

# The prevention and treatment effects of egg yolk high density lipoprotein on the formation of atherosclerosis plaque in rabbits

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## ABSTRACT

**Objective(s):** Atherosclerosis is the main leading cause of cardiovascular diseases. The purpose of this study was to assess the potential preventive effect of egg yolk HDL on the atherosclerosis plaque formation.

**Materials and Methods:** Thirty rabbits were divided into five groups: A; normal diet, B; hypercholesterolemic diet, C; hypercholesterolemic + 400 mg/kg egg yolk HDL D; hypercholesterolemic +100 mg/kg egg yolk HDL and E; 200 mg/kg egg yolk HDL. At the end of the experiment, the lipid profiles were measured by spectrophotometric method. The histological sections of thoracic aorta also were taken and analyzed under light microscope.

**Results:** At the end of the 2<sup>nd</sup> and the 4<sup>th</sup> weeks, there was a significant increase of cholesterol level in groups B, C, and D compared to group A ( $P<0.05$ ). Following HDL treatment, triglyceride (TG) levels increased significantly versus group A and also the TG level decreased significantly in group C, D, and E versus group B ( $P<0.01$ ). Egg yolk HDL significantly increased HDL-C in groups C, D, and E ( $P<0.01$ ) compared to groups A and B ( $P<0.05$ ). The surface area of the atherosclerotic plaque was increased significantly in group B versus group A ( $P<0.001$ ). Egg yolk HDL consumption reduced the plaque size significantly ( $P<0.001$ ).

**Conclusion:** Our findings indicated that treatment with egg yolk HDL increased serum HDL-C and decreased atherosclerotic plaque size in rabbits. Thus, egg yolk HDL may be considered as an anti-atherosclerotic treatment for cardiovascular diseases.

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## Introduction

Cardiovascular diseases (CVD) are the most common cause of morbidity and mortality in many countries. According to the World Health Organization (WHO), cardiovascular diseases account for 16.7 million deaths per year (1).

Atherosclerosis is a condition in which an artery wall thickens as a result of the increase of total cholesterol (TC), triglyceride (TG), and low density lipoprotein cholesterol (LDL-c). A complex chronic inflammatory and metabolic disease is caused largely by the accumulation of macrophages and white blood cells and is promoted by LDL (2).

HDL has some physiological activities. For example, mature HDL presents a hydrophobic core composed

of cholesteryl esters and TGs with proteins embedded in a lipid monolayer collected mainly of phospholipids and free cholesterol. HDL contains several apolipoproteins including apolipoprotein A-I (apoA-I) and apoA-II (the two main proteins) and a large number of less abundant proteins counting apoCs, apoE, apoD, apoJ (3, 4).

The most relevant function of HDL is to promote the efflux of excess cholesterol from peripheral tissues to the liver for excretion; a pathway known as reverse cholesterol transport (5, 6). The efflux of cholesterol is important for maintaining cellular cholesterol homeostasis (7).

Egg yolk contains high amount of HDL. Egg yolk HDL can be used to treat cardiovascular disease. The

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focus of egg yolk HDL treatment has been on its favorable actions in increasing HDL-c and reducing LDL-c, very low density lipoprotein cholesterol (VLDL-c), and lipoproteins (8).

On the basis of efficacy of Egg yolk HDL on the treatment of cardiovascular diseases and its other beneficial effects, the purpose of this study was to assess the clinical utility of egg yolk HDL on the lipid profiles and atherosclerosis plaque size in rabbits.

## Materials and Methods

### Preparation of egg yolk proteins

Eggs were purchased from Simorgh Company. To separate the egg yolk proteins, the eggs were homogenized in 5 volume of 0.15 M phosphate buffer (pH 7.4) containing 3.4 Mm ethylenediaminetetraacetic acid (EDTA) and 2 Mm Na<sub>2</sub>HPO<sub>4</sub> and NaH<sub>2</sub>PO<sub>4</sub>. The homogenate was centrifuged at 8500 rpm for 15 min at 4°C. After centrifugation, a clear supernatant was separated from a floating oily and a pellet. The clear supernatant was called "egg yolk proteins" and submitted for the isolation of lipoproteins (9).

### Isolation of egg yolk lipoproteins

Potassium bromide (KBr) was added to the egg yolk proteins to a final concentration of 8.9 g KBr/20 ml solution (44.5%). The solution was placed in a centrifuge tube and overlaid with 20 ml 0.75% NaCl. The tube was centrifuged (Beckman, model L-90) at 50,000 rpm for 6 hr at 15 °C. After centrifugation, fractions were collected and the density was determined by refractometry. The floating HDL fraction (1.15 g/ml ≤ density (d) ≤ 1.25 g/ml) was separated from the bottom fraction (lipid-free protein) and adjusted to a density of 1.30 g/ml by the addition of solid KBr in a final volume of 20 ml. The solution was then located in centrifuge tube, overlaid with 20 ml 22% KBr in 0.75% NaCl, and centrifuged at 50,000 rpm for 6 hr at 15 °C. The floating LDL fraction (1.03 g/ml ≤ d ≤ 1.10 g/ml) was brought to final KBr concentration of 22% and overlaid in a centrifuge tube with 20 ml 0.75% NaCl. The separated lipoproteins were dialyzed against 0.15 M NaCl-10 Mm phosphate buffer (pH 7.4).

### Animals and diets

Thirty adult New Zealand White male rabbits weighing 2-2.5 kg were used in this study (Pasteur Institute, Tehran, Iran). The animals were maintained in individual cages at 20-25 °C temperature and humidity level of 55-65%, under a 12 hr light-dark cycle. They were fed on a standard rabbit's diet (Javane Khorasan, Mashhad, Iran) consisted of 10% protein, 40-50% carbohydrates, 2% vegetable fat, and 15-25% fiber.

Animals were fasted for 12-14 hr and venous blood samples were taken to determine baseline levels. Then they were randomly separated into five

groups (n=6). Each group received one of the five experimental diets: A; normal diet for 4 weeks (as control group), B; 3% of cholesterol diet for 4 weeks (as hypercholesterolemic group), C; hypercholesterolemic rabbits that received egg yolk HDL in high dose (400 mg/kg) by gavage daily, D; hypercholesterolemic rabbits that received egg yolk HDL in low dose (100 mg/kg) by gavage daily, and E; normal diet rabbits that received egg yolk HDL in dose (200 mg/kg) by gavage daily.

The cholesterol rich diet was specially fabricated for the experiments, composed of additional 3% cholesterol added to the standard diet, and the rabbits were fed for 4 weeks. Then, egg yolk HDL was gavaged to rabbits for 6 weeks in groups C, D, and E.

### Biochemical analysis

After a 12-14hr fasting, blood samples were collected from the femoral artery of animals' leg at the baseline and every two weeks until the end of the study. For obtaining the serum, blood samples were centrifuged at 3500 rpm for 20 min. Serum TC, TG, and HDL-C were measured by using standard enzymatic methods (Pars Azmoon Company, Iran) and then were analyzed with an auto analyzer system (Hitachi 902, Japan) (10).

### Preparation of tissue samples

At the end of the experiment, all rabbits were anesthetized via inhalation of ether and thoracic aorta was harvested (from the left subclavian artery to the diaphragm). The aortic plaques were assessed blindly; the thoracic aortas were fixed in 10% formalin for 2 days. After tissue processing and embedding in paraffin, 5µm sections were prepared using a rotary microtome (Leitz 1512, Germany), and dried on a hot plate at 50-55 °C for 30 min; H&E were used to stain the tissue sections. The preparations were examined under light microscope using ×10 and ×40 objective lenses (UPlanFI, Japan) and images were transferred to computer using a high-resolution camera (BX51, Japan). All sections were digitally photographed and the surface areas of atherosclerotic plaque were estimated using a 1000 µm 2 counting frame. Plaque surface area was measured by point grid overlays  $A = P \times A(p)$ ; A= surface area, P = no of test points, and A(p)= area associated with one point in the grid (50 mm<sup>2</sup>). Ten sections including atherosclerotic plaque from each aorta were chosen and analyzed.

### Statistical analysis

Values were expressed as mean±SEM. Statistical analysis was performed with SPSS (version 13). Statistic calculations were carried out with One Way analysis of variance (ANOVA) for multiple pair wise comparisons of groups. It was followed by Tukey test. A significant difference was defined as P-value <0.05.

**Table 1.** The lipid profiles of groups A, B, C, D, and E measured in the different periods of the experiment (mg/dl)

Column1	W0	W2	W4	W6	W8	W10	W12
<b>Cholesterol</b>							
Group A	82.5025	42.78633	87.85873	91.31583	79.889	70.77897	60.12247
Group B	68.45821	1048.2***	723.33***	499.21***	280.381**	191.9155*	284.5417*
Group C	82.91348	884.7817***	430.3365*	192.049#	134.8423#	108.9495	81.35233
Group D	73.275	903.4***	552.829***	267.8918#	170.305	111.0195	73.24817#
Group E	51.65467	124.86###	78.145###	67.051###	84.50933#	70.33183#	49.52483#
<b>HDL</b>							
Group A	46.8703	35.622	38.7557	41.8708	38.5748	37.7126	38.8258
Group B	44.20033	77.04633	43.49133	52.158	25.049	38.64	26.20467
Group C	53.31933	62.81183	50.753	50.75817	57.078###	84.2####	52.19***
Group D	49.02917	40.05567	40.91783	45.48317	58.67###	74.36###	45.943**
Group E	49.60533	66.9445	66.38883*	32.9935	48.76633#	44.30583	31.4114
<b>TG</b>							
Group A	127.6217	75.04567	59.45367	103.014	86.83639	81.08606	77.40177
Group B	150.1043	397.4683	213.618***	195.9492	92.90767	248.898***	251.661***
Group C	148.6758	385.5633*	129.3335	87.61017	74.97483	137.394###	253.731***
Group D	124.9027	432.32***	118.1432#	118.2198	167.5743	208.434***	252.398***
Group E	101.3665	137.7457#	95.98467#	85.49833	93.89433	259.911***	263.905***

Group A: normal group, B: only atherosclerotic, C: atherosclerotic and gavaged dose 1 (400 mg/dl), D: atherosclerotic and gavaged dose 2 (100 mg/dl), E: only gavaged dose 3 (200 mg /dl). Results (mg/dl blood) are presented as mean±SEM. \*  $P$ -value<0.05, \*\*  $P$ -value<0.01, and \*\*\*  $P$ -value<0.005. #  $P$ -value<0.05, ##  $P$ -value<0.01, and ###  $P$ -value<0.005 (\* versus group A) (# versus group B). TC; total cholesterol, HDL-C; high density lipoprotein-cholesterol, TG; triglyceride

## Results

### Biochemistry: serum lipid profiles

#### Serum TC

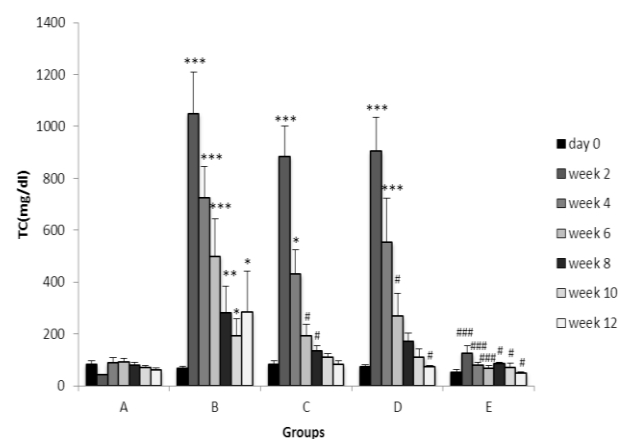
Figure 1 illustrates that the baseline serum levels of TC in group A and other groups were similar. Serum TC significantly increased in group B compared to group A in week 2 until week 12 ( $P$ -value <0.05). Serum levels of TC in groups B, C, and D that fed cholesterol diet from 2<sup>nd</sup> to 6<sup>th</sup> week significantly increased than group A and E ( $P$ -value < 0.005). In addition, there was a significant difference in the serum level of TC between the groups C, D, and E compared to group B during 8 to 12 weeks following egg yolk HDL treatment ( $P$ -value < 0.05) (Figure and Table 1 show details).

#### Serum HDL

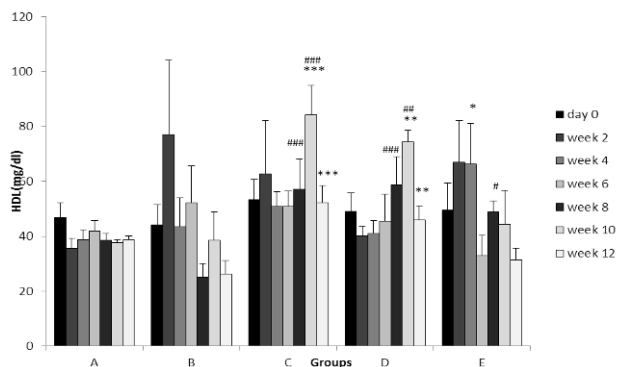
The baseline serum levels of HDL in all groups were similar. At the end of 8<sup>th</sup> and 10<sup>th</sup> weeks, the serum HDL was significantly increased by egg yolk HDL treatment in groups C and D ( $P$ -value <0.01) compared to groups B and A. In addition, there was significant increase in the serum level of HDL in group E in comparison with group B at 8<sup>th</sup> week ( $P$ -value <0.05). In groups C and D serum levels of HDL at weeks 0 to 6 were significantly different compared to weeks 8 to 12 ( $P$ -value <0.01). (Figure 2 and Table 1).

#### Serum TG

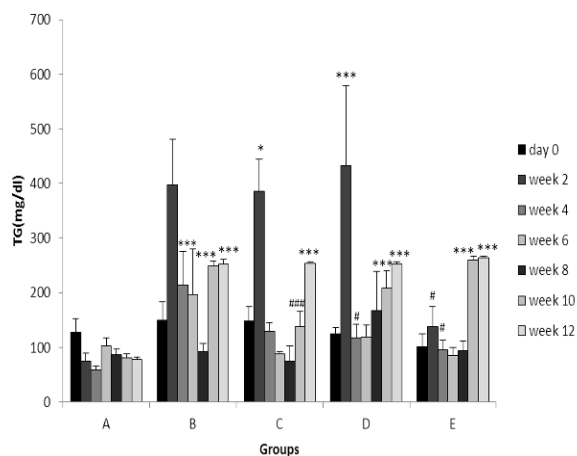
The baseline serum levels of TG in group A and other groups were similar. The serum TG level was significantly increased in 2<sup>nd</sup> week that fed high cholesterol diet (group C). The serum TG level was significantly increased at 10<sup>th</sup> and 12<sup>th</sup> weeks in groups C, D, and E



**Figure 1.** Total cholesterol in five groups of A, B, C, D, and E. Group A: normal group, B: only atherosclerotic, C: atherosclerotic and gavaged dose 1 (400 mg/dl), D: atherosclerotic and gavaged dose 2 (100 mg/dl), E: only gavaged dose 3(200 mg /dl). Values are expressed as mean±SEM. \*  $P$ -value< 0.05, \*\*  $P$ -value< 0.01, and \*\*\* $P$ -value<0.005. #  $P$ -value< 0.05, ##  $P$ -value< 0.01, and ###  $P$ -value<0.005 (\* versus group A) (#versus group B)



**Figure 2.** HDL-C in five groups of A, B, C, D, and E. Group A: normal group, B: only atherosclerotic, C: atherosclerotic and gavaged dose 1 (400 mg/dl), D: atherosclerotic and gavaged dose 2 (100 mg/dl), E: only gavaged dose 3 (200 mg /dl). Values are expressed as mean±SEM. \**P*-value < 0.05, \*\**P*-value < 0.01, and \*\*\**P*-value < 0.005. #*P*-value < 0.05, ##*P*-value < 0.01, and ###*P*-value < 0. (\* versus group A) (#versus group B)



**Figure 3.** Triglycerides in five groups of A, B, C, D, and E. Group A: normal group, B: only atherosclerotic, C: atherosclerotic and gavaged dose 1 (400 mg/dl), D: atherosclerotic and gavaged dose 2 (100 mg/dl), E: only gavaged dose 3 (200 mg /dl). Values are expressed as mean±SEM. \**P*-value < 0.05 and \*\*\**P*-value < 0.005. #*P*-value < 0.05 and ###*P*-value < 0. (\* versus group A) (#versus group B)

that fed egg yolk HDL compared to group A. At the end of 10<sup>th</sup> and 12<sup>th</sup> weeks, the serum TG was significantly decreased by egg yolk HDL treatment in group C (*P*-value<0.01) compared to group B. In group E serum level of TG at 10<sup>th</sup> and 12<sup>th</sup> weeks was significantly increased compared to first weeks (*P*-value <0.005) (Figure3).

#### Histological evaluation

Histopathological analysis: The thoracic aorta obtained from control rabbits revealed normal structural features histologically (Figure 4-A). However, there was an increase in atherosclerotic plaques in the sub-endothelial layer of hypercholesterolemic aorta (Figure 4-B). As shown in Figure 4-B, the tunica intima layers were completely occupied with atherosclerotic

plaques. The extracellular matrix, fibroblast-like cells, and smooth muscle cells in the atherosclerotic plaques were strongly present in the hypercholesterolemic animals.

Surface area of atherosclerotic plaque: The thoracic aorta of control animals (group A) had no evidence of atherosclerotic plaques. In hypercholesterolemic rabbits (group B), the surface area of the plaques was increased significantly (*P*-value<0.005). By contrast, the atherosclerotic plaque cross-sectional areas obtained from vessels of hypercholesterolemic animals received HDL of egg yolk supplementation (groups C & D) were reduced in comparison to those from animals that received the cholesterol diet alone (*P*-value <0.005) (Figure 5).

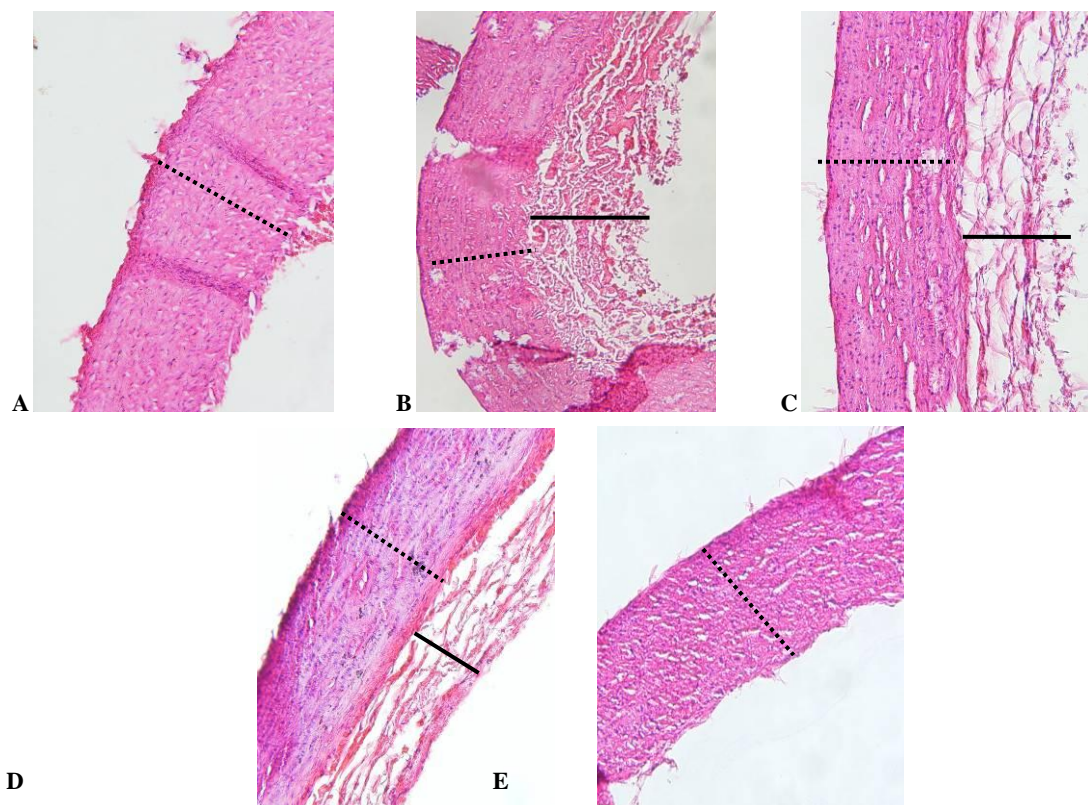
#### Discussion

In this study we demonstrated by chemical and histological evaluation that egg yolk HDL treatment resulted in a significant decrease in atherosclerotic plaque surface. Treatment with high and low doses of egg yolk HDL (400 and 100 mg/kg) was shown to increase plasma HDL levels. Increased HDL levels are correlated inversely with cardiovascular disease (11, 12), but low plasma levels of HDL are associated with increased risk of coronary heart disease (CHD) (13, 14). A strong therapeutic rationale therefore exists for increasing plasma HDL levels of patients at risk for CHD (15). The torcetrapib clinical trial, where patients taking the cholesteryl ester transfer protein (CETP) inhibitor showed an increase of 72% in HDL levels, was terminated prematurely as it resulted in an increased risk of mortality and morbidity of unknown mechanism (16,17).

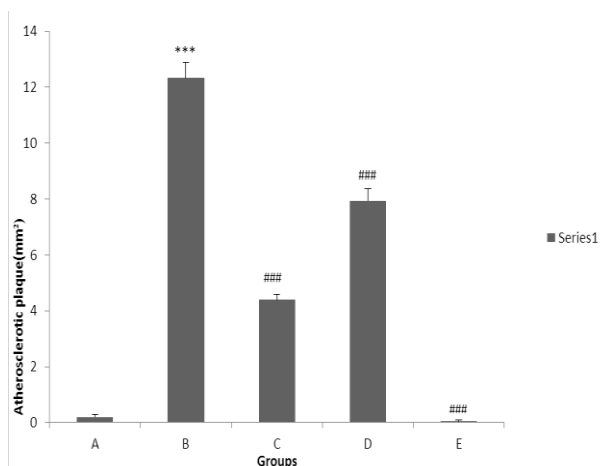
The present study also demonstrated the antiatherogenic effect of purified HDL from egg yolk on experimental atherosclerosis. In agreement with our study, it was shown that IV injection of the homologous HDL-vHDL fraction suppressed the progression of atherosclerosis in cholesterol-fed rabbits (18).

This study provides the first data that purified HDL egg yolk has an anti-atherogenic effect in experimental model of atherosclerosis. One of the important findings in our study is that egg yolk HDL does significantly affect plasma cholesterol and HDL plasma levels and does significantly decrease cholesterol content in the vascular walls which can inhibit the progression of atherosclerosis (11, 13, 19).

Omole and Ighodaro evaluated the effect of egg yolk consumption on the lipid profiles which implied an increase in serum cholesterol (20) Spence *et al*, also showed that regular consumption of eggs increases the risk of cardiovascular diseases. But in our study the HDL separated from egg yolk by the methods mentioned has lost a high percentage of fat in the egg yolks in the final product. It seems that the high percentage of HDL can impinge anti-



**Figure 4.** Hematoxylin & eosin stained arterial sections of thoracic aorta. An atherosclerotic plaque (P) can be observed in the group B in comparison to group A. These plaques occupy the intima layer completely in an aortic section which belongs to hypercholesterolemic rabbits. A: normal group, B: only atherosclerotic, C: atherosclerotic and gavaged dose 1 (400 mg/dl), D: atherosclerotic and gavaged dose 2 (100 mg/dl), E: only gavaged dose 3 (200 mg /dl), scale bars: 500  $\mu$ m.  $\times$ 20 ( — atherosclerotic plaque) ( ..... medial layer)



**Figure 5.** Mean of surface area of atherosclerotic plaque (mm<sup>2</sup>) in five groups of A, B, C, D, and E. Group A: normal group, B: only atherosclerotic, C: atherosclerotic and gavaged dose 1, D: atherosclerotic and gavaged dose 2, E: only gavaged dose 3. Values are expressed as mean  $\pm$  SEM. \*\*\*P-value < 0.001 and ###P-value < 0.001 (\*versus group A) (#versus group B)

atherogenic effect and reduce the atherosclerotic plaque size (19, 21). In turn, the aim of this study was to purify the HDL from egg yolk that could produce large

amounts of HDL for determining the oral effects. In our knowledge this is the first report on the preventive effect of the HDL egg yolk on lipid profile and atherosclerotic plaque size in an animal model.

The cardio-protective effects of HDL have become the topic of intense scientific scrutiny in recent years. One of the hypotheses suggested the key role of HDL in reversing cholesterol transport pathway (22, 23). On the other hand a high level of plasma HDL promotes the net movement of cholesterol from extra hepatic tissues back to the liver; consequently, reduction of the cholesterol accumulation was seen in peripheral tissues. This process may finally decrease atherosclerosis. The metabolic fate of HDL-associated cholesterol through the reverse cholesterol transport pathway is intimately linked to the metabolism of TG-rich lipoproteins (23). Indeed, one mechanism whereby the cholesteryl esters carried initially within the HDL particles are transported to the liver is through transferring to apo B-containing lipoproteins by CETP (24) with the eventual uptake of these lipoproteins within hepatocytes (25, 26).

HDL has also been shown to promote fibrinolysis (27, 28), to act as an anti-oxidant (29, 30) and to decrease LDL uptake in endothelial cells by challenging for the LDL receptor (31, 32).

Thus, the extent to which the reverse cholesterol transport pathway is involved in protecting against atherosclerosis remains to be clearly recognized, and it has to be mentioned that other antiatherogenic properties of HDL may also play an important function in this process (22). Plasma concentrations of HDL cholesterol and apo A-I, the major protein moiety of HDL are inversely correlated by plasma TG concentrations (33, 34, 23).

## Conclusion

Our findings indicated that treatment with egg yolk HDL increased serum HDL-C and decreased atherosclerotic plaque size in rabbits. Thus, egg yolk HDL can be considered as an anti-atherosclerotic agent in treatment of patients with CVD.

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## Conflict of interest

The authors declared no conflicts of interest.

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