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HYPOCHOLESTEROLEMIC EFFECT OF MILK FAT AND OLIVE OIL IN C57BL/6N MICE FED AN ATHEROGENIC DIET

Zommara, M. A.
Dept. of Dairy Sci., Fac. of Agric., Kafr El-Sheikh 33516, Tanta Univ., Egypt

ABSTRACT

In the present study, C57BL/6N mice, an atherosclerosis diet-inducible strain, were raised on atherogenic diets containing 15% of either butter oil or olive oil for 3 months. Compared to olive oil diet, butter oil diet enhanced mice growth, increased serum HDL-cholesterol concentration, reduced liver cholesterol content and also reduced the atherogenic index. Both experimental diets did not develop any atherosclerotic lesions in the aortic valve. The attained results revealed that consumption of butter oil altered blood lipid profiles in such a manner as not to elevate the risk factors for cardiovascular diseases.

Keywords: Butter oil, olive oil, atherosclerosis, cholesterol, C57BL/6N mice

INTRODUCTION

Many studies explored the relationship between dietary factors and risk for atherosclerosis, particularly, the regulation of cholesterol and lipoprotein metabolism by the type and amount of dietary fat (McNamara, 1987; Mancini and Parillo, 1991; Nishina *et al.*, 1993). In general, diets rich in mono- and polyunsaturated fat decrease plasma cholesterol levels; whereas, diets high in saturated fat, in particular lauric, myristic and palmitic, acids, are positively correlated to plasma cholesterol level (Frantz, 1981; Nordov and Goodnight, 1990). However it is well known that milk fat contains not only about 70% saturated fatty acids (Table 1) but also contains approximately 0.3% cholesterol, so it might be responsible for increased risk of coronary heart disease (CHD).

Table 1: Fatty acid composition of the dietary fat (%)

Fatty acid	Olive oil	Butter oil
Butyric	-	3.4
Caproic	-	2.5
Caprylic	-	1.6
Capric	-	3.3
Lauric	-	4.0
Miristic	-	12.9
Palmitic	10.0	30.4
Palmitoleic	0.6	2.1
Stearic	3.2	11.7
Oleic	74.8	24.3
Linoleic	10.5	3.1
α-linolenic	0.9	0.8
Arachidic	-	0.2
Saturated fatty acids (SFA)	13.2	69.7
Monounsaturated fatty acids (MUFA)	75.5	26.4
Polyunsaturated fatty acids (PUFA)	11.4	3.9

Olive oil, the predominant fat source in the Mediterranean diets, is rich in monounsaturated fatty acids (MUFA) and natural antioxidants (Owen, *et al.*, 2000). This leads to relatively low levels of oxidized lipoproteins and associated with decreased mortality from cardiovascular disease among Mediterranean populations (Galli and Visioli, 1999).

This study was designed to elucidate the effect of an atherogenic diet added with butter oil or olive oil on blood and liver lipids and the development of atherosclerosis in C57BL/6N mice. This strain has diet-inducible atherosclerosis as well known from the literature.

MATERIALS AND METHODS

Preparation of butter oil

Butter oil was prepared by extracting bovine milk butter (Snow Brand Milk Products Co. Ltd. Hokkaido, Japan) with n-hexane (1:3, v/v) for one hour at room temperature followed by evaporating the solvent under vacuum using a rotary evaporator. The extracted butter oil contained 290 mg cholesterol /100g fat as determined by the method described by Ikeda *et al.*,(2001).

Diets and animals

The animals used in this study were 7 weeks old C57BL/6N male mice with a mean body weight of 19-22g and were obtained from Seac Yochitomi Ltd. (Yochitomi-Cho, Chikujyo-gun, Fukuoka, Japan). The mice were housed individually in a plastic cages in a temperature controlled room (21-22°C) with a 12 h-light / 12 h-dark cycle. The animals were acclimatized under the previous conditions and maintained on a commercial non-purified diet (NMF, Oriental Yeast Co., Tokyo, Japan) for four weeks before starting the experiment. At the end of this period the rats were arranged in two groups with seven mice in each group.

The experimental diets were formulated according to the AIN-93G formula as described by Reeves *et al.*, 1993. As shown in Table 2, the final mixture of the diets contained 15% of either olive oil (Nacalai Tesque, Kyoto, Japan) or butter oil, about 1.25% cholesterol and 0.5% sodium cholate and, therefore, the diets are considered atherogenic according to Wang *et al.*, 1999. The fatty acid composition of the experimental diets is shown in Table 1. The diets contained equal amount of energy (about 4278 Kcal/kg diet). The mice were raised on these purified diets for 12 weeks. Food and deionized water were provided freely throughout the experimental period. The body weight and food intake were measured every other day. This experiment was carried out under the control of the guidelines for animal experiments of the Faculty of Agriculture and Graduate Course at Kyushu University and Law No. 105 and notification No. 6 of the Government of Japan.

Table 2: Composition of the experimental diets (g/kg)

Ingredients	Olive oil	Butter oil
Casein	200	200
Olive oil	150	-
Butter oil	-	150
Vitamin mixture	10	10
Mineral mixture	35	35
Choline bitartrate	2.5	2.5
L-cystine	3	3
Cellulose	50	50
α-Corn starch	132	132
Corn starch	300	300
Sucrose	100	100
BHQ*	0.014	0.014
Cholesterol	12.5	12.5
Sodium cholate	5	5

*Butylated hedroxyqunone

Tissue preparation

After food had been withheld for 12 h (from 9:00 pm to 9:00 am), the mice were killed by withdrawing blood from the right ventricle under anesthesia with an intraperitoneal injection of sodium pentobarbital (5 mg/g body weight). Blood was collected and centrifuged at 3000 rpm for 30 min for serum preparation. The liver, spleen, brain were excised, washed in saline solution and its weight was recorded. Livers and serum were immediately immersed in liquid nitrogen and kept at –25°C until analysis.

Morphometric determination of atherosclerosis

The heart was perfused, *in situ*, with 50 ml phosphate buffer saline (PBS) via a cannula inserted in the right ventricle, allowing unrestricted efflux from an incision in the vena cava. Perfusion was continued with 50 ml of a 10% neutral formalin buffer solution at pH 7.4 (Wako Pure Chemicals, Osaka, Japan). The heart was dissected and the bulk of the fat and tissue adhering to it was removed as much as possible. The heart was fixed in a 20 % neutral formalin buffer solution at pH 7.4 (Wako Pure Chemicals). To determine the cross-sectional lesion volume, hearts containing aortic roots were processed for quantitative atherosclerosis assay using a modification of the method described by Paigen *et al.*, (1987). Briefly, the heart was cut along a plane between the tip of the two atria and the top half was embedded in paraffin. Consecutive sections (10 µm thick) were prepared from the aortic arch until the disappearance of the tissue. The sections were mounted on glass slides and stained with hematoxylin-orcine as described by Ni *et al.*, (1998). Five sections of each heart were selected for intimal area determination; the first and most distal sections to the heart were where the aortic valve cusp was barely discernible. From this section, moving to the base of the heart, every other 150 µm section was photographed. The intimal area was measured using video camera mounted on Olympus LX70 light microscope and

analyzed using Adobe Photoshop and Nih image/68K 1.57 software (National institute of health, Bethesda, MD, USA) on a Power Macintosh Computer.

Analysis

Serum total cholesterol, triglycerides, high-density lipoprotein (HDL) cholesterol and phospholipids concentrations were measured using commercially available enzyme assay kits (Cholesterol C-test, triglyceride G-test, and phospholipids B-test purchased from Wako Pure Chemical Industries Ltd. Osaka, Japan, and HDL-C2 test was from Daiichi Chemicals, Tokyo, Japan). Serum low-density lipoprotein (LDL) cholesterol concentration was calculated using the equation of DeLong *et al.* (1986) as followes:

LDL-cholesterol =Total cholesterol-HDL-cholesterol-(0.16 x triglycerides)

Liver lipids were extracted as described by Folch *et al.*, (1957) and used for determination of cholesterol (Sperry and Weeb, 1950), triglyceride (Fletcher, 1968) and phospholipids (Bartlett, 1959). The fatty acid composition of the dietary fat was determined as described by Imaizumi *et al.* (1993). The atherogenic index was calculated using the following equation as described by Kawase, *et al.*, (2000).

Atherogenic index =[(Total cholesterol-HDL-cholesterol)/HDL-cholesterol]

Statistical analysis

Data are expressed as the mean value for seven mice per group, and statistical differences were determined by student's *t* test according to Fisher (1970).

RESULTS AND DISCUSSION

As shown in table 3, total and daily food intake were significantly higher in the mice fed butter oil diet than that fed the olive oil diet. However, the food efficiency (g body weight gain/g food intake) showed no significant differences and was comparable between the two groups.

Table 4 summarizes mice growth parameters. Initially, the mice used in this study weighed approximately 24 g. Although there were no statistically significant differences in average body weight between the two groups at the end of 3 months of feeding, the high fat diets, body weight gain was variable. Mice consuming the olive oil diet gained the least weight, while mice consuming the butter oil diet gained the highest weight. Out of seven mice in the olive oil group, three mice (42.9%) lost body weight during the experimental period and resulted in less final body weight when compared to their initial weight. However, the butter oil mice group did not show such observation. The lack of weight gain during the feeding period was anticipated, as the mice used were adult and fully-grown. Also, feeding diet with cholesterol and cholic acid may cause hepatocytes toxicity, which adversely affected mice growth. Fujino *et al.* (1987), demonstrated that feeding mice with a diet containing 1.5% cholic acid induced mouse mortality due to hepatocytes toxicity. In this respect, butter oil seems to ameliorate the side effect of the atherogenic diet. On the other hand, no significant differences were recorded in relative liver, spleen and brain weights (g tissue/100g body weight) between the two mice groups.

Table 3. Food intake of mice fed diets with olive oil or butter oil

Parameter		Olive oil	Butter oil
Total food intake (g)	Max	263.5	316.4
	Min	221.0	233.5
	Avg	**238.9**	**268.6***
	SD	14.2	25.3
Daily food intake (g)	Max	3.21	3.86
	Min	2.70	2.85
	Avg	**2.91**	**3.28***
	SD	0.17	0.31
Food efficiency (g body gain/ g food intake)	Max	0.021	0.019
	Min	-0.011	0.004
	Avg	**0.006**	**0.013**
	SD	0.012	0.006

Data are range and average of 7 mice per group.
(*) Significantly different at $P < 0.05$

Table 4: Growth parameters of mice fed diets with olive oil or butter oil

Parameter		Olive oil	Butter oil
Initial body weight (g)	Max	26.00	25.30
	Min	22.60	21.20
	Avg	**23.90**	**23.63**
	SD	1.21	1.38
Final body weight (g)	Max	23.60	25.80
	Min	28.10	29.10
	Avg	**25.40**	**27.10**
	SD	1.78	1.22
Body weight gain (g)	Max	5.50	5.10
	Min	-2.40	1.00
	Avg	**1.49**	**3.47**
	SD	2.85	1.86
Liver (%)	Max	7.97	8.31
	Min	5.82	5.47
	Avg	**6.85**	**6.65**
	SD	0.69	1.11
Spleen (%)	Max	0.413	0.722
	Min	0.256	0.307
	Avg	**0.339**	**0.470**
	SD	0.060	0.145
Brain (%)	Max	1.903	1.808
	Min	1.544	1.567
	Avg	**1.755**	**1.671**
	SD	0.122	0.091

Data are range and average of 7 mice per group.

Table 5 shows concentration of total serum cholesterol, lipoprotein cholesterol, triglycerides and phospholipids and the atherogenic index of mice. No significant differences were found in serum cholesterol, triglycerides and phospholipids between the two groups although the mice in the butter oil group tended to have lower serum cholesterol and higher phospholipids concentrations than the olive oil group. Kumer *et al.* (1999), postulated that rats fed diets containing greater than 2.5% of ghee had lower levels of serum cholesterol compared with rats fed diets containing groundnut oil. They demonstrated that the hypocholesterolemic effect of ghee is mediated by increasing the secretion of biliary lipids (Kumar *et al.*, 2000).

Table 5: Serum lipids (mg/dl) of mice fed diets with olive oil or butter oil

Lipids		Olive oil	Butter oil
Total cholesterol	Max	378.8	295.1
	Min	167.3	170.7
	Avg	242.3	231.3
	SD	75.0	45.1
HDL-cholesterol	Max	45.1	58.1
	Min	25.0	34.7
	Avg	33.8	45.6*
	SD	6.1	8.6
HDL-C/T-C	Max	0.21	0.26
	Min	0.09	0.15
	Avg	0.15	0.20
	SD	0.05	0.04
LDL-cholesterol	Max	337.7	233.4
	Min	133.4	129.9
	Avg	202.9	180.1
	SD	74.7	42.7
Atherogenic index	Max	10.2	5.3
	Min	3.8	2.8
	Avg	6.4	4.1
	SD	2.7	1.0
Triglycerides	Max	45.7	44.6
	Min	22.6	29.2
	Avg	34.9	35.2
	SD	7.7	5.7
Phospholipids	Max	205.2	276.6
	Min	122.6	130.6
	Avg	168.3	208.2
	SD	29.7	52.2

Data are range and average of 7 mice per group.
(*) Significantly different at P <0.05

It has been suggested that plasma HDL operates in the transfer of cholesterol from peripheral tissues through the plasma and clearance via the liver as bile acids. Therefore, there is a strong inverse relationship between plasma HDL-cholesterol level and incidence of atherosclerosis (Swenson,

1992; Nishina, 1993). In this respect, feeding butter oil diet resulted in a significant increase in mice serum HDL-cholesterol concentration compared to the olive oil diet (Table 5) suggesting a beneficial effect of milk fat in a hypercholesterolemic diet.

LDL is the major transport vehicle of plasma cholesterol in man since about 70-80% of total plasma cholesterol was reported to be carried by it. Oxidation of LDL is considered to be an important step in development of fatty streaks. Oxidized LDLs are readily taken up by monocyte-derived macrophages via scavenger receptors, a process that results in the formation of foam cells, which is an early event in the formation of the atherosclerotic plaque (Witztum and Steinberg, 1991). Although, serum LDL-cholesterol concentration was not significantly different between the two groups, it was lower by 11.2% in the butter oil group than that of the olive oil one (Table 5).

The atherogenic index is an indication for the susceptibility for atherosclerosis. No significant differences were found between the two groups in the atherogenic index, due to wide variation among the mice (Table 5), although it was lower by 35.7% in mice fed the butter oil diet than that fed the olive oil diet.

Aortic valve lesions were observed visually by using light microscope. After reviewing all cross sections of the aortic valve of the mice in both groups (35 slides, each), no lesions were found either in the butter oil or the olive oil mice. C57BL/6N mice are shown to develop atherosclerotic lesions when fed an atherogenic diet. (Paigen, 1987 ; Paigen, 1995). However, in the present study addition of olive oil and butter oil to the diets ameliorated the deleterious effects of the atherogenic diet containing high fat and cholesterol.

Table 6 shows concentration of lipids in mice livers fed the atherogenic diets. Mice fed the butter oil diet resulted in a significant reduction of liver cholesterol concentration than that fed the olive oil diet. However, triglycerides and phospholipids were comparable between the two groups. The atherogenic diets resulted in cholesterol gallstone development in 71.4% and 85.7% of the mice fed the olive oil and the butter oil diets, respectively. In contrast, Paigen (1995), found no significant correlation of gallstones and atherosclerosis using gallstone susceptible C57BL/6 mice fed an atherogenic diet.

Table 6. Liver lipids (mg/ g) of mice fed diets with olive oil or butter oil

Lipid		Olive oil	Butter oil
Total cholesterol	Max	40.85	20.77
	Min	18.69	11.97
	Avg	**28.41***	**14.90***
	SD	7.03	3.15
Triglycerides	Max	29.25	25.05
	Min	14.03	9.02
	Avg	**19.33**	**16.51**
	SD	5.80	9.03
Phospholipids	Max	29.77	30.78
	Min	24.23	24.71
	Avg	**26.80**	**27.67**
	SD	2.11	1.83

Data are range and average of 7 mice per group.
(*) Significantly different at P <0.05

In conclusion, the present study showed a hypocholesterolemic effect of butter oil compared to olive oil when incorporated to an atherogenic diet by 15% (w/w). This effect was demonstrated by a significant reduction in liver cholesterol concentration and lower incidence of serum and serum LDL-cholesterol. Furthermore, butter oil increased serum HDL-cholesterol by 35% and reduced the atherogenic index by 36% compared to the olive oil. The present findings didn't support linking of milk fat consumption to hypercholesterolemia and incidence of atherosclerosis. However, use of excess intake of milk fat as a means for lowering serum cholesterol is not recommended, but the study indicates that there is no reason for apprehension for consuming butter oil in the diet.

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تأثير دهن اللبن مقارنة بزيت الزيتون عند تغذية الفئران من سلالة C57BL/6N على عليقة مسببة لتصلب الشرايين

محسن عبد العزيز زماره

قسم الألبان، كلية الزراعه بكفر الشيخ، جامعة طنطا

يحتوى دهن اللبن على نسبة مرتفعة من الأحماض الدهنية المشبعه والكوليسترول والتى تعتبر من العوامل المسئولة عن امراض أوعية القلب الدموية. وعلى العكس من ذلك يحتوى زيت الزيتون على نسبة مرتفعة من الأحماض الدهنية وحيدة عدم التشبع ومضدات طبيعية للأكسدة والتى يعتقد أنها تحمى شعوب منطقة البحرالأبيض المتوسط من مرض تصلب الشرايين.

أهتمت هذه الدراسة بتغذية الفئران من نوع C57BL/6N- وهى سلالة حساسة للأصابة بمرض تصلب الشرايين بواسطة العليقه- على عليقه مسببه لتصلب الشرايين تحتوى على دهن اللبن أو زيت الزيتون بنسبة ١٥% من وزنها. أدت التغذية على دهن اللبن الى تحسين نمو الفئران وزيادة تركيز الكوليسترول فى البروتينات الدهنية عالية الكثافه بسيرم الدم وقللت من تركيز الكوليسترول فى الكبد وأحدثت أنخفاضا فىقيمة دليل تصلب الشرايين مقارنة بعليقة زيت الزيتون و لم تؤدى أى من العليقتين الى أحداث تصلب فى الصمام الأورطى بالقلب. أظهرت الدراسة أن أستهلاك دهن اللبن أثر على دهون الدم بطريقة لم تؤدى الى زيادة التعرض لأمراض أوعية القلب الدموية