Effect of ursodeoxycholic acid in an experimental colon cancer model

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ABSTRACT

Aims: the present study was designed to examine the effect of ursodeoxycholic acid as chemoprotective agent in experimental colon carcinogenesis in rats.

Material and methods: one hundred and ten 10-week-old, Sprague-Dawley rats were divided into five groups: group A (20), no treatment. Group B (20), receiving daily both ursodeoxycholic acid (UDCA) 4 mg/kg of body weight and ethanol 1.23 g/kg of body weight added to the drinking water from the beginning of the study through 24 weeks. Group C (30), receiving 18 weekly doses of dimethylhydrazine (DMH) 21 mg/kg of body weight subcutaneously from the beginning of the study, with the same doses of UDCA and ethanol as in group B. Group D (20), ethylen-diamin-tetracetic acid solution alone for 18 weeks. Group E (20), receiving the same doses of ethanol plus DMH injections as in group C. All experimental animals were sacrificed after 25-27 weeks.

Results: no tumors developed in dimethylhydrazine-free groups. No significant differences in number of tumor-free animals, number of tumors per rat, and macro-microscopic tumor findings were seen between animals in group C and animals in group E.

Conclusions: we concluded that such an ursodeoxycholic acid supplementation did not modify colorectal carcinogenesis using a dynamic DMH-induced model in rats.

Key words: Colon cancer. Carcinogenesis. Dimethylhydrazine. Tumors. Ursodeoxycholic acid.

INTRODUCTION

Colorectal cancer (CRC) is a frequent tumor with a high mortality in Western countries (1). It represents 13% of the total number of cancer cases diagnosed annually in Asturias (Northern Spain) (2). Environmental factors play a significant role in its induction (3); in fact, diets high in fats, which are associated with increased fecal concentration of secondary bile acids, are known to promote colon cancer (4,5).

Ursodeoxycholic acid (UDCA) is widely used for several disorders (6-10). This bile acid had been described as a cancer promoter both in epidemiological (11,12) and
experimental studies. On the other hand, several authors have confirmed its cytoprotective role in humans (13) and in experimental conditions (13-15). It has been considered a chemoprotective agent (16,17), thus the dose and timing required would have to be established.

Long-term prospective studies in humans are difficult to carry out (18). The induction of colorectal cancer with 1-2 dimethylhydrazine (DMH) in rats is a currently valid experimental model that may transferred to humans (19-21).

The present study was designed to examine the effect of ursodeoxycholic acid supplementation as chemoprotective agent on experimentally induced colon tumors in both male and female rats.

MATERIAL AND METHODS

This work belongs to the same experiment from which several articles have already been published. A minimum number of 20 rats per group was established in order to achieve tumor induction using the dose and time established for the DMH of 76-90% of rats (22,23); for a rate of tumors in the rats induced by DMH of 1.60 (24,25) and 1.87 (26-28); and a variable and high mortality according to previous studies. In group C (the most important group in this study) mortality was expected to be higher than in the other groups, so the number of animals in this group was increased related to availability.

UDCA administration alone was seen to produce insoluble residues in water, so as to induce aversion to water. Based on these experimental findings, UDCA was mixed with alcohol, so the risks of both a lower dose of UDCA per rat and a heterogeneous consumption in UDCA groups were avoided. Then, groups B, C and E were to be designed.

A DMH group versus UDCA plus ethanol plus DMH group design was not possible based on the uncertain effect of UDCA plus ethanol on colorectal carcinogenesis.

So, one hundred and ten 10-week-old “Sprague-Dawley” rats (Lab Letica®, Barcelona, Spain), both male and female and from the same genetic line, were distributed into five groups. None of the 20 rats in group A (control group) received any kind of treatment. The 20 rats of group B (UDCA group) received ursodeoxycholic acid (UDCA, Ursochol®, Zambon SA, Zambon Group, Barcelona, Spain), at 4 mg/kg of body weight, and ethanol 1.23 g/kg of body weight, both added to the drinking water from the beginning of the study until sacrifice. The 30 rats of group C (DMH and UDCA group) received 18 subcutaneous (sc) injections weekly (24,25) with 21 mg/kg of 1,2-dimethylhydrazine (DMH; Fluka Chemica A.G., Sigma Co.®, St. Louis, Missouri, USA) from the beginning of the study, and were treated with the same volume of ethanol and UDCA as those in group B. The 20 rats of group D (EDTA control group) received the same subcutaneous volume of EDTA solution as those in group C, mixed with only distilled water and without DMH. The 20 rats of group E (DMH + ethanol group) received the same sc injections of DMH, and were treated with the same volume of ethanol as the rats in group C.

The feeding was standard (ITM-R20 diet, Lab Letica®, Barcelona, Spain), containing 3% fat and 5% fiber. Fifty percent of animals from each group were weighed weekly until sacrificed. The volume of ethanol and UDCA consumed by rats were controlled (29), and the total consumption of drinking water was measured throughout the study.

Room conditions were controlled, as recommended by other authors (30,31). The animals were separated in cages, for a maximum of three per cage, in order to avoid autophagia (23-27). Animals of different gender were not mixed in order to avoid aggression.

The recommendations of both the European Ethics Committee (E.C. Directive number 609/86) and the text of Royal Decree 1201/2005 of October 10 (published on Oct. 25 in Boletín Oficial del Estado) for the treatment of experimental animals were followed throughout the study.

Surviving rats were sacrificed between study weeks 25 and 27. A fixed and equal number of rats from each group were sacrificed each week during the sacrifice period of time (23-25).

At autopsy the number of tumors, and their localization and size were all recorded. The tumor area was measured using the major and minor diameters (24,25,31,32), and presented as the mean ± standard deviation (SD) (in squared millimeters). Samples were later taken from the cecum and ascending colon (right colon), and the transverse and descending colon (left colon). Lesions in the colonic mucosa were classified (33,34) according to their degree of parietal invasion, differentiation, histological type, association with lymphoid tissue, size, and macroscopic appearance. Other extra-intestinal findings were also recorded.

Statistical analysis

A bivariate analysis was used for group comparisons, especially between the DMH and UDCA (C) and DMH (E) groups. For a comparison of mean values the ANOVA single-factor test was used. The SPSS-10.1 (Chicago-Illinois) computer program for Windows was used for statistical analysis. Differences between both groups were significant when p value was equal to or less than 0.05.

RESULTS

Five rats (4.5%) died prior to study completion, 1 rat from group C (3.3%), and 4 from group E (20%). Three male rats did not complete the study because of an occlusive tumor of the descending colon: one in group C, and
two in group E. Thus, in group E one male rat developed a colon tumor with metastases and digestive hemorrhage, these being confirmed microscopically; and one female rat died early with no tumor found. These animals were therefore excluded from the analysis.

Alcohol consumption was similar in all ethanol-fed groups (1.23 ± 0.0064 g/kg of body wt per day in group B, compared to 1.23 ± 0.0071 in group C, and 1.23 ± 0.0094 in group E; p = 0.77). UDCA consumption was similar in both UDCA-fed groups also (4 ± 0.0021 mg/kg of body wt per day in group B, compared to 4 ± 0.0032 in group C, p = 0.81). Neither diarrhea nor dehydration was observed in rats that finished the study, when adding alcohol to the drinking water.

No significant differences were found between the weight of animals in group C and group E: in male rats, 531.64 ± 78.71 g and 556.82 ± 27.68 g, respectively (p = 0.30); in female rats, 316.02 ± 23.30 g and 307.50 ± 35.90 g, respectively (p = 0.15).

No tumors were seen in the animals of DMH-free groups (A, B, and D).

Tumors developed in DMH-treated groups only: no differences were observed in the mean number of tumors or in the number of tumor-free rats (Table I).

No significant differences were found in tumor size, or in macroscopic morphology, or in tumor location for rats in group C as compared to rats in group E (Table II).

Microscopic findings (Table III) revealed no differences for animals in group C when compared to those in group E in terms of number or histological type, or regarding extent of invasion or differentiation, or in tumor association with lymphoid tissue.

However, rats in group C had a significantly higher number of mucinous carcinomas in the right colon (75%) when compared to those found in the left colon (13.5%; p = 0.003). For rats in group E this trend was also observed (p = 0.31) (Table IV). In group C, 2 female rats developed a mucinous and poorly differentiated colonic tumor with hepatic metastases, confirmed microscopically.

Two small-bowel tumors were also found in group C: one in a male rat and one in a female rat that developed peritoneal carcinomatosis.

### Table I. Tumor incidence and distribution

<table>
<thead>
<tr>
<th></th>
<th>DMH + UDCA + OH</th>
<th>DMH + OH</th>
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<tbody>
<tr>
<td>Group C (n = 29)</td>
<td></td>
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</tr>
<tr>
<td>Number of rats sacrificed, n (%)</td>
<td>29 (96.7)</td>
<td>6 (80)</td>
</tr>
<tr>
<td>Male/female, n</td>
<td>14/15</td>
<td>7/9</td>
</tr>
<tr>
<td>Number of tumor-free rats, n (%)</td>
<td>7 (24.1)</td>
<td>6 (37.5)</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>3/14 (21.4)</td>
<td>1/7 (14.3)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>4/15 (26.7)</td>
<td>5/8 (55.6)</td>
</tr>
<tr>
<td>Total number of tumors, n (%)</td>
<td>28 (96.5)</td>
<td>16 (100)</td>
</tr>
<tr>
<td>Male/female, n</td>
<td>15/13</td>
<td>12/4</td>
</tr>
<tr>
<td>Mean number tumor/rat, mean ± SD</td>
<td>1.45 ± 0.66</td>
<td>1.60 ± 1.02</td>
</tr>
<tr>
<td>Male</td>
<td>1.55 ± 0.82</td>
<td>2 ± 0.89</td>
</tr>
<tr>
<td>Female</td>
<td>1.18 ± 0.66</td>
<td>1 ± 0</td>
</tr>
</tbody>
</table>

UDCA: ursodeoxycholic acid; OH: ethanol; SD: standard deviation; DMH: dimethylhydrazine; n: number of cases.

### Table II. Macroscopic characteristics of tumors

<table>
<thead>
<tr>
<th></th>
<th>DMH + UDCA + OH</th>
<th>DMH + OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean tumor size, mm² ± SD</td>
<td>85.64 ± 113.05</td>
<td>45.87 ± 53.13</td>
</tr>
<tr>
<td>Male</td>
<td>57.73 ± 56.07</td>
<td>39.08 ± 49.69</td>
</tr>
<tr>
<td>Female</td>
<td>117.85 ± 65.76</td>
<td>66.25 ± 65.76</td>
</tr>
</tbody>
</table>

Macroscopic tumor findings, n (%):
- Polypoid: 19 (67.9) vs. 13 (81.3)
- Normal mucosa: 2 (7.1) vs. 2 (12.5)
- Other morphologies: 7 (25) vs. 1 (6.2)
- Tumor distribution in colon, n (%):
  - Right colon: 12 (42.9) vs. 7 (43.8)
  - Left colon: 16 (57.1) vs. 9 (56.2)

UDCA: ursodeoxycholic acid; OH: ethanol; SD: standard deviation; DMH: dimethylhydrazine; n: number of cases. Tumor size is expressed as the greatest tumor area in squared millimeters.

### Table III. Microscopic characteristics of tumors

<table>
<thead>
<tr>
<th></th>
<th>DMH + UDCA + OH</th>
<th>DMH + OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological type, n (%)</td>
<td>17 (60.7)</td>
<td>8 (50)</td>
</tr>
<tr>
<td>Adenocarcinomas</td>
<td>11 (39.3)</td>
<td>8 (50)</td>
</tr>
<tr>
<td>Mucinous adenocarcinomas</td>
<td>3 (25)</td>
<td>2 (28.6)</td>
</tr>
<tr>
<td>Degree of differentiation, n (%)</td>
<td>11 (39.3)</td>
<td>5 (31.3)</td>
</tr>
<tr>
<td>High</td>
<td>5 (17.9)</td>
<td>7 (43.7)</td>
</tr>
<tr>
<td>Moderate</td>
<td>12 (42.9)</td>
<td>7 (43.7)</td>
</tr>
<tr>
<td>Poor</td>
<td>16 (57.1)</td>
<td>11 (68.7)</td>
</tr>
<tr>
<td>Extent of invasion, n (%)</td>
<td>7 (25)</td>
<td>4 (25)</td>
</tr>
<tr>
<td>In situ carcinoma</td>
<td>5 (17.9)</td>
<td>5 (31.3)</td>
</tr>
<tr>
<td>Peritoneal involvement</td>
<td>16 (57.1)</td>
<td>11 (68.7)</td>
</tr>
<tr>
<td>Other</td>
<td>5 (17.9)</td>
<td>5 (31.3)</td>
</tr>
</tbody>
</table>

UDCA: ursodeoxycholic acid; OH: ethanol; SD: standard deviation; DMH: dimethylhydrazine; n: number of cases.

### Table IV. Tumor classification in relation to location

<table>
<thead>
<tr>
<th></th>
<th>DMH + UDCA + OH</th>
<th>DMH + OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right colon, n</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>Tumor size, mm² ± SD</td>
<td>116.67 ± 108.64</td>
<td>45.43 ± 57.42</td>
</tr>
<tr>
<td>Adenocarcinomas, n (%)</td>
<td>3 (25)</td>
<td>2 (28.6)</td>
</tr>
<tr>
<td>Mucinous carcinomas, n (%)</td>
<td>9 (75)</td>
<td>5 (71.4)</td>
</tr>
<tr>
<td>Left colon, n</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td>Tumor size, mm² ± SD</td>
<td>62.38 ± 114.03</td>
<td>46.22 ± 53.11</td>
</tr>
<tr>
<td>Adenocarcinomas, n (%)</td>
<td>14 (87.5)</td>
<td>6 (66.7)</td>
</tr>
<tr>
<td>Mucinous carcinomas, n (%)</td>
<td>2 (13.5)</td>
<td>3 (33.3)</td>
</tr>
</tbody>
</table>

*p = 0.003 (adenocarcinomas versus mucinous carcinomas of left colon in DMH and UDCA group).

### DISCUSSION

The induction of colorectal cancer in the rat with 1-2 dimethylhydrazine (DMH) is currently a valid experimental model that may be transferred to humans given that tumors induced with DMH are similar in these two species, both in their macro-microscopic and clinical behavior (19-21).
An incidence of spontaneous experimental colonic carcinogenesis below 2-3 rats/100,000 rats observed is reported (35). In the absence of carcinogen (DMH) no rats developed tumors, as expected.

Diets high in fat, which are associated with increased fecal concentration of secondary bile acids, are known to promote colon cancer (4,5). Ursodeoxycholic acid (UDCA), the 7-B-epimer of chenodeoxycholic acid, is a bile acid widely used in humans (6-10).

With the amounts of ethanol (25) and UDCA described, animals showed no natural aversion to them, and we did not expect any differences in consumption between groups, as it was. Thus, we supplemented them using the designed dose and timing required by this study.

This work stems from the same experiment from which several articles have already been published. Based on them, in a dynamic model of concomitant administration of ethanol and procarcinogen (36-38), at the dose and timing established (25,39-41), this relatively low dose of ethanol would not have been dangerous enough to modify colorectal carcinogenesis.

In human studies (11) UDCA is metabolized to the 7-B-epimer of chenodeoxycholic acid, whose mechanism of action resides in its conjugation with nitrosamines at the intestinal epithelium, with mutagenic properties, by the luminal route. In experimental studies in rats this effect could be explained by an increased permeability mediated by mast cells (42), increased cell proliferation rate (12,30), and diminished activity of alkaline sphingomyelinase in the epithelium (43), which is also observed in human pathology. This was not observed in our rats.

In epidemiologic studies a significant reduction in stool deoxycholic acid concentrations (as a biomarker of cell damage) has been described at a dose of 4 mg/kg body wt per day for 3 weeks (13). Other authors (44) found a significant reduction of the risk for colon cancer at 13-15 mg/kg body wt per day for 12 years. Moreover, it has been reported some kind of prevention for colon cancer at 9-10 mg/kg body wt per day for 6-14 years (5), and for high-grade dysplasia at 8-10 mg/kg body wt per day for 3 years (16).

Taking these studies together, data suggest that chemoprevention seems to be time- and dose-response-related.

Nowadays, its applicability to cancer prevention remains uncertain, and the dose and critical timing of UDCA supplementation would have to be established (17). In experimental studies in rats this protective effect could be explained by the inhibited expression of both K-ras oncogene and cyclooxygenase-2 activity in tumor cells (15); thus, a slower progression of the adenoma-carcinoma sequence has also been involved (14,45).

In our study, the minimum dose schedule of 4 mg/kg body wt per day was used as a chemopreventive dose against colon cancer. Our results show no significant differences either in the number or in the macro-microscopic characteristics of tumors in DMH-induced rats (groups C and E), so that this relatively low dose of UDCA was not effective enough to show any chemopreventive effect. Nevertheless, there were some parameters, including tumor size, that have shown no significant differences between their mean values, despite a high gap between them; and others, including tumor location, that have attained statistical significance without this apparent gap of difference. It seems that the number of animals per group could have altered our results, and avoided demonstrating the possible protective effect of UDCA.

Moreover, a dynamic model of concomitant administration of UDCA and procarcinogen is a controversial design for studying colorectal carcinogenesis. UDCA when supplemented during the initiation phase of carcinogenesis significantly decreased the total number and size of aberrant crypt foci (ACF, as a cell hyperproliferation marker) and was effective in preventing tumor occurrence, but given in promotion/progression and post-initiation phases, at the same doses as in our study for 2 weeks, failed to alter the number or size of ACF (14). Based on these findings the concomitant administration used in our study could also have inhibited the chemoprotective effect.

As in human pathology, a mucinous adenocarcinoma is considered a poorly differentiated tumor with a behavior different to that of a non-mucinous one (33). As in previous studies (25), a significantly greater number of mucinous adenocarcinomas were found in the right colon of rats within group C when compared to the left colon of these same animals.

In our study, as suggested by some authors (46-49), we have been able to demonstrate a different behavior between both sides of the colon. Right tumors therefore showed their own and worse behavior when compared to left tumors and this effect was not modified by exogenous ethanol and UDCA delivery. Our results are not comparable to those of other authors (28,50) due to the different histological criteria used (33,34), following our previous studies (24,25,51).

Finally, we conclude that oral UDCA supplementation at a dose of 4 mg/kg body wt per day for 24 weeks did not modify colon cancer in a dynamic model of carcinogenesis induced by DMH in Sprague-Dawley rats. And secondly, that a different histological behavior did exist in tumors located in the right colon compared to left ones, and the external agents used (UDCA and ethanol) did not modify it.

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