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Chemopreventive Effects of Pomegranate Seed Oil on Skin Tumor Development in CD_1 Mice

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ABSTRACT Pomegranate seed oil was investigated for possible skin cancer chemopreventive efficacy in mice. In the main experiment, two groups consisting each of 30, 4–5-week-old, female CD_1 mice were used. Both groups had skin cancer initiated with an initial topical exposure of 7,12-dimethylbenzanthracene and with biweekly promotion using 12-*O*-tetradecanoylphorbol 13-acetate (TPA). The experimental group was pretreated with 5% pomegranate seed oil prior to each TPA application. Tumor incidence, the number of mice containing at least one tumor, was 100% and 93%, and multiplicity, the average number of tumors per mouse, was 20.8 and 16.3 per mouse after 20 weeks of promotion in the control and pomegranate seed oil-treated groups, respectively ($P < .05$). In a second experiment, two groups each consisting of three CD_1 mice were used to assess the effect of pomegranate seed oil on TPA-stimulated ornithine decarboxylase (ODC) activity, an important event in skin cancer promotion. Each group received a single topical application of TPA, with the experimental group receiving a topical treatment 1 h prior with 5% pomegranate seed oil. The mice were killed 5 h later, and ODC activity was assessed by radiometric method. The experimental group showed a 17% reduction in ODC activity. Pomegrante seed oil (5%) significantly decreased ($P < .05$) tumor incidence, multiplicity, and TPA-induced ODC activity. Overall, the results highlight the potential of pomegranate seed oil as a safe and effective chemopreventive agent against skin cancer.

KEY WORDS: • ***ornithine decarboxylase*** • ***Punica granatum*** • ***initiation*** • ***promotion*** • ***prostaglandins*** • ***punicic acid***

INTRODUCTION

SKIN CANCER is the most common type of cancer in the United States,[1] with more than a million reported cases[2] and 9,000 deaths per year.[3] Increasing incidence of these cancers due to constant exposure of skin to environmental carcinogens, including both chemical agents and ultraviolet radiation, provides a strong basis for chemoprevention with both synthetic and natural, and internal and topical, remedies.[4] Further, skin cancer chemoprevention is a useful model for cancer chemoprevention in general.[5]

Chemical and UVB radiation-induced skin carcinogenesis in murine skin and possibly human skin is a stepwise process of at least three distinct stages: initiation, promotion, and progression. Experimental initiation *in vivo* is accomplished by the topical application of a single dose of a skin carcinogen such as 7,12-dimethylbenzanthracene (DMBA), and is essentially irreversible. However, an initiation dose of carcinogen may not produce visible tumors, resulting only following prolonged and repeated application of a tumor promoter such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) to initiated skin.[6,7] Promoters like TPA induce ornithine decarboxylase (ODC), the rate-limiting enzyme in the synthesis of polyamines[8] and an important molecular target for skin cancer chemoprevention.[9] Other targets may also involve promotion, or initiation or progression events in the multistage process of neoplastic development.

Our previous work has highlighted the efficacy of topically applied natural products derived from onion and garlic oils,[10] and more recently sandalwood oil[11,12] and its constituent,[13] in preventing skin tumors in CD_1 and SENCAR mice. In the present work, we bring this experience to bear on the study of pomegranate seed oil as a potential skin cancer chemopreventive product.

Pomegranate fruit (*Punica granatum*) has been used worldwide as an item of diet and medicine for millenia, and has also been regarded as an important symbol in world religions and mythologies and of medicine itself.[14] We previously demonstrated potent antioxidant and prostaglandin-inhibitory activities for polyphenols extracted from pomegranate seed oil and pomegranate fermented juice,[15] as well as a wide range of human breast cancer suppressive properties *in vitro*, including promotion of apoptosis and inhibi-

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tion of proliferation and invasion by the seed oil, and inhibition of DMBA-initiated carcinogenesis in a mouse mammary organ culture (MMOC) by the fermented juice polyphenols.[16] We recently showed chemopreventive activity of the whole seed oil in the MMOC to be even stronger, weight per weight, than that of the purified fermented juice polyphenols.[17]

Pomegranate seed oil consists of >80% conjugated fatty acids, the most important of which is the octadecatrienoic acid, punicic acid. Punicic acid, like the <1% polyphenols in pomegranate seed oil, is an inhibitor of prostaglandin biosynthesis.[18] Punicic acid is also cytotoxic to mouse leukemia cells, possibility related to inhibition of lipid peroxidation.[19] Pomegranate is one of only about a half dozen plants known to contain conjugated fatty acids. A possible relationship between the relative botanical isolation of pomegranate and its singular chemistry and anticancer properties has been noted.[20]

The purpose of the present investigation was to study the chemopreventive effects of pomegranate seed oil on DMBA-initiated and TPA-promoted skin tumor development during the initiation and promotion phases in CD_1 mice. Further, the effects of pomegranate seed oil on weight gain and ODC activity in the experimental animals were also evaluated.

MATERIALS AND METHODS

Pomegranate seed oil

Pomegranate seed oil was provided by Rimonest Ltd. (Rimonest Ltd., Haifa, Israel; www.rimonest.com) from pomegranates of the "Wonderful" cultivar, organically grown at Kibbutz Sde Eliahu, Israel, in the year 2000. Seeds were separated from their juice sacs, washed in water, and dried in a convection current solar dryer. Oil extrusion was by "cold press" at 80°C, using a Type 40A electric screw press (Skeppsta Maskin, Orebro, Sweden). The oil was assayed by an independent laboratory (Mylnfield Research Services, Invergowrie, Dundee, Scotland) and shown to contain not less than 80% conjugated fatty acids as triglycerols, diglycerols, and monoglycerols.

Tumorogenesis protocol

The skin cancer protocol of Dwivedi *et al.*[13] was used. In brief, 4–6-week-old CD_1 mice were divided into two groups, each group containing 30 mice, as indicated in Table 1. The mice were kept in an environmentally controlled room with temperature, humidity, and light regulated. The backs of the mice were shaved carefully with an electric clipper to avoid cuts. The mice were allowed to rest for 2 days before carcinogenesis was initiated.

Table 1. Topical Treatments Received by the Mice in the Two Experiments

Group 1	100 μL of acetone
Group 2	100 μL of 5% pomegranate seed oil in acetone

Carcinogenesis was initiated with DMBA (200 nmol in 100 μL of acetone) applied topically. One week later, carcinogenesis was promoted with TPA (5 nmol in 100 μL of acetone), applied topically twice weekly. TPA treatment continued throughout the duration of the experiment (20 weeks). Mice in group 1 served as the control and were pretreated topically with 100 μL of acetone 1 h prior to each TPA application. Mice in group 2 were pretreated topically with 100 μL of 5% pomegranate seed oil in acetone 1 h prior to each TPA application. Tumor counts and group weights were taken on a weekly basis. Tumor incidence and multiplicity were calculated and analyzed statistically.

ODC assay

Mice were divided into two groups, each containing three mice. The backs of the mice were shaved carefully with an electric clipper to avoid cuts. Mice in group 1 received 100 μL of acetone before TPA (5 nmol in 100 μL of acetone) treatment topically. Mice in group 2 received 100 μL of 5% pomegranate seed oil in acetone, before topical TPA (5 nmol in 100 μL of acetone) treatment.

Mice were killed 5 h after the topical applications of TPA. The dorsal epidermis was removed and homogenized in phosphate buffer (pH 7.2) containing 0.1 m*M* pyridoxal phosphate and 0.1 m*M* EDTA. The homogenate was centrifuged at 105,000 *g* for 90 min and the supernatant collected and used for the ODC assay. The assay mixture in the main part of a Warburg flask was composed of 40 μL of phosphate buffer (pH 7.2), 25 μL of pyridoxal phosphate, 25 μL of dithiothreitol, 25 μL of EDTA, 10 μL of L-ornithine containing 0.5 μCi of DL[1-^{14}C]ornithine, and 200 μL of epidermal supernatant.

The center well of the Warburg flask contained 400 μL of ethanolamine and methoxyethanol used to absorb the $^{14}CO_2$ produced in the main compartment. After incubation at 37°C for 1 h, the reaction was stopped by the addition of 500 μL of citric acid. The mixture was stored in a dark place overnight to ensure complete absorption of $^{14}CO_2$ in the center well. The contents of the center well were transferred to a scintillation vial. The center well was washed with 0.5 mL of ethanol four times, and the wash also added to the scintillation vial, along with 10 mL of scintillation fluor. Radioactivity was counted with a Beckman LS6000SE liquid scintillation counter. The disintegrations per minute were quantified. Assessment of ODC activity was accomplished by measuring the production of $^{14}CO_2$ from DL-[1-^{14}C]ornithine.

Protein assay

Protein was assayed in the supernatant with a Bio-Rad Protein Assay Kit. A standard curve was obtained using bovine serum albumin. Absorbance values at 595 nm were determined using the spectrophotometer. Protein concentra-

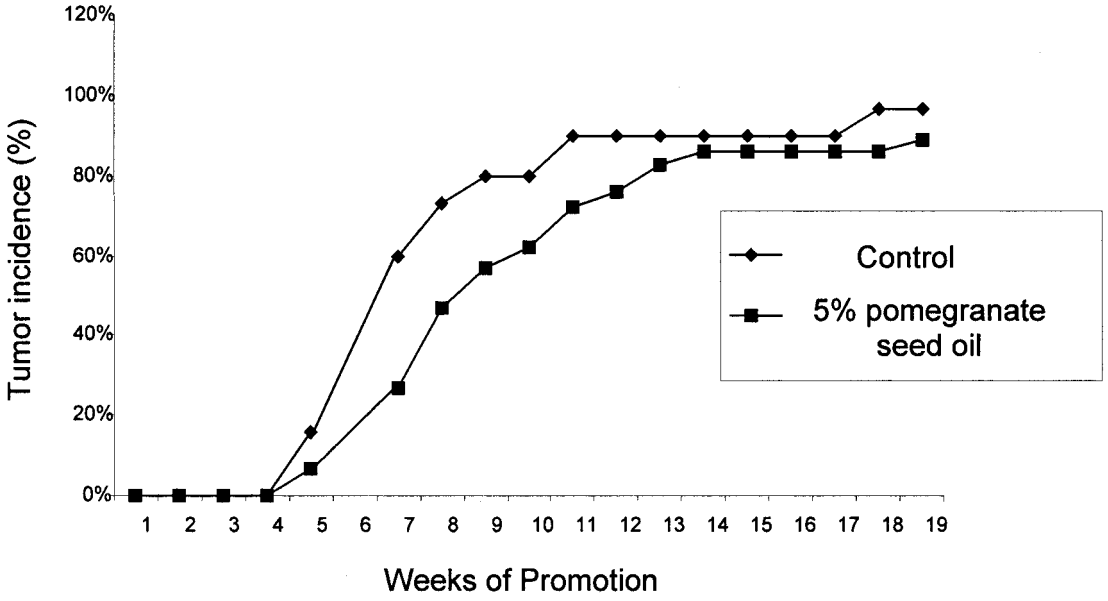

FIG. 1. The effects of pomegranate seed oil treatment on tumor incidence in CD_1 mice.

tions of the supernatant were extrapolated from the standard curve data.

Statistical analysis

The INSTAT software (GraphPad, San Diego, CA, U.S.A.) was used for the data analysis. χ^2 was used for the comparison of papilloma incidence and Student's t test for tumor multiplicity and ODC activity. Significance was considered at $P < .05$.

RESULTS

The effects of pomegranate seed oil treatment on the incidence of skin tumors in CD_1 mice are shown in Fig. 1. Skin tumors appeared in the sixth week of promotion after the initial DMBA application in the control and treated groups. Pomegranate seed oil treatment did not delay the appearance of tumors, but significantly decreased ($P < .05$) the rate at which the tumors developed. Skin tumor incidence after 20 weeks of promotion was 100% and 93% for

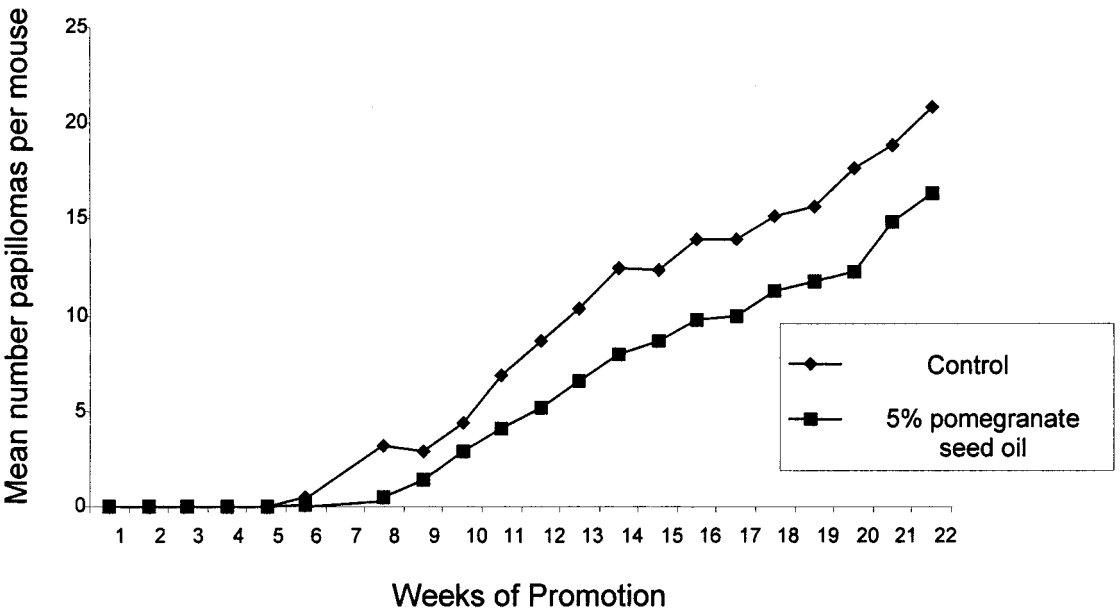

FIG. 2. The effects of pomegranate seed oil treatment on tumor multiplicity in CD_1 mice.

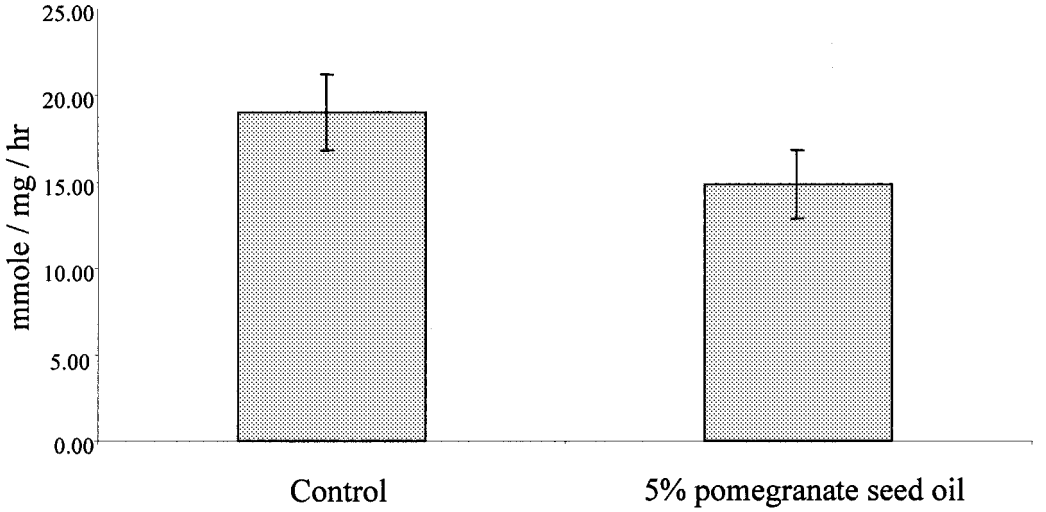

FIG. 3. The effects of pomegranate oil treatment on TPA-induced epidermal ODC activity in CD_1 mice. ODC activity is expressed as nmol of $^{14}CO_2$ produced/μg of protein/h. The 5% pomegranate seed oil (only) group did not have detectable ODC activity (data not shown).

the control and 5% pomegranate seed oil-treated groups, respectively.

The effects of pomegranate seed oil treatment on tumor multiplicity in CD_1 mice are shown in Fig. 2. Pomegranate seed oil treatment significantly decreased ($P < .05$) the tumor multiplicity throughout the 20 weeks of promotion. The mean number of tumors per mouse was 20.8 and 16.3 for the control and 5% pomegranate seed oil-treated groups, respectively.

Topical application of 5% pomegranate seed oil also significantly inhibited ($P < .05$) TPA-induced epidermal ODC activity. Fig. 3 illustrates the effects of pomegranate seed oil treatment on TPA-induced epidermal ODC activity. The ODC activity was 18.49 and 14.84 nmol of $^{14}CO_2$/mg/h in the control and 5% pomegranate seed oil-treated groups, respectively. The pomegranate seed oil group has significantly ($P < .05$) decreased ODC activity. Topical application of 5% pomegranate seed oil alone did not induce any epidermal ODC activity. Topical application of 5% pomegranate seed oil also did not have any effect on weight gain, as indicated in Fig. 4.

CONCLUSIONS

Pomegranate seed oil (5%) topical applications significantly decreased the incidence of skin tumor development,

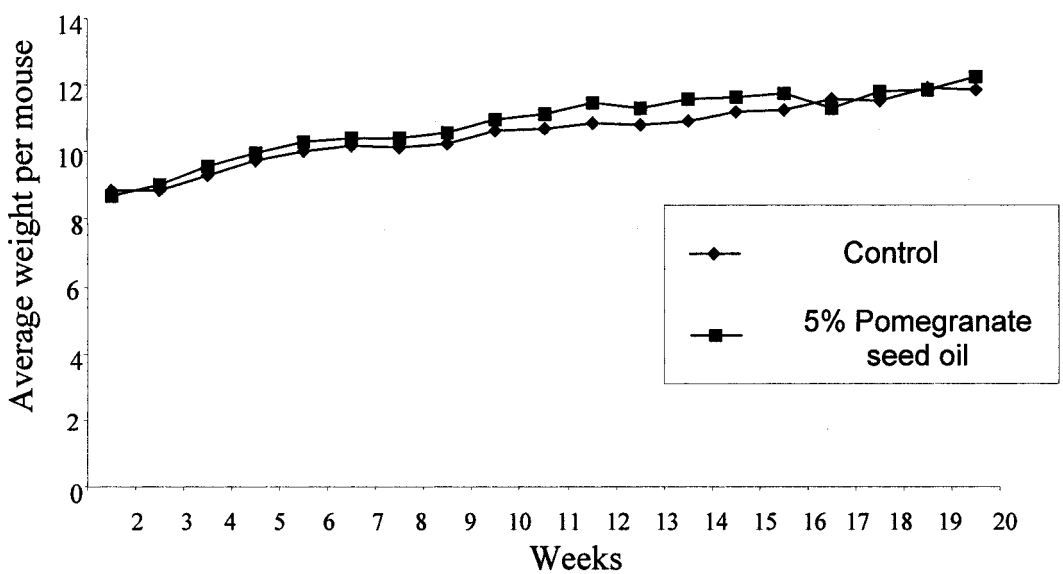

FIG. 4. The effects of pomegranate seed oil treatment on weight gain.

skin tumor multiplicity, and ornithine decarboxylase activity during 20 weeks of promotion. It is thus likely that the inhibition of ornithine decarboxylase by the pomegranate seed oil was at least partially responsible for the chemopreventive effect.

As noted, pomegranate seed oil is very rich in punicic acid, a known inhibitor of prostaglandin biosynthesis, specifically by inhibiting cyclooxygenase (Cox 1 and Cox 2) and lipoxygenase.[21] Pomegranate seed oil also inhibits the upstream eicosanoid enzyme, phospholipase A2, expressed by human prostate cancer cells.[22] That prostaglandins at very low concentrations promote ornithine decarboxylase[23] suggests that the inhibition of prostaglandin biosynthesis by pomegranate seed oil might also contribute to its inhibition of ornithine decarboxylase and, ultimately, to inhibition of skin cancer promotion.

Overall, pomegranate seed oil appears to be a benign natural product with potential as a topical chemopreventive agent against skin cancer. More in-depth investigations, including clinical studies, are warranted to evaluate this hypothesis further.

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