Clear (CleA) and cloudy (CloA) apple juices containing different amounts of analyzed procyanidins and pectin were investigated for protective effects of colon cancer and underlying molecular mechanisms in F344 rats given intraperitoneal injections of 1,2-dimethylhydrazine (DMH; 20 mg/kg body wt) once a week for 4 weeks. Rats received either water (Cont), CleA or CloA (ad libitum) for 7 weeks starting 1 week before the first DMH injection. CloA inhibited DMH induced genotoxic damage in mucosa cells of the distal colon compared with Cont as investigated by single-cell microgel electrophoresis assay. The mean tail intensity in mucosa cells of DMH-treated controls (Cont/DMH: 6.1 ± 0.9%) was significantly reduced by CloA (2.4 ± 0.8%; P < 0.01) but not by CleA intervention (4.1 ± 1.2%; P > 0.05). The crypt cell proliferation index induced by DMH (Cont/NaCl: 10.0 ± 0.7%; Cont/DMH: 19.9 ± 1.0%; P < 0.001) was significantly decreased by CleA (15.7 ± 0.7%; P < 0.001) and CloA intervention (11.9 ± 0.4%; P < 0.001). CloA but not CleA significantly reduced the number of large aberrant crypt foci (ACF) consisting of more than four aberrant crypts (AC) (Cont/DMH: 37.4 ± 5.4; CleA/DMH: 32.8 ± 4.4, P > 0.05; CloA/DMH: 18.8 ± 2.5 ACF; P < 0.05) and the overall mean ACF size in the distal colon (Cont/DMH: 2.31 ± 0.09; CleA/DMH: 2.27 ± 0.05; CloA/DMH: 2.04 ± 0.03 ACF; P < 0.05). After treatment with DMH and/or apple juices there were no changes in transcripts of colonic cyclooxygenase isoforms (COX-1, COX-2) or glutathione-associated enzymes (GST-M2, GST-P), the spleenocyte natural killer cell activity and plasma antioxidant status. However, CloA but not CleA prevented the DMH-induced reduction of spleenocyte CD4/CD8 (T-helper cells to cytotoxic lymphocytes) ratio. Since both formulations contained comparable concentrations and types of monomeric polyphenols, complex polyphenols or non-polyphenolic compounds, such as pectin might be responsible for the stronger cancer-preventive effect by CloA.

Introduction

Epidemiologic studies suggest that the consumption of vegetables and fruits is inversely associated with colorectal cancer risk (1). Much of this protective effect of vegetables and fruits has been attributed to biologically active secondary plant metabolites, which are the non-nutrient plant constituents such as the carotenoids, phenolic acids and flavonoids.

Until now, >6500 structurally different, naturally occurring flavonoids have been described. Various flavonoid compounds have been found to possess a range of cancer preventive activities including prevention of oxidative DNA damage (2), inhibition of carcinogen activation (3,4), induction of carcinogen detoxifying systems (5), interaction with cellular signaling pathways and modulation of gene expression controlling proliferation, differentiation and apoptosis of cancer cells (6).

Apples are a significant source of flavonoids in human diets in Europe (7,8) and epidemiological data correlated apple consumption with a reduced lung cancer risk (9). To initially characterize mechanisms by which apples may prevent cancer, studies using apple formulations derived from different apple tissues have shown antioxidative and antiproliferative activity in vitro (10–12). Although no attempt has been made to identify the relevant bioactive component(s) in apples, the observed in vitro effects were discussed to be attributed either to the high concentrations of flavonoids in apple peel and flesh as well as apple procyanidins or non-flavonoid apple fibre such as apple pectin (13).

More detailed studies on the cancer preventive effects of different fruit or vegetable varieties have usually been conducted in complex animal models using either the lymphoblasted fruit or vegetable itself (14), juices (15), polyphenol enriched extracts (16,17) or specific constituents (18). However, comparable in vivo studies using apple preparations in rodent models for colon carcinogenesis are still lacking.

Besides genetic models of intestinal carcinogenesis (19), the rodent model of dimethylhydrazine (DMH)- or azoxymethane (AOM)- induced colon cancer has been widely used in studies to evaluate anticarcinogenic properties of dietary factors by analyzing colonocyte DNA damage (20), epithelial hyperproliferation (21), tumor/cell-cycle related gene expression (17), signal transduction (22), and initiation and growth of putative premalignant lesions called aberrant crypt foci (ACF) (14).

Owing to the strong in vitro support of cancer preventive activity by apple constituents, the aim of the present study was to investigate the potential effects of apple juice on markers of colon carcinogenesis in the rat model of DMH-induced carcinogenesis. As the variety of apple cultivars and procedures of apple processing significantly determine the content and spectrum of polyphenols and non-polyphenolic compounds in apple juices, we used polyphenol-rich apple varieties for the production of two different juice preparations. Both the cloudy (CloA) and the clear apple juice (CleA) contained high and comparable amounts of monomeric polyphenols.

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; CleA, clear apple juice; CloA, cloudy apple juice; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; DMH, dimethylhydrazine; PGE2, prostaglandin E2; SCFA, short chain fatty acids.

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Clear (CleA) and cloudy (CloA) apple juices containing different amounts of analyzed procyanidins and pectin were investigated for protective effects of colon cancer and underlying molecular mechanisms in F344 rats given intraperitoneal injections of 1,2-dimethylhydrazine (DMH; 20 mg/kg body wt) once a week for 4 weeks. Rats received either water (Cont), CleA or CloA (ad libitum) for 7 weeks starting 1 week before the first DMH injection. CloA inhibited DMH induced genotoxic damage in mucosa cells of the distal colon compared with Cont as investigated by single-cell microgel electrophoresis assay. The mean tail intensity in mucosa cells of DMH-treated controls (Cont/DMH: 6.1 ± 0.9%) was significantly reduced by CloA (2.4 ± 0.8%; P < 0.01) but not by CleA intervention (4.1 ± 1.2%; P > 0.05). The crypt cell proliferation index induced by DMH (Cont/NaCl: 10.0 ± 0.7%; Cont/DMH: 19.9 ± 1.0%; P < 0.001) was significantly decreased by CleA (15.7 ± 0.7%; P < 0.001) and CloA intervention (11.9 ± 0.4%; P < 0.001). CloA but not CleA significantly reduced the number of large aberrant crypt foci (ACF) consisting of more than four aberrant crypts (AC) (Cont/DMH: 37.4 ± 5.4; CleA/DMH: 32.8 ± 4.4, P > 0.05; CloA/DMH: 18.8 ± 2.5 ACF; P < 0.05) and the overall mean ACF size in the distal colon (Cont/DMH: 2.31 ± 0.09; CleA/DMH: 2.27 ± 0.05; CloA/DMH: 2.04 ± 0.03 ACF; P < 0.05). After treatment with DMH and/or apple juices there were no changes in transcripts of colonic cyclooxygenase isoforms (COX-1, COX-2) or glutathione-associated enzymes (GST-M2, γ-GCS, GST-P), the spleenocyte natural killer cell activity and plasma antioxidant status. However, CloA but not CleA prevented the DMH-induced reduction of spleenocyte CD4/CD8 (T-helper cells to cytotoxic lymphocytes) ratio. Since both formulations contained comparable concentrations and types of monomeric polyphenols, complex polyphenols or non-polyphenolic compounds, such as pectin might be responsible for the stronger cancer-preventive effect by CloA.
Beside the heterogeneous cloud fraction exclusive to CloA, the juices further differ in their content of procoyandins and non-polyphenolic dietary fibre ingredients, such as pectin. Using these apple juice preparations with different content of native apple compounds gives the opportunity to relate potential cancer preventive effects to the distinct composition of either CloA or CleA or both juices.

Materials and methods

Apple juice preparations

The apple juices were produced in the Research Institute Geisenheim from the 2002 harvest. The following apple varieties were used: cv. Topaz (25%), cv. Bohnagel (17.5%), cv. Winterambour (22.5%), cv. Bittenfelder (15%) and mixed table-apple varieties (20%). The fruits were crushed in a hammer mill (Beller; Niefern, Germany) and extracted in an HP-L 200 horizontal press (Bucher; Niederweningen, Switzerland). The resulting juice was centrifuged with a separator (SA R 3036, Westfalia, Oelde, Germany) and finally pasteurized (85 °C, 30 s). After a further separation, the CloA was hot-filled (85 °C) into 0.75 l glass bottles. CleA was produced from CloA by pectinase (50 μl/kg, Fructzym P, Erbslöh, Geisenheim, Germany) treatment. After depectinization, the juice was cross-flow filtered (Bucher-Abcor, Niederweningen, Switzerland, Supercor membrane, nominal cutoff 18 KDa) and hot-filled. Bottled apple juices were stored at 4 °C until further use.

HPLC analysis of apple juice polyphenols

Apple polyphenols were separated on a 1090 HPLC/PDA system (Hewlett-Packard; Böblingen, Germany) equipped with a 250 × 4.6 mm Aqua 5 μm C18 column and protected with a 4 mm × 3 mm C18 ODS security guard (Phenomenex; Aschaffenburg, Germany). Gradient elution was applied with an acetonitrile/acetic acid gradient according to a previously published protocol (23). Detection wavelengths were 280, 320 and 360 nm. The juices were injected directly after centrifugation and 0.45 μm filtration with cellulose acetate membranes. Quantification was carried out using peak areas from external calibration curves. Analysis and polyphenol quantification were done immediately before experiments started. Further, the pectin content was analyzed according to a previously published protocol (24).

Animals

Male Fischer 344 (F344) rats (n = 90; 118 g on arrival) were obtained from a licensed animal supplier (Harlan Winkelmann, Barchen, Germany) and housed in a temperature- and humidity-controlled animal unit under ambient temperature of 21 ± 2 °C and a 12-12 h light-dark cycle. The rats were allowed to acclimatize for 9 days during which they consumed ad libitum tap water. Owing to the described effects of isolavones on AOM-induced development of ACF (25) a standard rat diet free of soyprotein was used for the present study (Sniff, Soest, Germany) and was further HPLC-analyzed to confirm the absence of potential isoflavone contamination (data not shown). The basal diet consisted of 3.3% fat, 19.0% protein, 4.9% fibre, 3.1% minerals and 56.7% carbohydrates (12.2 kJ/g).

Experimental protocol

After a period of 10 days from arrival, the rats were randomly assigned to three treatment groups (n = 30/group) receiving either tap water (control; Cont), CleA or CloA ad libitum until the end of the experiment. Juices were provided daily and juice or water consumption was recorded. Juices had the same ascorbic acid content (59 mg/l) immediately before filling into the drinking bottles. Body weight was recorded twice every week. One week after starting the intervention half (n = 15) of the intervention group received intraperitoneal injections of either DMH (20 mg/kg body wt) or 0.9% NaCl, the mucosa was pinned flat on a parafilm wax block in a Petri dish, mucosal side up and fixed in 4% PBS-buffered formalin solution for minimum 1 h (BrdU assay) or 16 h (ACF assay).

The 5 mm strip of the colonic mucosa specimen was resected for BrdU assay and after a further overnight fixation the remaining colonic mucosa not utilized for proliferation assay was rinsed in PBS, stained with 0.2% methylene blue, dehydrated, and examined under inverted microscope (Axioskop, Axiovert 100) at 100× magnification. Crypts were considered aberrant, if they were visibly enlarged and protruding when compared with surrounding crypts, having elongated openings and increased pericryptal zones. The number of ACFs observed per distal colon and the number of aberrant crypts per focus were recorded.

For processing the BrdU assay the resected strip was fixed and dehydrated, embedded in Paraplast plus (WVR International, Bruchsal, Germany) and cut into 5 μm thick sections (Microm HM 500 O, Heidelberg, Germany) mounted on silanized glass slides and dried overnight at room temperature. After heating the sections at 97 °C for 30 min in 10 mM sodium citrate (pH 6.0), DNA-synthesizing epithelial cells were identified by immunohistochemistry with an anti-BrdU monoclonal antibody (DakoCytomation, Glostrup, Denmark) diluted 1:200 and with the Vectastain Elite ABC kit (Alexis, Grünberg, Germany). Immunocomplexes were visualized with diaminobenzidine. Labeled cells were recognizable under the microscope by the dark-brown nuclear pigment. Tissue sections were then counterstained for nuclear staining with hematoxylin and examined at 400× magnification under a Zeiss Axiovert 100 microscope. Well-oriented crypts (n = 25 per animal) with the lumen visible from the bottom to the mucosal surface and with a single layer of cells along each crypt column were selected for counting BrdU labelled and BrdU unlabelled epithelial cells randomly selected from entire tissue sections.

Immunological analysis

Immune cell suspensions from resected spleens were prepared with RPMI-1640 culture medium containing 50 ml/l of heat-inactivated fetal bovine serum, 1-glutamine (2 mM/l), penicillin (1 × 105 U/l), streptomycin (100 mg/ml) and HEPES (25 mM/l); all components were purchased from Life Sciences (Eggenstein-Leopoldshafen, Germany). Splenocytes were isolated as described previously (28). Fifty cells of each slide (150 cells per animal) were analyzed using the imaging software of Perceptive Instruments (Halstead, UK). The amount of damaged DNA was expressed as the percentage of DNA in the tail (tailing intensity).

mRNA analysis

The distal colons of n = 5 animals/group were cleared of the contents by flushing with 37 °C prewarmed 0.9% NaCl in diethylycarbonate-treated water and mucosa cells were collected in 1.8 ml of phosphate buffered-saline (1 × PBS) by carefully scraping the luminal colonic mucosa with a glass slide. Total RNA was extracted from the cells using the guanidinium isocynoacetate/acid phenol method (27). The reverse transcription (RT) and the polymerase chain reaction (PCR) were performed in a PCR thermocycler (PTC 200, MJ Research Inc., Waltham, MA) by using the Ready-to-Go RT-PCR kit in accordance with the kit manual (Amersham Pharmacia Biotech, Freiburg, Germany). PCR cycling conditions were: initial denaturation at 95 °C for 5 min, subsequent amplification over 25–30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s, final extension at 72°C for 5 min. The respective sequences used for generation of sense and antisense primers were nt 959–982 and 1683–1662 of the rat COX-2 cDNA (GenBank accession no. NM_017232.2), nt 1091–1110 and 1775–1752 of the rat COX-1 cDNA (accession no. NM_017043.1), nt 904–925 and 1758–1736 of rat γ-GCS (accession no. NM_012815.2), nt 1–20 and 768–749 of rat GST-M2 (accession no. M13350.1), in 2–22 and 480–460 of GST-P cDNA (accession no. NM_012577.1) and at 71–94 and 570–547 of rat GAPDH cDNA (accession no. XM_235039.2) used as internal standard for normalization. Primer sequences for amplification of the GHR-related enzyme cDNAs were taken from a previously published protocol (17).

Primers were tested for linearity over cycle number and analyses were all carried on the linear part of the curve, thus allowing semi-quantitative analysis of mRNA amount. PCR products were run on 1.5% agarose gel in 1× TBE buffer and ethidium bromide-stained bands were visualized and quantified using an automated computer-based image analysis system (QuantityOne/FluorS Image) (Biorad, München, Germany). Quantities of each PCR product were normalized by dividing the average gray level of the signal by that of the corresponding GAPDH amplimph.

Assays of ACF and colonic cell proliferation

To investigate the proliferative activity of epithelial cells, n = 5 rats per group received a 30 mg/kg intraperitoneal injection of 5-bromodeoxyuridine (BrdU) 1 h before killing. After excision of the distal colon and carefully flushing with prewarmed 0.9% NaCl, the mucosa was pinned flat on a parafilm wax block in a Petri dish, mucosal side up and fixed in 4% PBS-buffered formalin solution for minimum 1 h (BrdU assay) or 16 h (ACF assay).

The 5 mm strip of the colonic mucosa specimen was resected for BrdU assay and after a further overnight fixation the remaining colonic mucosa not utilized for proliferation assay was rinsed in PBS, stained with 0.2% methylene blue, dehydrated, and examined under inverted microscope (Zeiss Axiosvert 100) at 100× magnification. Crypts were considered aberrant, if they were visibly enlarged and protruding when compared with surrounding crypts, having elongated openings and increased pericryptal zones. The number of ACFs observed per distal colon and the number of aberrant crypts per focus were recorded.

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The expression of cell-surface markers on the immune cells of spleen was investigated by immunofluorescence using phycoerythrin-conjugated mouse anti-rat monoclonal antibodies to CD4 (Phaltag, Hamburg, Germany) and fluorescein-conjugated mouse anti-rat monoclonal antibodies to CD8 (Caltag) with appropriate isotype controls. Samples were analyzed with a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany).

Natural killer (NK) cell activity of immune cells derived from spleen was assessed by flow cytometry. The mouse Moloney leukemia cell line, YAC-1, was used as target cell line (28).

Antioxidant status
Total plasma antioxidant activity in rats (n = 5-7/group) was determined by using the FRAP assay with minor modifications as described before (29) and the improved decolorization TEAC assay (30). Interassay and intraassay variability were <5%.

Statistical analysis
All data are presented as the mean ± SEM. Statistical analysis was accomplished using ANOVA and Newman–Keuls multiple group comparison test to identify significant differences between groups. P-values <0.05 were considered significant.

Results

General observations
During the experiment, body weight curves were similar in all groups independent of DMH treatment or intervention with either of the different apple juices. Without considering the available energy from the apple pectin the mean daily caloric intake by the apple juice intervention was 23.6 kJ (CleA; 21.5 ml/animal/day) and 27.8 kJ (CloA; 22.9 ml/animal/day) per animal. Rats fed on apple juices compensated the increased juice-derived energy by a decreased food intake compared with Cont receiving tap water (P < 0.001, Cont: 17.7 ± 1.9 g, CleA: 15.5 ± 1.0 g and CloA: 15.0 ± 0.9 g), which resulted in a comparable daily total energy intake (Cont: 215.3 kJ, CleA: 212.7 kJ and CloA: 210.8 kJ) and growth rate independent of the treatment group.

Polyphenol and pectin content of apple juice preparations
Table I shows the concentrations of polyphenolic compounds present in CleA and CloA. Both apple juice preparations contained a similar mixture and quantity of dihydrochalcones, hydroxycinnamic acids and quercetin glycosides. When focused on the contents of procyanidins B1 and B2, CloA contained ~30% more procyanidins B1/B2 than CleA (Table I). The composition and the amounts of the different polyphenols reflect the typical pattern of mixed cider apple juices. Further analysis showed that the pectin concentration in CloA is ~4-fold that of CleA and CloA (Table I), which is due to the enzymatic pectin degradation and the ultrafiltration during the processing of clear juices.

Genotoxicity
DNA damage in colon mucosa cells isolated from rats receiving injections of 0.9% NaCl instead of DMH was low as evaluated by the comet assay (Figure 1). Juice intervention had no effect in groups treated with NaCl. The treatment with DMH induced a significant increase in DNA damage in the water group (Cont) when compared with Cont/NaCl. This strong genotoxic effect of DMH was significantly reduced by the intervention with CloA (P < 0.01) but not with CleA (P > 0.05). The level of DNA damage in the group of CloA/DMH did not significantly differ from that of CloA/NaCl (Figure 1).

Epithelial proliferation
In all the animal groups analyzed, BrdU was exclusively incorporated in crypt cell nuclei located at the bottom of the colon crypts. Neither of the juices affected the low proliferation index in colon crypts of NaCl treated control animals (Figure 2). Compared with the Cont/NaCl group, DMH significantly increased the epithelial proliferation in the Cont group (P < 0.001). This hyperproliferative effect of DMH observed, was significantly reduced by intervention with CleA (P < 0.001) and almost abolished by CloA (P < 0.001). Furthermore, the proliferation lowering effect of CloA was significantly stronger when compared with CleA (P < 0.001; CloA/DMH versus CleA/DMH). However, when compared with the respective controls receiving NaCl instead of DMH, the proliferative activity in the colon epithelium of DMH-treated groups with either juice intervention was still significantly elevated (P < 0.001; CleA/DMH versus CleA/NaCl; P < 0.05, CloA/DMH versus CloA/NaCl).

Table 1. HPLC analysis of polyphenolic compounds and pectin in CleA and CloA

<table>
<thead>
<tr>
<th>Polyphenols</th>
<th>CleA (mg/l† (mg/kg body wt*)</th>
<th>CloA (mg/l† (mg/kg body wt*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procyanidin B1</td>
<td>4.2 (0.43)</td>
<td>5.8 (0.64)</td>
</tr>
<tr>
<td>Procyanidin B2</td>
<td>15.5 (1.59)</td>
<td>20.9 (2.29)</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>17.5 (1.80)</td>
<td>17.9 (1.96)</td>
</tr>
<tr>
<td>Phloretin-2'-galactoside</td>
<td>6.0 (0.62)</td>
<td>6.3 (0.69)</td>
</tr>
<tr>
<td>Phloretin-2'-xyloglucoside</td>
<td>60.9 (6.28)</td>
<td>59.8 (6.55)</td>
</tr>
<tr>
<td>Phlorizin</td>
<td>23.9 (2.46)</td>
<td>19.7 (2.16)</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>154.0 (15.83)</td>
<td>155.9 (17.08)</td>
</tr>
<tr>
<td>3-Coumaroyl quinic acid</td>
<td>1.9 (0.20)</td>
<td>1.9 (0.21)</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>2.3 (0.24)</td>
<td>0.6 (0.07)</td>
</tr>
<tr>
<td>4-Coumaroyl quinic acid</td>
<td>75.1 (7.72)</td>
<td>76.7 (8.40)</td>
</tr>
<tr>
<td>Quercetin-3-rutinoside</td>
<td>0.8 (0.08)</td>
<td>0.5 (0.05)</td>
</tr>
<tr>
<td>Quercetin-3-galactoside</td>
<td>1.9 (0.20)</td>
<td>1.7 (0.19)</td>
</tr>
<tr>
<td>Quercetin-3-glucoside</td>
<td>1.1 (0.11)</td>
<td>1.0 (0.11)</td>
</tr>
<tr>
<td>Quercetin-3-hamnoside</td>
<td>3.2 (0.33)</td>
<td>2.5 (0.27)</td>
</tr>
<tr>
<td>Total</td>
<td>368.3 (37.89)</td>
<td>371.2 (40.67)</td>
</tr>
<tr>
<td>Pectin</td>
<td>220 (22.63)</td>
<td>834 (91.38)</td>
</tr>
</tbody>
</table>

*Data represent the mean of three independent HPLC measurements.
†The mean daily polyphenol uptake during the experiment as detailed in parentheses is calculated with 209 g as the mean body weight and a mean daily juice intake of 21.5 ml CleA and 22.9 ml CloA during the intervention.
Aberrant crypt foci (ACF)

There were no ACF in the distal colon of any of the groups treated with NaCl. CleA given to the DMH-treated animals had no effect either on the number of ACF (Figure 3A) or the estimated number of AC per ACF (Figure 3C). In contrast, the total number of ACFs per colon was consistently reduced in animals with CloA intervention when compared with the Cont/DMH group (Figure 3A). This effect, however, was only statistically significant for the largest ACFs (≥4 crypts/foci) with an ~50% reduction of ACF number when compared with either the Cont (Figure 3D; 37.4 ± 5.4 versus 18.8 ± 2.5; P < 0.05) or the CleA group (32.8 ± 4.4 versus 18.8 ± 2.5; P < 0.05). As a result of reduced number of large ACFs, the intervention with CloA significantly reduced the mean ACF multiplicity when compared with Cont (Figure 3C; 2.3 ± 0.1 versus 2.0 ± 0.03; P < 0.05).

Gene expression

The treatment with DMH only showed the tendency to increase COX-2 mRNA levels but did not significantly change mucosal cyclooxygenase isoforms COX-1 and COX-2 mRNA levels in the distal colon of control animals. Furthermore, none of the juices used for intervention leads to significant gene expression changes in groups treated with NaCl or DMH (Table II). Moreover, besides mRNAs of cyclooxygenases, we also analyzed transcripts coding for the glutathione-related enzymes γGCS, GST-P and GST-M2, which were previously shown to be modulated by black tea and red wine polyphenols applied in an AOM post-initiation protocol (17). Of the GSH-related enzyme mRNAs analyzed, the gene expression of GST-M2, γGCS and GST-P was not affected by DMH. Furthermore, none of the apple juices showed any effect on gene expression levels of GSH-related enzymes (Table II).

**Fig. 2.** Proliferation index in the distal colon as the percentage of BrdU-positive cells (mean ± SEM) determined within 25 randomly chosen crypts per animal. The treatment with DMH significantly increased the percentage of BrdU-positive cells (mean ± SEM) within the crypts of the water receiving control (Cont) group (P < 0.001). When compared with Cont, the intervention with CleA or CloA significantly (P < 0.001) reduced hyperproliferation by DMH with a stronger antiproliferative effect of CloA (P < 0.001; CloA/DMH versus CleA/DMH).

**Fig. 3.** Analysis of ACF in the distal colon mucosa of DMH-treated animals (mean ± SEM; n = 5/group). The total number of ACFs (A) as well as the total number of aberrant crypts per colon (B) remained unchanged by intervention with either the clear (CleA) or cloudy apple juice (CloA). (D) As further detailed with respect to ACF size (AC/ACF) the constant number of ACFs irrespective of juice intervention was restricted to small sized ACFs (AC/ACF ≤ 3) while the mean number of larger ACFs (AC/ACF ≥ 4) was significantly reduced by CloA but not CleA (D, insert; *P < 0.05). This resulted in an overall decrease of ACF size by CloA (C, P < 0.05).
Results are presented as the mean ± SEM on n = 5 animals per group.

**Immunological parameters**

To evaluate the potential effects of the apple juice preparations on parameters of systemic immunity which might be implicated in eliminating tumor cells, we analyzed the lytic activity of NK cells isolated from spleen and quantitated cytotoxic CD4-positive and CD8-positive subsets of spleen lymphocytes.

There was no statistically significant effect on NK cell activity of either the DMH treatment or the apple juice intervention in any of the groups (data not shown).

The CD4:CD8 ratio was significantly reduced by DMH in the groups with water (Cont; P < 0.05) or CleA (P < 0.05) when compared with the respective controls treated with NaCl (Figure 4). This reduction of CD4:CD8, which was due to increased CD4 and decreased CD8, was not observed in the DMH group receiving CloA, leading to a normalization of CD4:CD8 when compared with the Cont/NaCl group (P > 0.05) and a significantly elevated CD4:CD8 ratio when compared with the Cont and also the CleA groups receiving DMH (P < 0.05).

**Antioxidant status**

As determined with the TEAC assay, the plasma total antioxidant capacity was not modified by DMH or any of the juices used for interventions (data not shown). However, results of the FRAP assay showed slightly but not significantly elevated ferric reducing capacity in groups receiving CleA when compared with the respective water receiving controls (Figure 5). Furthermore, this increase was significant for the CloA/DMH group when compared with the Cont/DMH group (P < 0.05).

**Discussion**

Previous *in vitro* studies have suggested that polyphenolic compounds in apple have cancer-preventive properties since bioactivity toward cellular parameters including proliferation, apoptosis and differentiation was shown in different cell lines (13). However, until now *in vivo* data on protective effects using crude apple preparations were not available. In the present *in vivo* study, we have identified the cancer preventive potential of a cloudy apple juice which reduced genotoxicity, hyperproliferation and the development of aberrant crypt foci by DMH, with a further modulation of immune parameters. This effect was stronger for CloA than for CleA (anti-proliferation) or exclusive to CloA (antigenotoxicity, ACF development, systemic immune parameter).

Since DNA damage is recognized as the initial step in chemical carcinogenesis, the blocking of DNA damage would be the first line of defense against cancer caused by carcinogens. Furthermore, it is assumed that DNA-alkylation may also be important in human cancer formation (31) and thus antigenotoxic effects observed in chemical carcinogenesis by DMH or AOM in rodent models might be representative for the human system. Accordingly, by using comet assay based detection of DNA single strand breaks, our results showed that CloA but not CleA attenuated the genotoxic effect of DMH in enterocytes of the distal colon.

The apple juice preparations used in the current study contained similar amounts of well characterized monomeric polyphenols. However, CloA provided a higher content of procyanidin B1/B2 and of pectin and an additional cloud...
fraction lacking in CleA. These differences might explain the greater antigenotoxic effect exerted by CloA.

Pectin as the main dietary fibre source, present in cloudy apple juices has been shown to be a good fermentation substrate for intestinal bacteria releasing distinct spectra of short chain fatty acids (SCFA) (acetate, propionate, butyrate) (32). SCFA exert a trophic function on colonic mucosa (33) and further influence mucosal proliferation and apoptosis in the colon (34,35). However, when using a mixture of SCFA potentially arising in the colon from bacterial fermentation of pectin, DNA breaks by \( \text{H}_2\text{O}_2 \) could not be inhibited in rat colonic epithelial cells \textit{in vitro} (36).

DMH is an indirect carcinogen, which is metabolized by \( \text{P}450 \text{~2E1} \) and \( \text{1A2} \) isozforms to methylidazonium to exert its carcinogenic effect by giving rise the main DMH adduct \( \text{O}^\circ\)-methylguanamine (37). Other results suggested that beside the alkylation effect on DNA, radical oxygen species may also play a role in DMH carcinogenesis (38). However, the involvement of superoxide anion radical as reported by this study using short-term protocols which terminate 24 h after a single carcinogen application (38) appear to be a part of the very initial mechanism of DMH initiation since comparable effects were not observable by comet assays in context with our study terminating 3 weeks after the final DMH dose (K.Briviba, unpublished data). Interestingly, in our experimental design significantly elevated DNA single strand breaks in the DMH treated water group could be detected as late as 3 weeks after the final DMH application. These observations might be due to the relatively high total DMH dose (80 mg/kg) which result in a persistence of adduct formation as already shown for a susceptible mouse strain treated with variable doses of DMH (39).

The mechanism by which CloA and CleA impair the initial genotoxic event of DMH can only be hypothesized. It cannot be deduced from our results, whether the protection from DMH-mediated initial oxidation or prolonged DNA- adduct formation is due to interaction with mucosal/bacterial glucuronidase (3), DMH activating \( \text{P}450 \text{~isoenzymes} \) (4), an increased rate of 8-OhdG repair or antioxidant enzyme activity (38,40). In this context, the unchanged levels of GST-related enzyme expression as analyzed in the present study might exclude an apple juice mediated induction of phase II detoxification by those isoforms, which were shown to be involved in the chemopreventive activity of red wine and black tea polyphenols (17). Moreover, free radical scavenging during the time of DMH treatment might also be excluded as the underlying mechanism which determined the antigenotoxic effect of the juices. Although the apple juices of the present study represented a significant antioxidant capacity of \(-5.5 \text{ mM trolox equivalent, the trolox equivalent plasma antioxidant capacity (TEAC)}\) in the rats remained unchanged irrespective of apple juice intervention. This might be based on the low bioavailability of apple polyphenols as already suggested for humans (41).

In the rodent DMH model, DNA alkylation leading to DNA damage has been shown to be closely associated with increased epithelial proliferation, which taken together result in enhanced tumor induction in the distal colon (39). In the present study, in accordance with the observed antigenotoxic activity of both apple juice preparations, the DMH-induced hyperproliferation was significantly inhibited by the intervention with CloA and to a significantly lesser extent with CleA.

It is well documented, that pectin as the major dietary fibre component in apple affects epithelial hyperproliferation in different animal models of colon carcinogenesis (22,42). In these studies pectin was provided at 6% by weight in the experimental diets corresponding to an estimated total daily uptake of 720-1200 mg pectin in 12-20 g diet per animal. According to our juice analysis the mean daily pectin uptake per animal was 19.1 mg and 4.73 mg of apple pectin by CloA and CleA uptake, respectively. Owing to the relatively low pectin dosage provided by the juices when compared with pectin containing diets, we suppose that the observed antiproliferative effect of the juice preparations is not solely mediated by pectin but the relatively higher amount of pectin in CloA might further promote antiproliferative mechanisms when compared with CleA.

Consistent with our \textit{in vivo} results of antiproliferative capacity by apple juice preparations, apple polyphenol extracts exhibited strong antiproliferative activity \textit{in vitro} (12). As recently characterized by others, this \textit{in vitro} antiproliferative activity of apple polyphenolic compounds might be related to procyanidins rather than flavonoid monomers. A procyanidin-enriched apple extract showed growth inhibiting properties in a human metastatic cell line without any antiproliferative effect of comparable concentrations of flavonoid monomers (43). These data suggest that in our \textit{in vivo} experiment the apple procyanidins might be also responsible for the significant reduction of colonocyte proliferation. However, \textit{no in vivo} data are available on the antiproliferative capacity of apple procyanidins. Until now, \textit{in vivo} studies have favored enriched extracts derived from grapes (16) or grape seeds (44) and showed distinct results depending on different extract composition or intervention protocols used. Applied in a post-initiation protocol, chronic administration of complex wine extracts containing significant amounts of procyanidins did not affect epithelial proliferation in the colon (16). When applied in a short-term pre-initiation experiment, a daily dose of grape seed procyanidin extract or purified grape seed procyanidin B2 significantly reduced AOM-mediated colonic proliferation as analyzed by PCNA labelling (44). Procyanidin B2 is a major procyanidin compound of different apple cultivars (45) and is also found as a major constituent in the apple juices used for the present study. Interestingly, the estimated dosage of \(-2 \text{ mg/kg body weight of pure procyanidin B2} \) significantly reduced the AOM-mediated epithelial hyperproliferation (44) is comparable to the procyanidin dosage by the average daily uptake of the apple juices used in the present experiment.

It is well described that the growth inhibiting activity of plant polyphenols is often accompanied by enhanced apoptosis (46) which represents a parameter depending on cellular concentrations of COX-2 derived prostaglandin E2 (PGE2). Alterations in COX-2 activity and PGE2 concentrations determine resistance to apoptosis or apoptosis promotion (47). Phytochemicals with the potential as chemopreventive agents show COX-2 inhibiting activities (17). In the present pre-initiation experiment COX-2 mRNA level in the colonic mucosa of Cont was slightly but not statistically significantly elevated by DMH when compared with Cont/NaCl. This might be on account of the short-term protocol giving rise to early stages of ACFs without COX-2 overexpression when compared with the normal colon mucosa (48). Moreover, none of the apple juices further reduced the level of mucosal COX-2 mRNA after DMH treatment. Thus, the present finding of...
constant COX-2 expression in the colon mucosa irrespective of treatment with DMH and/or intervention with apple juices suggests that the observed alterations of colonic proliferation might not be directly associated with or mediated by changes in basal COX-2 expression.

In addition, we also analyzed effects of apple juice intervention on the initiation and growth of ACF. We assessed ACFs by recording their total number and the number of ACFs consisting of four or more aberrant crypts, since it has been postulated that mainly the larger ACF are more predictive for tumor development than the total number of ACF (49). However, more recently it has been considered that neither number nor size of ACFs are suitable biomarkers for colorectal cancer and thus, other ACF-related characteristics, such as mucin content (50) or beta-catenin expression (51) may be more predictive to carcinogenesis. Furthermore, it could be demonstrated that more than the general histopathological features of ACFs, the gene expression profiles are distinct and predictive for high- or low-risk ACFs with different tumorigenic potential (52). Although we are aware of the inconsistency of ACFs in their predictive value for growing out into later adenoma and further carcinoma stages, we could clearly show that the uptake of CloA but not CleA reduced the number of ACFs in the distal colon. This CloA effect was significant for ACFs which contained four or more ACFs with an ~50% reduction of large sized ACFs and a significantly reduced ACF multiplicity suggesting that the large ACFs might be more responsive to intervention with CloA.

In addition to the role of pectin in modulating proliferation and apoptosis in rat colon epithelium (22,42), diets high in fibre (e.g. 10% pectin) significantly suppressed AOM induction of ACF development (53). However, owing to the relatively low dosage of pectin provided daily by the juices in the present study, it is unclear whether pectin or other juice components such as procyanidins are primarily responsible for the anticarcinogenic properties. Others have recently reported that a procyanidin enriched extract purified from apple reduced the total number of colonic ACFs by 50% without affecting ACF multiplicity when applied in drinking water at a final concentration of 0.01% over 5 weeks post-initiation (43). Monomeric apple polyphenols did not show any significant effects in the ACF assay at comparable doses. Although we did not analyze the amount of polymeric procyanidins (degree of polymerization ≥3) in juices, the mean daily dose of procyanidin B1/B2 by CloA intake exceeded that of the CleA groups by ~45%.

Furthermore, besides these parameters locally restricted to the distal colon, we could also show that CloA had an impact on the immune system. As already demonstrated for AOM (28), DMH also significantly reduced the spleen CD4:CD8 ratio in control animals owing to a drop in CD4 and a rise in CD8 positive spleenocytes. In humans, it has been demonstrated for different types of cancers, that an increase in peripheral CD8 and a decrease in CD4 positive T-cells is generally accompanied by malignant tumor progression and further, is a valuable prognostic marker with respect to tumor recurrence or survival rate (54). As a consequence, a normalization of DMH-mediated decreased CD4:CD8 ratio through CloA intake would imply a modulation of immunoological processes associated with tumor promotion and progression.

In summary, the data presented here provide evidence that primarily CloA reduced colonic precancerous events in rats initiated for colon carcinogenesis with DMH. At present, we only can speculate about the components in CloA which could be responsible for the anticancer effects including antigno-toxic, antiproliferative and growth inhibiting properties on ACFs and immunomodulatory effects. Many apple ingredients, shown to modify cancer related processes in vitro have already been identified. However, it is difficult to evaluate the relative importance of individual constituents for the anticancer effects of the complex apple juices seen in the present study in vivo. We suggest, that the strong effect obtained by CloA is mainly due to the distinct diversity of polyphenols and non-polyphenolic compounds. These might alter their cancer preventive properties and their capacity to modulate immune parameters by antagonistic, additive and/or synergistic mechanisms.

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References

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