Title: Inhibition of serum cholesterol oxidation by dietary vitamin C and selenium intake in high fat fed rats.

Authors: Menéndez-Carreño, M., Ansorena, D*., Milagro, F. I., Campión, J., Martínez, J. A., Astiasarán, I.

Address: Department of Nutrition, Food Science, Physiology and Toxicology.
University of Navarra, C/ Irunlarrea s/n 31008-Pamplona, Spain.

*To whom correspondence should be addressed

Phone: 948-425600. Fax 948-425649.

E-Mail: dansorena@unav.es

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ABSTRACT

Cholesterol oxidation products (COPs) have been considered as specific *in vivo* markers of oxidative stress. In this study, an increased oxidative status was induced in Wistar rats by feeding on a high-fat diet (cafeteria diet). Another group of animals received the same diet supplemented with a combination of 2 different antioxidants, ascorbic acid (100 mg/kg rat/day) and sodium selenite (200 μg/kg rat/day) and a third group fed on a control diet. Total and individual COPs analysis of the different diets showed no differences among them. At the end of the experimental trial, rats were sacrificed and serum cholesterol, triglycerides and COPs were measured. None of the diets induced changes in rats body weight, total cholesterol and tryglicerides (TG) levels. Serum total COPs in rats fed on the high-fat diet were 1.01 μg/ml, two fold the amount of the control rats (0.47 μg/ml). When dietary antioxidant supplementation was performed, serum total COPs concentration (0.44 μg/ml) showed the same levels than those of the rats on control diet. 7β-hydroxycholesterol, formed non–enzymatically via cholesterol peroxidation in the presence of reactive oxygen species, showed even slightly lower values in the antioxidant-supplemented animals compared to the control ones. This study confirms the importance of dietary antioxidants as protective factors against the formation of oxysterols

INTRODUCTION

Cholesterol is an unsaponifiable lipid prone to oxidation by reactive oxygen species, light, UV light, ionizing radiation, chemical catalysts, enzymatic reactions and other oxidizing conditions, leading to the formation of cholesterol oxidation products (COPs) [1-2]. Some studies pointed out that COPs could be absorbed from the diet in rats [3-6].

It has been demonstrated that COPs may produce a wide range of adverse biological outcomes including cytotoxicity, apoptosis, mutagenesis, carcinogenesis, diabetes and metabolic disorders, being their main negative effect the development of atherosclerosis [7-13]. Also, it has been shown that the presence of COPs in diet promotes fatty streak lesions formation in several different animal models of atherosclerosis [6].

In this context, certain oxysterols, 7β-hydroxycholesterol and 7-ketocholesterol, have been suspected not only as oxidative stress inducers, which are associated to an increase of risk of atherosclerosis [14-16] but also as specific markers of oxidative stress [17]. There are evidences that oxidized lipoproteins play a key role in the pathogenesis of atherosclerosis and especially both oxysterols are found at significantly increased levels in atherosclerotic lesions [14, 18-20].

The endogenous formation of oxysterols in relation to the influence of the type of diet and the consumption of antioxidants has been scarcely studied out so far [21-22]. Some data suggest that a greater intake of antioxidative vitamins such as vitamin E, vitamin C and β-carotene are associated with reduced risk of atherosclerotic vascular disease [23], which may happen through different mechanisms such as inhibition of LDL oxidation, cell lipid peroxidation and cell-mediated oxidation of LDL, and

reduction in blood cholesterol levels [24]. In this sense, ascorbic acid could limit lipid peroxidation, protecting partially oxidized LDL against further oxidative modification [25]. On the other hand, selenium is known to play an important role in cytoprotection against cholesterol oxide-induced vascular damage in the vascular wall of rats [26]. Combinations of selenium and vitamin E have been shown to contribute to protect the vascular endothelium and to control oxidative status and altered lipid metabolism in liver [27-28]. No data have been found about the combination of selenium and vitamin C.

In the current article, we hypothesized that the dietary intake of a combination of two different antioxidants (ascorbic acid and selenium) could prevent cholesterol oxide formation in rats fed on a high fat diet.

MATERIALS AND METHODS

Animals, diets and experimental design - Male Wistar rats, supplied by the Applied Pharmacobiology Center (CIFA, Pamplona, Spain), were housed at 21-23ºC with a 12 hours light cycle (8 a.m. to 8 p.m.) and assigned into three different dietary groups. A group of animals (Control group, C) were fed on standard pelleted diet (Harlan Iberica, Barcelona, Spain) containing 16.62 % of energy as protein, 73.16 % of energy as carbohydrate and 10.22 % of energy as lipid by dry weight. The second group of animals (Cafeteria, Caf) was fed on a high-fat diet in order to generate a diet induced obesity model as previously reported [29]. High-fat diet components were paté, bacon, chips, cookies, chocolate and chow with proportions 2:1:1:1:1:1 and the diet was given to each rat daily. The composition of this Cafeteria diet was 9.26 % of energy as protein, 31.58 % of energy as carbohydrates and 59.15 % of energy as lipids by dry

weight (430 Kcals per 100 g in the Cafeteria diet vs. 350 Kcals per 100 g in the pelleted diet). Cafeteria diet was supplemented in a third group of animals (Caf+Aox) with a daily dose of vitamin C (100 mg/Kg rat) + Sodium Selenite (200 µg/Kg rat) both mixed into the food. Animals had *ad libitum* access to water and food during the experimental trial. Body weight was recorded daily. At the end of the experimental period (72 days), animals were sacrificed by decapitation and blood and tissue samples were immediately collected and weighed. All the procedures were performed according to national and institutional guidelines of the Animal Care and Use Committee at the University of Navarra.

Diet Composition - Moisture was determined according to the Association of Official Analytical Chemists (AOAC) method [30]. Total fat was determined by an extraction with petroleum ether according to the AOAC [31]. Protein was analysed using the Kjeldahl method for the determination of nitrogen [32], using 6.25 as the factor to transform nitrogen in protein. Ashes were determined by incineration using the method of the AOAC [33]. Quantitative fat extraction was made with a chloroform/methanol mixture using the method of Folch et al. [34]. Cholesterol content was analyzed by gas chromatography according to Kovacs et al. [35]. Derivatization to obtain the trimethyl silyl ethers of cholesterol was performed. A Perkin-Elmer Autosystem XL gas chromatograph equipped with an HP1 column (30m x 0.25mm x 0.1µm) was used. The oven temperature was 265ºC. The temperature of both the injection port and detector was 285ºC. Cholesterol identification and quantitation was done by using pure 5α-cholestane (Sigma, St. Louis, MO, USA) as an internal standard, where 1 ml was added to the sample as a solution (2 mg/ml), previously to the extraction procedure. A Perkin-Elmer Turbochrom programme was used for quantitation. COPs determination in diets was performed using 0.5 g of the Folch

extract of the correspondent diet adding 1 ml of 19-hydroxycholesterol (20 µg/ml) as internal standard. Ten mL of 1N KOH solution in methanol were then added to perform a cold saponification at room temperature for 20 h, in darkness and under continuous agitation in an orbital shaker (Rotaterm P; Selecta, Barcelona) at 100 rpm. The unsaponifiable material was extracted with diethyl ether and purified by silica SPE according to Guardiola et al. [36]. COPs were finally eluted from the cartridge with acetone. Samples solutions of COPs were derivatised to trimethylsilyl (TMS) ethers according to a modified version of the method described by Dutta et al. [37]. After drying the solvent, 400 µl of Tri-Sil reagent were added and the tubes were kept at 60ºC for 45 min. The solvent was evaporated under a stream of nitrogen and the TMS-ethers derivates were dissolved in 400 µl of hexane. Dissolved samples were filtrated prior to GC-MS analysis in order to avoid the damage of the capillary column. Gas chromatography-Mass spectrometry analysis was performed on a CG 6890N Hewlett Packard coupled to a 5975 Mass Selective Detector (Agilent Technologies, Inc., CA, USA). The TMS-ethers derivates of COPs were separated on a capillary column Varian VF-5ms CP8947 (50 m x 250 µm x 0.25 µm film thickness) (Varian, France). Oven temperature conditions were optimized in order to achieve a proper separation of the individual compounds. Oven was programmed with an initial temperature of 75ºC, heated to 250ºC at a rate of 30ºC/min, raised to 290ºC at rate of 8ºC/min, and finally, it was raised to 292ºC at a rate of 0.05ºC/min. High purity helium was used as a carrier gas at a flow rate of 1 ml/min. The inlet pressure used was 13.40 psi. The injector temperature was 250ºC and the transfer line to detector at 280ºC. The samples were injected in a splitless mode with an injection volume of 1µl. The mass spectrometer was operated in electron impact ionization (70 eV). Identification of the peaks was made by

the characteristic ion fragmentation of the standard substances and the quantification was made using select ion monitoring program using the internal standard method.

Serum measurements - Serum triacylglicerides were determined with the RANDOX kit (Randox Laboratories LTD, UK) and cholesterol level with the Cholesterol-CP kit (Haribo, ABX diagnostic, Montpellier, France). All these measurements were adapted for COBAS MIRA equipment (Roche, Switzerland). COPs in serum were analyzed following the method described in Menéndez-Carreño et al. (in press) [37]. Lipids from serum were extracted from serum according to a modified version of the method described by Folch et al. [34] using chloroform-methanol (2:1; v/v). 1 ml serum was introduced into a centrifuge tube, and 6 ml of chloroform and 3 ml of methanol were added. After shaking on vortex for 1 minute, samples were then centrifuged at 4000 rpm for 15 minutes to facilitate phase separation using a Hermle Z320 centrifuge (Apeldoorn, The Netherlands). The chloroform (lower) layer was transferred to a test tube taking care of not transferring any solid material which remains at the interface between the upper and lower phases. 10 mL of 1N KOH solution in methanol and 1 ml of stock solution of the internal standard, 19-hydroxycholesterol (20 μg/ml), were then added to perform a cold saponification at room temperature for 20 hours, in darkness and under continuous agitation in an orbital shaker (Rotaterm P; Selecta, Barcelona) at 100 rpm. 100 mg/Kg of butylhydroxytoluene (BHT) was added as antioxidant in order to avoid COPs artifact formation during the saponification. The extraction of the unsaponifiable material, the purification, and further analysis of COPs was exactly the same as explained above for the determination of COPs in diets.

Statistical analysis - All results are expressed as mean ± standard deviation of the mean. The differences between the groups were evaluated by one-way ANOVA, and

Tukey post-hoc test was applied when suitable (SPSS 15.0 packages for Windows, Chicago, IL, USA). A level of probability up at $p<0.05$ was set up as statistically significant. A Pearson correlation test was performed between COPs content in the diets and in the serum samples.

RESULTS

Control (C) and cafeteria diets (Caf and Caf+Aox diets) presented different macronutrients profile, as shown in table 1, especially in relation to the fat content, 7 times higher in cafeteria diets. This diverse macronutrient distribution of the diets led to 20 % more energy in both high-fat diets in comparison to the standard one. Regarding the cholesterol content, it was significantly lower in chow diet than in the two cafeteria ones, and a similar trend was detected for the COPs content, being 7.5 times higher in the cafeteria diets. Analyzing specifically oxysterols, 7-ketocholesterol was the main oxysterol in all the three diets (table 2), being less abundant in the control one and more abundant in the cafeteria diets.

These results led to percentages of cholesterol oxidation significantly higher in C (0.68%) than in Caf and Caf+Aox diets, which did not show significant differences between them (0.25 % and 0.24 %, respectively). However, the total oxysterol content of the diet was much higher in both cafeteria diets with no differences between them, suggesting that the antioxidant supplementation of the diet does not affect the amount of oxysterols ingested.

Whereas the body weight of the animals at the beginning of the experimental trial was similar within the 3 groups, the two groups fed on cafeteria diets gained significantly more weight than rats fed chow pellets (table 3). As no significant

differences were found for weight gain between the two cafeteria diets and the weights of their white adipose tissue depots were similar (data not shown), we can conclude that antioxidant addition did not affect animals weight, adiposity and growth.

Regarding the analysis of serum lipid metabolites, neither total cholesterol nor triglyceride levels differed among the three groups of rats (table 4). Although HDL-cholesterol was not measured in C group, no differences were found between the two cafeteria-fed groups.

In this study, seven different COPs were analyzed in serum rats from the three experimental groups; 7α-hydroxycholesterol, 7β-hydroxycholesterol, 5,6β-epoxycholesterol, 5,6α-epoxycholesterol, cholestanetriol, 25-hydroxycholesterol and 7-ketocholesterol, but only 7α-hydroxycholesterol, 7β-hydroxycholesterol, cholestanetriol and 7-ketocholesterol could be quantified in all the serum samples (table 4). Cholestanetriol was detected in all the cafeteria-fed animals, but only in 7 and 6 out of 10 samples in Caf+Aox and C groups, respectively.

As a result of this dietary treatment during 72 days, the most abundant serum oxysterol in all the three groups was 7-ketocholesterol. Its levels reached 38.3% of total COPs in the case of control group and 52.2% and 47.7% for the Caf and Caf+Aox animals. The total amount of serum oxysterols was significantly increased in rats fed on cafeteria diet compared to the C group, with significant increments in all the oxysterols quantified (table 5). However, the Caf+Aox diet decreased the different serum oxysterol concentrations to the levels of the control rats, which in the case of 7β-hydroxycholesterol, was even significantly lower than that of the control animals.

In summary, as shown in figure 1, animals fed on the cafeteria diet showed a higher percentage of serum oxysterols than the control rats, indicating a pro-oxidant effect of the diet. However, the supplementation of this diet with antioxidants resulted

in a normalization of the circulating oxysterol levels, demonstrating a protective effect of the supplementation that could have beneficial effects on different metabolic features related to glycemia control and atherogenesis.

DISCUSSION

High-fat diets have been traditionally used for generating overweight in rats, being cafeteria diet a variation of them [29]. As a result, animals might develop obesity, insulin resistance, hyperglycemia, fatty liver, oxidative stress, and, in some cases, lipid metabolism disturbances. Our model of obesity in rats, in agreement with previous results of our own group, achieves adiposity and weight gain but does not induce fasting hypertriglyceridemia or hypercholesterolemia [29, 38].

In this paper, it is found that oxidative stress has been effectively induced by the high fat diet, as the levels of total COPs were significantly higher than those of the control group. This pro-oxidant effect of the cafeteria diet has been associated to an impairment of some obesity-related features such as nonalcoholic fatty liver, diabetes and atherogenesis [39], and the supplementation of this diet with a high dose of ascorbic acid has recently been reported to protect against body weight gain and adiposity [40]. However, in this study, the diet supplementation with a lower dose of vitamin C induced no changes in body weight and adiposity during the assayed period. The use by other authors of similar or higher doses of vitamin C during long periods has shown no toxic or deleterious effects in similar animal models [41-44].

Rats fed on very high cholesterol diets usually present increased serum oxysterol levels [22]. Other studies have shown that, although a cholesterol-rich diet supplemented with vitamins E and C induces hypercholesterolemia in rabbits, it was

able to reduce lipid peroxidation and lipoprotein modification [45]. Ringseis & Eder [21] pointed out that, as a consequence of antioxidant-induced inhibition of lipid peroxidation, it is reasonable to assume that the formation of COPs in animal tissues would be inhibited by these compounds, although this effect requires more studies. On the other hand, *in vitro* trials have demonstrated that cholesterol is susceptible to oxidation under selenium deficiency-induced oxidative stress [46]. In that study, a significant increase in the amounts of 7-hydroxycholesterol was detected due to the selenium deficiency. In fact, selenium has been widely used as antioxidant, with doses up to 2 μg/g body weight in rats [47-48], and has been reported to protect from vascular damage induced by COPs [32]. The effect of dietary selenium on the reduction of COPs formation in serum could be mediated by GSH-dependent selenoperoxidases, such as GPx, which have been implicated in the defence against oxidized low density lipoproteins [49]. In this sense, different papers showed a significant correlation between dietary selenium concentration and GPx levels [50-53].

This study also showed that the control group, which had a standard feeding, led to COPs concentrations in serum significantly lower than those animals fed on a high-fat diet (cafeteria group). The values for total COPs both for control and modified groups were similar to those found by Mahfouz and Kummerov [22] in rats fed on high cholesterol diets for 4 months. The cafeteria diet did not affect the total serum cholesterol and TG levels as expected, but increased the serum COPs levels. Increasing dietary vitamin C and selenium in a high-fat diet prevented the oxidation of circulating cholesterol.

Thus, the intake of the antioxidants mixture included in the cafeteria diet can efficiently decrease the oxidation process affecting cholesterol. As total cholesterol

levels were not modified by the different dietary treatments, the percentage of cholesterol oxidation also showed significant differences between the cafeteria diet and the others.

One remarkable finding is the fact that serum 7β-hydroxycholesterol levels in the Caf+Aox diet group were even slightly lower than those obtained for the control group. Ringseis & Eder [21] demonstrated that insufficient vitamin E in the diet increased the formation of 7β-hydroxycholesterol in rats fed on salmon oil. Most of the 7β-hydroxycholesterol is formed non-enzymatically via cholesterol peroxidation in the presence of reactive oxygen species (ROS) [54]. As 7β-hydroxycholesterol is formed in the presence of ROS, it is likely influenced by the antioxidative status [21]. Yoshida et al. [55] measured 7α-hydroperoxycholesterol, 7β-hydroperoxycholesterol and 7-ketocholesterol as well as 7α-hydroxycholesterol and 7β-hydroxycholesterol were measured as total 7-hydroxycholesterol in samples of human plasma because their usefulness for the assessment of oxidative stress in animal and in vivo experiments. They concluded that 7β-hydroxycholesterol may be a better biomarker for oxidative stress than 7α-hydroxycholesterol. As the consumption of selenium and antioxidants suppresses ROS formation by different mechanisms [56], it is reasonable to suppose that endogenous formation of 7β-hydroxycholesterol could have been suppressed by the high intake of vitamin C and selenium. In fact, no correlation was found between the level of 7β-hydroxycholesterol in the diet and in its content in the serum samples ($r = 0.26$, $p<0.05$) pointing to an endogenous formation of this compound.

Arca et al. [57] found that familial combined hyperlipidemia patients are prone to a condition of oxidative stress, as shown by the presence of increased levels of COPs. Thus, oxidative stress should be taken into account to explain the increased cardiovascular risk in these patients. Moreover, 7β-hydroxycholesterol and 7-

ketocholesterol are biomarkers for oxidative stress in patients with atherosclerosis [17], being possible the interconversion of these compounds in humans [58]. 7-ketocholesterol was the most abundant oxysterol in the 3 groups of rats, showing a positive correlation [r = 0.66, ($p<0.01$)] with the data obtained from the diet.

In conclusion, the concentrations of oxysterols were influenced by the dietary fat, but also by the vitamin C and selenium intake. The fact that the concentration of COPs was reduced by increasing dietary vitamin C and selenium supply confirms the importance of antioxidants as a protective factor against the formation of oxysterols from high-fat dietary pattern.

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Table 1. General composition (g/100 g) and energy value (Kcal/100 g) for the three tested diets.

	Control Diet (C)	Cafeteria Diet (Caf)	Cafeteria Selenium Vitamin C diet (Caf + Aox)
Moisture (g/100 g)	12.13±0.10a	25.55±0.10b	25.33±0.04b
Total fat (g/100 g)	4.03±0.4a	28.32±0.14b	28.33±0.08b
Protein (g/100 g)	14.75±0.08c	9.97±0.10a	9.98±0.07a
Carbohydrates (g/100 g)	64.93±0.11c	33.90±0.17a	34.02±0.13a
Ashes (g/100 g)	4.16±0.07b	2.25±0.08a	2.35±0.04a
Energy (Kcal/100 g product)	354.99±0.37a	430.45±1.87b	430.38±0.84b

Results are expressed as mean ± standard deviation. n=10. Values in the same row bearing different letters are significantly different ($p < 0.05$).

Table 2. Cholesterol content and cholesterol oxidation products of the three tested diets.

	Control Diet (C)	Cafeteria Diet (Caf)	Cafeteria Selenium Vitamin C diet (Caf + Aox)
7α-Hydroxycholesterol (µg/g fat)	0.11±0.01a	0.57±0.01b	0.55±0.02b
7β-Hydroxycholesterol (µg/g fat)	0.07±0.00a	0.44±0.01b	0.44±0.03b
5,6β-Epoxycholesterol (µg/g fat)	n.d.	1.44±0.08a	1.47±0.10a
5,6α-Epoxycholesterol (µg/g fat)	n.d.	0.33±0.02a	0.36±0.04a
Cholestanetriol (µg/g fat)	0.04±0.00a	0.18±0.01b	0.21±0.0b
25-Hydroxycholesterol (µg/g fat)	n.d.	n.d.	n.d.
7-Ketocholesterol (µg/g fat)	0.47±0.0 a	2.42±0.11b	2.31±0.09b
Cholesterol (mg/100g)	2.75±0.15a	58.02±4.07b	57.95±5.13b
Total COPs (µg/g fat)	0.70±0.02a	5.39±0.11b	5.34±0.38b
Percentage of cholesterol oxidation (%)	0.68±0.02b	0.25±0.03a	0.24±0.03a

Cholesterol and individual COPs were analyzed in quadruplicate in the three different diets. Total COPs is the sum of all individual COPs detected. Percentage of cholesterol oxidation %=(Total COPs diet/Cholesterol diet)*100. Results are expressed as mean ± standard deviations. n=10. Values in the same row bearing different letters are significantly different ($p < 0.05$). n. d. means non determined compound.

Table 3. Body weight changes of rats fed on three different diets

	Control Diet (C)	Cafeteria Diet (Caf)	Cafeteria Selenium Vitamin C diet (Caf + Aox)
Initial weight (g)	260.6±16.4a	263.9±21.87a	268.1±21.82a
Final weight (g)	427.6±32.4a	533.5±79.78b	512.1±62.26b
Weight gain (g)	167.0±18.9a	269.6±62.65b	244.0±45.01b

Results are expressed as mean ± standard deviations. n=10. Values in the same row bearing different letters are significantly different ($p < 0.05$).

Table 4. Lipid metabolites and cholesterol oxidation products in serum of rats fed on three different diets.

	ontrol iet (C)	eria Caf)	a Selenium in C diet + Aox)
olesterol (mg/dL)	6±10.15a	12.59a	±12.93a
HDL (mg/dL)	n.a.	3.80a	1±4.35a
TG (mg/dL)	.89±4.25a	3.73a	±20.71a
oxycholesterol (µg/mL)	4±0.04a	.06b	±0.04a
oxycholesterol (µg/mL)	9±0.03b	.03c	±0.03a
oxycholesterol (µg/mL)	n.d.	l.	.d.
oxycholesterol (µg/mL)	n.d.	l.	.d.
estanetriol (µg/mL)	n=6)±0.03a	.04b	=7) ±0.03a
oxycholesterol (µg/mL)	n.d.	l.	.d.
ocholesterol (µg/mL)	8±0.04a	.15b	±0.07a
tal COPs (µg/mL)	7±0.10a	.17b	±0.11a

Total COPs is the sum of all individual COPs detected. Results are expressed as mean ± standard deviations. n=10. Values in the same row bearing different letters are significantly different ($p < 0.05$). n.a. means non analyzed compound. n. d. means non detected compound.