Abstract: Ellagic acid has been shown to have chemopreventive effects in various experimental cancer models. We wanted to see whether pure ellagic acid and natural ellagitannins from cloudberry (Rubus chamaemorus) seed and pulp have any effect on adenoma formation in Apc-mutated Min mice. From the age of 5 wk, the mice were fed either a control diet, a diet containing pure ellagic acid at 1,564 mg/kg, or diets containing 4.7% (wt/wt) cloudberry seeds or 5.3% cloudberry pulp. The concentrations of ellagitannins and free ellagic acid in the seed diet were 807 and 42 mg/kg and in the pulp diet 820 and 34 mg/kg, respectively. After the 10-wk feeding period, ellagic acid had no effect on the number or size of adenomas in the distal or total small intestine, but it increased adenoma size in the duodenum when compared with the control diet (1.50 ± 0.29 vs. 1.16 ± 0.31 mm; \( P = 0.029 \)). Neither cloudberry seed nor pulp diets had any effect on the adenoma formation. Chemopreventive effects and mechanisms of whole cloudberry and other similar sources of phenolic compounds should, however, be studied, further taking into account food matrix and interactions with other dietary constituents that may be involved in the bioavailability and metabolism of ellagitannins.

Introduction

Ellagic acid, a plant phenolic compound, has been studied extensively in various experimental cancer models. It has been shown to decrease, for example, the incidence of chemically induced lung (1), small intestinal (2,3), mammary (4), and esophageal tumors (5) and also to reduce tumor multiplicity (6) and chemically induced mutagenesis in rat esophageal tissues (7). One mechanism behind these effects may be an inhibition of bioactivation of procarcinogens in tissues because ellagic acid has been found to decrease hepatic cytochrome P450 activity (8) and to increase the activities of several phase II enzymes (8–10). Based on in vitro studies, other suggested chemopreventive mechanisms include cell cycle arrest, reduced proliferation and angiogenesis, and induction of apoptosis (11–15).

However, in foods ellagic acid is mainly found as polymeric ellagitannins, the main sources being strawberries, raspberries, blackberries, and, especially in the northern areas of Scandinavian countries, cloudberries (Rubus chamaemorus). Thus, the results of studies using free ellagic acid should be interpreted with caution. It is apparent that ellagic acid and ellagitannins are poorly absorbed, and the absorbed proportion is quickly eliminated from the blood (16–18). So far, there is only one study reporting that ellagitannins could be absorbed as such in rats (19). In humans, only ellagitannin-derived microbial metabolites and, in some cases, ellagic acid have been found in the plasma and/or urine (20–22). In light of these studies, the possible biological effects of ellagitannins could be attributed to the metabolites of colonic microflora rather than ellagitannins as such.

With regard to colon cancer, no evidence of chemoprevention by ellagic acid has been found in vivo; ellagic acid had no effect on the incidence of chemically induced colon tumors (2–4) or the number of aberrant crypt foci in the rat colon (23). However, there are no data on the effects of ellagic acid in animal models of intestinal tumorigenesis, where the cytochrome P450 has not been activated. Because the adenomatous polyposis coli (APC) gene is referred to as a gatekeeper gene for both germline and sporadic colorectal tumors (24), we wanted to see the effects of pure ellagic acid and, on the other hand, the effects of natural ellagitannins on adenoma formation in Apc-mutated Min mice. Cloudberry seeds and pulp were used as ellagitannin sources because cloudberry is rich in ellagitannins (25,26), which are distributed equally between the seeds and the pulp.

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Because our previous study suggested that a diet containing freeze-dried cloudberry prevents adenoma formation in Min mice (27), it was interesting to see whether cloudberry seeds and pulp have distinct effects on intestinal tumorigenesis.

**Materials and Methods**

**Diets**

The diets were modified AIN93-G diets (28). The composition and content of fat (20 g fat/100 g diet) were designed to approximate those in a typical Western-type diet and were a mixture of butter, rapeseed oil, and sunflower oil providing the intake of saturated, monounsaturated, and polyunsaturated fatty acids in a ratio close to that from the Western-type diet. Pure ellagic acid (Sigma E-2250, Sigma-Aldrich, St. Louis, MO) was administered at 1,565 mg/kg in the diet, equal to its concentration in the previous whole cloudberry diet that contained 10% (wt/wt) freeze-dried cloudberry (27). In the present study, cloudberrys (Kiantama, Suomussalmi, Finland) were freeze-dried and ground before the seeds and pulp were separated from each other by sieving. The freeze-dried cloudberry contains 47% (wt/wt) seeds and 53% (wt/wt) pulp. To maintain the same concentrations of seeds and pulp in the diets as in the previous whole cloudberry diet, the seed diet contained 4.7% (wt/wt) seeds and the pulp diet contained 5.3% (wt/wt) pulp (Table 1). Contents of ellagitannin and free ellagic acid in the cloudberry seeds and pulp were analyzed as described previously (25,26). Polymeric ellagitannins were converted to ellagic acid in 20-h acid hydrolysis (1.2 M HCl, 85°C) and quantified as ellagic acid equivalents. The concentrations of ellagitannins and free ellagic acid in the seed diet were 807 and 42 mg/kg and in the pulp diet 820 and 34 mg/kg, respectively. Based on the estimated food consumption of 2.5 g per mouse per day, the average ellagic acid intake was 3.9 mg per mouse per day in the ellagic acid group. The intakes of ellagitannins and free ellagic acid were 2.0 and 0.11 mg per mouse per day in the seed group and 2.1 and 0.08 mg per mouse per day in the pulp group. The control diet was a similar high-fat diet without any berry components or phenolic compounds (Table 1). The diets were vacuum packed in weekly portions and stored at −20°C.

**Animals**

The Laboratory Animal Ethics Committee of the University of Helsinki (Helsinki, Finland) approved the study protocol. Male and female C57BL/6J Min/+ (Min) mice were bred at the Experimental Animal Unit of the University of Helsinki from inbred mice originally obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were genotyped by using a polymerase chain reaction assay (29). At 5 wk of age, the mice were stratified by litter and sex and assigned randomly to the control or experimental diets with 12 mice per group. The duration of the feeding period was 10 wk. Animals were housed in plastic cages in a temperature- and humidity-controlled animal facility with a 12-h light/dark cycle. They had free access to the semisynthetic diets and tap water. Body weights of the animals were recorded weekly. At the end of the feeding period mice were killed by CO₂ asphyxiation. The small intestine, cecum, and colon were removed and opened along the longitudinal axis, and the number, diameter, and location of adenomas were determined as previously described (30). Briefly, the small intestine was divided into five sections and opened longitudinally. The colon and cecum were kept together. The number, size, and location of adenomas were determined microscopically at a magnification of ×67.

**Statistical Analysis**

Adenoma data were analyzed for the colon, the total small intestine, and all parts of the small intestine separately. Adenoma data between the control group and the experimental groups were compared by the nonparametric Mann-Whitney U test (SPSS, version 10.0, Chicago, IL). Data were considered significant at \( P < 0.05 \).

**Table 1. Composition of Diets (g/kg diet)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Ellagic Acid Diet</th>
<th>Cloudberry Seed Diet</th>
<th>Cloudberry Pulp Diet</th>
<th>Control Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloudberry seeds</td>
<td></td>
<td>46.6</td>
<td></td>
<td>53.4</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>1.565</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>235.8</td>
<td>222.6</td>
<td>229.4</td>
<td>236.2</td>
</tr>
<tr>
<td>Dextrose</td>
<td>478.3</td>
<td>459.2</td>
<td>440.7</td>
<td>479.0</td>
</tr>
<tr>
<td>Butter</td>
<td>148.7</td>
<td>142.7</td>
<td>144.6</td>
<td>148.9</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>13.3</td>
<td>11.4</td>
<td>12.9</td>
<td>13.3</td>
</tr>
<tr>
<td>Rapeseed oil</td>
<td>62.1</td>
<td>59.6</td>
<td>60.4</td>
<td>62.2</td>
</tr>
<tr>
<td>Mineral mix AIN-93-MX</td>
<td>41.5</td>
<td>39.9</td>
<td>40.4</td>
<td>41.6</td>
</tr>
<tr>
<td>Vitamin mix AIN-93-VX</td>
<td>11.8</td>
<td>11.3</td>
<td>11.5</td>
<td>11.8</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3.6</td>
<td>3.5</td>
<td>3.5</td>
<td>3.6</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.014</td>
<td>0.013</td>
<td>0.013</td>
<td>0.014</td>
</tr>
</tbody>
</table>

\( a \): The diets were isocaloric, containing 41% of energy from fats, 39% from carbohydrates, and 19% from proteins.

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Results

The animals grew well in all dietary groups (Fig. 1), and no adverse effects of feeding could be detected. Pure ellagic acid had no effect on the number or size of adenomas in the distal or total small intestine (Figs. 2 and 3A), but it increased adenoma size in the duodenum when compared with the control diet (Fig. 3B, 1.5 ± 0.3 vs. 1.2 ± 0.3 mm; \( P = 0.029 \)). Neither cloudberry seed nor pulp had any effect on adenoma formation when compared with the control diet (Figs. 2 and 3).

Discussion

In the present study, pure ellagic acid at the concentration of approximately 1,500 mg/kg diet failed to decrease adenoma formation in the distal and total small intestine but increased the size of adenomas in the duodenum. Rao et al. have shown (2) that ellagic acid at clearly higher concentrations of 4,000 and 8,000 mg/kg diet had no effect on azoxymethane-induced colon tumor incidence, but the higher dose, however, decreased the incidence of small intestinal adenocarcinomas. In another study, ellagic acid, at a concentration comparable with ours (1,000 mg/kg diet), had no effect on the incidence of 2-amino-1-methyl-6-phenylimidazo[4,5-6]pyridine-induced colon tumors (4). Tumors in Min mice are mainly located in the small intestine and not in the colon, but, due to the fidelity of this model to human experience (31), it is regarded as a valuable tool for studying APC-mediated colon carcinogenesis. Thus, the results of the present study support the previous in vivo findings that ellagic acid is not an effective chemopreventive agent against intestinal tumorigenesis, even though it may have beneficial effects in some other tissues (1,4,5). In this respect, our results with Apc-mutated mice do not differ from those obtained by using carcinogen-treated animals.

The pure ellagic acid that is not bound to the food matrix may also have harmful effects, especially in the proximal part of the small intestine where the local concentration of ellagic acid is at its highest. This could explain the increase in adenoma size in the most proximal fifth of the small intestine in the pure ellagic acid group. However, in the cloudberry seed and pulp, ellagic acid was bound to the food matrix and was mainly present as ellagitannins, which may have prevented any adverse effects on adenoma growth in the duodenum. In Caco-2 cells, free ellagic acid easily penetrated the apical membrane, but further transport across the basolateral membrane was highly restricted, leading to the accumulation of ellagic acid inside of the cells where it bound to the proteins and especially to the DNA (32). This accumulation could explain the beneficial but also the possible harmful effects of free ellagic acid in the epithelial cells. Certain flavonoids have also been shown to have cytoprotective effects at low but adverse effects at high concentrations in rat H4IIE hepatoma cells (33). The purity of the ellagic acid preparation used in the present study was 95%, and the possibility that the impurities had some unknown effects on adenoma formation cannot, however, be completely excluded.

Cloudberry seeds and pulp had no effect on adenoma formation in this study. They were used as natural sources of ellagitannins because our previous study suggested that the diet containing 10% (wt/wt) freeze-dried cloudberry prevents the adenoma formation in Min mice. In the present study, the concentration of ellagitannins in the seed diet was 807 mg/kg and in the pulp diet 820 mg/kg. Thus, the ellagitannin concentrations in these diets were equal but only half when compared with the 1,565-mg/kg concentration in...
the previous whole cloudberry diet (27). However, we were
interested to see whether the seeds and the pulp have an effect
of their own on tumorigenesis. The reason that neither of
these diets was able to prevent adenoma formation may be
due to the lower ellagitannin concentration in these diets than
in the previous whole cloudberry diet. The amount of dietary
ellagitannins in this study was much lower than the amount
used in the ellagitannin bioavailability studies. This raises a
possibility that the level of ellagitannins or its metabolites in
the epithelial cells and systemic circulation may have been
too low for any beneficial effects. The results also suggest
that the direct effect of ellagitannins from the luminal side of
the epithelium does not show any significant protection at the
concentration used. We cannot, however, exclude the possi-
bility that the chemopreventive effect of cloudberry in our
previous study (27) was due to the higher concentration of
ellagitannins.

The oil content of cloudberry seeds is 11.9% on a dry
weight basis, the most abundant fatty acids being linoleic and
α-linolenic acids (41.1% and 35.6% of total fatty acids) (34).
Thus, the cloudberry seed diet contained 2.0 g/kg diet more
α-linolenic acid than the control diet. The independent effect
of this small amount of α-linolenic acid on adenoma forma-
tion is unlikely.

Ellagic acid together with quercetin or resveratrol syner-
gistically reduced proliferation and induced apoptosis in vi-
trou through 21wanf1/cip1, p53, and the mitogen-activated protein
kinases p38 and JNK1,2 (35–37). This synergy may also ex-
plain the observation that total pomegranate juice containing
ellagitannins and various other polyphenolics reduced prolif-
eration in vitro more effectively than pure ellagic acid or
ellagitannin fractions separated from pomegranate (38). In
addition to ellagitannins, whole cloudberry contains
flavonols, hydroxycinnamic acids, and p-hydroxybenzoic
acid (39), which also may interact synergistically with each
other. Thus far, the distribution of these compounds between
seed and pulp has not been studied.

Group size in the present study was 12 mice per group.
The Min mouse strain is very sensitive to changes in the diet,
and, according to our previous experience from several feed-
ing experiments, 12 mice per group is sufficient to provide
the statistical power necessary to find a real difference be-
tween the control and experimental groups.

Our results do not support further studies on the effects
of pure ellagic acid in the prevention of colon cancer. However,
the chemopreventive effects of whole cloudberry and other
similar sources of phenolic compounds warrant more re-
search. Food matrix and interactions with other dietary con-
stituents may be involved in the bioavailability, metabolism,
and possible chemopreventive effects of dietary compounds,
and these should also be taken into account in future studies.

Acknowledgments and Notes

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