Chemopreventive effect of orange oil on the development of hepatic preneoplastic lesions induced by N-nitrosodiethylamine in rats: An ultrastructural study

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Orange peel oil is used extensively as an approved flavour enhancer in fruit drinks, carbonated beverages and as a scenting agent in soaps and cosmetics. Limonene, which is a monocyclic monoterpenoid is present in orange peel oil from 90 to 95% (w/w). Monoterpenes have been shown to be very effective chemopreventive agents against several rodent tumors and are currently in clinical trials. However, not much information is available regarding the ultrastructural changes associated with the chemopreventive effects of the monoterpenes. The effect of orange oil on the suppression of preneoplastic hepatic lesions during N-nitrosodiethylamine (DEN) induced hepatocarcinogenesis was studied electron microscopically. Rats were administered 200 ppm DEN through drinking water for a period of 1 month. After an interval of 2 weeks, the animals were administered orange oil by gavage for a period of 5 ½ months. The chemopreventive effect of orange oil was monitored on the basis of liver weight profile, histological pattern by light microscopy and ultrastructural alterations by electron-microscopy. Orange oil administration following DEN treatment showed decreased liver weights, increased intercellular gap junctional complexes, cell density and polarity when compared with only the DEN treated rats. In the present study chemopreventive effect of orange oil on DEN-induced hepatic preneoplasia in rats which is associated with the restoration of the normal phenotype and upregulation of junctional complexes has been demonstrated.

A wide variety of chemical agents have been shown to possess chemopreventive properties against a broad spectrum of tumors. These agents function through different mechanisms which include carcinogen detoxification, suppression of genetic mutation, inhibition of cellular signal transduction pathways and induction of apoptosis1. Several epidemiological studies have shown a close relationship between food intake and cancer. Also studies carried out on experimental animal models have demonstrated that daily ingestion of some vegetables and fruits could undoubtably contribute to cancer prevention2. A number of non-nutrient chemicals from edible plants and fruits have also been reported to possess anticancer activity3. Suppressing agents such as β-carotene, vitamin A, vitamin E and monoterpenes have been of special interest because these agents act at all stages of chemical carcinogenesis4.

Orange peel oil is used extensively as an approved flavor enhancer in fruit drinks, carbonated beverages and as a scenting agent in soaps and cosmetics. Limonene is a monocyclic monoterpenoid (Fig. 1) which is found in essential oils of citrus fruits, spices and herbs. The limonene content of orange peel oil ranges from 90 to 95% (w/w). Because of its citrus fragrance, limonene is a component of many soft drinks, juices, cosmetics and perfumes. Limonene has been reported to modulate initiation and promotion/progression stages of carcinogenesis5. Neither orange peel oil nor d-limonene showed tumour promoter activity when given via diet in which tumours were initiated with 7,12-dimethylbenz(a)anthracene in a two-stage skin carcinogenesis6. Both monoterpenoids, d-limonene and its source, orange oil were found to prevent rat mammary carcinoma, induced by direct-acting carcinogen, nitrosomethylurea7. This chemopreventive effect was restricted to the promotion/progression stages in this model8. The anti-carcinogenic activity of d-limonene during initiation stage of DMBA-induced

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Fig. 1 — Chemical structure of d-limonene
Carcinogenesis was mediated through induction of phase II hepatic conjugating enzymes glutathione transferase and UDP-glucuronyl transferase. However, the mechanisms of action of both orange oil and limonene are still not very clear. Limonene was shown to inhibit isoprenylation of 21-26 kDa proteins including p21RAS and other subunits of GTP-binding proteins in dose dependent manner. Ren and Gould recently reported inhibition of small G protein isoprenylation by anti-cancer monoterpenes through inhibition of coenzyme Q and geranylgeranyl-protein transferases (GGPTases).

Several anti-tumour promoting agents such as retinoids and dexamethasone which maintain epithelial cell differentiation, have antipromotional properties and modify the growth and differentiation of transformed cells in culture involving mechanisms of upregulation of intercellular gap junctions. In this study we have made an attempt to investigate the possible upregulation of intercellular gap junctions during chemoprevention of orange oil which is rich in d-limonene using N-nitrosodiethylamine (DEN) induced rat hepatocarcinogenesis as a model system. In the present paper we wish to report that chemopreventive properties of orange oil are associated with an increase in the number and frequency of intercellular gap junctions, analysed electron microscopically suggesting a role for upregulation of intercellular gap junctions.

**Materials and Methods**

**Chemicals**—N-Nitrosodiethylamine (DEN) was purchased from Sigma Chemical Company (St. Louis, MO, USA), Orange oil was a gift from S.H. Kelkar and Co. (Mumbai, India).

**Experimental animals**—A total of 32 male WR strain rats from the Rat Colony of Cancer Research Institute, aged 2-3 months weighing 170-200 gm were used for the experiment. These animals were randomised and housed four per cage with rice husks for bedding. Food and water were provided *ad libitum*. The experimental protocol followed is shown in Fig. 2. The animals were divided into 4 groups (eight per group). Groups 1 and 2 served as vehicle control (sunflower oil) and orange oil control respectively. In Groups 3 and 4 animals were administered 200 ppm of DEN in deionized water as described earlier. After a period of 1 month, DEN administration was discontinued and animals were given plain deionized water without DEN for 2 weeks as a recovery interval. Groups 3 and 4 were further administered either vehicle (sunflower oil) or orange oil for 7 months. At the end of the experiment, animals were sacrificed and livers were removed, weighed, and photographed. Tissue slices taken from mid-lobe of all the livers were fixed in 10% neutral buffer formalin and processed for histopathology. Sections were stained with Haematoxylin and Eosin for light microscopy.

**Electron microscopy**—Control and treated liver tissues taken from the mid-lobes were minced into tiny pieces of 1 mm-2 mm in size and first fixed in...
Fig. 3—Liver weight profile during in vivo suppression of DEN-induced hepatic preneoplasia by orange oil (data are mean ± SE).

3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 hr at 4°C, then washed in the same buffer and post fixed in 1% osmium tetroxide (OsO₄) for 1 hr at 4°C. Dehydration was carried out through graded alcohols, then embedded in araldite mixture and incubated for 48-72 hrs at 60°C for polymerization. Ultrathin sections of thickness 600-800Å were cut using glass knives on a LKB 2088 Ultratome ® V and mounted on double coated (formvar and carbon) 200 mesh copper grids. Sections stained with uranyl acetate and lead citrate were examined and photographed under Zeiss EM 109 transmission electron microscope operating in the 80 kv mode.

Administration of orange oil—Orange oil was administered after dilution with sunflower oil (1:1) prepared freshly every day. The diluted solution was administered to rats by gavage at a dose of 3 ml/kg/day for 5 days in a week. This dose of orange oil was selected after preliminary experiments (unpublished observations). Rats were observed for signs of toxicity, behavioural changes and death. Body weights were recorded every week till the end of the experiment.

Fig. 4—Histological appearance of the livers of rats during in vivo suppression of DEN-induced hepatic preneoplasia by orange oil. (X 160). Sections stained with hematoxylin and eosin. (A) Vehicle control. (B) DEN. (C) Orange oil control. (D) DEN orange oil
Fig. 5—(A) Hepatocytes of vehicle treated rats showing well differentiated organelles and junctional complexes between neighbouring cells (X 18,480); (B) Hepatocytes of only orange oil treated rats showing normal features of differentiated hepatocytes with junctional complexes (X 18,840); (C) Poorly differentiated HCC in DEN administered rats showing loss of cell polarity and density and architecture of organelles (X 18,480); (D) Cells from HCC in DEN administered rats showing changes such as disintegration of cell membranes with clustering of organelles (X 27,300) and (E) Cells from HCC induced in DEN administered rats showing wide intercellular oedema with inflammatory exudate and ill-defined organelles (X 12,600).
Results

Liver weights—Our earlier findings indicated that preneoplasia could be induced in rats by administering N-nitrosodimethylamine (DEN) for a period of 1 month. Accordingly, one month of DEN exposure was used as the period for inducing hepatic preneoplasia to test the chemopreventive properties of orange oil. At the end of the experiment i.e. after 7 months, visibly all animals were in good health and no mortality was observed. Also, no significant difference in body weights of DEN administered animals was noted compared to control groups. However, the mean liver weight was 14 g in DEN administered group whereas it ranged between 8.5 to 9.5 g in other groups (Fig. 3).

Histopathological observations—Histologically, the livers of DEN treated rats showed large basophilic lesions surrounded by hepatic parenchymal cells (Fig. 4B) associated with the loss of polarity of cells. Also, hyperplasia was observed in these livers. Administration of orange oil following DEN treatment resulted in the reversal of DEN induced alterations. The livers showed normal histological changes (Fig. 4D) similar to that of the only vehicle or only orange oil administered groups (Figs 4A, C).

Ultrastructural observations—Electron microscopically both vehicle control and plain orange oil administered controls showed more compact and intense intercellular junctional complexes (Figs 5A, B). In DEN administered rats, hepatic cells showed poorly

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Fig. 6—(A) Hepatic cells during in vivo suppression of DEN induced hepatic preneoplasia by orange oil showing an increased cell polarity and with increased cell organelles along with abundant junctional complexes (X 10,800); (B) Hepatic cells during in vivo suppression of DEN induced hepatic preneoplasia by orange oil showing well defined membranes and restitution of gap junctional complexes with narrowed intercellular space between adjacent cells (X 12,800) and (C) Hepatic cells during in vivo suppression of DEN induced hepatic preneoplasia by orange oil showing well defined tight junctions and adherens junction forming a junctional complex between adjacent cells (X 12,800).
differentiated cytoplasm with pleomorphism of nuclei and disorganised cellular plates (Fig. 5C). The membranes appeared impaired, with widened intercellular spaces besides complete loss of cell-to-cell contact (Fig. 5D). Few undistinguished organelles were also observed (Fig. 5E). However, in rats administered orange oil following DEN treatment, hepatic cells showed increased cell density and retained cell polarity with many junctional complexes and intact bile canaliculi (Fig. 6A). The membranes appeared compact and normal with increased intercellular gap junctional complexes (Fig. 6B). Also tight junctions and adherens junctions were observed (Fig. 6C).

**Discussion**

Chemoprevention of cancer involves cancer control where the induction of carcinogenesis can be totally prevented or the rate of development slowed or reversed partially or substantially by the administration of one or more naturally occurring or synthetic chemical agents. Fruits, vegetables and common beverages, as well as several herbs and plants with diverse pharmacological properties, have been shown to be rich sources of chemicals with potential for chemoprevention of various cancers. Among these, naturally occurring monoterpenoids have received increasing attention in recent years. Among the monoterpenoid compounds, d-limonene has been shown to have anticarcinogenic and antimutagenic activity. D-limonene administration showed inhibition of chemical carcinogenesis by DEN, NNK and NMU. Dietary limonene has also been shown to inhibit DMBA-induced mammary tumor development in rats. D-limonene induces both phase I and phase II hepatic detoxification enzymes in rats. All these studies suggest the potential use of d-limonene in cancer prevention. However, not many studies have been done to study the alterations at the ultrastructural levels during reversal of carcinogen induced changes by monoterpenoids. Accordingly, an attempt was made in this to study ultrastructural changes during reversal of DEN-induced hepatocarcinogenesis. Orange peel oil which contains about 90-95% of d-limonene was used as the chemopreventive agent.

In the present study, DEN-administered rat livers showed loss of cell polarity with gradual widening of gaps between altered phenotypes due to absence of cell-to-cell contact disturbing the morphological structure (Figs 5 C-E). However, orange oil administered rats following DEN treatment showed the reversal of the polarity lost in carcinoma cells, enhanced adhesiveness among the adjacent cells, and restored morphological architecture and intimate cellular contacts with well defined cell boundaries resembling that of normal hepatocytes (Fig. 6). Yamashaki has pointed out the importance of cell-to-cell communication for regulating cell growth and described a system that involves contact between cells called gap junctions. Gap junctions are composed of pore-like structures (connexin) which allow molecules to diffuse between connecting cells. Tumor cells are extremely deficient in the gap junctions. Zhang et al. while investigating possible mechanism of carotenoid action reported that β-carotene upregulated the expression of the connexin 43 gene responsible for the production of one of the important components of the gap junction. In this study orange oil restored the tight junctions which were lost in DEN-induced liver tumors. It is possible that orange oil also is involved in the upregulation of connexin genes and restoration of gap junctions. This aspect requires to be further investigated.

It is now well established that several solid tumors have disturbed homologous or heterologous intercellular gap communications and drugs that could upregulate gap junctions should also be considered for chemotherapy. Upregulation of gap junctions is involved in the restoration of growth control and other non-tumorigenic phenotypes by retinoids and carotenoids. It is likely that orange oil along with retinoids and carotenoids may prove as one of the chemopreventing candidates for upregulation of gap junctions and provide defense against carcinogenesis in vivo—a concept that has been central to growth control hypothesis.

In summary, the results of this study demonstrates that orange oil significantly inhibits growth of liver tumors which is associated with the restoration of the normal phenotype and upregulation of junctional complexes. In addition, these results provide further evidence for the modulatory role of gap junctional intercellular communication during carcinogenesis. Studies are in progress to understand the mechanisms involved in the upregulation of gap junctions during chemoprevention by orange oil.

**References**