May an alcohol induced increase of HDL be considered as atheroprotective?

Ivana Kralova Lesna[1] , Pavel Suchanek, Petr Stavek and Rudolf Poledne

IKEM, Centre for Cardiovascular Research

Vídeňská 1958/9, 140 21 – Prague 4-Krč

Short title: Consumption of alcohol and atherogenesis

Summary:

It is well known that the consumption of moderate doses of alcohol leads to increase of HDL cholesterol (HDL-C). Atheroprotectivity of HDL particles is based primarily on their role in reverse cholesterol transport (RCT). In the study with a cross-over design 13 male volunteers were studied in two different regimens: i) drinking of 36 gr alcohol daily and ii) drinking only non-alcoholic beverages, to test whether alcohol induced increase of HDL cholesterol can affect cholesterol efflux (CHE) from cell culture of labelled human macrophages. Alcohol consumption induced significant ($p<0,05$) increases of HDL cholesterol (in mmol/l) from 1,25 ±0,32 to 1,34 ±0,38 and Apo A1 (in g/l) from 1,34 ±0,16 to 1,44 ±0,19. These changes were combined with a slight increase of cholesterol efflux (in %) from 13,8±2,15 to 14,9±1,85, $p=0,059$. There were significant correlations between individual changes of HDL-C and Apo A1 concentrations and individual changes of CHE (0,51 and 0,60, respectively).

Conclusion: Moderate alcohol consumption changes the capacity of plasma to induce CHE only at a border line significance.

Key words: reverse cholesterol transport, cholesterol efflux, lipoproteins, alcohol consumption

[1]) Corresponding author, ivka@medicon.cz

Introduction

Several studies have revealed that life style factors including moderate consumption of alcohol positively influence the incidence of cardiovascular disease (Rimm 1999). Both case-control and cohort studies have shown that light and moderate drinkers have higher concentration of HDL particles than non-drinkers (Rimm 1999, Bazzano 2008) and HDL cholesterol has been suggested to be the mediator of 40—60% of the positive effect of alcohol in the incidence of coronary heart disease (Grønbaek 2002).

Reverse cholesterol transport is based on the specific property of a sub-fractions of HDL particles to induce free cholesterol efflux (CHE) from cells, to bind and esterify free cholesterol and to transport it to the liver for further metabolism or excretion in the stool. This pathway is crucial for protection of peripheral cells from the negative effect of cholesterol.

In the first step of RCT unesterified cholesterol is carried out to the plasma membrane and then is taken up into extracellular space. Although some non-specific processes may be involved (Rothblat 1982), the removal of cellular cholesterol is realized almost entirely through interaction between cellular receptors such as ABCA1, ABCG1 and SR-BI and plasma acceptors. It has been proved (Linn 1999) that a part of intracellular cholesterol leaves macrophages together with endogenously synthetized ApoE. The accepted cholesterol leads to enlargement of the HDL particles. Free cholesterol on the surface of discoidal pre-β particles is esterified through LCAT and migrates to the centre of particle and promotes further cholesterol efflux. HDL cholesterol is then transported to the liver both directly through interaction of HDL with hepatic SR-BI and indirectly via the transfer of HDL cholesterol esters to triglyceride rich lipoproteins by CETP action. These cholesterol esters are brought to the liver in LDL or in chylomicron remnants.

Studies analysing the effect of alcohol consumption on RCT are rare. Three independent studies identified the positive influence of alcohol intake on CHE from rat hepatoma cells (van Gaag 2001, Beulens 2004, Sierksma 2004) and one of these studies (Beulens 2004) confirmed the increase of CHE from mouse macrophages.

In this study, a method of cholesterol efflux (CHE) measurement from human macrophages was used to determine possible changes of an alcohol induced increase of HDL-C. The crucial role of macrophages in the process of atherosclerosis as well as substantial differences in cholesterol cell metabolism between rodents and humans (Oschry 1982) lead us to choose this cell model.

The complexity of RCT process makes it difficult to evaluate the RCT as a whole from the separate studies of particular steps of RCT. However, to observe such a complexity, the methods that use the diluted serum or plasma as acceptor of cholesterol from cultivated cells may be used.

We therefore measured cholesterol efflux in healthy men in whom the HDL cholesterol and Apolipoprotein A1 were influenced by consumption of moderate doses of alcohol.

Abbreviations:

ABCA1	adenosine triphosphate binding cassette transporter A1
ABCG1	adenosine triphosphate binding cassette transporter G1
SR-BI	scavenger receptor B1
LCAT	lecithin:cholesterol acyl transferase
CETP	cholesterol-ester transfer protein
PLTP	phospholipids transfer protein

Methods:

Diets and lifestyle

The study had a randomized crossover design. Two 4-week diet modifications were carried out in succession without a washout period. The cross over design of the study was used to minimize all confounding effects. Inclusion criteria were males only, self-motivation to

participate in the study, age between 18 and 50 years, body mass index < 30 $kg.m^{-2}$ and a normal concentrations of lipoproteins. Exclusion criteria were: use of any medication that could affect study outcomes, consumption of alcohol more than 30 gr daily, diabetes mellitus and/or other major illness as assessed by medical history. None of the subjects used any medication 4 weeks before and during the study. Seven volunteers consumed 36 gr of alcohol (one litre of beer) per day in the first period followed by period of abstinence and the other 6 participants consumed only non-alcoholic beverages in the first part, followed by consumption of one litre of beer per day in succession.

Subjects were asked to continue their normal eating and living habits (except the provided alcohol) with exception of certain decrease of energy intake during period with moderate alcohol consumption to keep constant body weight. Compliance with the protocol was checked by a daily questionnaire. Body weight, waist and hip circumference were measured on the first and last days of the each study period.

The study was approved by the Local Ethic Committee and conducted in accordance with the Helsinki Declaration. All participants provided written informed consent prior to enrolment into the study.

Biochemical data

Blood samples were obtained by venous puncture at the beginning of the study and at end of each experimental period after a 12-h overnight fast into vacutainer tubes. Samples for lipid analysis were allowed to coagulate at room temperature, separated, portioned, and frozen in duplicate and stored at –80 °C until the analysis at the end of the study. Blood samples for cholesterol efflux measurements were taken into tubes containing EDTA as an anticoagulant, placed immediately in ice water and centrifuged at 3700 rpm at 4 °C for 10 minutes. The plasma was separated, portioned, and frozen in duplicate and stored at –80 °C until the

analysis at the end of the study. Plasma triglyceride concentrations, total and low density cholesterol concentrations were determined enzymatically by commercial kits (Roche diagnostics) with autoanalyzer (Cobas Mira Plus). High density lipoprotein cholesterol (HDL-C) concentration was measured after phosphotungstate precipitation of apolipoprotein B (Apo B) containing lipoproteins. The coefficients of variation for lipoprotein assay were less than 3,0 %. Non-esterified fatty acid (NEFA) concentrations were measured by enzymatic test (Wako Chemicals GmbH, Neuss, Germany) and Apo A1 and Apo B concentrations by imunoturbidimetric assay (Orion Diagnostica, Espoo, Finland). The coefficients of variation for NEFA and apoliproproteins analyses were less than 7,0 %. All biochemical analyses and CHE measurements were performed at the end of the study to eliminate inter-assay variation. All lipoprotein analysis methods in the laboratory were under permanent control by the Center for Disease Control (CDC), Atlanta, GA, USA. LDL cholesterol concentration was also calculated from Friedewald´s formula.

Anthropometric data

Anthropometric data (height, weight, waist circumference and body mass index (BMI=weight.height^{-2}) were determined at the beginning of the study and at the end of each study period.

Cell culture and efflux measurements

RCT was measured using human macrophages pre-labeled in the medium with [^{14}C] cholesterol as described recently in detail (Kralova Lesna 2008). Briefly, THP-1 human monocytes (human monocytic leukemia cells, ECACC 88087201) were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 2mM glutamine and 1% penicillin/streptomycin (PAA Laboratoires) at 37 °C, 5% CO_2.

THP-1 monocytes were seeded into 24-well plates at a density approximately 2×10^5 cells/ml in the presence of phorbol 12- myristate 13-acetate (100 ng/ml) (Sigma-Aldrich) for 72 h to induce differentiation into macrophages. Before each of the succeeding steps, the cells were washed with phosphate-buffered saline (PBS) (PAA Laboratories), containing 0.1% fatty acid free bovine serum albumine (FAFA) (PAA Laboratories). THP-1 macrophages cells were labelled during a 48-hour incubation in a medium containing [^{14}C] cholesterol (specific activity 0,2 μCi/ml) (PerkinElmer Life Sciences, Inc.).

These macrophages were subsequently labelled during 48-hr incubation in a medium containing [^{14}C] cholesterol. To measure cholesterol efflux, cells were incubated for 240 minutes in a RPMI medium containing 5% of plasma of the study subjects (efflux measured between 15 and 240 min). Cholesterol efflux (%) was expressed as the radioactivity of the efflux media divided by total radioactivity of the sample (media plus lysed cells). Each plasma sample was analysed in triplicate and presented data are means of triplicates. The coefficient of variation for CHE assay was 9,95 %.

The measured values of efflux were not corrected to efflux to albumin alone (6,57 % (SD 0,57)) and they may include the efflux of labelled cholesterol carried with endogenously synthetized ApoE. These parameters should not vary within wells and subjects.

Statistical analysis

All results are expressed as mean ± standard deviation (SD). The differences between periods were evaluated using a paired t-test. The relationship between changes of CHE and changes of lipoprotein parameters were analyzed by a simple linear regression.

Results

The participants of the study were 13 young males (age 32,31 ± 5,9 years). No significant changes of body weight and waist circumference were documented during both dietary regimens (Tab.1) and it may be considered as good proof of regular weight controls during both experimental periods.

As expected, the alcohol consumption increases HDL concentration by 7,2 % and ApoA1 concentration by 7,5% (Tab.2). Surprisingly an increase of concentration of total cholesterol and LDL cholesterol (8,6 % and 8,3 % respectively) was found. Similarly, a non-significant trend was observed in ApoB and concentrations of triglycerides, NEFA and glycemia were not affected by the alcohol consumption.

CHE was non-significantly higher at the end of drinking period (14,9 ±1,85 %) compared to non-drinking period (13,8 ±1,66%) (p=0,059). These results were not affected even after adjustment to HDL-C and/or Apo A1 concentrations.

On the other hand, positive correlations were found between changes of CHE and changes of HDL cholesterol and ApoA1, see the figures 1 and 2, respectively. The correlation between CHE change and change of ApoA1 reached a correlation coefficient of 0,60 and the coefficient of correlation to change of HDL concentration was 0,51.

Table 1

Antropometric parameters:

	Baseline	Abstinence period	Alcohol period	Statistical significance
Weight (kg)	79,04 (14,15)	78,67 (13,91)	79,14 (14,12)	n.s.
Body mass index ($kg.m^{-2}$)	24,75 (4,01)	24,64 (3,9)	24,79 (3,99)	n.s.
Waist (cm)	84,00 (8,32)	83,86 (8,46)	84,04 (8,2)	n.s.

Table 2

Biochemical parameters

	Baseline	Abstinence period	Alcohol period	
Total cholesterol (mmol/l)	4,88 (0,74)	4,72 (0,4)	5,13 (0,88)	p<0,01
Triglycerides (mmol/l)	1,19 (0,68)	1,24 (0,67)	1,24 (0,48)	n.s.
LDL cholesterol (mmol/l)	3,27 (0,69)	3,23 (0,67)	3,50 (0,74)	p<0,01
HDL cholesterol (mmol/l)	1,29 (0,30)	1,25 (0,32)	1,34 (0,38)	p<0,01
Apo B (g/l)	0,94 (0,15)	0,93 (0,17)	0,98 (0,20)	n.s.
Apo A1 (g/l)	1,35 (0,17)	1,34 (0,16)	1,44 (0,19)	p<0,01
NEFA (mmmol/l)	0,28 (0,12)	0,26 (0,10)	0,27 (0,17)	n.s.
Glucose (mmol/l)	5,5 (0,32)	5,68 (0,51)	5,57 (0,46)	n.s.
CHE (%)	Not measured	13,80 (2,15)	14,90 (1,85)	p=0,059

Discussion:

Controlled alcohol consumption leads to a significant increase of HDL-C and ApoA1 concentrations. Furthermore, individual changes of HDL-C and ApoA1 clearly correlated with individual changes of cholesterol efflux.

The mechanism of alcohol influence on HDL concentration is not clear yet. The study of de Silva (de Silva 2000), based on investigation of transformed human hepatocytes (Tamm S-P 1992, Dashi 1996) suggests that a moderate consumption of alcohol rises HDL-C primarily by increasing the transport rates of ApoA I and ApoA II. The increase of lipoprotein lipase activity has been repeatedly reported after alcohol consumption (Taskinen 1985, de Silva 2000, Kovar 2004, Mudrakova 2007), whereas the hepatic lipase activity is lowered (Goldberg 1984, Taskinen 1985) or unchanged (Kovar 2004) after alcohol intake. Van Tol (van Tol 1995) found a tendency to an increased esterification rate in the postprandial phase in middle aged men after red wine consumption. Van der Gaag (Van der Gaag 2001) confirmed this result in a controlled study when he found an increased HDL cholesterol esterification rate by approximately 11% after 3 weeks of moderate alcohol consumption. On the contrary, Riemens (Riemens 1997) found no effect on plasma LCAT in persons with a higher consumption of alcohol.

In our study the inclusion of alcohol to diet resulted in a highly significant change of HDL cholesterol but the change of cholesterol efflux was only of border line significance.

To the best of our knowledge, our study is the first to demonstrate that an increase of HDL cholesterol elicited by a controlled moderate alcohol consumption correlates with cholesterol efflux from human macrophages. Our results are in an agreement with the similar studies in the literature (Van der Gaag 2001 and Beulens 2004) which have described an increase of CHE via ABCA1 receptor to plasma from rat hepatoma cells and mouse macrophages. This increase was confirmed in a group of post-menoupausal women (Sierksma 2004). The study

of Hoang (Hoang 2008) found higher CHE to plasma of men with moderate consumption of alcohol compared to abstainers, but a retrospective design with alcohol consumption obtained by questionnaire is a limitation of this study. The results of studies focused on cholesterol efflux in humans with chronic abuse of alcohol lead to less uniform results (Rao 2000, Marmilott 2007).

The measurement of CHE may be affected by the method used. We decided to use modified macrophages because they play a crucial role in the pathogenesis of atherosclerosis. This cell model has been used in similar studies frequently (Palmer 2004, Mweva 2006, Berrougui 2006), and the involvement of receptors ABCA1, ABCG1 and SR-BI has been proved in these cells (Uehara 2007, Sporstøl 2007). Both the whole diluted sera and the isolated lipoproteins have been used in studies of cholesterol efflux as acceptors of cellular cholesterol. We preferred the use of the whole serum because it avoids the pitfall changes of HDL or their sub-fractions during isolation. Although the use of the whole serum does not allow for discrimination between the relative roles of different pathways and receptors involved in CHE, it is likely that it has the best chance to resemble the situation as it would appear in vivo.

The limitation of our study is in an increase of total and LDL cholesterol after alcohol consumption. These results are not in agreement with the above mentioned studies. An apparently feasible explanation is the diet composition. The volunteers were asked to maintain their usual diet and their body weight was controlled weekly. As no changes of body weight and other anthropometric parameters were recorded, we might suppose that the energy content of diets (including alcohol energy content) did not substantially differ between both periods. In the Czech male eating pattern, the alcohol consumption in pubs is usually combined with a consumption of diet high in saturated fats. Thus this difference between the compositions of diets during both periods might be responsible for the increase of total and

LDL cholesterol. These increases of the total and LDL cholesterol could be based on increase of LDL particles, as no change of ApoB concentration was detected.

An extensive meta-analysis of more than forty studies aimed at the influence of alcohol in humans (Rimm 1999) convincingly concluded that the alcohol intake is causally related to a lower risk of coronary heart disease through the changes in lipids and hemostatic factors. The correlation between the individual changes of apolipoprotein A1 concentration and the cholesterol efflux found in our study suggests that the changes in HDL cholesterol as a result of moderate alcohol intake might relate to the changes in reverse cholesterol transport, although results obtained were only on the border line of significance.

Several recent published studies (de Vries 2005, Berroughi 2007, Kralova Lesna 2008) supposed that serum levels of HDL – C and apolipoprotein A1 may not be an adequate indicator of efficacy of RTC and direct measurement of cholesterol efflux to natural acceptor might represent an improvement of the atherosclerosis risk estimate.

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Figure captions

Figure 1.

Relationship between the change of cholesterol efflux (CHE) after i) 4 weeks of moderate consumption of alcohol and after ii) 4 weeks of drinking only non-alcoholic beverages and the change of HDL cholesterol.

Figure 2.

Relationship between the change of cholesterol efflux (CHE) after i) 4 weeks of moderate consumption of alcohol and after ii) 4 weeks of drinking only non-alcoholic beverages and the change of ApoA1.

Fig 1.

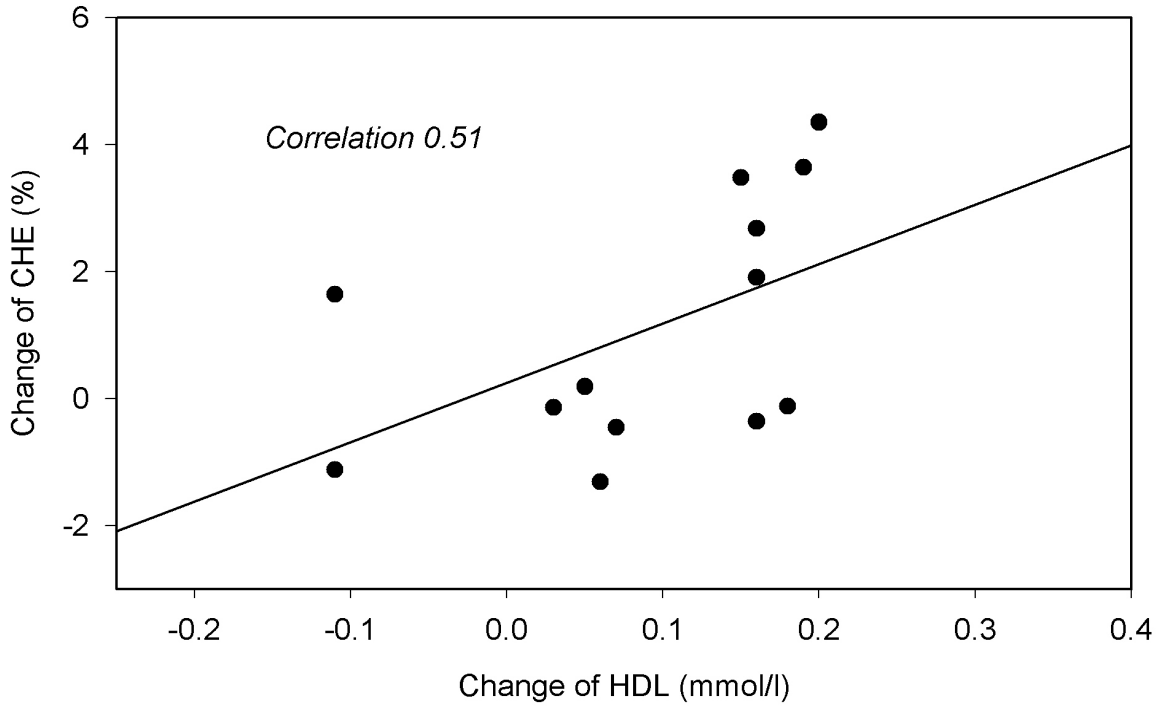
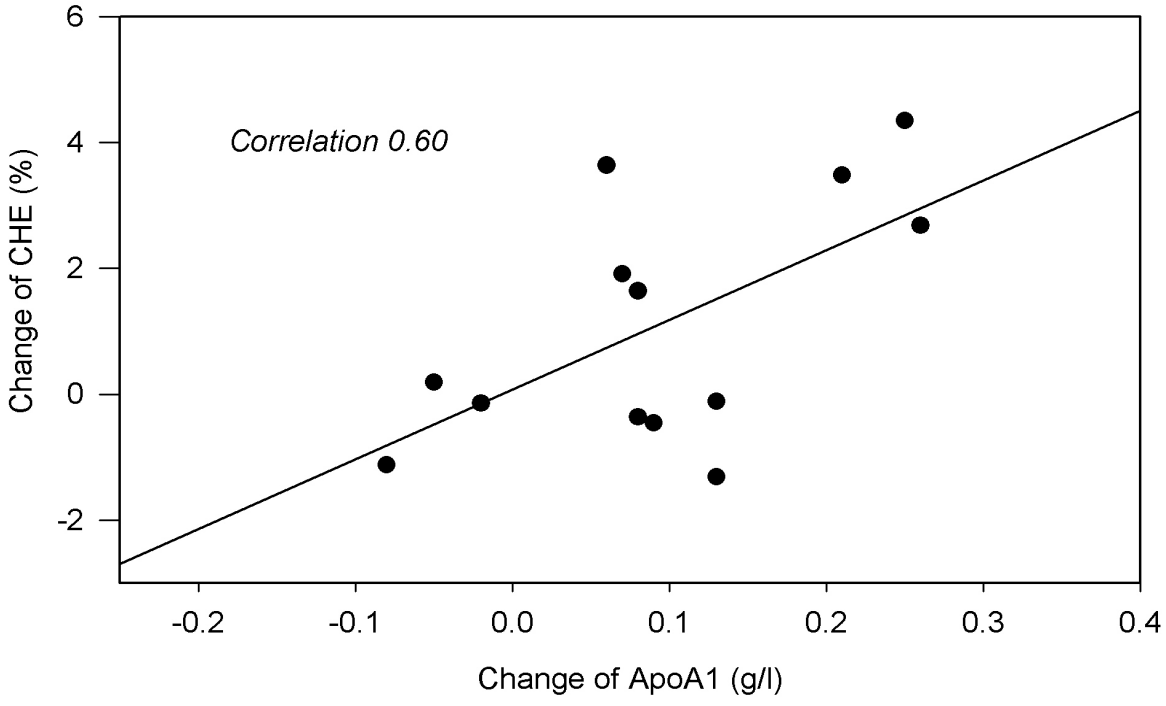

Correlation 0.51
Change of CHE (%)
Change of HDL (mmol/l)
6
4
2
0
-2
-0.2
-0.1
0.0
0.1
0.2
0.3
0.4

Fig 2.


Correlation 0.60
Change of CHE (%)
Change of ApoA1 (g/l)
6
4
2
0
-2
-0.2
-0.1
0.0
0.1
0.2
0.3
0.4