to investigate the protective effects conferred by atorvastatin (AVT) meditated by the *HMGCR* gene in diabetic rats with atherosclerosis.

*Methods:* Serum triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), very-low-density lipoprotein cholesterol (VLDL-C), fasting blood glucose (FBG) and serum insulin (INS) were all determined by means of in vivo experiments. Following the establishment of the diabetic model of atherosclerosis, the expressions of HMGCR, low density lipoprotein receptor (LDLR), fatty acid synthase (FASN) were detected by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and Western blot analysis in the vitro experiments. Flow cytometry was adopted in order to detect cell cycle and apoptosis.

*Results:* The in vivo experiments results indicated that FBG and INS among the diabetic arteriosclerosis rats exhibited markedly higher levels; after injected with AVT and HMGCR, decreased contents of TC, TG, LDL-C and VLDL-C, while increased contents of HDL-C as well as an increased positive rate of HMGCR protein expression were observed. In *vitro* experiment, the mRNA and protein expression of LDLR were increased and FASN were decreased in cells transfected with HMGCR and AVT; with a greater number of cells arrested at the S phase and less in the G0/G1 phase, as well as data indicating the rate of apoptosis was inhibited after HMGCR and AVT transfection processes.

*Conclusion:* The key findings of the present study suggested that the protective effect conferred by AVT in diabetic rats with atherosclerosis was associated with the overexpression of the HMGCR gene, thus presenting a novel target for atherosclerosis treatment.

## 1. Introduction

Atherosclerosis is a chronic and progressive disease that may lead to diabetes mellitus, endothelial dysfunction, hypertension, and hypercholesterolemia [1]. Statistics have revealed that atherosclerosis accounts for approximately 50% of all deaths among western nations [2]. Atherosclerosis arises in the intima of medium-sized arteries with disturbed blood flow [3], with a chronic inflammation a significant potentiality resulting in the incidence of an acute clinical event as a consequence of plaque rupture and thrombosis [4]. Diabetes mellitus represents a chief risk factor for various cardiovascular diseases, acting to accentuate the inflammatory process stimulating the formation of new vessels which accelerates the development of atherosclerosis [5]. Reports have highlighted atherosclerosis as the foremost prevailing complication associated with diabetes mellitus [6], while diabetes mellitus has been linked with disorders in relation to antioxidant defenses and pro-oxidant enhancement, leading to an increased susceptibility of atherosclerosis, endothelial dysfunction, insulin-resistance and damaged pancreatic β-cell function [7]. From a clinical treatment perspective, the current existing therapeutic approaches for subclinical coronary atherosclerosis in patients with asymptomatic diabetes are largely inadequate [8]. Therefore, the identification of more effective therapeutic methods is required for patients suffering from diabetic atherosclerosis.

Statins owing to its ability to reduce the synthesis of endogenous cholesterol as well as lower blood levels of low-density lipoprotein

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cholesterol (LDL-C) by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) are a widely employed drug regimen of choice used to both prevent and treat atherosclerotic cardiovascular disease [9]. HMGCR is a rate-limiting enzyme involved in cholesterol biosynthesis [10], and is encoded as part of the statin-binding domain of the enzyme [11]. The HMGCR enzyme is found in eukaryotes and prokaryotes, including the enzymes of eukaryotes with certain archaea and enzymes of eubacteria with certain other archaea [12]. The activity of HMGCR is regulated at both post-transcriptional and post-translational levels [10], with a previous report suggesting that HMGCR could be used as a predictive marker coupled with beneficial anti-tumor effects in relation to statin treatment in patients with breast cancer [13]. Atorvastatin (AVT), a member of the stain family, is the most widely used pharmaceutical drug, applied for hypercholesterolemia prevention purposes [14]. A study revealed that statins could decrease the incidence rate of cardiovascular events in patients with diabetes mellitus [15]. AVT has been reported to influence the regulation of blood lipids resulting in enhanced cardiac function in diabetic patients with coronary heart disease [16]. However, the effects of AVT meditated by the HMGCR gene on diabetic atherosclerosis are yet to be explored. Hence, the central objective of the present study was to investigate the effects of AVT meditated by the HMGCR gene on diabetic rats complicated with atherosclerosis.

## 2. Materials and methods

### 2.1. Ethics statement

All animal experiments in this study were conducted with the approval of the Ethics Committee in PLA 254 Hospital and Affiliated Hospital of Jining Medical University. Significant efforts were made in order to minimize both the number of animals used as well as their respective suffering.

### 2.2. Establishment of diabetic atherosclerosis models

A total of 120 Wistar rats (female and male in half, and 20 rats were standby) aged 10 weeks and weighing 125 ~ 240 g were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, Hunan, China). The rats were raised in the specific pathogen-free (SPF) animal laboratory at 22 ~ 25 °C with 55% ~ 65% humidity, with conventional fodder (73.5% corn, 20% wheat bran, 5% fish flour, 1% cereal mash, and 0.5% salt). Fifty HMGCR knockout rats (the first generation rats were prepared in Shanghai Biomodel Organism Science & Technology Development Co., Ltd, Shanghai, China and the offspring of the rats were bred in our laboratory) and eighty normal rats were employed for the preparation of the diabetic atherosclerosis models. An additional 20 normal rats were randomly selected as control.

In order to establish the models, the rats were permitted free access to high fat fodder composed of 20% sucrose, 10% lard, 1% cholesterol, 10% egg yolk powder, 0.5% sodium cholate and 58.5% conventional fodder. After 3 d had elapsed, the rats were intraperitoneally injected with 255 mg/kg nicotinamide (NA) solution (Shanghai General Pharmaceutical Co., Ltd., Shanghai, China), and administered a dose of 50 mg/kg streptozotocin 15 min later (STZ, Sigma, Santa, Clara, CA, USA) dissolved in 0.1 mmol/L citric acid buffer (pH = 4.5) via the tail vein. Meanwhile, the rats subject to gavage with vitamin D3 (VD3) injection (Shanghai General Pharmaceutical Co., Ltd., Shanghai, China) (the total dose of 550,000 IU/kg for 3 d). The rats in the control group were granted with access to free feeding with conventional fodder, and injected once with 50 mg/kg saline and NA solution, with the same dose as the model group administered.

### 2.3. Identification of diabetic atherosclerosis models

After 4 weeks, the model rats were anesthetized by means of an intraperitoneal injection of 3% sodium pentobarbital under fasting conditions. Aortic blood (2 mL) samples were collected and immediately centrifuged for 5 min at 1610 *g*. The plasma was separated, with fasting blood glucose (FBG) content determined using an automatic biochemical analyzer (model: Hitachi 7150) while serum insulin (INS) was detected using an insulin tester (model: Roche 2010). Blood test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) applied in order to measure the serum triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), LDL-C, and very-low-density lipoprotein cholesterol (VLDL-C) respective contents. In the event that FBG ≥ 7.8 mmol/L, TG ≥ 0.58 mmol/L, and TC ≥ 1.96 mmol/L, the rat models were considered to have been successfully established [17]. The success rate of the rat model was determined, and the experiment was repeated 3 times.

### 2.4. Animal grouping and transfection

Ten normal rats were randomly selected as the normal group, while the diabetic atherosclerosis rats were regarded as the model group, AVT-treated group (with 20 mg/kg AVT in the stomach, AVT was purchased from Pfizer Inc, NY, USA), $HMGCR^{-/-}$ group (HMGCR knockout), HMGCR group (HMGCR rats were injected of liposome (Invitrogen, Carlsbad, CA, USA) enwrapped recombinant eukaryotic expression plasmid of HMGCR into the hind limb muscles of rats), $HMGCR^{-/-}$ + AVT group (rats were injected of HMGCR knockout and intervened with AVT) and HMGCR + AVT group (rats were injected of GeneSHUTTLE-40 liposome enwrapped recombinant eukaryotic expression plasmid of HMGCR into the hind limb muscles of rats, with the rats then treated with AVT), with 10 rats in each group. The blood glucose and blood lipid content of the rats were determined in each group after 4 weeks.

### 2.5. Histopathological observation

The abdominal cavity of the rats was opened in order to separate the abdominal aorta. Hematoxylin-eosin (HE) staining methods were applied in order to observe the histopathological changes of the aorta. Neutral formalin solution (Chongqing Beibei Chemical Reagent Factory, Chongqing, China) was used to fix tissues for 1 d. After ethanol dehydration, xylene transparency and paraffin embedding, the tissues were subsequently sliced into 5 μm sections, dried at 60 °C, and stained using HE methods (NanchangYulu Experimental Equipment Co., Ltd., Nanchang, Jiangxi, China). Thereafter, the tissue sections were stained with hematoxylin for 6 min, rinsed using tap water for 1 min, treated with 1% dilute hydrochloric acid for a few seconds, washed in distilled water for 10 min and stained blue by ammonia. The tissue sections were then rinsed under tap water for 1 min, washed 3 times with distilled water, stained by 1% eosin for 3 min, rinsed in distilled water for 10 min, dehydrated by ethanol and mounted by neutral resin. The samples were observed using an optical microscope (Olympus CX40, Olympus Optical Co., Ltd, Tokyo, Japan) with 100 × magnification. The experiment was repeated 3 times.

### 2.6. Immunohistochemical staining

The positive expression rate of HMGCR protein in rat liver tissues was detected by means of immunohistochemical staining. The rat livers were promptly under aseptic condition. Serial paraffin sections of the tissues (5 μm) were dewaxed with xylene, and hydrated using gradient alcohol. Antigen retrieval was performed in a microwave oven, while endogenous peroxidase blockade was performed using 3% hydrogen peroxide. The tissues were then placed at room temperature under dark conditions for 10 min, then added with monoclonal HMGCR primary antibody (1:100, ab202685, Santa Cruz, Santa Cruz, CA, USA) and incubated overnight at 4 °C. The sections were subsequently washed 3 times with phosphate buffered saline (PBS) (3 min per time), and

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