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Lowering of body iron stores by blood letting and oxidation resistance of serum lipoproteins: a randomized cross-over trial in male smokers

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Abstract. Salonen JT, Korpela H, Nyyssönen K, Porkkala E, Tuomainen T-P, Belcher JD, Jacobs DR Jr, Salonen R (Research Institute of Public Health, University of Kuopio, Kuopio, Finland, and Division of Epidemiology, University of Minnesota, Minneapolis, MN, USA). Lowering of body iron stores by blood letting and oxidation resistance of serum lipoproteins: a randomized cross-over trial in male smokers. *J Intern Med* 1995; **237**: 161–68.

Objectives. The purpose of this study was to test the hypothesis that the reduction of body iron stores by venesection (blood letting) would reduce the susceptibility to oxidation of atherogenic serum lipoproteins.

Design. This is a randomized, controlled cross-over trial in 14 regularly smoking men with elevated serum ferritin concentration. The study design comprised two 14-week study periods, with a 14-week wash-out period in between, with either blood donations or control.

Setting. The study site was the Research Institute of Public Health, University of Kuopio. Investigators from the Division of Epidemiology, University of Minnesota, Minneapolis, participated in the planning of the study.

Subjects. Fourteen volunteers who were heavy smokers and had previous experience in blood letting were recruited for the study.

Interventions. During the intervention periods, the subjects donated 450 mg (500 mL) of blood three times in 14 weeks.

Main outcome measurements. Oxidation resistance of very low density lipoprotein (VLDL)/low density lipoprotein (LDL) was measured after inducing oxidation with haemin and H_2O_2.

Results. Serum ferritin concentration was reduced by 44% [95% confidence interval (CI) 8–82%, $P = 0.021$] during the venesection periods, the maximal oxidation velocity was decreased by 20% (95% CI 3–30%, $P = 0.032$), and the lag time to start of oxidation was lengthened (oxidation resistance increased) by 33% (95% CI 1–64%, $P = 0.036$).

Conclusions. These observations indicate that the reduction of body iron stores by venesection can increase the oxidation resistance of serum VLDL/LDL in regularly smoking men.

Keywords: antioxidants, clinical trials, ferritin, iron, lipid peroxidation, venesection.

Introduction

Iron is a transition metal which can easily become oxidized and thus act as an oxidant. Halliwell & Gutteridge [1] have proposed that the general effect of catalytic iron is to convert poorly reactive free radicals such as H_2O_2 into highly reactive ones, such as the hydroxyl radical. To be able to promote free radical production in the human body, iron needs to be liberated from proteins such as haemoglobin, transferrin or ferritin. It is thought that oxidative stress itself can provide the iron necessary for the formation of reactive oxygen species [2]. For example, superoxide radical can mobilize iron from ferritin [1–3].

Oxidative stress due to oxygen free radicals promotes the oxidation of lipids. Redox-active forms of iron catalyse free radical production and can thus promote the oxidation of lipids. Free radical formation and lipid peroxidation can be prevented by the iron-

chelating agent desferrioxamine [1]. Recently, Balla *et al.* demonstrated that the combination of activated neutrophils and haemin or physiological concentrations of hydrogen peroxide and haemin induce a rapid peroxidation of low density lipoprotein (LDL) *in vitro* and that free iron is released from the degraded haem ring [3]. Transition metal ions have been found to be required for the peroxidation of LDL by neutrophils, monocyte/macrophages and smooth muscle cells [4–6].

There are very few clinical data concerning the effect of iron on lipid peroxidation. We observed in 60 eastern Finnish men a positive association between blood haemoglobin concentration and titre of autoantibodies against malondialdehyde (MDA) modified LDL ($r = 0.27$, $P < 0.05$), suggestive of a role of haem iron or the haemoglobin itself in lipid peroxidation *in vivo* in men [7].

High body iron stores have been associated with the risk of both cancer [8] and coronary heart disease [2, 9, 10]. Sullivan has proposed a role for iron sufficiency in the etiology of coronary heart disease [11]. Salonen *et al.* observed an association between elevated serum ferritin concentration and high dietary iron intake and the risk of acute myocardial infarction in a cohort of 1931 middle-aged eastern Finnish men [9]. This finding was recently confirmed in a longer follow-up of the same cohort [12]. In the US Health Professionals Study in 44933 men aged 40–75 with no history of cardiovascular disease, dietary intake of haem iron (but not that of total iron) had a consistent and statistically significant association with an increased risk of myocardial infarction [13]. Magnusson *et al.* reported an association between a low iron-binding capacity and an increased risk of myocardial infarction in 2036 men and women from Iceland [14]. Hypothetically, the possible coronary disease promoting effect of high iron stores could be through increased susceptibility to oxidation of lipids by elevated iron stores and consequent chronic exposure of lipids to redox-active iron [2].

We found no previous controlled clinical trials of the effect of iron depletion on either serum lipid levels or on lipid peroxidation. For that reason we carried out a clinical trial to test the effect of the reduction of stored iron by blood letting on oxidation resistance of atherogenic serum lipoproteins in healthy regularly smoking men.

Materials and methods

Subjects

Fourteen men from Kuopio, eastern Finland, aged 30–63 years, smoking regularly between eight and 50 cigarettes daily, were randomly allocated either to blood letting or control, seven men in each group. The subjects were screened from a group of 36 volunteers who responded to a newspaper advertisement recruiting men who smoked regularly and who had experience in blood donations. All men with any conditions or medications possibly influencing blood lipids or lipid oxidation were excluded. Sixteen men with the highest serum ferritin concentration were selected for the trial. Of these, two were excluded because of expected lack of adherence.

All subjects gave informed consent after oral and written information.

Experimental design

This study was a randomized cross-over trial with a Latin square design. The blood letting intervention consisted of three venesections at 6-week intervals at study weeks 0, 6 and 12. Each time, 450 g (500 mL) of blood was drawn. The subjects were advised not to donate blood during the control periods. All subjects were instructed to keep their smoking, exercise, alcohol intake and diet unchanged during the 10-month study period. Blood lipids and the oxidation resistance of the combined fraction of very low density lipoprotein (VLDL) and LDL were measured just before the first venesection and again in 14 weeks, 2 weeks after the last blood letting to allow the haemodilution to recover. After a wash-out period of 14 weeks, the same experimental procedure was repeated after switching the subjects' groups from blood letting to control and vice versa.

All blood samples were drawn after a fast of 12 h and abstinence from smoking of 1 h. After the study, the subjects were advised to stop smoking. Those who accepted were invited to visit a smoking cessation therapist or provided with a self-help smoking cessation programme. The study protocol was approved by the ethical committee of the University of Kuopio, Kuopio, Finland.

Clinical and chemical measurements

Measurement of VLDL/LDL resistance to oxidation.

Materials. The susceptibility of VLDL/LDL to oxidation was assessed by using a modification of the method developed by Balla *et al.* [3]. Potassium bromide, H_2O_2 (30%), solid sodium chloride and sodium hydroxide were from Merck (Darmstadt, Germany). Haemin chloride, N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES) and Tween 20 were obtained from Sigma (St Louis, MO, USA). Haemin chloride was dissolved daily with 0.02 M NaOH to a stock solution of 1 mM, and working solution of haemin (7.5 μM) was prepared by diluting the stock solution with 10 mM HEPES in 150 mM NaCl (pH 7.4). Ninety-six-well microtitre plates were from Biohit (Kajaani, Finland).

Isolation of VLDL/LDL. Combined VLDL and LDL were isolated from 3.0 mL of fresh ethylenediaminetetraacetic acid (EDTA) plasma by ultracentrifugation (in Beckman XL-90 ultracentrifuge) for 23 h at 4°C (32000 r.p.m., Beckman 50.4 Ti rotor, Palo Alto, CA, USA) using potassium bromide gradient. The top layer from the tube (< 1.063 g mL^{-1} density) was collected with a pipette and frozen at −80°C until assayed. The deep-freezer was kept oxygen-free by the regular addition of dry ice.

Measurements. Cholesterol content of the VLDL/LDL fraction was determined by an enzymatic colorimetric method (Kone Oy, Espoo, Finland), and the fraction was diluted to a cholesterol concentration of 0.78 mM with 10 mM HEPES in 150 mM NaCl, pH 7.4. The microtitre plate was preincubated with 0.1% Tween 20 for 1 h at room temperature for coating the wells to inhibit adsorption of haemin and LDL to the walls of the well. The wells were allowed to dry. Fifty μL of diluted VLDL/LDL fraction, 50 μL of haemin working solution and 50 μL of 1.5 mM H_2O_2 was pipetted into the wells. Each sample was assayed in duplicate. Blank determinations were included in every row of the microtitre plate. The reaction was followed by a microtitre plate reader (Easy Reader 400, SLT Instruments, Salzburg, Austria) at the wavelength of 405 nm at 8-min intervals for 7 h. Because haemin is degraded during the reaction, the absorbance curve was decreasing. Maximum reaction velocity (V_{max}) and the time from the start of the reaction to the beginning of the greatest slope (lag time) was computed by mean of a least squares regression equation. The measurements were in four batches from VLDL/LDL samples that had been kept frozen. To rule out any effects of storage or between-batch variation on the estimates of treatment effects, all four samples of each subject were always included in the same batch. The between-batch coefficient of variation (CV) of a frozen serum pool, ultracentrifuged and measured daily identically with the study samples, was 14.8% for V_{max} and 9.6% for the lag time ($n = 11$).

Determination of thiobarbituric acid reactive substances. Serum thiobarbituric acid reactive substances (TBARS) were determined according to Yagi [15] with the exception that we measured the butanol extracted TBARS spectrophotometrically (Hitachi U-2000, Tokyo, Japan) instead of fluorometrically. The between-batch CV for TBARS measurements was 17% at the level of 0.824 μg L^{-1} ($n = 6$).

Other measurements. Weight was measured and the current number of cigarettes smoked daily was obtained by interview at the time of blood drawing. Haemoglobin and haematocrit were determined from fresh blood samples using a whole-blood cell counter (Cell-Dyn 600, Mountain View, CA, USA). Ferritin concentration was measured from frozen serum samples in two batches immediately after each of the two study periods with a radioimmunoassay (Amersham International, Amersham, UK). Neither serum iron nor transferrin concentrations were measured because of the large analytical variability in these measurements [16]. Ascorbic acid and dehydroascorbic acid (DHA) [17] and α-tocopherol [18] were determined by high-performance liquid chromatography (HPLC) methods. Albumin concentrations were assayed from frozen serum samples photometrically (Kone Oy, Espoo, Finland). The between-batch coefficient of variation for albumin was 1.9% at the level of 32 g L^{-1}. Haemopexin was determined in two batches by an immunodiffusion technique (Nor-Partigen; Behringwerke, Marburg, Germany).

Statistical analysis

In the statistical analysis, the blood letting periods for both groups of seven men were pooled and the same was done for the control periods. Thus, each subject served as his own control in the comparison between the blood letting and the control periods. Confidence intervals (CI) for the mean differences between

Table 1 Mean (SE) baseline values and changes of indicators of iron status, antioxidants and the oxidation susceptibility of lipoproteins before and during blood letting and control in 14 male smokers

	Venesection		Control		*P* for difference between periods*
	Before	Change	Before	Change	
Blood haemoglobin (g L^{-1})	160.8 (2.1)	−9.1 (2.1)	159.9 (2.9)	−1.5 (2.0)	0.064
Blood haematocrit	0.47 (0.01)	−0.04 (0.01)	0.47 (0.01)	−0.02 (0.01)	0.066
Serum ferritin (μg L^{-1})	209 (37)	−135 (27)	171 (45)	−52 (23)	0.021
Body weight (kg)	89.6 (4.3)	−1.0 (0.5)	88.4 (4.3)	0.9 (0.3)	0.018
Smoking (cigarettes day^{-1})	24.9 (2.2)	−0.4 (1.0)	26.1 (2.8)	−2.1 (1.2)	0.082
Plasma α-tocopherol (μmol L^{-1})	21.3 (1.6)	−1.0 (2.0)	19.0 (2.2)	2.2 (1.7)	0.275
Lipid-adjusted† α-tocopherol (μmol $mmol^{-1}$)	3.57 (0.29)	−0.13 (0.20)	3.32 (0.40)	0.34 (0.29)	0.192
Plasma ascorbate (μmol L^{-1})	49.5 (6.7)	−3.4 (4.0)	51.9 (6.3)	−7.9 (4.7)	0.557
Plasma dehydroascorbic acid (μmol L^{-1})	0.61 (0.41)	1.11 (0.74)	1.09 (0.66)	1.81 (1.15)	0.552
Serum albumin (g L^{-1})	42.4 (0.9)	−0.36 (0.84)	42.7 (0.9)	−1.14 (0.58)	0.444
Serum haemopexin (g L^{-1})	0.90 (0.07)	0.00 (0.07)	0.88 (0.07)	0.03 (0.07)	0.803
VLDL/LDL oxidation susceptibility					
Maximal slope (mAbs min^{-1})	0.888 (0.075)	−0.080 (0.052)	0.762 (0.092)	0.081 (0.045)	0.032
Maximal slope cigarettes	0.041 (0.007)	−0.005 (0.004)	0.039 (0.010)	0.004 (0.002)	0.018
Lag to start (min)	146 (17)	49 (18)	187 (31)	−7 (19)	0.036
Lag/cigarettes	6.28 (0.83)	2.41 (0.80)	7.82 (1.15)	0.19 (0.84)	0.044
Serum TBARS (μmol L^{-1})	0.88 (0.085)	−0.17 (0.057)	0.79 (0.089)	−0.08 (0.076)	0.344
Serum TBARS/cigarettes	0.038 (0.004)	−0.0078 (0.003)	0.033 (0.004)	0.0005 (0.004)	0.058

VLDL, very low density lipoprotein; LDL, low density lipoprotein; TBARS, thiobarbituric acid reactive substances.

* Based on paired *t*-tests comparing changes in venesection and control periods ($n = 14$ pairs).

† Plasma α-tocopherol divided by serum total cholesterol concentration.

periods were estimated using the pooled standard error of changes during periods. Differences between periods, subtracting change during blood letting minus change during control, were tested for statistical significance by the paired *t*-test. In addition, Wilcoxon matched-pairs signed rank tests were used to confirm the significance levels for the main outcome variables by a non-parametric analysis.

Results

Changes in weight, smoking and serum ferritin

There was a mean reduction in body weight of 1 kg ($P < 0.05$ for change) during blood letting, whereas during the control periods the body weight increased on average by 0.9 kg ($P < 0.01$ for change), as shown in Table 1. No other reason for the weight reduction was found except the blood letting itself. The number of cigarettes smoked daily tended to decrease during both periods and more so during the control periods. No subject stopped smoking during the study. Mean serum ferritin concentration was reduced as a consequence of three venesections from 209 μg L^{-1} to 74 μg L^{-1} (by 66% $P < 0.001$ for change). Serum ferritin also decreased, although less, during the control periods (by 30%; $P < 0.05$ for change). The net effect (change during blood letting minus change during control periods) of blood letting on serum ferritin concentration was a reduction of 44% (95% CI 8–82%; $P = 0.021$).

Changes in lipid peroxidation

The slope of the haemin degradation curve after the induction of oxidation (V_{max}), reflecting the speed of oxidation, was reduced by 9% (NS) during the venesection periods and increased by 11% (NS) during the control periods (Table 1). The net effect of blood letting on this slope was a 20% decrease (95% CI 3–30%; $P = 0.032$ in *t*-test and $P = 0.028$ in Wilcoxon test). The lag time from the induction to the start of haemin degradation, reflecting the resistance of the lipoproteins to oxidation, was increased by 32% ($P < 0.05$) during the blood letting periods and did not change significantly during control periods. The net effect of blood letting on lag

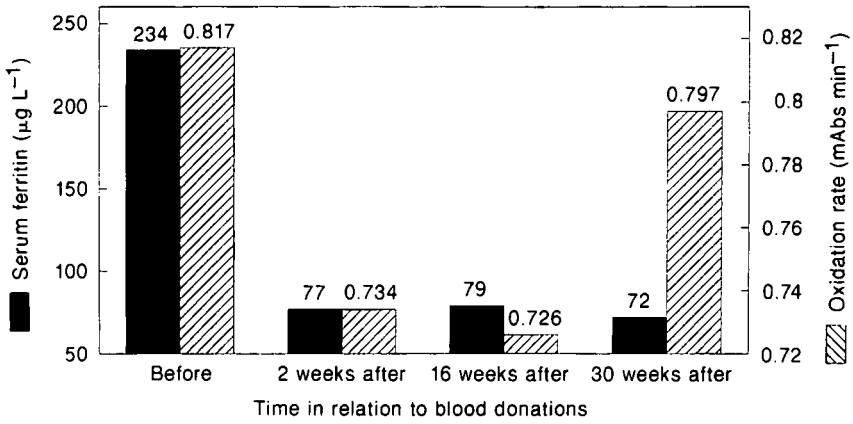

Fig. 1 The mean serum ferritin concentration and the maximal velocity of very low density lipoprotein/low density lipoprotein oxidation before blood letting and 2, 16 and 30 weeks after the last blood donation in seven men who donated blood in the first cross-over period.

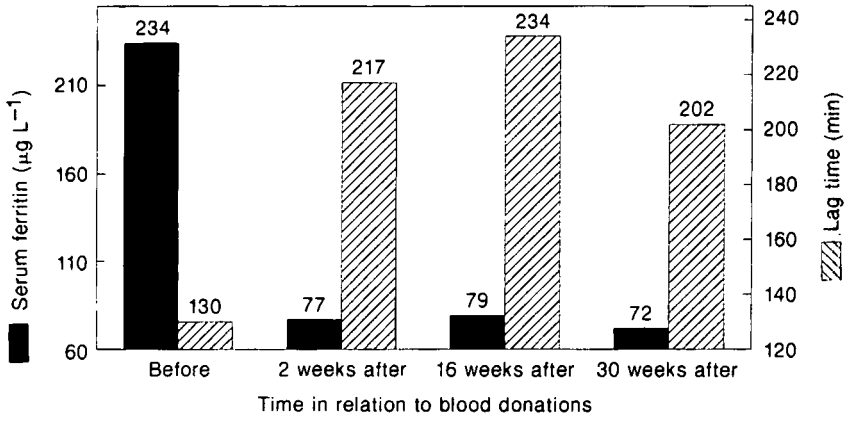

Fig. 2 The mean serum ferritin concentration and the lag time to the start of very low density lipoprotein/low density lipoprotein oxidation before blood letting and 2, 16 and 30 weeks after the last blood donation in seven men who donated blood in the first cross-over period.

time was a 33% increase (95% CI 1–64%; $P \doteq 0.036$ in t-test and $P = 0.041$ in Wilcoxon test).

As the change in the number of cigarettes smoked daily changed differentially during blood letting and control periods, the ratio of variables concerning lipid oxidation to the daily number of cigarettes was also compared between periods. The ratio of the maximal oxidation velocity to the daily number of cigarettes smoked was reduced by 12% (NS) during the blood letting periods and increased by 10% (NS) during the control periods. The net effect of the blood donations on this ratio was a decrease of 22% (95% CI 1–44%; $P = 0.018$ in t-test and $P = 0.030$ in Wilcoxon test). The respective net effect on the ratio of lag time to the number of cigarettes was also 31% ($P = 0.044$ in t-test and $P = 0.041$ in Wilcoxon test).

To explore the longer-term effect of blood letting, the changes after the venesection period were analysed in the seven men who donated blood during the first cross-over period and acted as controls during the second period (Figs 1–3). The change in both the maximal oxidation rate and in the lag time took place in parallel with the change in serum ferritin (Figs 1 and 2). The maximal change in serum ferritin concentration and in both lipid oxidation parameters was seen within 2 weeks of the third and last blood donation. Whereas the increase of lag time was sustained over a period of 30 weeks after the last blood donation (Fig. 2), the maximal oxidation rate started to rebound between 16 and 30 weeks after the last venesection. There was an increase in the lag time in all seven subjects during the blood letting period, ranging from 5–167% (mean 67%) and no further increase in the subsequent seven months (Fig. 3).

Serum TBARS was reduced during the venesection

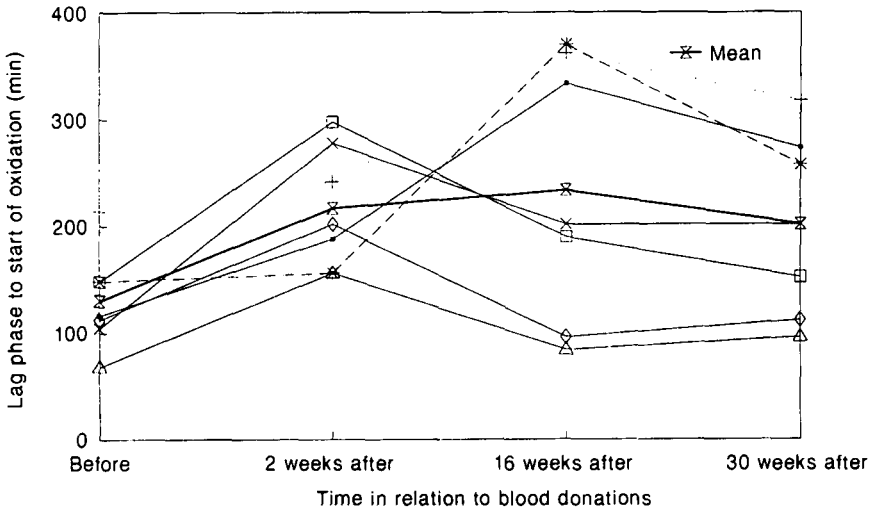

Fig. 3 The lag time to the start of very low density lipoprotein/low density lipoprotein oxidation before blood letting and 2, 16 and 30 weeks after the last blood donation in seven men who donated blood in the first cross-over period.

periods by 19% ($P < 0.01$ for change) and did not change significantly during control periods. However, the difference between periods was not statistically significant. As the change in the number of cigarettes smoked daily changed differentially during blood letting and control periods, the ratio of serum TBARS to the daily number of cigarettes was also compared between periods. There was a 21% decrease ($P < 0.01$) during blood letting and no significant change during control periods, the net effect being 23% (95% CI $-4\%...51\%$; $P = 0.058$).

Changes in plasma antioxidants

Neither plasma ascorbate nor dehydroascorbate concentration changed significantly during either venesection or control periods, even though there was a trend towards a net reduction of plasma dehydroascorbate concentration during blood letting (Table 1). Plasma α-tocopherol concentration decreased non-significantly by 5% during blood letting and increased non-significantly by 11% during control periods ($P = 0.275$ for difference). Changes in both serum albumin and serum haemopexin concentrations were very small and were not statistically significant (Table 1).

Associations between changes in indices of lipid peroxidation and antioxidants

Pooled over all periods ($n = 28$), the change in lipid oxidation velocity correlated inversely ($r = -0.36$) with the change in the lag time and in plasma ascorbic acid concentration ($r = -0.36$) and positively with the change in plasma dehydroascorbate concentration ($r = 0.29$). The change in lag time had inverse correlations with the changes in serum TBARS concentration ($r = -0.31$) and a positive correlation with the change in plasma ascorbic acid concentration ($r = 0.38$). There were no other significant correlations between changes in variables measured.

Discussion

The present study is the first clinical trial on the effect of iron depletion on the susceptibility of serum lipids to oxidation. The findings of this randomized controlled cross-over study indicate that the reduction of body iron stores by blood letting can increase the oxidation resistance of serum VLDL and LDL in regularly smoking men. Even though the magnitude of the effect could not be estimated with great certainty because of the small sample size of the present study, the size of the effect does not appear trivial. On the basis of the observations presented here, decreasing body iron stores may have an impact on lipid oxidation that could have both clinical and public health importance. Our findings need to be repeated in larger trials concerning the effects of both iron depletion and iron supplementation on lipid oxidation both in smoking and non-smoking men and women.

In previously reported *in-vitro* studies, Balla *et al.*

found that haem, a physiologically widespread hydrophobic iron compound, can rapidly generate oxidized LDL which becomes cytotoxic to cultured vascular endothelial cells [3]. From these studies it has been hypothesized that haem iron might be a critical oxidant of LDL and endothelial cells *in vivo* [3].

The exact mechanism through which reduced iron stores could increase the oxidation resistance of VLDL/LDL remains open. In our trial, plasma ascorbic acid concentration tended to increase and the concentration of the oxidized form of ascorbic acid, dehydroascorbate, tended to decrease during the venesection periods. The changes in plasma ascorbate and oxidized ascorbate correlated with the changes in the oxidation resistance of VLDL/LDL. This suggests that iron depletion could have increased the oxidation resistance of lipoproteins in part through the elevation of the plasma concentration of the antioxidatively active form of vitamin C, the reduced ascorbic acid.

The plasma α-tocopherol concentration tended to decrease during the blood letting periods and to increase during control periods. As in heavy smokers in general, the mean plasma level of α-tocopherol was very low in our subjects, only 20 μmol L^{-1}. Also, there were no changes in the concentrations of iron and haem binding proteins, albumin and haemopexin, during blood letting. This speaks against the possibility that other incidental changes during the study periods would have introduced the observed effects. Most probably, the reduction of the availability of catalytic iron could have attenuated the oxidation reactions in the lipoproteins, thus reducing the amount of lipid hydroperoxides present in VLDL and LDL in the circulation.

As a consequence of red blood cell loss due to blood donations, the production of new erythrocytes is accelerated to replace the lost ones. This consumes iron stores and leads to a reduction in serum ferritin concentration. This also means that red blood cells are on average younger after three blood donations than after the control periods. It is possible that older erythrocytes leak both ferritin-bound and other chelated iron more than younger red blood cells. Thus one can speculate that the effect of blood donations on the oxidation resistance of lipids could be, besides through the decrease of body iron stores, also through the reduction of leakage of iron from red blood cells.

The observation that serum ferritin concentration remained low during the control period following the blood letting period and the 3-month wash-out period probably reflects a carry-over effect from the preceding blood letting period to the subsequent control period, in spite of the more than 3-month wash-out period between the blood letting and control periods. This phenomenon tended, if anything, to attenuate the observed effect of blood letting on serum lipids and their oxidation susceptibility.

By showing lipid oxidation susceptibility reducing effect of iron depletion, the present study provides indirect support for the role of iron overload in the aetiology of coronary heart disease. Larger clinical trials are warranted to retest our findings in different kinds of subjects and to investigate the efficacy of iron depletion therapies in the prevention of atherosclerotic progression, and possibly, coronary heart disease. If the antioxidative effect of blood donations is confirmed, venesection, a safe and inexpensive therapy, could be used in the prevention and treatment of all free radical induced diseases, especially in persons with high iron stores.

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