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The role of unsaponifiable components in the lipidemic property of olive oil.

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ABSTRACT

Pure olive oil triglycerides (POLO), free from all unsaponifiable matter, were isolated from Virgin Spanish olive oil (COLO) by alumina-charcoal column chromatography. COLO and POLO were used as sources of dietary fat in two animal studies. The responses of serum and liver lipids to the two types of dietary fat were examined. Our results show that animals fed POLO-diet gave somewhat higher serum total and LDL cholesterol levels as compared to those on COLO-diet. The increase in serum cholesterol level is followed by a parallel increase in liver cholesterol content. These results indicate that the hypocholesterolemic effect of olive oil was partly due to the presence of the unsaponifiable matter. Supplement of the POLO-diet separately with a-tocopherol and squalene resulted in serum lipid responses similar to that observed with the COLO-diet. The serum and liver triglyceride levels are not affected by the removal of unsaponifiable components but addition of a--T and squalene to the POLO-diet appeared to lower both the cholesterol and triglyceride levels in the serum but increased only the liver cholesterol content. These results show that the unsaponifiable components modulate the hypocholesterolemic effect of olive oil

INTRODUCTION

People in the Mediterranean region generally consumed high fat diet rich in olive oil, yet they have low incidence of cardiovascular disease as compared to other Western countries (Keys, 1970). Olive oil is highly enrich in oleic acid and epidemiologists assumed that oleic acid was the reason for the low cardiovascular disease incidence in the Mediterranean countries. Mattson and Grundy (1985) using a liquid formula diet showed that high-oleic-acid safflower oil was as hypocholesterolemic as high-linoleic-acid safflower oil in male patients in a metabolic ward. Gustafsson et al (1994) showed a diet rich in high-oleic-acid rapeseed oil effectively reduced serum lipoprotein cholesterol concentrations in hyperlipidemic subjects. Berry et al,

(1992) showed that a diet rich in monounsaturated fatty acid (olive oil) lowered serum total and LDL cholesterol levels in young healthy students. In laboratory animal models, Rudel et al (1990) confirmed that high-oleic-acid safflower oil exhibited hypocholesterolemic property as compared to saturated fats. The general deduction from all these results was that the major fatty acid, oleic acid, was responsible for the hypocholesterolemic effect in the high-oleic acid oils. However, Perez-Jimenez et al (1995) found differences in serum lipid responses between the olive oil and high-oleic-acid sunflower seed oil in normolipidemic males. They suggested that the unsaponifiable matter might be the cause of the differences in serum lipid responses. The content of unsaponifiable matter in oils varies considerably (Shahidi & Shukla, 1996). Analysis of olive oil revealed that it contains substantial amounts of unsaponifiable compounds in addition to the major components, the triglycerides (Gunstone et al, 1986). Since previous studies used high-oleic-acid oils as the source of oleic acid, it is not possible to ascertain how much of the hypocholesterolemic effect of olive oil was due to its oleic acid and how much of that effect could be due to the unsaponifiable components. Therefore this study was designed to differentiate the hypocholesterolemic effect of olive oil between its triglycerides and its unsaponifiable fraction, and to study the cholesterolemic effect of some of the unsaponifiable components in olive oil.

MATERIALS AND METHODS

Male Golden Syrian hamsters were purchased from the Animal Breeding Unit, Faculty of Medicine, University of Malaya. They were divided into groups of approximately equal average body weights. They were housed in stainless cages in a air-conditioned room with temperature of 25±0.2 °C. They were fed on semi-synthetic diets (Table 1) differing only in the nature of dietary fat for 4-6 weeks. Water was given *ad libitum*. At the end of the experimental period the animals were sacrificed after overnight fasting. Blood was collected by cardiac

Table 1. Formulation of the semi-synthetic diets

Ingredients	g/100 g diet	Ingredients	g/100g diet
Corn flour	29.0	Oil*	20.5
Dextrose	17.5	Choline bitartrate	0.2
Cellulose	5.0	Cholesterol	0
Casein	22.0	Mineral mix	4.5
DL-Methionine	0.3	Vitamin mix	1.0

* Commercial Virgin olive oil or isolated pure olive oil triglycerides

puncture and sera were prepared by centrifugation at 1500 rpm for 10 minutes. Liver was excised, cleansed and frozen at – 20 °C until analysis.

Serum lipids were analysed by enzymatic procedures using Sigma diagnostic kit. LDL cholesterol was determined from the supernatant fraction after HDL precipitation (Khor & Chieng, 1996). Liver lipids were extracted with chloroform-methanol (2:1,v/v) according to Folch et al (1957) and analysed by TLC using solvent system consisting of hexane-diethyl ether-formic acid (80:20:2,v/v/v). The liver cholesterol, cholesterol esters and triglycerides were estimated by an acid-charring method (Marsh & Weinstein, 1966). Standard calibration graphs for each lipid was constructed using commercial lipid standards. Fatty acid methyl esters of olive oil was analysed by GLC using a wide bore capillary column (Carbowax 10, 30m x 0.75mm) and the unsaponifiable components were analysed by capillary GLC using a DB-17HT column. GLC peaks were identified by comparing their retention times with authentic standards.

Alumina-charcoal column chromatography was used to isolate pure olive oil triglycerides from commercial Virgin olive oil (Khor & Chieng, 1996). The fatty acid profile of the isolated triglycerides was comparable to that of the commercial olive oil (Table 2). The isolated olive oil triglycerides were also completely free of all unsaponifiable components.

STATISTICAL ANALYSIS

Student t-test was used to evaluate the significance of differences ($P<0.05$) between means of sample groups

RESULTS AND DISCUSSION

Two separate experiments were carried out. All animals appeared healthy during the experimental period and there were no significant differences in body weight gains. The

Table 2: Fatty acid composition (%) of commercial Virgin olive oil (COLO) and isolated pure olive oil triglycerides (POLO).

Fatty acids	COLO	POLO
16:0	8.96	8.93
16:1	1.36	1.67
18:0	1.79	1.68
18:1	76.26	76.13
18:2	8.29	8.56
18:3	1.53	1.56
20:0	0.21	0.16
20:1	0.59	0.39
20:2	0.39	0.50

commercial Virgin olive oil contains about 1.036% of unsaponifiable matters. These unsaponifiable matters consist of mainly short-chain hydrocarbons, squalene, sterols, tocopherols and other unidentified components as analyzed by capillary GLC. These results are comparable to those reported previously (Gunstone et al, 1986; Kiritsakis A & Markakis, 1987).

In the first study, two groups of animals were used. One group was fed on diet containing commercial Virgin olive oil (COLO) as the source of dietary fat and the other group was fed on diet containing pure olive oil triglycerides (POLO) free of all unsaponifiable matter. The serum lipid responses of these two groups of animals were shown in Table 3. The group fed on diet containing POLO gave somewhat higher level of serum total cholesterol than the group fed on diet containing COLO. The difference was not statistically significant due to small sample size and big standard deviations ($P<0.10$). However, a significant difference was seen between the LDL-cholesterol level of the two groups ($P<0.05$). No changes were observed in HDL-cholesterol and serum triglyceride levels between the dietary groups. These results indicate that the unsaponifiable components in olive oil complement the cholesterol-lowering effect of the oleic acid in olive oil. Huang et al (1991) reported that the unsaponifiable components of olive oil showed cholesterol-lowering effect when fed to Sprague-Dawley rats. The complementary role of the unsaponifiable components on the non-hypercholesterolemic effect of palm oil was also observed (Khor & Tan, 1992). Shahidi & Shukla (1996) recently reviewed the importance of the unsaponifiable matter in oils and fats in nutrition and in health.

When the liver lipids were analyzed (Table 4) it was noticed that the POLO group had significantly higher total lipids and total cholesterol, free and esterified cholesterol levels than those of the COLO group. Beynen (1988) reported that feeding olive oil to rats and

Table 3: Effects of dietary Virgin olive oil (COLO) and pure olive oil triglycerides (POLO) on serum lipid levels (mg/dL).

Dietary groups	Total Cholesterol	LDL-Cholesterol	HDL-Cholesterol	Triglycerides
COLO	114.72±7.68	24.51±4.12[a]	25.35±1.95	54.99±6.15
POLO	123.65±5.63	38.99±2.12[b]	25.61±2.85	55.08±0.98

Results are expressed as Mean ± S.E.M. n=4.
COLO = commercial Virgin olive oil.
POLO = Isolated pure olive oil triglycerides, free from all unsaponifiable components
Mean with superscript a is significantly different ($P<0.05$) from mean with superscript b in the same column

Table 4: Effect of dietary olive oil and isolated pure olive oil triglycerides on liver lipids

Dietary groups	Total lipids (mg/g)	Triglycerides (mg/g)	Cholesterol (mg/g)		
			Total	Free	Esterified
COLO	47.88±1.70[a]	9.83±1.17	3.12± 0.39[a]	1.99±0.24[a]	1.13±0.15[a]
POLO	67.27±3.41[b]	10.71±1.38	5.08±0.69[b]	2.37±0.19[b]	2.71±0.50[b]

Results are expressed as Mean±S.E.M. n=4.
Means with superscript a are significantly different (P<0.05) from means with superscript b in the same column

rabbits increased liver total cholesterol level as compared to other oils. There was no significant difference in liver triglyceride level between the two groups. These results show that the unsaponifiable components of olive oil affect mainly cholesterol metabolism and have little effect on triglyceride metabolism in the liver.

In the second study, the amounts of α-tocopherol and squalene equalled to the total tocopherols and squalene present in COLO were added separately back to POLO and used as sources of dietary fat. The results of this study (Table 5) once again show that the POLO group had higher serum total and LDL cholesterol levels than the COLO group, and the two groups had similar HDL cholesterol and serum triglyceride levels. These findings confirm our earlier observations that removing the unsaponifiable

Table 5: Effect of unsaponifiable components of olive oil on serum lipids (mg/dL) in the hamster

Dietary groups	Total cholesterol	LDL-Cholesterol	HDL-Cholesterol	Triglycerides
COLO	78.33±2.11	24.24±0.37[a]	24.77±0.70	76.87±4.33[a]
POLO	85.18±6.68	28.84±0.18[b]	24.98±0.49	76.45±4.77[a]
POLO-T	79.53±3.94	23.83±0.10[a]	23.16±1.18	54.92±3.08[b]
POLO-SQ	77.26±6.08	23.64±0.24[a]	23.64±1.64	59.78±2.45[b]

Results are expressed as Mean±S.E.M. n=4
Means in the same column with different superscripts are significantly different (P<0.05).
T = α-Tocopherol, 20mg/100g diet
SQ = squalene, 90mg/100g diet

Table 6: Effect of unsaponifiable components of olive oil on liver lipids in the hamster

Dietary groups	Total lipids (mg/g)	Cholesterol (mg/g)			Triglycerides (mg/g)
		Total	Free	Esterified	
COLO	17.9±0.03[a]	12.57±0.12[a]	6.43±0.01[a]	6.14±0.01[a]	3.99±0.01
POLO	23.8±0.05[c]	14.78±0.14[b]	8.20±0.01[b]	6.58±0.01[a]	4.10±0.01
POLO-T	40.0±0.05[d]	17.86±0.09[b]	14.09±0.03[c]	3.76±0.01[b]	3.51±0.01
POLO-SQ	31.7±0.06[e]	18.46±0.10[b]	6.15±0.01[d]	12.308±0.03[c]	4.74±0.01

All results are expressed as Mean ± S.E.M. n=4
Means with different superscripts are significantly different (P<0.05)

components in Virgin olive oil raised the serum total and LDL cholesterol level as compared to the COLO (Table 3). However, when α-tocopherol (α-T) was added back to POLO, the group, which consumed the POLO-T diet, gave similar results as the COLO group. Similarly when squalene (SQ) was added back to POLO, the group which consumed the POLO-SQ diet also gave similar results as the COLO group. These results clearly show that α-T and SQ possess cholesterol-lowering effect and could complement the hypocholesterolemic action of oleic acid in olive oil. The cholesterolemic effect of α-T has been studied previously but the results are controversial, as slightly positive (Chase et al, 1981; Howard et al, 1982) and neutral effect (Tsai et al, 1978; Stampfer et al, 1983; Kesaniemi & Grundy, 1982; Ehnholm et al, 1982; Schwartz & Rutherford, 1981) of α-tocopherol on serum cholesterol level were observed. Olive oil has much more squalene than tocopherols (Gunstone et al, 1986). The cholesterol-lowering property of dietary sqaulene has been studied previously but the results are inconsistent as Strandberg et al (1990) observed no effect of dietary squalene on serum cholesterol level whereas Miettinen and Vanhanen (1994) observed an increase in serum cholesterol level in man. On the other hand, Khor and Chieng (1997) recently reported a cholesterol-lowering effect for dietary squalene in the hamster.

When the liver lipids were analyzed, the results show that the POLO group had significantly higher liver total lipids than that of the COLO group, confirming our earlier findings (Table 3). The increase in total lipids was partly due to an increase in total cholesterol and free cholesterol contents in the liver. No effect was seen in the liver triglycerides. Addition of α-tocopherol and squalene to POLO in the diet resulted in an increase liver total lipids and total cholesterol level, a phenomenon also observed in the hamster when α-tocopherol and squalene were added to the palm oil triglycerides (POTG) (Khor & Chieng, 1997). Huang et al (1991) also reported that addition of squalene to

the diet resulted in an increased liver cholesterol level in the rat. Squalene is a important intermediate in cholesterol biosynthesis and dietary squalene was shown to inhibit the regulatory enzyme, liver HMG CoA reductase, in the rat (Strandberg et al, 1989). Therefore it is easy to conceive that dietary squalene supplement would interfere with the cholesterol metabolism. However, it is still unresolved how the interference in cholesterol metabolism by dietary squalene would alter serum cholesterol levels as reports from different laboratories are contradictory (Strandberg et al, 1990; Miettinen & Vanhaen, 1994; Khor & Chieng, 1994).

In conclusion, our results show that oleic acid is not the only hypocholesterolemic factor in olive oil and that one or more components of the unsaponifiable fraction are required for the full manifestation of the hypocholesterolemic effect of olive oil.

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REFERENCES

Berry EM, Eisenberg S, Friedlander Y, Harats D, Kaufmann NA, Norman Y & Stein Y. (1992). Effects of diets rich in monounsaturated fatty acids on plasma lipoproteins – the Jerusalem Nutrition Study. II. Monounsaturated fatty acids vs carbohydrates. *Am J Clin Nutr* 56:394-403.

Beynen AC (1988). Dietary monounsaturated fatty acids and liver cholesterol. *Artery* 15:170-5.

Chase HP & Dunpont MM (1980). Vitamin E and diabetes mellitus. *Pediatr Res* 14: 569.

Ehnholm C, Huttumen JK, Kostiainen E, Likka M & Aho K (1982). Vitamin E does not influence plasma lipoprotein metabolism in healthy subjects with normal nutritional status. *Clin Chim Acta* 121: 321-325.

Folch J, Lees M & Sloane-Stanley GH (1957). A simple method for the isolation and purification of total lipids from an animal tissue. *J Biol Chem* 226:10544-50.

Gunstone FD, Harwood JL & Padley FB (1986). The lipid handbook, Chapman and Hall, London and New York.

Gustafsson IB, Vessby B, Ohrvall M & Nydahl M. (1994) A diet rich in monounsaturated content of n-3 fatty acids in serum in hyperlipidemic subjects. *Am J Clin Nutr* 59:667-74.

Howard DR, Rundell CA & Batsakis JG (1982). Vitamin E and serum lipoproteins: a non-correlation. *Am J Clin Pathol* 77: 243-244.

Huang YS, Redden P, Lin X, Smith R, Mackinnon S & Horrobin DF (1991). Effects of dietary olive oil non-glyceride fraction on plasma cholesterol level and liver phospholipid fatty acid composition. *Nutr Res* 11:439-48.

Kesaniemi YA & Grundy SM (1982) Lack of effect of tocopherol on plasma lipids and lipoproteins in man. *Am J Clin Nutr* 36: 224-228.

Keys A (1970). Coronary heart disease in seven countries. *Circulation* 41: 1-21.

Khor HT & Chieng DY (1996). Effect of dietary supplementation of tocotrienols and tocopherols on serum lipids in the hamster. *Nutr Res* 16:1393-1401.

Khor HT & Chieng DY (1997). Effect of squalene, tocotrienols and tocopherol supplementations in the diet on serum and liver lipids in the hamster. *Nutr Res* 17:475-83.

Khor HT & Tan DTS (1992). The triglycerides in palm oil are not hypercholesterolemic in the hamster. *Nutr Res* 12:621-28.

Kiritsakis A & Markakis P (1987). Olive oil: A review. *Ad Food Sci* 31:453-82.

Marsh JB & Weinstein DB (1966). A simple charring method for determination of lipids. *J Lipid Res* 7:574-6.

Mattson FH & Grundy SM (1985). Comparison of the effects of dietary saturated, monounsaturated and polyunsaturated fatty acids on plasma lipids and lipoproteins in man. *J Lipid Res* 26:194-202.

Miettinen TA & Vanhanen H (1994). Serum concentration and metabolism of cholesterol during rapeseed oil and squalene feeding. *Am J Clin Nutr* 59: 356-63.

Rudel LL, Haines JL & Sawyer JK (1990). Effects on plasma lipoproteins of monounsaturated, saturated and polyunsaturated fatty acids in the diet of African green monkeys. *J Lipid Res* 31:1873-82.

Schwartz PJ & Rutherford IM (1981). The effect of tocopherol on high density lipoprotein cholesterol. *Am J Clin Pathol* 76: 834-844.

Shahidi F & Shukla VKS (1996). Nontriacylglycerol constituents of fats, oil. *Inform* 11:1227-1232.

Stampfer MJ, Willet W, Castelli WP, Taylor JO, Fine J & Hennekens CH (1983). Effect of vitamin E on lipids. *Am J Clin Nutr* 79:714-716.

Strandberg TE, Silvis RS & Miettinen TA (1989) Effect of cholestyramine and squalene feeding on hepatic and serum plant sterols in the rat. *Lipids* 24:705-8.

Strandberg TE, Silvis RS & Miettinen TA (1990). Metabolic variables of cholesterol during squalene feeding in humans: comparison with cholestyramine treatment. *J Lipid Res* 31: 1637-43.

Tsai AC, Kelly JJ, Peng B & Cook N (1978). Study on the effect of mega-vitamin E supplementation in man. *Am J Clin Nutr* 31: 831-837.