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·ORIGINAL ARTICLES·

Effects of Human High Density Lipoprotein Injection on Atherosclerotic Lesions in New Zealand White Rabbits Fed with Cholesterol-Rich Diet and the Comparison with Lovastatin

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MeSH Lipoprotein, HDL; Disease Models, Rabbit; Atherosclerosis; Lovastatin;

Aim The effects of in vivo administration of human high density lipoprotein (HDL) injection on the development of aortic streaks were studied in cholesterol-fed rabbits.

Methods The rabbits received a 1% cholesterol-rich diet for 8 weeks. During this period, the HDL group was intravenously administered with 50 mg/week of HDL injection; the lovastatin group was administered with 10 mg/day of lovastatin via subcutaneous injection; the placebo group received normal saline (0.9% NaCl). The HDL injection was manufactured and provided by Tsinghua Unisplendour Guhan Bio-pharmaceutical Corporation Ltd. During the study, plasma lipid levels followed a similar profile in all groups to cholesterol-rich diet.

Results At the completion of study, atherosclerotic-like lipid-rich lesions covered 32.6% ±21.7% ($\bar{x} \pm s$), of the intima aortic surface in the placebo group, 9.1% ±7.8% in the HDL group, and 20.8% ±13.1% in the lovastatin group. The levels of plasma total cholesterol were 10.05 ±2.30, 2.93 ±1.41, and 3.74 ±1.73 g/L in placebo, HDL, and lovastatin groups, respectively. The levels of plasma high density lipoprotein cholesterol (HDLC) were 0.43 ±0.12, 0.62 ±0.23, 0.23 ±0.14 g/L in placebo, HDL, and lovastatin groups, respectively. The value of total cholesterol deposited within vessel wall were significantly lower in the aortas of the HDL group than those in the placebo group and lovastatin group. Human HDL injection showed a more efficient inhibition of atherosclerotic lesions than lovastatin did.

Conclusion The administration of human HDL injection to cholesterol-fed rabbits dramatically inhibits the extent of aortic fatty streaks, modifies plasma lipid, and lipid deposition in the arterial wall.

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1 INTRODUCTION

Atherosclerosis is a slowly progressive disease characterized by the accumulation of cholesterol within the vessel wall. Low density lipoprotein (LDL) and high density lipoprotein (HDL) are the major cholesterol-carrier lipoproteins[1,2]. LDL is responsible for the delivery of lipids (cholesterol) from liver to the tissue[2,3]. Compelling evidence supports the concept that the lipids deposited in the atherosclerotic lesions are derived primarily from plasma LDL[3]. Reverse cholesterol transport seems to be the major route for removal of the exchangeable cholesterol deposited in the extrahepatic tissue[4,5]. It has been postulated that a major role of the plasma HDL particles is act as a scavenger of tissue cholesterol[6]. Lovastatin has been proved to be effect on plasma cholesterol-lowering not only in animal experiments and also in clinical trials[7-9]. However, the compared effects of HDL and lovastatin on atherosclerotic lesion prevention are not determined.

2 MATERIALS AND METHODS

2.1 Experimental design

Forty health New Zealand White rabbits were selected by body weight and plasma lipid levels. The animals were divided into 4 groups. Group 1 served as placebo group; group 2 was intravenously administered human high density lipoprotein (HDL) injection (50 mg/week/rabbit, HDL group); group 3 received lovastatin (10 mg/day/rabbit) via subcutaneous injection (Lovastatin group); group 4 was kept on normal chow. At the beginning of the experiment, each group contained 10 rabbits which were randomly allocated. At the ending of

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the experiment all animals were killed and the aortic fatty streak formation and cholesterol deposition were studied.

The rabbits in groups 1 to 3 received 1% cholesterol-rich diet for 8 week. During this period, the HDL group was intravenously administered with 50 mg/week every rabbit of 20 mL human HDL injection; the lovastatin group received 10 mg/day every rabbit of subcutaneously administered in volume of 200 μL lovastatin solution (0.5 kg/L) with dimethyl sulfonate as solvent; the placebo group received 20 mL normal saline (0.9% NaCl). The normal chow group provided the baseline for plasma lipid levels served the negative atherosclerotic lesion placebo.

2.2 Animal model

Adult New Zealand White rabbits (1.9±0.21 kg body weigh) were housed in the Experimental Animal Center of Nanhua University. They were individually caged stainless steel wire-bottomed cages in room placeboled to 20±2℃ temperature, 50% ±14% humidity. Our animal care facility is accredited by the Hunan Association for Accreditation Animal Laboratory Care, and all procedures were reviewed by the Institutional Animal Care and Use Committee.

Atherosclerosis was induced by feeding the animals a 1.0% cholesterol-rich diet at a daily amount of 150 g. The atherogenic diet was prepared by step-by-step mixing normal rabbit chow with cholesterol powder. Water was provided ad lib. Human HDL injection was provided by Tsinghua Unisplendour Guhan Bio-pharmaceutical Corporation Ltd. in the People's Republic of China.

2.3 Morphological and histological evaluation of atherosclerosis

The rabbit were anesthetized with Ketamine plus Dompus (5 and 35 mg/kg, respectively, given intramuscularly). The carotid artery was cannulated, and the animals were exsanguinated. After laparotomy and exposure of the aorta, the animals were killed by an overdose of anesthetic, and the aortas were perfused in situ with 0.2 mol/L phosphate-buffered saline. The aortas were removed intact from the aortic arch to the iliac bifurcation and were stripped of all adventitial debris. Each aorta was fixed in 10% buffered formalin. The fixed aortas were stained by Sudan Ⅳ to reveal sudanophilic plaques.

After staining, the aortas were pinned open to flatten them. A template was then made for each aorta by tracing the outlines of the aorta and the atheromatous lesions on a clear plastic sheet. Each template was photographed. Morphometric assessment of the percentage of total aorta covered with lipid deposits (Sudan-positive area) was determined by three observers individually. Each of three blinded observers prepared a template for each aorta, and each then performed planimetric measurements on his own templates. Inter-observer variation was less than 2%. All measurements were averaged for each aorta.

For light microscopy sections, segments of the thoracic aortas after Sudan Ⅳ staining were embedded in paraffin and stained with hematoxylin-eosin.

2.4 Plasma lipid analysis

Blood samples were collected in EDTA (1.5 g/L) tube by ear bleeds before initiation of the assigned diet and just before sacrifice. To placebo for possible diurnal differences in measured blood values within the same rabbit and between rabbits, all bleeds were performed approximately the same time of day (9:00 to 11:00 AM). All the animals were tested for their baseline and killing levels of total and HDL cholesterol and triglycerides after 12-16 h fasting. Plasma was immediately obtained by low speed centrifugation at 4℃. Aliquots were separated and kept frozen at −70℃ until assayed. Cholesterol content was determined using an enzymatic kit (Dong'ou Biological Technology Limited Company, Xiamen, China). Triglyceride content of plasma was measured using an enzymatic kit (Dong'ou Biological Technology Limited Company, Xiamen, China).

2.5 Biochemical analysis of aortic wall

Aortas were homogenized at 4℃ in 5 mL of 0.13 mol/L tris HCl (pH 7.4), 0.01% NaN_3 using a homogenizer.

The total lipids were extracted from the homogenates in 10 vol of chloroform/methane (2∶1 vol/vol).

The lipid-containing was dried under nitrogen and then resuspended in isopropyl alcohol. The total cholesterol level was measured by the specific enzymatic assays mentioned for plasma lipid analysis.

2.6 Animal surveillance

Animals were closely followed during the experimental period. Their daily food intake was recorded and their weight taken once a week to detect any sudden weight loss.

2.7 Statistical analysis

Results are expressed as mean $\bar{x} \pm s$ unless otherwise stated. Multiple group means were compared by single factor analysis of variance (ANOVA) with factorial analysis and differences between groups analyzed by Fisher protected least significant difference and Scheffe F tests. The data were analyzed by SPSS (version 9.0).

3 RESULTS

3.1 Plasma lipids

All the animals responded to the atherogenic diet with

similar, marked increase in total plasma cholesterol compared with predict values (predict baseline 6.1 ±1.6 mg/L). At 60 d, plasma cholesterol levels in placebo, HDL and lovastatin groups were significantly higher than normal chow group. More than, the plasma cholesterol levels in both HDL group and lovastatin group was significantly lower than placebo group.

Table 1. Plasma levels of blood lipids and lipoprotein each group.

Group	TC	TG	HDLC	LDLC	VLDLC	TC/HDLC
Normal	0.68 ±0.10	0.87 ±0.18	0.17 ±0.03	0.34 ±0.08	0.34 ±0.05	4.04 ±0.54
Placebo	10.05 ±2.29[a]	1.16 ±0.45[a]	0.42 ±0.12[a]	8.83 ±2.29[a]	1.22 ±1.56[a]	25.9 ±10.2[a]
HDL	2.92 ±1.41[ab]	0.94 ±0.24	0.62 ±0.23[abc]	2.16 ±1.49[ab]	0.76 ±0.25[abc]	
Lovastatin(7) *	3.74 ±1.73[ab]	1.12 ±0.30	0.22 ±0.13[a]	3.05 ±1.71[ab]	0.68 ±0.17[b]	18.3 ±7.39[ab]

TC: total cholesterol, HDLC: HDL cholesterol, LDLC: LDL cholesterol, VLDLC: VLDL cholesterol, TG: triglycerides, TC/HDLC: ratios of TC to HDLC. (n= 10, g/L) Values are expressed as gram per liter ($\bar{x} \pm s$). a. $P< 0.05$, compared with group normal chow. b, $P< 0.05$, compared with group placebo. c, $P < 0.05$, compared with group lovastatin. * The numeric in parenthesis indicates the number of rabbits.

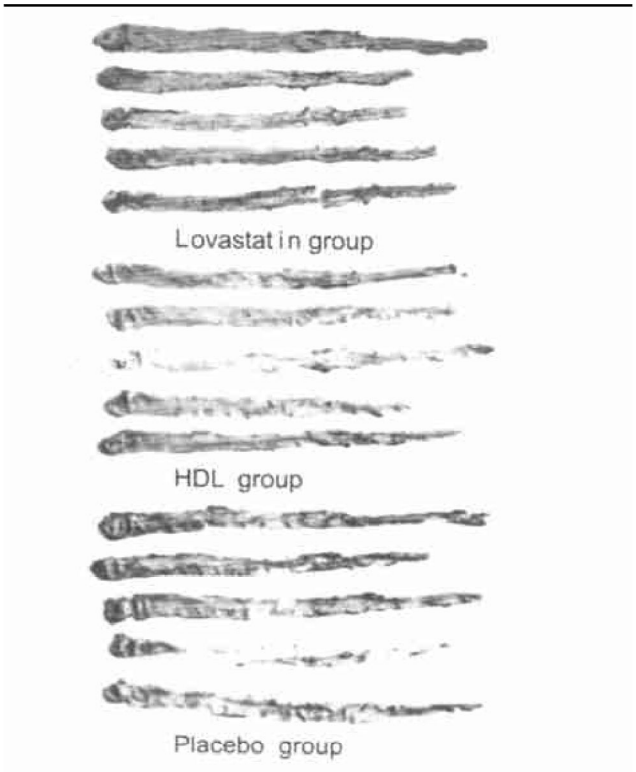

Figure 1. Sudan IV stained aortas demonstrating the atherosclerotic fatty streaks. Placebo group developed severe atherosclerotic lesions, in HDL and lovastatin groups the lesions were reduced by treatment of human HDL injection or lovastatin.

3.2 Morphological and histological analysis

Placebo group animals were killed 60 d after beginning the atherogenic diet and served as a reference point for the level of atherosclerotic lesions achieved in the other 2 groups before the HDL or saline administration. When the three groups (placebo, HDL, lovastatin) were analyzed for the presence of aortic lipid-rich lesions (fatty streaks), all groups showed the atherosclerotic lesion distribution and characteristics typical of experimental model. Lesions consisted of fatty streaks, affecting mainly the aortic arch and the descending thoracic aorta. The bifurcation points, such as the intercostals ostia, were affected most, the abdominal aorta much less so.

The aortic atherosclerosis involvement was evaluated by computerized planimetry of the Sudan IV-positive areas, expressing the results as a percentage of the total aortic surface covered by fatty lesion (Table 1). Placebo group showed 32.6% ±21.7% of the aortic surface affected. The aortic surface of lovastatin group was 20.8% ±13.1% involved. HDL group had only 10% ± 7.9% of their aortic surface covered by fatty streak, a statistically significant difference not only compared with placebo group, but also when compared even with lovastatin group.

Histological examination of the aortas of the three groups of animals revealed similar lesions characterized by accumulation of lipids in the hyperplastic areas of the inner layer of the intimae. A decrease in lesion thickness in the HDL and lovastatin animals is evident in comparison with the animal in normal chow group.

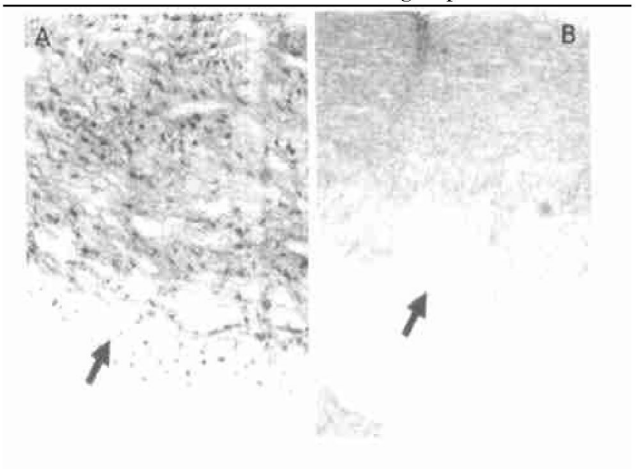

Figure 2. Photomicrographs of representative aortic sections from HDL-treated (panel A), placebo (panel B), 90 days on atherogeinc diet rabbits. [Hematoxylin-eosin stain; original magnification, (A) × 200, (B) × 100].

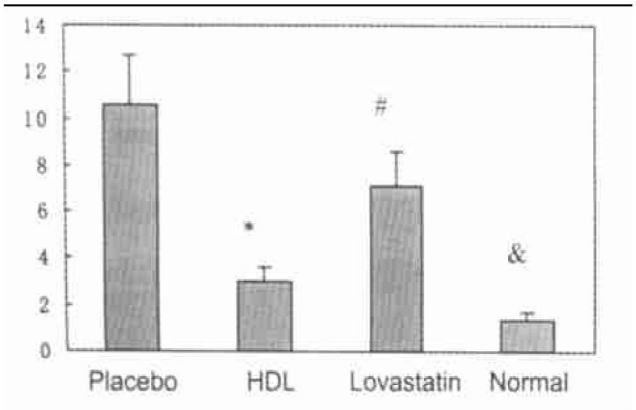

Figure 3. Extent of atherosclerosis as measured by percent of total area of aortic wall. Results are expressed as percentage of aortic fatty streaks (Sudan IV-positive areas); $\bar{x} \pm s$. * $P < 0.05$ compared with Placebo group, # $P < 0.05$ compared with Lovastatin group.

3.3 Lipid accumulation in the aortic wall

To analyze lipid infiltration, a chemical analysis of the lipid extraction from the aortic wall was performed. Values of total cholesterol accumulated within the aortic wall are presented in Fig 3. Placebogroup showed heavier lipid deposition in the aortic wall than HDL group and louastain group. Group 2 had a 60% reduction of total cholesterol. When HDL group was compared with lovastatin group significant decreases were also observed.

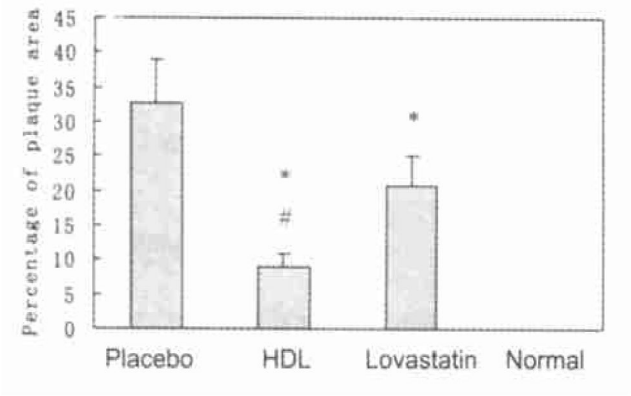

Figure 4. Bar graph showing lipid deposition in aortic wall. Values are expressed as mg/g aorta, $\bar{x} \pm s$. (* $P < 0.05$ compared with placebo, lovastatin and normal group, respectively, # $P < 0.05$ compared with placebo group and normal group, respectively, and $P < 0.05$ compared with placebo group.

3.4 Effect of diet ant treatment

All groups had similar body weight gain throughout the experimental period. The HDL injection treated rabbits gained an average of 0.65 ± 0.2 kg, and placebo group gained an average of 0.61 ±0.2 kg, and HDL group gained an average of 0.63 ± 0.3 kg. No difference in food intake was observed among groups. The animals showed good physical appearance and normal behavior during the entire experiment.

Table 2. Body weights of the experimented animals during study period ($\bar{x} \pm s$).

Group	Baseline	1 month	2 months	2.5 months
Placebo	1.91 ±0.11	2.13 ±0.09	2.42 ±0.11	2.37 ±0.12
HDL	2.01 ±0.12	2.10 ±0.21	2.40 ±0.21	2.38 ±0.23[b]
Lovastatin	1.98 ±0.12	2.21 ±0.21	2.38 ±0.19	2.20 ±0.19[a]
Normal	1.95 ±0.28	2.18 ±0.09	2.33 ±0.10	2.47 ±0.12

a: $P < 0.05$, vs group normal. b: $P < 0.05$, vs group lovastatin.

4 DISCUSSION

The present study sought to determine if human high density lipoprotein injection administration could inhibit atherosclerosis lesions or reverse its further development.

After the high-cholesterol diet was given for 60 d, the animals had 35% of their aortic surface covered by fatty streaks containing 18 ±4 mg of cholesterol/g aorta.

Our results showed that administration of human HDL injection inhibited the progression established atherosclerotic lesions and cholesterol deposition when compared with placebo (group). Therefore, human HDL injection have been shown to reverse the extent of arterial damage and lipid infiltration induced by 60 d of atherogenic diet to levels significantly lower than those achieved after 60 d on diet. HDL-treated animals developed 65% less aortic infiltration with fatty streaks, as determined by computerized planimetry of the aortic wall, than the placebo animals. Their aorta also contained 70% less than cholesterol than those placebo.

In the present study, as expected, all the animals rapidly developed hypercholesterolemia upon atherogenic diet ingestion. Statistical difference in plasma lipids was observed between groups. Human HDL injection treatment induced a significant reduction in the extent of aortic atherosclerotic involvement when compared with placebo and lovastatin groups, respectively. Plasma lipid levels were also modified by the treatment, there seems to be both plasma lipid lowering effect and direct role for HDL in aortic cholesterol metabolism. However, Badimon and his colleagues[6] reported that inhibition of cholesterol-induced atherosclerosis without change in plasma lipid levels has observed in rabbits. It is possibly reasonable explanation that we used human HDL injection which prepared from human plasma fraction IV, and got the reagent cholesterol from different source, in which the cholesterol content and residues should be different from that Badimon used.

Lovastatin is a lipid-lowering drug which inhibits cholesterol synthesis. In this study the results demonstrated that human HDL injection more efficiently inhibited development of atherosclerotic lesions in cholesterol-fed rab-

bits than lovastatin did, even though both of them showed a similar cholesterol lowering effect. We can postulate that the inhibitory efficacy of atherosclerosis by HDL mainly attributes to its reverse cholesterol effect. However, the inhibition of atherosclerosis of statins results from its lipid-lowering effect.

Accumulating evidence supports the concept that the stimulatory effect of HDL in the cholesterol efflux from extrahepatic cells is mediated by reversible binding of HDL apoprotein to high-affinity binding sites on the cell surface. Li et al[10] suggested that apoprotein binding appears to transducer intracellular signals involving protein kinase C and, perhaps, other kinases that in turn induce the translocation of the free cholesterol to the cell membrane, making it more accessible to the cholesterol acceptor HDL. Rubin et al[11] reported that overexpression of human apo AI in transgenic mice raises plasma HDL and protects against atherosclerosis. Apo AI transgenic rabbits were generated and it was shown that overexpression of human apo AI reduced development and diet induced atherosclerosis[12].

In summary, this study demonstrates that human high density lipoprotein injection inhibits aortic lipid deposition and extent of fatty streaks in cholesterol-fed rabbits accompanying decrease of plasma lipid levels. These observations suggest that human HDL might have a significant role in enhancing the reverse cholesterol transport from extrahepatic tissues to the liver for its metabolization and excretion. Therefore, human HDL injection may have more potential inhibition of atherosclerosis than lovastatin does.

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人血高密度脂蛋白注射液对高胆固醇饲料所致新西兰白兔动脉粥样硬化病变的作用及与洛伐他汀的比较

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[主题词] 脂蛋白, 高密度; 动脉粥样硬化; 洛伐他汀; 疾病模型, 家兔;

目的 研究人血高密度脂蛋白(HDL)注射液对喂高胆固醇饲料家兔主动脉粥样指纹病变的影响。**方法** 实验家兔给与 1% 胆固醇饲料 8 周。在此期间, HDL 组每只家兔每周静脉注射 50 mg 人血 HDL; 洛伐他汀组每只家兔每天皮下注射 10 mg 洛伐他汀; 安慰剂组每只家兔每天静脉注射 20 mL 生理盐水。人血 HDL 注射液由清华紫光古汉生物制药集团有限公司生产并提供。**结果** 在试验期间, 各组实验动物喂高胆固醇饲料后血脂水平均有升高。第八周后, 实验动物主动脉内膜表面富脂粥样病变面积比分别为: 安慰剂组 32.6% ±21.7% ($\bar{x}$ ±s, 下同); HDL 组 9.16% ±7.87%; 洛伐他汀组 20.8% ±13.1%。血清总胆固醇水平分别为: 安慰剂组 10.0 ±2.30 g/L; HDL 组 2.92 ±1.41 g/L; 洛伐他汀组 3.74 ±1.73 g/L。血清 HDL 胆固醇水平分别为: 安慰剂组 0.43 ±0.12 g/L; HDL 组 0.62 ±0.23 g/L; 洛伐他汀组 0.23 ±0.14 g/L。HDL 组血管壁胆固醇含量比安慰剂组和洛伐他汀组都明显降低, 说明人血 HDL 具有比洛伐他汀更有效的抗动脉粥样硬化病变作用。**结论** 人血 HDL 能够有效地抑制喂胆固醇饲料家兔主动脉粥样病变的作用, 并能够调节血脂, 减少动脉壁脂质沉积。

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