Original Article

Long-term lactobacillus ATCC4356 supplementation prevents formation of atherosclerosis via P65-mediated reducing of inflammation and oxidative stress in apoE~(-/-) mice

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Abstract: Lipid-reducing therapies have been widely used for the prevention and treatment on cardiovascular disease. However, common lipid-reducing medications have limited effects and are always accompanied with side effects. Recently, reducing the incidence of cardiovascular disease by the administration of lactobacillus has become a focus but the mechanism remains unclear. In this study, apolipoprotein E-knockout (ApoE~(-/-)) mice fed with different concentrations of Lactobacillus acidophilus ATCC4356 were used to investigate the effects of ATCC4356 on the development of atherogenesis and NF-κB p65-mediated signal pathway. The results showed that ATCC4356 had no impact on weight and blood lipids. However, ATCC4356 effectively relieved atherosclerotic lesions, decreased serum levels of oxidative stress factors (ox-LDL, MDA, SOD) and inflammatory cytokines (TNF-α, IL-10) in ApoE~(-/-) mice after being fed with high fat diet. These results suggest that the inhibitory effect of ATCC4356 on the atherosclerotic formation was not dependent on the reduction of cholesterol level but associated with the decreasing inflammatory reactions and oxidative stress effects via suppressing p65-mediated signaling pathway.

Keywords: Atherosclerosis, oxidative stress, hypercholesterolemia, inflammatory cytokines

Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity in the modern society and largely caused by complications of clinical atherosclerosis [1]. Chronic hyperglycemia and reactive oxygen species (ROS) are major risk factors for the development of various cardiovascular diseases, particularly atherosclerosis (AS) [2]. We know that the formation of atherosclerosis is a pathologic process including multiple inflammatory reactions. Microbial infection is a causative factor of AS and a variety of inflammatory cytokines are involved in the formation of the disease.

Concerning the side effects and limited effect of lipid-reducing medications, novel therapies are urgently needed in clinics to manage AS. One of the promising managements is to reduce cholesterol level in patients. Lacking atherogenic lipoproteins in enterocyte cells can cause atheroprotective effects for reduction of intestinal absorption of cholesterol. Relieving hypercholesterolemia is helpful to decrease clinical risk of atherosclerosis [1, 3]. Lactobacillus acidophilus ATCC4356 is one of the important microorganisms in the intestines and virginal mucosa of healthy humans [4]. Lactic acid bacteria are considered to be beneficial microorganisms and associated with multiple potential health effects for humans. Additionally, the cholesterol-reducing effect of Lacto acid bacular, such as Lactobacillus acidophilus ATCC4356, has been recently confirmed the potential utility in the prevention of atherosclerosis [5]. Ying Huang et al. reported Lactobacillus acidophilus ATCC4356 could effectively prevent atherosclerosis via inhibition of intestinal cholesterol absorption in apolipoprotein ApoE knockout (ApoE~(-/-)) mice. Uchida M. et al. reported that the polysaccharides, extracted from Lactobacillus kefiranofaciens, could significantly inhibit aortic atherosclerotic plaque lesions.
of male New Zealand white rabbits fed with high cholesterol diet. Mann et al. found that the habit of drinking yogurt fermented by *Lactobacillus acidophilus* significantly weaken the risk of hypercholesterolemia development, indicating that *Lactobacillus* had potential lipid-regulating function [5].

However, the cholesterol-reducing effect of *Lactobacillus* on atherosclerosis and its mechanism remains unclear. Only a few studies have focused on the effect of *Lactobacillus* intervention on the development of atherosclerosis in animal models. Apart from high level of cholesterol causes AS, ROS and associated oxidation products causes endothelial cells damage. Various pro-inflammatory cytokines are released during this process, which further initiate AS. In the present study we selected ApoE-/- mice to establish atherosclerosis model. ApoE-/- mice were fed with high-fat and high-cholesterol diet daily and 10⁸ CFU/mL *Lactobacillus* intragastrically; high-dose group of *Lactobacillus acidophilus* (LA. H): ApoE-/- mice were subjected to high-fat high-cholesterol diet daily and 10⁹ CFU/mL *Lactobacillus* intragastrically; high-dose group of *Lactobacillus acidophilus* (LA. H): ApoE-/- mice were subjected to high-fat high-cholesterol diet daily and 10⁹ CFU/mL *Lactobacillus* intragastrically; normal control (WT) group: C57BL/6J mice were treated with daily normal diet and NS intragastrically. Each group of mice was given a daily volume of 0.5 mL via intragastric gavage for 12 weeks continuously.

**Analysis of plasma lipoprotein and inflammation**

Blood samples were obtained from fasting mice through retro-orbital. Blood samples were left at room temperature for 30 min, and then centrifuged to harvest serum. Total serum cholesterol (TC), triglycerides (TG), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), glucose, ALT, AST, TBIL and DBIL were analyzed using enzyme-based colorimetric assay (Hitachi 7170 automatic biochemical analyzer). The levels of interleukin (IL)-10, C-reactive protein (CRP), and tumor necrosis factor (TNF)-α were assayed using enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s instruction.

**Atherosclerotic lesion analysis**

Heart, liver and aortic arch were dissected after blood collection. Tissues were frozen with O.C.T. The lesions were visualized by staining with oil red O followed by a hematoxylin & eosion counter-stain the cross-sections. The cross-section atherosclerotic lesions area and aortic lumens were determined by computer-assisted morphometry. Atherosclerotic lesions were normalized to aortic lumen area.

**Detection of NF-κB and IκB-α protein expression of the aorta by western blotting**

Total protein of frozen aorta was extracted by homogenizing in RIPA lysis buffer. Nucleoprotein and cytoplasm protein were extracted by Nc-nucleus protein and plasma protein extraction kit according to the manufacturer’s instruction. Then 50 mg of total protein was loaded onto 10% SDS-PAGE, and western blot analysis was performed with antibody against Iκ-B (1:500), NF-κB P65 (1:200), histone (1:500), tubulin (1:500) or GAPDH (1:6000). Specific signals of Iκ-B, NF-κB and histone were quantified on an imaging system and normalized to internal control tubulin or GAPDH.

**Statistical analysis**

Statistics analysis was performed using the SPSS19.0 software. Data are presented as means ± standard deviation (SD). Differences
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between groups were analyzed using the one-way ANOVA, and multiple comparisons were analyzed by the LSD method. *P*-value <0.05 was considered statistically significant.

**Results**

*Effects of ATCC 4356 on hematology indexes in ApoE−/− mice*

Firstly, we defined the basic hemodynamic measurement on all mice. The weight of ApoE−/− mice is higher (*P*<0.05) compared to WT group (Table 1). ApoE−/− mice in vehicle group exhibited a higher liver (*P*<0.05) and spleen index (*P*<0.01). However, feeding with *Lactobacillus acidophilus ATCC4356*, the spleen index decreased significantly in La. H group compared to vehicle group (*P*<0.05) (Table 1). We also found that high-fat diet (HFD) significantly increased serum TC, HDL-C and LDL-C levels (*P*<0.01) while TG, TBIL, DBIL and ALT, AST remained the same (Table 1). In La. L group and La. H group, HDL-C level had a tendency to rise, and blood glucose, ALT, DBIL and TBIL had a trend to decrease. However, it was not statistically significant (Table 1).

*Lactobacillus acidophilus ATCC 4356 reduces the incidence of atherosclerotic lesion in ApoE−/− mice*

Next we evaluated the atherosclerotic lesions area. We calculated the proportion of atherosclerotic lesions area in aortic intima by imaging the section of aortic arch and thoracic aorta. After 12 weeks’ treatment, atherosclerotic lesions in aortic arch and thoracic aorta were rarely observed in WT group while atherosclerotic plaques were observed clearly in vehicle group. In *lactobacillus* treated group, atherosclerotic lesions area on aortic arch intima decreased to (17.14±2.92)% in La. L group and (6.82±1.88)% in La. H group (*P*<0.05) (Figure 1A).

In the aorta thoracic segments, similar trend was observed. In vehicle group, the atherosclerotic lesions area was (12.11±3.60)%, while it was decreased to (9.28±1.34)% in La. L group and (4.40±0.27)% (*P*<0.05) in La. H group (Figure 1B). The results suggested that *lactobacillus acidophilus ATCC 4356* treatment in ApoE−/− mice decreased the atherosclerotic lesions area, which indicated the treatment prevented the formation of atherosclerotic lesion.

In WT group, a morphologically intact endothelium covering the cell-free subendothelial space could be found, and there was no plaque formation. In vehicle group, plaques and foam cells accumulated, and extracellular lipid pools deposited and intima atrophy were observed (Figure 1C). In addition, the structure of blood vessel was unclear, and part of intima irregularly thickened. Besides atheroma was developed and large amount of foam cells were presented. Compared to the vehicle group, the plaque amount, the intima thickness, the foam cells number and the inflammatory cells infiltration of the group treated with *lactobacillus* was down regulated.
Effects of lactobacillus acidophilus ATCC4356 on inflammatory cytokines and ROS level

As described above that feeding the mice with Lactobacillus acidophilus ATCC4356 reduces the incidence of atherosclerotic lesion. Based on these findings, we then determined the effects of Lactobacillus acidophilus ATCC4356 on the inflammatory cytokines and the ROS levels in ApoE<sup>-/-</sup> mice. We selected glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA) to assess the oxidative stress. These molecules are involved in various steps of ROS processes [REFS]. In the WT group treated with normal diets, the ox-LDL level was 1553.98±204.87 nmol/L. But after fed with HFD, the ox-LDL level was 2964.78±577.83 nmol/L, which was significantly increased compared to the WT group (P<0.05) (Figure 2A). Lactobacillus treatment decreased ox-LDL level to 1648.07±361.50 nmol/L (P<0.05) (Figure 2A). No significance was observed in mouse serum GSH levels (Figure 2B).

Compared with the mice of WT group fed with ordinary diet, the serum MDA level in ApoE<sup>-/-</sup> mice of vehicle group fed with high-fat high-cholesterol diet increased significantly (P<0.05). After the oral administration of L. acidophilus strain ATCC4356, the MDA level of the mice decreased, and the MDA level of La. H group was significantly different from that of the vehicle group (P<0.01), as shown in Figure 2C. However, the trend of serum SOD activity was the opposite. Compared with WT group, the SOD activity of ApoE<sup>-/-</sup> mice fed with high-fat high-cholesterol diet declined considerably by 66.03% (vehicle group, P<0.01). The SOD activity increased after oral administration of L. acidophilus ATCC4356, by 31.13% in La. H group as compared with the vehicle group (P<0.05) (Figure 2D).

The serum level of IL-10 in ApoE<sup>-/-</sup> mice fed with high-fat high-cholesterol diet was significantly lower than that of the WT group (P<0.05). Although the oral administration of L. acidophilus strain ATCC4356 tend to be increased the serum level of IL-10 in ApoE<sup>-/-</sup> mice, the difference was not significant (Figure 2F). Compared with WT group, the TNF-α level in the vehicle
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The serum TNF-α level in ApoE/−/− mice declined after oral administration of *L. acidophilus* strain ATCC4356, compared with the vehicle group (P<0.05) (Figure 2E). The serum TNF-α level in ApoE/−/− mice fed with high-fat high-cholesterol diet was decreased compared with the mice in WT group (Figure 3A and 3B). After oral administration of *L. acidophilus* strain ATCC 4356, there was an upregulation of IκB-α in the aorta compared with vehicle group (P<0.01, Figure 3A and 3B). NF-κB p65 was mainly found in the cytoplasm in WT group and very rare in the nuclei (Figure 3C and 3D). The NF-κB p65 nuclear translocation was enhanced in ApoE/−/− mice that fed with high-fat high-cholesterol diet. In the vehicle group, the NF-κB p65 expression was greatly up-regulated in the nuclei (Figure 3E and 3F). These results showed that oral administration of *L. acidophilus* strain ATCC 4356 inhibited...
NF-κB p65 nuclear translocation (Figure 3C and 3D).

Discussion

The cholesterol reduction ability of Lactobacillus is now widely recognized and researched, the mechanisms involved are remained to be further investigated. We found that after gastric lavage with bacterial liquid of L. acidophilus strain ATCC4356 at the dose of $5 \times 10^7$ CFU/day and $5 \times 10^8$ CFU/day in ApoE$^{-/}$- mice fed with high-fat high-cholesterol diet for 12 weeks, the serum levels of LDL-C, TC and HDL did not change significantly compared with the ApoE$^{-/}$ mice receiving normal saline. This result indicates that L. acidophilus ATCC4356 administration does not impact on serum cholesterol level of ApoE$^{-/}$ mice fed with high-fat high-cholesterol diet. Moreover, the body weight and liver index of ApoE$^{-/}$ mice receiving gastric lavage with L. acidophilus strain ATCC4356 were not significantly different from that of mice receiving gastric lavage with normal saline.

Yoo Heon et al. performed gastric lavage with L. acidophilus strain ATCC 4356 in pigs fed with high-fat high-cholesterol diet [6]. They found neither body weight nor food intake amount was changed compared with the pigs not administered with probiotics. Hatakka K et al. performed 4-week randomized double-blinded 2-period trial for 38 healthy male subjects with normal average cholesterol levels [7, 8]. For the experimental group, 2 tablets of P. Freudenr-
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eichii (US) were administered daily and the blood lipid level was measured regularly. Throughout the entire course of the experiment, the levels of TC, HDL-C, LDL-C and TG did not change significantly in the experimental group. This result is consistent with our findings, but the effects of Lactobacillus on body weight, cholesterol level and the relevant mechanism should be further studied.

There are more studies about the effect of Lactobacillus on blood lipid rather than the effect of Lactobacillus on atherosclerosis. Tang et al. found that Lactobacillus casei produced by Yakult had an inhibitory effect on atherosclerosis. The present study found no significant effect of L. acidophilus ATCC4356 on blood lipid and body weight of mice, but the atherosclerotic plaque formation was alleviated [9]. Typical atherosclerosis was induced in ApoE-/mice fed with high-fat high-cholesterol diet for 12 weeks. It was supposed that the inhibitory effect of L. acidophilus ATCC4356 on atherosclerotic plaque formation may be not due to the reducing cholesterol effect.

Ox-LDL is the product of LDL oxidation and one of the main reasons of endothelial cell damage. It can also induce the expression of proinflammatory cytokines by endothelial cells. Steinberg’s theory of oxidation showed that the production of reactive oxygen species and ox-LDL oxidative stress played a crucial role in atherosclerosis [10]. Ox-LDL can inhibit the activity of iNOS and NO production, which leads to vasomotor dysfunction, facilitates the proliferation and migration of endothelial cells and smooth muscle cells, and promotes atherosclerosis [2, 11, 12]. Therefore, clearing and reducing ox-LDL is an important management to treat atherosclerosis. In our study, the serum level of ox-LDL increased significantly in ApoE-/mice fed with high-fat high-cholesterol diet, and it decreased significantly after oral administration of L. acidophilus strain ATCC4356. TiuKullisaar et al. found that compared with the 5 subjects who drank fresh goat milk, the serum oxLDL levels of 16 healthy subjects who drank fermented goat milk with L. fermentum ME-3, L. buchneri S-15 and L. plantarum LB-4 were lower. They also discovered that the total antioxidant activity of the subjects drank fermented goat milk was higher than the control subjects [13, 14]. These findings suggest that L. acidophilus ATCC4356 delays the formation and progress of atherosclerosis.

In this study, we found that the serum SOD activity decreased, while serum MDA level increased in ApoE-/mice fed with high-fat high-cholesterol diet as compared with WT group. After oral administration of L. acidophilus ATCC4356, the serum SOD activity was increased, and the MDA level was declined. These results demonstrated the strong antioxidative ability of L. acidophilus strain ATCC4356 which reduced the serum level of ox-LDL. Many reports have discussed about the antioxidative ability of Lactobacillus. T. Virtanen et al. determined the free radical clearing ability of Lactobacillus by fading spectrophotometry and the inhibitory effect on lipid peroxidation using liposome model by fluorescent staining [15]. They assessed the antioxidant ability of 25 Lactobacillus strains and found that L. acidophilus ATCC4356 had the highest antioxidant ability.

TNF-α and IL-10 are multi-effect cytokines, which act as potential pro-inflammatory cytokines in atherosclerosis and other metabolic disorders [16, 17]. TNF-α can be found in atherosclerotic plaques and higher level of TNF-α in circulation can increase the incidence of heart attack and atheroscleroticas well as tri-glyceride and glucose metabolism disorders [18, 19]. Caligiuri et al. reported the level of LDL and leness of plaques increased in ApoE/IL-10 double-knockout mice [20]. However, atherosclerosis was relieved after transfection with IL-10 cDNA in ApoE-/mice. We found that the serum TNF-α level in ApoE-/mice fed with high-fat high-cholesterol diet increased significantly while the IL-10 level decreased. Oral administration of L. acidophilus strain ATCC4356 reduced TNF-α level in the La. H group and Vehicle group (P<0.05); the serum IL-10 level showed an increasing trend. NF-κB is important in regulating the expressions of genes related to immune and inflammatory responses. The p65 subunit is responsible for the strong transcription activating potential of NF-κB, and the activation of NF-κB can be reflected by detecting p65 expression. We found that the NF-κB was activated in the aorta of ApoE-/mice fed with high-fat high-cholesterol diet. In atherosclerosis model group, IκB-α was degraded significantly, and nuclear p65 expression was...
upregulated, indicating p65 nuclear translocation. The administration of L. acidophilus strain ATCC4356 can inhibit the degradation of IκB-α, reduce p65 nuclear translocation and achieve anti-inflammatory and antioxidant effect [21].

In conclusion, L. acidophilus strain ATCC4356 had no impact on the body weight and blood lipid of ApoE-/- mice fed with high-fat high-cholesterol diet, though it alleviated atherosclerosis. It is suggested that the inhibitory effect of L. acidophilus strain ATCC4356 on atherosclerosis may not be exerted by reducing cholesterol level. Instead, the oxidative stress and the expression of inflammatory cytokines are inhibited by L. acidophilus strain ATCC4356 through suppressing the activation of NF-κB signaling pathway.

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Disclosure of conflict of interest

None.

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