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SHORT COMMUNICATION

Inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis by dietary olive oil and squalene

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Epidemiological studies have suggested that frequent olive oil consumption may be a protective factor against lung cancer formation. Squalene, a characteristic compound in olive oil, is an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and has been proposed to inhibit the farnesylation of *ras* oncoproteins. The present study investigated the effect of dietary olive oil and squalene in a mouse lung tumorigenesis model. Female A/J mice were fed AIN-76A diets containing 5% corn oil (control), 19.6% olive oil, or 2% squalene starting at 3 weeks before a single dose of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (103 mg/kg, i.p.). Animals were maintained on their respective diets throughout the study. At 16 weeks after NNK administration, 100% of the mice in the control group had lung tumors with a tumor multiplicity of 16 tumors per mouse. The olive oil and squalene diets significantly ($P < 0.05$) decreased the lung tumor multiplicity by 46 and 58%, respectively. The squalene diet significantly ($P < 0.05$) decreased lung hyperplasia by 70%. In mice fed a diet containing 2% squalene for 3 weeks, the activation of NNK was increased by 1.4- and 2.0-fold in lung and liver microsomes, respectively, but its relationship to the inhibition of carcinogenesis is not clear. These results demonstrate that dietary olive oil and squalene can effectively inhibit NNK-induced lung tumorigenesis.

Various dietary compounds have been found to be protective against chemical carcinogenesis (1). Epidemiological and experimental studies have suggested that an increased dietary intake of olive oil plays a beneficial role in the prevention of certain cancers (2–8). The mortality rate for lung cancer was lower in southern Italy than in northern Italy, although the proportion of smokers and the total intake of dietary fat was the same (7). In southern Italy, the traditional diet is rich in olive oil and fish, whereas in northern Italy the diet is rich in red meats and butter (7). The increased intake of olive oil may partly account for the low lung cancer mortality rate. In a case-control study in Italy, a protective association was found between daily use of olive oil as a salad dressing and lung cancer, but not when olive oil was used as a cooking oil (8). Presently there are no experimental studies to support the effect of olive oil on lung tumorigenesis and the mechanism(s) of action involved. Olive oil contains ~73% oleic acid (an omega-9 fatty acid) and 0.2–0.7% squalene (2,9). The presence of squalene and phenolic compounds in olive oil are responsible for the low susceptibility of olive oil to oxidation (2). Squalene, a triterpene, is an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA*) reductase activity (10) and has been proposed to inhibit the farnesylation of *ras* oncoproteins (11). Squalene has been shown to have anti-tumor promoting activities in rodents (12,13), to inhibit hyperproliferation in a mammary cell line (14), and to have a radioprotective effect in mice exposed to lethal whole-body irradiation (15). Since high olive oil consumption may have a protective effect for lung cancer, and squalene may be a constituent in olive oil that could be the potential protective factor, the present study was undertaken to determine the effect of dietary olive oil and squalene on lung tumorigenesis in mice.

Cigarette smoking is known to be the leading cause of lung cancer. Tobacco and tobacco smoke contain several classes of carcinogens. The tobacco-specific nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine, are formed from the nitrosation of nicotine during tobacco processing and cigarette smoking and have been suggested to play an important role in human tobacco-related cancers (16,17). Of these tobacco-specific nitrosamines, NNK is the most potent lung carcinogen in laboratory animals (16,17). In order for NNK to exert its carcinogenicity, it must be metabolically activated. Studies by us and other investigators have demonstrated that P450 enzymes are involved in the activation of NNK (18–22). The activation of NNK leads to the formation of highly reactive species that can methylate and pyridyloxobutylate DNA. In mouse lung tumors induced by NNK, activation of the K-*ras* gene caused by GC→AT transitions in the second base of codon 12 have been detected (23,24). The activation of the K-*ras* gene is then followed by proliferation and progression to a malignant tumor (23). The induction of lung tumors by NNK in A/J mice is a well-established model for studying lung carcinogenesis and its modulating factors. Therefore, we used this animal model to investigate the inhibition of NNK-induced lung tumorigenesis by dietary olive oil and squalene, as well as the effect of these dietary components on NNK metabolism.

NNK was purchased from Chemsyn Science Laboratories (Lenexa, KS). Squalene with a purity >98% was obtained from Sigma Chemical Company (St Louis, MO). The diets were formulated based on the AIN-76A diet. Diet formulation is shown in Table I. Based on a preliminary study, the percentage composition of the olive oil diet was adjusted so that the animals in all of the diet groups would consume approximately the same amount of protein, minerals, vitamins and fiber per kcal. The high olive oil diet supplied 46% of the total calories as fat. Corn oil (5%) was added to the high olive

***Abbreviations:** HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; P450, cytochrome P450.

Table I. Percentage of components in experimental diets[a]

Diet ingredient	Corn oil	Olive oil	Squalene
Casein	20.00	24.62	20.00
Corn oil	5.00	5.00	5.00
Olive oil	0.00	19.61	0.00
Cornstarch	48.75	28.86	47.75
Sucrose	16.25	9.60	15.25
Cellulose	5.00	6.15	5.00
D,L-Methionine	0.30	0.37	0.30
Mineral mix	3.50	4.31	3.50
Vitamin mix	1.00	1.23	1.00
Choline bitartrate	0.20	0.25	0.20
Squalene	0.00	0.00	2.00
Calculated energy value (kcal/g)	3.90	4.80	3.83

[a]The diet was formulated on the basis of the American Institute of Nutrition standard reference diet. Olive oil was added at the expense of the cornstarch and sucrose. The composition of the olive oil diet was adjusted so that all animals in the different diet groups would consume approximately the same amount of protein, minerals, vitamins and fiber per kcal.

oil diet in order to prevent essential fatty acid deficiency. In order to incorporate squalene into the diet, squalene was added to the corn oil, and the squalene-corn oil mixture was then thoroughly mixed into the diet using a mechanical mixer. The powdered diets were prepared by Research Diets, Inc. (New Brunswick, NJ) and stored in vacuumed-sealed bags at 4°C.

Female A/J mice 6 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in an air-conditioned room with a 12-h light/dark cycle. Mice were given the AIN-76A diet (containing 5% corn oil) and water *ad libitum* for 1 week. After the acclimation period, mice were randomly placed into four groups. The mice in groups 1 and 2 were maintained on the AIN-76A diet (control), mice in group 3 were given the high olive oil diet, and mice in group 4 were given the 2% squalene diet. Mice in groups 1–4 were fed their respective diets for 3 weeks before a single dose of NNK (103 mg/kg, i.p.) or saline. The dietary treatments were continued until the end of the experimental period. Mice were weighed weekly and food consumption was measured every other day. At 16 weeks after NNK dosing, the mice were killed and the lungs were removed and fixed in 10% buffered formalin. The tumors on the surface of the lungs were counted and the tumor volume was measured. The lungs were analyzed further by histopathology. The formalin-fixed lungs were transferred to 80% ethanol, embedded in paraffin, and serial sections (5 μm) were cut and mounted on glass slides. The sections were stained with hematoxylin and eosin for histopathological analysis.

In the present study, the mice fed the high olive oil diet consumed ~20% less food (wt) than the NNK control group (2.38 versus 3.04 g/mouse per day); however, there was no significant ($P > 0.05$) difference in the average caloric intake (11.40 versus 11.84 kcal/mouse per day). The decreased food intake by the mice in the high olive oil group was because the diet had a high nutrient density. Squalene itself (group 4) had no significant effect on the food and caloric intake. This pattern of food and caloric intake resulted in similar body wt gains in all dietary groups.

Table III. Histopathological analysis of pulmonary lesions in NNK-treated mice fed diets containing olive oil or 2% squalene[a]

Treatment	Number of mice	Adenomas/mouse	Hyperplasias/lung
Control + NNK	29	9.0 ± 3.5	0.97 ± 1.21
Olive oil + NNK	28	5.9 ± 3.9* (34%)	0.71 ± 1.15 (27%)
Squalene + NNK	28	4.3 ± 2.7* (52%)	0.29 ± 0.60* (70%)

[a]One slide from every 10 sections was used for the analysis. Four slides were read for each mouse. Values are the mean ± SD per mouse. Numbers in parentheses are the percent inhibition as compared with the NNK control group. *Significantly ($P < 0.05$) different from the NNK control group as determined by the Student's *t*-test.

Administration of a single dose of NNK (103 mg/kg, i.p.) to mice in the control group (group 2) resulted in a 100% lung tumor incidence, a tumor multiplicity of 16 tumors/mouse, and a lung tumor volume of 1.84 mm^3 (Table II). Dietary olive oil and squalene had no significant effect on lung tumor incidence. However, tumor multiplicity and volume was significantly ($P < 0.05$) decreased by 46–58% and 33–47%, respectively (Table II). These results suggest that dietary olive oil and squalene decreased the development and growth rate of the lung tumors. Olive oil and squalene may be exerting their anticarcinogenic effect during the post-initiation stage of carcinogenesis. Further studies are needed to determine the stage of carcinogenesis at which these two dietary components are effective. Histopathological analysis of the lungs identified the pulmonary lesions as hyperplasia (Figure 1) and adenoma. Squalene significantly ($P < 0.05$) decreased hyperplasia by 70% (Table III), which indicates that squalene can inhibit NNK-induced lung cell proliferation. Dietary olive oil and squalene significantly ($P < 0.05$) decreased adenoma formation by 34 and 52%, respectively (Table III). The histopathological analysis results confirmed the gross lung tumor analysis results, in that diets that contained olive oil or squalene effectively decreased NNK-induced lung tumorigenesis. Studies have suggested that the protective or tumor-promoting effects of dietary fats may be caused by fatty acid composition (3–5). Although olive oil is high in oleic acid, this fatty acid is also present in other oils, such as corn oil (30%), and in the fat of

Table II. Effect of dietary squalene and olive oil on gross tumor incidence, multiplicity and volume[a]

Treatment	Number of mice	Tumor incidence (%)	Tumor multiplicity (tumors/mouse)	Tumor volume (mm^3) per mouse
Control	25	20	0.2 ± 0.4	0.14 ± 0.30
Control + NNK	30	100	16.0 ± 6.8	1.84 ± 1.11
Olive oil + NNK	29	100	8.7 ± 5.0* (46%)	1.24 ± 0.86* (33%)
Squalene + NNK	30	97	6.8 ± 4.1* (58%)	0.97 ± 0.63* (47%)

[a]Tumor volume (mm^3) was measured using the formula $V = 4/3\pi r^3$, where r is the radius of the tumor determined by the mean values of the longest and shortest diameters. Values are the mean ± SD. Numbers in parentheses are the percent inhibition as compared with the NNK control group. *Significantly ($P < 0.05$) different from the NNK control group as determined by the Student's *t*-test.

Table IV. NNK metabolism in lung and liver microsomes from mice fed diets containing 0% and 2% squalene[a]

Group	Keto aldehyde	NNK-*N*-oxide *pmol/min per mg protein*	Keto alcohol	NNAL
Lung microsomes				
Control	13.98 ± 0.78	15.31 ± 0.92	10.08 ± 0.53	2.40 ± 0.15
2% Squalene	18.01 ± 2.86	18.75 ± 3.12	13.80 ± 2.02*	2.81 ± 0.20*
Liver microsomes				
Control	52.34 ± 5.85	ND[b]	25.54 ± 3.25	20.15 ± 1.72
2% Squalene	94.20 ± 8.18*	ND	51.48 ± 5.28*	25.07 ± 3.53

[a]Incubations contained 10 μM NNK (containing 1 μCi [5-^{3}H]NNK), 0.1 mg microsomal protein, an NADPH-generating system and 5 mM sodium bisulfite. Reactions were carried out for 30 min at 37°C. Values are the mean ± SD of three pooled samples in duplicate. *Significantly ($P < 0.05$) different from the control group as determined by the Student's *t*-test.
[b]ND, metabolite was not detectable.

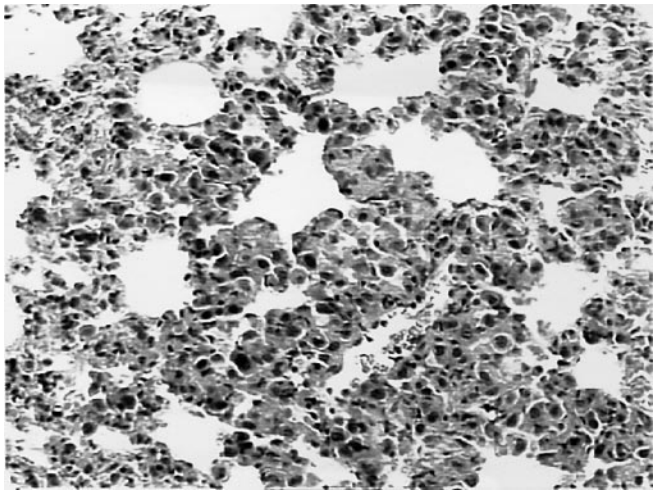

Fig. 1. NNK-induced alveolar hyperplasia from an NNK control mouse (hematoxylin and eosin stain) (magnification ×500). Hyperplasia occurred in the alveolar region, showing single or multiple layers of proliferative epithelial cells along intact alveolar septae with irregular and non-discrete margins of lesion, but continuous alveolar spaces were not obliterated by proliferative epithelial cells.

mammals and poultry (30–45%) (2). In the present study, the diet containing 19.6% olive oil contributed ~0.15% squalene to the total diet. Therefore, other factors in addition to squalene are playing a role for the inhibitory effect of olive oil on NNK-induced lung tumorigenesis. Nevertheless, our results suggest that squalene could be an inhibitory component in olive oil.

To determine whether the inhibition of NNK-induced lung tumor formation by squalene was caused by the inhibition of NNK activation, the effect of dietary squalene on the metabolism of NNK in the lung and liver microsomes was examined. Groups of 10 mice were fed AIN-76A diets containing 0 or 2% squalene for 3 weeks. At the end of the third week, mice were killed and NNK metabolism was determined in the lung and liver microsomes as previously described (19). Feeding diets containing 2% squalene to mice for 3 weeks significantly ($P < 0.05$) increased the rate of formation of keto alcohol (an α-hydroxylation product) by 1.4-fold in the lung microsomes (Table IV). Dietary squalene increased the rate of formation of NNK-*N*-oxide (a detoxification product) by 1.25-fold, but it was not statistically significant ($P > 0.05$). The rate of total α-hydroxylation of NNK (keto aldehyde and keto alcohol) was 1.7-fold higher than the rate of detoxification of NNK in the lung microsomes of mice fed the 2% squalene diet. In the liver microsomes, the rate of formation of keto aldehyde and keto alcohol (α-hydroxylation products) were significantly increased 2.0-fold by dietary squalene (Table IV). The increased activation of NNK suggests that squalene (or a metabolite of squalene) is inducing the specific P450s involved in the activation of NNK. It is possible that an increase in the metabolism of NNK by the liver of the mice fed the squalene diet may result in a decreased amount of NNK that reaches the lung, thereby partly contributing to the inhibition of NNK-induced lung carcinogenesis. The contribution of these changes on tumorigenesis is not clear and remains to be investigated.

A high percentage of NNK-induced lung tumors have activated K-*ras* (23,24). A possible mechanism by which squalene could be inhibiting NNK-induced lung tumorigenesis is by decreasing the farnesylation of *ras* because of its inhibitory effect on HMG CoA reductase. Dietary squalene has been demonstrated to be an inhibitor of hepatic HMG CoA reductase activity in rats (10). Inhibition of HMG CoA reductase activity results in decreased mevalonate synthesis, which would lead to decreased farnesyl pyrophosphate synthesis (25,26). The farnesyl moiety of farnesyl pyrophosphate is required for the farnesylation of oncoproteins, such as ras, in order to become biologically active (27). A second mechanism by which squalene may be inhibiting NNK-induced lung tumorigenesis is by scavenging for free radicals or reactive oxygen species. Squalene is an efficient quencher of singlet oxygen (28). The radioprotective effect of squalene in irradiated mice and the anti-tumor promoting activity of squalene in skin carcinogenesis has been suggested to be due to its antioxidant properties (12,15).

In the United States, the average intake of squalene is ~30 mg/day, whereas in Mediterranean countries where the cancer mortality rates are low, the intake of squalene can be 10–20 times higher because of the high consumption of olive oil (2,9). The present study demonstrates that olive oil can effectively inhibit NNK-induced lung tumorigenesis and that squalene could be an inhibitory component. Further studies are needed to determine the mechanism(s) of action for olive oil and squalene in tobacco-related lung carcinogenesis.

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