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ORIGINAL CONTRIBUTION

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Olive oil- and sunflower oil-fried sardines in the prevention of rat hypercholesterolemia

Zur Eignung von in Olivenöl und Sonnenblumenöl gebratenen Sardinen in der Prevention von Hypercholesterolemie bei Ratten

Zusammenfassung In Versuchen von 4 Wochen Dauer wurde der Einfluß cholesterolangereicherter Diäten, die in Olivenöl (Diät 1) oder in Sonnenblumenöl (Diät 3) gebratene Sardinen enthielten, auf die Erhöhung des Cholesterolspiegels bei wachsenden Wistar Ratten untersucht. Die Ergebnisse von Diät 1 wurden mit denen von Diät 2 (Kasein plus Olivenöl) und die Resultate von Diät 3 mit denen von Diät 4 (Kasein plus Sonnenblumenöl) verglichen. Alle Diäten enthielten Cholesterol und Rindergalle als cholesterolerhöhende Agentien. Der hypercholesterolämische Effekt war in den Diäten mit den gebratenen Sardinen (eine Erhöhung des Cholesterolspiegels (TC) von je 0.9 mmol/L ($p < 0.05$) und 0.4 mmol/L (nicht signifikant) in den Gruppen 1 und 3) auffallend kleiner als in den Kontrollgruppen 2 und 4 mit Kasein (eine Erhöhung des TC von je 13.9 mmol/L ($p < 0.01$) und 18.2 mmol/L ($p < 0.05$)). Die Serumtriglyceride fielen bei Aufnahme der Sardinen ($p < 0.05$) und stiegen bei Aufnahme der Kasein-Diäten ($p < 0.05$). Das HDL-Cholesterolgehalt war niedriger mit Diät 1 als mit Diät 2 ($p < 0.05$) aber ähnlich bei den Diäten 3 und 4. Die HDL-Fraktion betrug in den Diäten 1, 2, 3 und 4 je 13 %, 4 %, 53 % und 5 % des TC. Diese Ergebnisse zeigen, daß Diäten mit gebratenen Sardinen eine große Hemmwirkung auf den cholesterolerhöhenden Effekt des Cholesterols haben.

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Summary The effect of diets containing olive-oil-fried sardines (diet 1) or sunflower-oil-fried sardines (diet 3) upon the serum cholesterol-raise induced by dietary cholesterol was studied after a 4-week experiment in growing Wistar rats. Results of diet 1 were compared to those obtained in diets containing casein plus olive oil (diet 2), whereas results of diet 3 were compared to those obtained with casein plus sunflower oil (diet 4). All diets contained cholesterol and bovine bile as a cholesterol-raising agent. The hypercholesterolemic effect of dietary cholesterol in fried-sardine groups (a total cholesterol (TC) increase of 0.9 mmol/L ($p < 0.05$ and 0.4 mmol/L (not significant) in groups 1 and 3, respectively) was markedly lower than in groups 2 and 4 (a TC increase of 13.9 mmol/L ($p < 0.01$) and 18.2 mmol/L ($p < 0.01$), respectively). Serum triglyceride levels decreased in fried-sardine diets ($p < 0.05$) while they increased in casein diets ($p < 0.05$). HDL-cholesterol levels appear lower in diet 1 than in diet 2 ($p < 0.05$), but similar in diets 3 and 4. However, HDL-fraction carries in diets 1, 2, 3 and 4, 13 %, 4 %, 53 % and 5 % of TC, respectively. Results showed that fried-sardine diets exert a powerful check effect on the cholesterol-raising effect induced by dietary cholesterol.

Schlüsselwörter gebratene Sardinen – Diäten mit Cholesterol bereichert – Cholesterolspiegel – Serumtriglyceride – Olivenöl – Sonnenblumenöl

Key words Fried sardines – cholesterol-enriched diets – serum cholesterol – serum triglycerides – olive oil – sunflower oil

Abbreviations *CHD* = coronary heart disease · *EPA* = eicosapentaenoic acid · *HDL* = high density lipoproteins · *LDL* = low density lipoproteins · *MUFA* = monounsaturated fatty acids · *PUFA* = polyunsaturated fatty acids · *SFA* = saturated fatty acids · *TC* = total cholesterol · *VLDL* = very low density lipoproteins

Introduction

Dietary fats appear to be involved in the etiology and progress of CHD, atherosclerosis, stroke, cancer and allergies, and it is known that saturated fats and cholesterol precipitate and even accentuate CHD (9, 12–14, 26).

Data from the Seven Countries Study suggest that the traditional Mediterranean diet was associated with a low rate of CHD and total mortality (11). However, as regards to lipid intake, the peculiar characteristics of the "Mediterranean diet" are not only the nutrient composition of the foodstuff (10, 29) with lesser amounts of SFA and greater amounts of the unsaturated fatty acids (in particular, MUFA and the PUFA from the n-3 series, due to the high intake of olive oil and fish), but also the way in which these foodstuffs are usually consumed. In the Mediterranean diet about 50 % of total fat intake is derived not from the food itself but, rather, from the cooking fat (28).

Interest has grown in fish and fish products as sources of polyunsaturated fatty acids, mainly of the n-3 family. This interest has risen to a large extent from studies suggesting that n-3 fatty acids may have an important role in prevention and management of cardiovascular and cerebrovascular diseases (9, 13, 14, 21). Recent evidence suggests that n-3 fatty acids, by competing with n-6 fatty acids, may modify the effects of the latter fatty acids, thereby ameliorating the undesirable effects of excessive eicosanoid production (12, 14, 26). N-3 fatty acids added to the diet could inhibit both intimal hyperplasia and atherosclerosis in animals made hypercholesterolemic by diets rich in SFA and cholesterol (31).

In Spain, a marked increase in the consumption and use for frying purposes of sunflower oil and a decrease in the use of olive oil have been described (17). Economical reasons are involved in the use for frying of sunflower oil instead of olive oil. The latter oil, however, is shown to be more adequate for frying purposes because of its thermal stability.

Deep-fat frying of sardines either in olive oil or sunflower oil deeply changed the fatty acid composition of this oily-fish, increasing the oleic and linoleic acid content (23). Thus, the beneficial effects of the fatty fish related to its fat content would be deeply modified by frying.

The hypolipemic effect of fish consumption could mainly be attributed to its PUFA n-3 contents, but fish protein would also change cholesterol levels (29, 33). On the other hand, information is scarce on the effect of consumption of fried fish on the prevention of dietary hypercholesterolemia. In a previous work serum cholesterol-raising effect of diets was found to be markedly reduced by different kinds of olive-oil-fried sardines (22).

Taking into account the high daily consumption of fatty fish, fried food-stuff, and therefore of fried fatty fish, the aim of this work is to investigate the possible preventive effect of diets containing sardines fried in olive oil or in sunflower oil upon the induction of hypercholesterolemia by dietary cholesterol.

Materials and methods

Materials

Olive oil and sunflower oil (Carbonell trade mark, Córdoba, Spain) and sardines (*Sardina pilchardus*, WALB) were purchased at a local store. Acid casein, wheat starch, and mycrocrystalline cellulose were purchased from Central Ibérica de Drogas, S.A. (Madrid, Spain). Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), benzene, hexane, boron trifluoride in methanol, sucrose and all minerals and vitamins were obtained from Merck (Darmstadt, Germany). Chloroform, methanol, cholesterol and bovine bile were from Farmitalia Carlo-Erba (Madrid, Spain). Gas chromatography column was obtained from Supelco (Barcelona, Spain). Gas chromatography standards were from Sigma (St. Louis, Mo).

Methods

Performance of frying

The fried sardines of diets given to groups 1 and 3 were prepared as follows: sardines (600–700 g) head, scales, viscera and fishbones removed, were opened into a fan shape and fried either in olive oil or sunflower oil for 4 min at 180 °C. Domestic friers with a capacity of 3L were used as cooking receptacles. Once fried, the sardines from the first and second frying in olive oil or in sunflower oil were freeze-dried and kept at -20 °C under nitrogen atmosphere until both analysis and diet preparation were made. Details of the frying performance have already been published (23).

Animals and maintenance

Male Wistar rats (Instituto de Nutrición y Bromatología (CSIC-UCM). Facultad de Farmacia. Universidad Complutense de Madrid) weighing approximately 65 g at the outset were randomly divided into groups of 6 rats each. The animals were housed individually in metabolic cells and were kept in a room under controlled temperature (22.3 ± 1.8 °C and with a 12 h light/dark cycle).

Dietary treatments

The rats were fed with commercial rat-pellets (Panlab, Barcelona, Spain) after weaning and then switched, without any adaptive period, to the experimental diets. Water and food were provided *ad libitum* (except for group 4) over a 4-week experimental period. Group 1 was fed with a mixture of sardines fried in an olive oil that had been used one or two times to fry sardines, as a combined source of protein and fat. Group 3 was fed with a mixture of fried sardines from the first and second fryings in sunflower oil as its combined protein/fat source. Groups 2 and 4 were fed with defatted casein supplemented with 2 g/kg DL-methionine, and olive oil or sunflower oil, respectively. Diets contained roughly 150 g/Kg dry matter (DM) of protein, 170 g/Kg DM of lipid (fat plus cholesterol) and 360 g/Kg DM of wheat starch. Cholesterol (20 g/Kg DM) plus 0.5 g/Kg DM of bovine bile was used as a serum cholesterol-raising agent. Other basic dietary components (in g/Kg DM of food) are shown in Table 1. To achieve protein/fat source in diets of groups 1 and 3, freeze-dried fried sardines were thawed and ground before mixing with other ingredients. In order to facilitate the diets' preparation, and the diet and sample analyses, dietary treatment of groups 1 and 2 started 7 days before than those 3 was in the first days, significantly lower than that in groups 3 and 4. As the food intake for group 3 was, in the first days, significantly lower than that in groups 1 and 2, it was decided to make group 4 pairfed to group 3. Caloric densities of the diets, estimated according to their protein, fat, and carbohydrate contents and multiplied by 4, 9, and 3.75, respectively (1 Kcal corresponding to 4.184 KJ) were 1728 KJ/100 g DM for group 1, 1712 KJ/100 g DM for group 2, 1646 KJ/100 g DM for group 3, and 1694 KJ/100 g DM for group 4.

General procedures

Food intake was checked every day while body weight variations were measured on alternate days. Blood samples were collected under fasting conditions (16–18 h) from the rat's tail at the start of the experiment (basal value) and by carotic puncture 4 weeks later. Sera were separated and analyzed.

Table 1 Composition (g/kg DM) of the experimental diets containing sardines fried in olive oil (Diet 1), casein plus olive oil (Diet 2), sardines fried in sunflower oil (Diet 3) and casein plus sunflower oil (Diet 4)

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4
Protein content, intended in DM	~ 150[a]	~ 150[c]	~ 150[b]	~ 150[c]
Fat content, intended in DM	~ 150[a]	~ 150[d]	~ 150[b]	~ 150[e]
Sucrose	220.0	220.0	220.0	220.0
Microcrystalline cellulose	50.0	50.0	50.0	50.0
Mineral premix[f]	37.7	37.7	37.7	37.7
Vitamin premix[g]	1.2	1.2	1.2	1.2
Cholesterol	20.0	20.0	20.0	20.0
Bovine bile	5.0	5.0	5.0	5.0
BHT[h]	2.5	2.5	2.5	2.5
BHA[i]	2.5	2.5	2.5	2.5
Wheat Starch	ad 1000	ad 1000	ad 1000	ad 1000

[a] Olive-oil-fried sardines as the only source of protein and fat
[b] Sunflower-oil-fried sardines as the only source of protein and fat
[c] Acid casein, diethylether-deffated, plus 0.2 % DL-methionine, casein (89.4 % protein fresh matter, 7.1 % moisture)
[d] Olive oil
[e] Sunflower oil
[f] Mineral mixture. Composition (in mg/kg dry weight of food): KI, 0.21; Na_2SeO_3, 0.24; $Na_2CrO_4.4H_2O$, 1.58; NaF, 2.43; $CuSO_4.5H_2O$, 24.72; $ZnCO_3$, 25.5; $MnSO_4.H_2O$, 169.2; $FeSO_4.7H_2O$, 199.0; $MgCO_3$, 769.7; NaCl, 906.3; $MgSO_4.7H_2O$, 2,250; $Na_2PO_4.2H_2O$, 2,930; KCO_3H, 6,100; $CaPO_4H.2H_2O$, 8,590; KPO_4H_2, 8.200; $CaCO_3$ 10,000
[g] Vitamin mixture. Composition (in mg/kg dry weight of food): Choline dihydrogen citrate, 1,111; folic acid, 1.11; niacin, 22.22; calcium pantothenate, 8.88; riboflavin, 3.33; thiamine, 4.44; pyridoxine HCl, 6.66; retinyl palmitate, 4,400 IU; α-tocopheryl acetate, 33.33 IU; cholecalciferol 220 IU; menadione, 0.055
[h] BHT: Butylated hydroxytoluene
[i] BHA: Butylated hydroxyanisole

Nitrogen analyses

Nitrogen content of casein, sardines and diets was measured by the method of Kjeldalh using a Tecator Unity Auto 1030 (Sweden). The conversion factor for protein was N x 6.25.

Fat analyses

Fish fat was extracted by the method of Bligh and Dyer (1), saponified with 0.5N of sodium hydroxide, and then methylated following the method of Metcalfe et al. (16).

The fatty acid methyl esters of olive oil and sunflower oil and the fish-fat were analyzed by gas chromatography. A Hewlett Packard 5710 chromatograph (Palo Alto, California) with a steel column packed with 10 % Supelcoport 2330 on 100/120 Chromosorb W AW, 2 m per 1.175 mm i.d. was used. The temperature of the column was held 8 min at 170 °C and then increased to 240 °C at 2 °C/min. The temperature of the injector was 250 °C and that of the detector 300 °C. Sample size was 0.5 μL. Peak-areas were measured using a Perkin-Elmer Minigrator M-2 integrator (Norwalk, Ct.). Fatty acids were identified by comparing their relative and absolute retention times with those of commercial standards. The major fatty acid content of diets are presented in Table 2 and were calculated on the basis of percentage in the fat and the proportion of fat in the diets, using the conversion factors suggested for fish (20). To ascertain the value of these conversion factors (20) samples with and without pentadecanoic acid as internal standard were studied.

Peroxide value of fats contained in the different diets were measured according to norma UNE (27).

Serum analyses

TC in both serum and HDL after precipitation of VLDL + LDL with phosphotungstic acid and magnesium chloride (2) was analyzed according to the enzymatic cholesterol esterase-cholesterol oxidase method proposed by Boehringer Mannheim (Germany). Triglycerides were determined according to the enzymatic glycerol-phosphate oxidase method (GPO-PAP) proposed by Boehringer Mannheim.

Statistical analyses

Results obtained in diets 1 and 2, and those of diets 3 and 4 were compared, respectively, by Mann-Whitney U test (6). Basal and 28-day values were compared by the Wilcoxon *T*-test (6). Differences were considered statistically significant at $p < 0.05$.

Table 2 Major nutrient and fatty acid composition analysis of the experimental diets containing sardines fried in olive oil (Diet 1), casein plus olive oil (Diet 2), sardines fried in sunflower oil (Diet 3) and casein plus sunflower oil (Diet 4)

	Diet 1	Diet 2	Diet 3	Diet 4
Protein content (Nx6.25) *g/kg* DM	155.1	143.3	143.1	145.0
Lipid[a] content *g/kg* DM	177.0	170.4	163.7	169.2
Major fatty acids:	Amount (% of total fatty acids)			
Myristic	1.92	–	3.39	–
Palmitic	14.29	10.31	14.41	7.85
Palmitoleic	2.20	0.58	3.26	0.10
Stearic	3.97	3.88	4.68	4.96
Oleic	58.85	79.95	24.31	29.92
Linoleic	6.23	4.85	33.10	54.68
Eicosapentaenoic	3.78	–	5.41	–
Docosahexaenoic	3.93	–	4.71	–

[a]Fat plus cholesterol

Results and discussion

Fatty acid diet composition

The fatty acid content of fried sardine diets (Table 2) clearly indicates that the fat composition of the fried sardines tends to be similar to that of the respective frying oils. This fact is in agreement with data of other studies (15, 23). Fried sardines used in diets 1 and 3 contained similar and modest amounts of eicosapentaenoic and docosahexaenoic acids.

Food intake

Table 3 shows the daily food and cholesterol intake of the experimental groups during 4 weeks. Although a high cholesterol and bovine bile supplement was used, the intake of groups 1 and 2 was similar to the results obtained with unsupplemented casein diets in other experiments (30). As was already stated in *Materials and Methods*, groups 3 and 4 were made pair-fed because the food intake of group 3 was significantly lower than that in group 1 or 2 since the beginning of the experiment.

Factors such as taste, smell, and texture have been primarily suggested to influence diet intake (18). Oxidation and hydrolysis of the fat affect its palatability, spoiling the taste of food even when the food only contains very small quantities of fat. The peroxide values of sardines used in the current study were rather low (~6) and are apparently not related to the different food intakes seen (Table 3). However, as is well-known, hydroperoxides are very unstable and break down to produce many types of secondary products (8). The residual water in the fish must have contributed to the oxidation of the fat, even when stored under the conditions described. This fact might influence the taste and smell of sardines and in turn the dietary intakes.

Body weight gains

Final body weights and body weight gains were similar in diets 1 and 2, and in diets 3 and 4 (Table 3). Animals fed the diets 1 or 2 show a body weight 20 % higher than those fed with diets 3 or 4. These results seem to be a

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Table 3 Food and cholesterol intake, and body weight gains in rats fed the experimental diets containing sardines fried in olive oil (Diet 1), casein plus olive oil (Diet 2), sardines fried in sunflower oil (Diet 3), and casein plus sunflower oil (Diet 4)

		Diet 1	Diet 2	Diet 3	Diet 4
Intake	Food *g/day*	12.1±0.4	11.0±0.2	9.6±0.4	9.6±0.0
	Cholesterol *mg/day*	242.9±8.4	221.4±3.0	192.0±8.2	192.0±0.0
Body weight	Initial *g*	64.6±1.8	65.8±0.9	66.1±1.3	65.5±1.3
	Final *g*	190.7±4.7	182.1±4.1	158.7±8.1	155.9±8.5
	Gain *g*	126.1±5.1	116.3±3.1	92.6±8.1	90.4±9.0

Values are expressed as the mean ± SEM of six animals
No significant differences were found between diets 1 and 2, and between diets 3 and 4 (Mann-Whitney U-test)

consequence of the food intake observed in the different groups.

Serum lipid levels

Except in group 3, TC increased with regard to its respectively basal value after the 4-week experiment (Table 4). Therefore, the cholesterol-raising effect of diets in groups 2 and 4 (13.9 mmol/L and 18.2 mmol/L, respectively) was more noticeable than in sardine groups (1 mmol/L and 0.4 mmol/L for diets 1 and 3, respectively) (Table 4). The hypercholesterolemic effect of diets consumed by groups 2 and 4 concurs with others who pointed out that cholesterol-casein-diets produced a significant cholesterol increase (5, 7, 24).

Durand et al. (7) found that replacing half the olive oil in the diet by sardine oil suppressed and even reversed the hypercholesterolemic effect of diet.

The hypocholesterolemic effect of fried sardine-diets could mainly be attributed to its PUFA n-3 content, but protein composition would also change cholesterol levels (30, 33). Furthermore, in a review over n-3 fatty acids, the following question was asked: Is fish or fish oil protective? (19).

HDL-cholesterol levels appear lower in diet 1 than in diet 2, but similar in diets 3 and 4 (Table 4). Recently it has been suggested that oleic acid increases, by a synergistic action, the PUFA n-3 uptake by the cells (26). This effect would substantially potentiate the effect of PUFA n-3 in diet 1 and thereby would decrease HDL-cholesterol as larger doses of PUFA n-3 would. Large doses of PUFA n-3 may quite substantially lessen HDL-cholesterol and HDL-apoprotein A concentrations (19).

Dietary cholesterol has been shown to stimulate production of VLDL rich in esterified cholesterol, changing the rat's normal cholesterolemic profile, where the major proportion of TC lies in HDL (3–5, 7). With regard to the percentage of TC carried by the HDL, TC seems to be mainly related to VLDL + LDL in casein groups, because HDL fraction transports only the 4 % and 5 % of TC in groups 2 and 4, respectively. However, in the olive-oil-fried sardine group or in the sunflower-oil-fried sardine group HDL fraction carries the 13 % or the 53 % of TC, respectively.

According to Nestel (19), fish oils may modify the cholesterol-raising effect of dietary cholesterol reducing out flow of VLDL from the liver.

Final triglyceride levels were lower in diet 1 than in diet 2, and in diet 3 than in diet 4 (Table 4). Moreover, in sardine diets triglyceride level decreased with regards

Table 4 Plasma lipid concentration in rats fed the experimental diets containing sardines fried in olive oil (Diet 1), casein plus olive oil (Diet 2), sardines fried in sunflower oil (Diet 3) and casein plus sunflower oil (Diet 4)

			Diet 1	Diet 2	Diet 3	Diet 4
Serum cholesterol mmol/L	Total	Basal	2.43 ± 0.34	2.36 ± 0.18	2.28 ± 0.32	2.41 ± 0.24
		Day 28	3.39 ± 0.23^{a}	16.25 ± 2.93^{b}**	2.71 ± 0.19	20.64 ± 2.94^{b++}
	HDL	Basal	ND	ND	ND	ND
		Day 28	0.43 ± 0.04	0.65 ± 0.07*	1.44 ± 0.21	1.04 ± 0.12
Triglycerides mmol/L		Basal	1.00 ± 0.20	0.77 ± 0.21	1.16 ± 0.25	0.98 ± 0.15
		Day 28	0.43 ± 0.10^{a}	1.00 ± 0.10^{a}*	0.47 ± 0.02^{a}	1.25 ± 0.15^{a+}

Values are expressed as the mean ± SEM of six animals
Asterisks indicate statistical differences between diets 1 and 2 (* $p < 0.05$; **: $p < 0.01$)
Croisses indicate statistical differences between diets 3 and 4 (+: p 0.05, ++ $p < 0.01$)
Values bearing a letter were significantly different with respect to its respective basal value (Wilcoxon T test; a: $p < 0.05$; b: $P < 0.01$)
ND: not determined

to its respectively basal value after the 4-week experiment (Table 4). These results are in agreement with the lowering effect of fish oil indicated elsewhere in the literature (19, 25). However, in casein diets triglyceride levels increased after 4 weeks. These results are also in agreement with others (7).

Rats fed 15 wt% Max EPA for 2 weeks, had a 40 % lower concentration in plasma triglycerides than those fed the same amount of safflower oil; the rates of VLDL-triglyceride and of VLDL-apoprotein B production were significantly reduced by large doses of fish oils (12, 19, 29). In rat liver, additional factors such as diminished fatty acid synthesis and increased fatty acid oxidation have been demonstrated (32).

Our results provide that, in comparison with casein diets, fried-sardine diets showed a powerful check effect on the cholesterol-raising effect induced by dietary cholesterol.

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