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Pinus koraiensis seed oil (PinnoThin™) supplementation reduces body weight gain and lipid concentration in liver and plasma of mice

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ABSTRACT

Pinus koraiensis seed oil (pine nut oil), containing pinolenic acid, has been proposed as a beneficial fat by virtue of its effects on some lipid variables. In this study the effects of a pine nut oil-supplemented diet in mice, in comparison to control animals fed with a maize oil enriched diet, were investigated. Pine nut oil caused a significant reduction in body weight gain and liver weight (37.4 and 13.7%, respectively). An impressive decrease in plasma triglycerides and total cholesterol (31.8 and 28.5%, respectively) was also found in pine nut oil-fed animals. Liver lipids were also positively influenced by pine nut oil. The mitochondrial and cytosolic enzyme activities involved in hepatic fatty acid synthesis were strongly reduced both in pine nut oil and maize oil-fed animals, thus suggesting that the beneficial effects of pine nut oil are not due to an inhibition of hepatic lipogenesis.

KEY WORDS: body weight, liver lipids, plasma lipids, fatty acid synthesis, pine nut oil (PinnoThin™), mice

INTRODUCTION

Vegetable oils from the seeds of some conifers (Wolff et al., 2000), such as *Pinus pinaster* and *Pinus koraiensis,* are currently under investigation for their use in the feed industry and/or as dietary supplements (Sugano et al., 1994; Asset et

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al., 1999; Lee et al., 2004). These oils contain, to a certain degree, some unusual polyunsaturated fatty acids (PUFA) which are characterized by polymethylene-interrupted double bonds. In particular, the oil from the seeds of *Pinus koraiensis* (hereafter simply referred to as pine nut oil) contains about 15% pinolenic acid (all-cis-5,9,12-18:3) (Imbs et al., 1998).

Previous studies, mainly carried out on animal models such as rats, revealed that a diet enriched with pine nut oil had some beneficial effects influencing, in particular, haematic lipid parameters (Sugano et al., 1994; Asset et al., 1999). Some uncertainty, however, still exists regarding the hypolipidaemic properties of this oil. In addition, a possible influence of pine nut oil on body weight gain of treated animals was never observed (Sugano et al., 1994; Matsuo et al., 1996; Asset et al., 1999). The supplementation of the diet with pine nut oil therefore requires further investigation both for its nutritional properties and for the molecular mechanisms by which this fat influences lipid metabolism. Interestingly, it has been suggested that one possible mechanism, among others, by which conifer oils may influence lipid metabolism is a modulation of the *de novo* fatty synthesis (Asset et al., 1999), even if this aspect has remained practically unexplored. This anabolic pathway, mainly occurring in liver, is quite complex because it requires the concerted action of the mitochondrial citrate carrier (CIC) (Palmieri et al., 1972) and of the cytosolic enzymes acetyl-CoA carboxylase (ACC) and fatty acid synthetase (FAS) (Zara et al., 2001; Ferramosca et al., 2006). Furthermore, recent studies carried out in humans have demonstrated that PinnoThin™ stimulates the release of the satiety gut hormones cholecystokinin (CCK) and glucagon like peptide-1 (GLP-1), thereby leading to a reduced feed intake (Hughes et al., 2008; Pasman et al., 2008). The suggestion that PinnoThin™ could be used as an appetite suppressant in humans has therefore emerged (Hughes et al., 2008; Pasman et al., 2008).

In this study we have investigated the influence of a diet enriched with pine nut oil (PinnoThin™) on the body weight gain and on the level of some lipid classes in the liver and in the plasma of mice. Furthermore, we have also studied a possible modulation of the *de novo* fatty acid synthesis in the liver of the pine nut oil-fed animals.

MATERIAL AND METHODS

Materials

Bio-Rad protein assay kit was purchased from Bio-Rad (Richmond, CA, USA); 1,2,3-benzenetricarboxylate (1,2,3-BTA), acetyl-CoA, phosphoenol-pyruvate, ATP, NADH, NADPH, pyruvate kinase, lactate dehydrogenase, malonyl-CoA, were

from Sigma (St. Louis, MO, USA); PinnoThin™ TG (a triglyceride mixture from pine nut oil of *Pinus koraiensis)* was a generous gift from Lipid Nutrition B.V. (The Netherlands), a producer involved in research/development and marketing/sales of PinnoThin™ as a satiety ingredient; [1,5-^{14}C]citrate was from GE Healthcare (Buckinghamshire, UK), maize oil from Carapelli (Firenze, Italy). Kits for the assay of triglycerides, total cholesterol and phospholipids were purchased from Futura System (Formello, Italy). All other reagents were of analytical grade.

Animals

Male ICR mice were obtained from Harlan (Carezzana, Italy) at 5 weeks of age and housed individually in animal cages at a temperature of 22±1°C with a 12:12 h light-dark cycle and 30-40% humidity. The mice body weight, at the beginning of the dietary treatment, was 32.29±0.91 g for the control group and 32.07±0.86 g for the pine nut oil-treated group. Mice were divided into two groups and were fed *ad libitum* up to 8 weeks with a standard diet (Global Diet 2018S from Harlan Teklad, Madison, WI, USA) supplemented with 7.5% maize oil (control) or with the same diet containing 7.5% PinnoThin™ (pine nut oil-treated). The two diets (Table 1) were isocaloric and showed a similar fatty acid composition, except for the presence of 1.12% pinolenic acid in the pine nut oil-enriched diet. Table 1 reports the composition of both diets that were prepared each week and stored frozen until use. Energy content was calculated using 16.8 kJ/g for proteins and carbohydrates and 37.8 kJ/g for lipids. Body weight gain, liver weight and feed

Table 1. Composition of diets, %

Item	Maize oil (control)	Pine nut oil
Proteins	17.39	17.39
Lipids	12.77	12.77
Fatty acids		
16:0	1.55	1.02
18:0	0.39	0.28
9-18:1	3.50	2.96
9,12-18:2	6.63	6.52
9,12,15-18:3	0.35	0.27
5,9,12-18:3	-	1.12
other fatty acids	0.25	0.55
Carbohydrates	52.96	52.96
Sugars	4.54	4.54
Mineral + vitamin mix	3.08	3.08
Crude fibre	3.51	3.51
Ash	5.58	5.58
kJ/100 g	1743	1743

intake were recorded throughout the study, ranging from 2 to 8 weeks of dietary treatment. At each time period 5 mice were sacrificed by decapitation. For the determination of plasma lipids, control and treated mice were starved overnight before sacrifice. Blood was collected and centrifuged to separate plasma. All procedures were performed according to the guidelines for the care and use of experimental animals of the Italian Committee for Experimental Animals.

De novo fatty acid synthesis

Mice liver mitochondria were prepared using standard procedures. Freshly isolated mice liver mitochondria were resuspended in 100 mM KCl, 20 mM Hepes, 1 mM EGTA, 2 μg/ml rotenone, pH 7.0, at a concentration of about 5 mg protein/ml and loaded with L-malate as previously described (Zara and Gnoni, 1995). The citrate transport assay, carried out at 9°C, was initiated by the addition of 0.5 mM [^{14}C]citrate to malate-loaded mitochondria and stopped by adding 12.5 mM 1,2,3-BTA.

Mitochondria were then reisolated by centrifugation (Beckman JA-20 Centrifuge, JA 25.50 rotor) at 18000 *g* for 10 min at 2°C, washed once and extracted with 20% $HClO_4$. The mixture was centrifuged and the radioactivity in the supernatant was counted.

Mice liver cytosol was obtained by centrifuging (Beckman JA-20 Centrifuge, JA 25.50 rotor) the post-mitochondrial supernatant at 20000 *g* for 20 min at 2°C. The pellet was discarded and the supernatant was then centrifuged at 105000 *g* (Beckman Optima L-80 XP Ultracentrifuge, 90 Ti rotor) for 1 h. On the resulting cytosol the activities of acetyl-CoA carboxylase (ACC) and fatty acid synthetase (FAS) were measured spectrophotometrically (Amersham Biosciences ULTROSPEC 1100pro spectrophotometer) as previously described (Linn, 1981; Wagner et al., 2004).

Chemical analysis

Liver lipids were extracted using a 1:1 mixture of chloroform and methanol as described by Bligh and Dyer (1959). The extracts were dried under nitrogen flow and resuspended in 0.1% Triton X-100 before carrying out the individual assay of triglycerides, cholesterol and phospholipids using commercial kits. For the determination of plasma lipids, mice were starved overnight before sacrifice. Blood was collected and centrifuged to separate plasma. Plasma triglycerides, cholesterol and phospholipids were then measured using commercial kits (colorimetric analysis using an Amersham Biosciences ULTROSPEC 1100pro spectrophotometer). Protein was determined by the Bradford (1976) method.

Statistical analysis

Experimental data represent the means ± SE. The Student's t-test was performed to detect significant differences between the control and pine nut oil-treated animals. Differences were considered statistically significant at $P<0.05$.

RESULTS

Feed intake, body weight gain, liver weight and feed conversion efficiency. Whereas the feed intake was identical in the two groups of mice, the body weight gain was significantly lower in treated animals with respect to the control ones at all the times tested (Table 2). In particular, at the 8th week of feeding the body weight gain resulted 37.4% lower in treated animals. Interestingly, the liver weight was also lower in pine nut oil-treated mice (Table 2). Finally, a decrease in the feed conversion efficiency was also found in treated mice (decrease of 36.4% at the 8th week).

Table 2. Effect of dietary treatments on feed intake, body weight gain, liver weight and feed conversion efficiency; mean ± SE, n=5

Weeks	Feed intake, g/day		Body weight gain, g	
	control	pine nut oil	control	pine nut oil
0	-	-	-	-
2	8.53 ± 1.75	8.36 ± 1.37	2.22 ± 0.10	1.92 ± 0.04*
4	8.65 ± 1.90	8.69 ± 1.74	4.44 ± 0.24	3.90 ± 0.26*
6	8.43 ± 1.69	8.47 ± 1.54	6.69 ± 0.60	4.95 ± 0.30*
8	8.22 ± 2.05	8.17 ± 2.13	10.06 ± 0.94	6.30 ± 0.51*

Weeks	Liver weight, g/100 g body weight		FCE, g gained/g feed eaten	
	control	pine nut oil	control	pine nut oil
0	3.75 ± 1.30	3.75 ± 1.30	-	-
2	4.33 ± 0.38	4.43 ± 0.37	0.018 ± 0.002	0.016 ± 0.002
4	4.15 ± 0.45	3.97 ± 0.90	0.018 ± 0.002	0.016 ± 0.002
6	4.20 ± 0.36	3.85 ± 0.31	0.019 ± 0.002	0.014 ± 0.001*
8	4.29 ± 0.25	3.70 ± 0.26*	0.022 ± 0.003	0.014 ± 0.002*

*P<0.05

Liver and plasma lipid levels.The liver lipid content was investigated in order to determine whether the pine nut oil-supplemented diet was able to selectively influence the lipid composition of this organ.

The levels of hepatic triglycerides, total cholesterol and phospholipids were significantly lower in pine nut oil-fed mice with respect to control animals at the

8th week (Table 3). Further experiments carried out in liver revealed that the protein content also decreased by about 10% in treated mice (data not shown).

Table 3. Lipid analysis of livers from control and pine nut oil-fed mice, mg/g liver; mean ± SE, n=5

Weeks	Triglycerides		Total cholesterol		Phospholipids	
	control	pine nut oil	control	pine nut oil	control	pine nut oil
0	3.69 ± 0.28	-	1.93 ± 0.23	-	9.25 ± 0.40	-
2	6.70 ± 0.03	5.56 ± 0.43*	3.34 ± 0.15	3.29 ± 0.38	11.88 ± 1.66	12.06 ± 0.78
4	6.73 ± 0.60	6.06 ± 0.52	2.75 ± 0.25	2.50 ± 0.65	11.12 ± 0.68	11.46 ± 0.36
6	6.00 ± 0.57	5.88 ± 0.47	2.67 ± 0.15	2.47 ± 0.24	11.78 ± 0.90	11.32 ± 0.67
8	4.17 ± 0.16	3.76 ± 0.25*	2.47 ± 0.18	1.96 ± 0.31*	8.82 ± 0.33	7.93 ± 0.52*

*P<0.05

More prominent changes were found in the plasma lipid levels. A strong and significant decrease in pine nut oil-treated mice was indeed found in the triglyceride (Figure 1A) and cholesterol (Figure 1B) plasma levels. At 8 weeks of this dietary treatment a decrease of 31.8 and 28.5% in the plasma triglyceride and cholesterol concentration was, respectively, found in

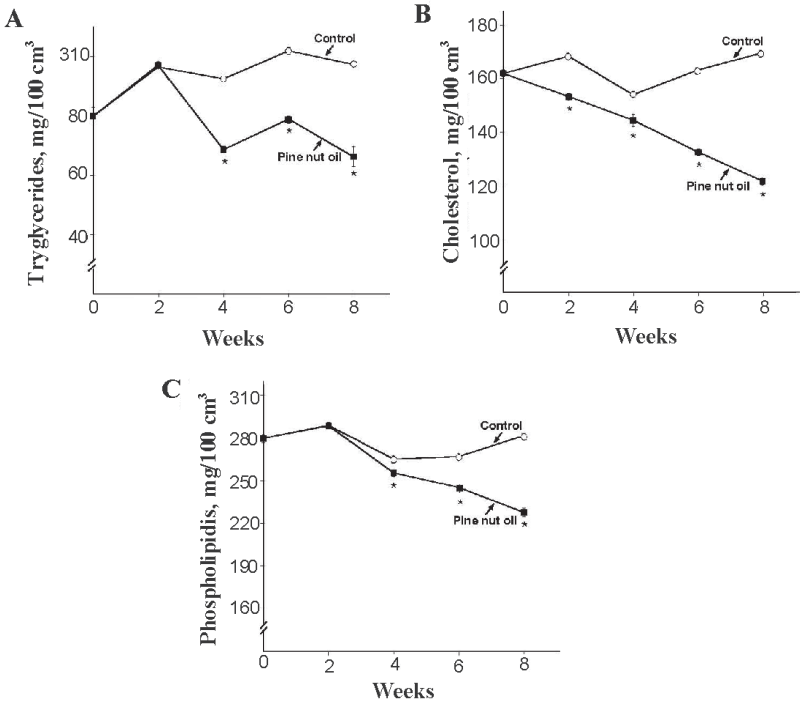

Figure 1. Contents of triglycerides (A), cholesterol (B) and phospholipids (C) in the plasma of control and pine nut oil-treated mice, mg/100 cm3

comparison to the values of control animals. Also in the case of plasma phospholipids there was a significant decrease in pine nut oil-fed animals (Figure 1C), even if the observed changes were less dramatic in comparison to triglyceride and cholesterol levels.

Pine nut oil supplementation of the diet and hepatic fatty acid synthesis. The activity of the mitochondrial citrate carrier (CIC) was investigated in liver mitochondria freshly isolated from control and treated mice. Figure 2A shows that after a lag-phase of about two weeks there was a progressive decrease in the citrate transport activity which went down to 41% of the starting activity after 8 weeks of dietary treatment. This decrease, however, was exactly the same in control animals.

Citrate transported outside mitochondria by the CIC represents the carbon fuel for the *de novo* fatty acid synthesis. We then measured the activity of both enzymes implied in this anabolic process, i.e. the ACC (Figure 2B) and the FAS (Figure 2C). A time-dependent decrease in both enzymatic activities was found in treated animals.

At the 8th week, the ACC and the FAS activities represented 41% and 36.9%, respectively, of the starting activities found at the beginning of the

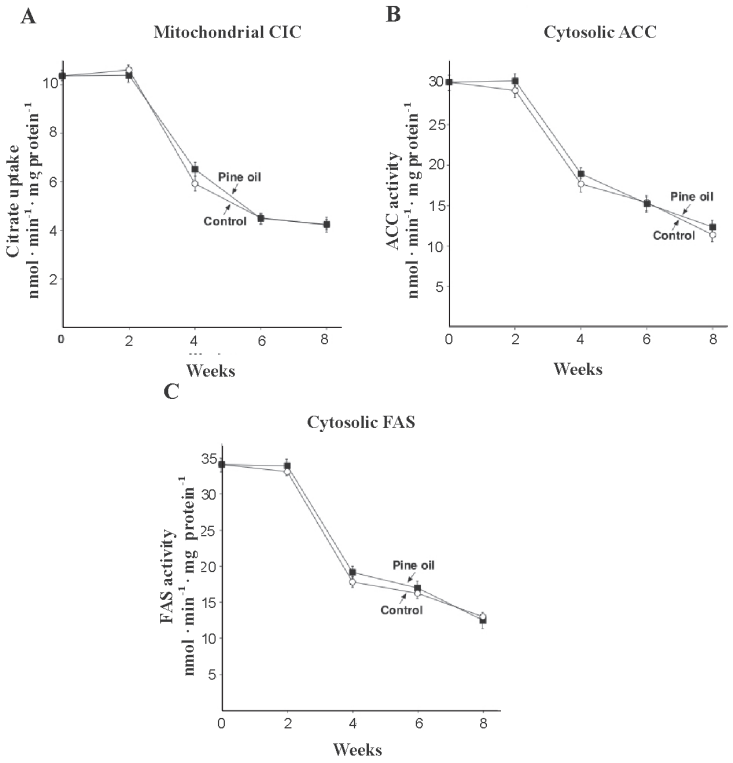

Figure 2. Hepatic fatty acid synthesis in pine nut oil-fed mice

pine nut oil treatment. However, parallel changes were detected in control animals over time.

DISCUSSION

The fatty acid composition of the diet is an important factor capable of modulating lipid metabolism (Clarke, 2004; Jump et al., 2005). Many studies have been carried out on this topic, especially in the case of the canonical n-3 and n-6 PUFA, such as linoleic and linolenic acid. Comparatively less is known in the case of some unusual PUFA, like pinolenic acid (Imbs et al., 1998). The *Pinus koraiensis* seed oil, containing a significant amount of pinolenic acid, has been studied relatively little and investigations on its effects on lipid metabolism are only preliminary (Sugano et al., 1994; Ide et al., 1995; Asset et al., 1999; Lee et al., 2004).

Our experiments clearly demonstrated that mice fed with a diet containing 7.5% pine nut oil, despite sharing the same feed intake as the control animals, reproducibly weighed less than the control mice (Table 2). Furthermore, the liver weight was also lower in pine nut oil-treated animals. Both these aspects are noteworthy because a dietary fat capable of decreasing body weight gain is desirable and a specific influence on the liver weight might also be of interest in the case of hepatic steatosis. These effects are probably due to the pinolenic acid, or to some of its metabolites, because this unusual fatty acid is the main difference in the fat composition of the two experimental diets. In addition, it must be considered that other bioactive compounds present in the pine nut oil diet, such as polyphenolic constituents, may have a role in triggering the effects observed in this study. To our knowledge, this is the first time that pine nut oil has affected body weight gain and liver weight. In previous investigations, the diet supplementation with 10% (Sugano et al., 1994; Matsuo et al., 1996) or 5% pine nut oil (Asset et al., 1999) in comparison to other dietary fats, did not influence the growth performance of treated animals at all. This discrepancy could be due to the different experimental design which, in this study, was extended up to 8 weeks and included mice instead of rats (Sugano et al., 1994; Matsuo et al., 1996; Asset et al., 1999). It is indeed known that mice and rats can respond differently to dietary fatty acids (Moya-Camarena and Belury, 1999). A future area of research will be to extend the investigation on the effect of pine nut oil to other species in order to have greater insight into the nutritional properties of this fat.

Another interesting aspect of this investigation is the strong decrease in triglycerides and cholesterol content in the plasma of pine nut oil-treated

animals (Figures 1A and 1B). A moderate reduction in the level of the same lipid classes was also observed in liver (Table 3). These effects are comparable to the modulation of the lipid profile in blood serum and liver by the n-3 PUFA contained in fish oil.

However, it appears that n-3 PUFA essentially reduce plasma triglycerides, with only inconsistent effects on total cholesterol, at least in humans (Vanschoonbeek et al., 2003). Further studies both in animal models and in humans are, in any case, necessary before recommending the use of pine nut oil as a protective fat in patients with dyslipidaemia. In particular, the effect of pine nut oil on lipoprotein metabolism (Asset et al., 1999, 2000) both in animals and humans merits further investigation. It is also evident from this study that the effects of pine nut oil on the lipid variables are not due to a decreased fatty acid synthesis. In fact, a significant inhibition of the mitochondrial CIC and of the cytosolic enzymes involved in the *de novo* fatty acid synthesis was also found in the control mice which were fed with a diet having a fat composition similar to that of treated animals, except for the absence of pinolenic acid. This finding suggests that the PUFA present in both diets, and mainly linoleic acid which represents about 51% of total dietary fatty acids, are the factors capable of decreasing the hepatic fatty acid synthesis.

CONCLUSIONS

We can summarize that a PinnoThin™ supplemented diet reduces body weight gain without any influence on the feed intake and decreases lipid concentration in liver and plasma without any influence on the enzymes of the *de novo* fatty acid synthesis in mice.

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