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Cloudy Apple Juice Is More Effective than Apple Polyphenols and an Apple Juice Derived Cloud Fraction in a Rat Model of Colon Carcinogenesis

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As recently shown, a cloudy apple juice (CloA) was effective to modulate colon cancer associated parameters in rats treated with 1,2-dimethylhydrazine (DMH). To identify the bioactive substance classes in CloA, we fractionated CloA to yield a total polyphenol (PF) and a cloud (CF) fraction consisting of proteins, fatty acids, polyphenols, and cell wall polysaccharides. Rats received water (control (Cont)) or CloA, PF, and CF separate or combined (PF−CF) ad libitum for 7 weeks starting one week before the first DMH-injection. As determined by comet assay, the DMH-induced genotoxicity in colonocytes of controls (Cont/DMH: 7.7 ± 0.5%) was significantly reduced by CloA (3.3 ± 0.3%) but not by any of the fractions. The crypt cell proliferation induced by DMH (Cont/NaCl: 7.5 ± 0.6%; Cont/DMH: 14.9 ± 0.8%) was significantly decreased by CloA (9.4 ± 0.4%), PF (12.4 ± 0.7%), CF (11.6 ± 0.4%), and PF−CF (12.4 ± 0.6%). Although not statistically significant, CloA tended to reduce the number of large aberrant crypt foci (ACF) (Cont/DMH: 19.0 ± 3.7; CloA/DMH: 12.3 ± 1.9), while none of the fractions affected ACFs. Neither CloA nor the fractions changed mRNAs of colonic cyclooxygenases (COX-1, COX-2), glutathione-associated enzymes (GST-M2, γ-GCS, GST-P), the splenocyte CD4/CD8 ratio, natural killer cell activity, and plasma antioxidant status. These results demonstrate that CloA had a higher cancer-preventive potential than the fractions and further, besides PF, identified CF as an additional bioactive fraction of CloA.

KEYWORDS: Dimethylhydrazine; genotoxicity; proliferation; aberrant crypt foci; rat; cloudy apple juice

INTRODUCTION

Estimates of the worldwide incidence and mortality of cancer clearly demonstrate that colorectal cancer is the second leading cause of cancer death with more than 500 000 deaths in 2002 (*1*). Epidemiological studies have consistently shown that a high dietary intake of fruits and vegetables is associated with a reduced risk of developing cancer (*2*). About one-third of all cancer deaths could be avoided through appropriate dietary modification by increasing the consumption of fruits, vegetables, and whole grains (*3*). Therefore, there has been growing interest to identify the bioactive components in plant foods. As fresh and processed fruits and vegetables contain high concentrations of diverse phytochemical compounds such as the carotenoids, phenolic acids, and flavonoids, much of the cancer-protective effect has been attributed to these biologically active secondary plant metabolites (*4*).

Driven by this suggestion, until now numerous in vitro studies have already shown the antioxidant (*5*−*7*), antiinflammatory (*8*), antiproliferative (*9, 10*), and apoptosis-inducing (*10, 11*) activities of polyphenols derived from flavonoid-rich foods. However, activities observed in cell culture with supraphysiological doses of the dietary polyphenols may not be relevant for the in vivo status because of the intestinal or systemic formation of (in-)active metabolites (*12*) and the limited colonic (*13*) and systemic availability of polyphenols (*14*). Because of these difficulties in extrapolating data from cell culture to in vivo cancer prevention, the possible targets of secondary plant metabolites and the mechanisms by which they act in vivo as single substances, as part of extracts or as the complete food, are unclear.

Several vegetable and fruit products including juices are reported to increase the systemic antioxidant levels (*15*−*18*), to modulate distinct systemic immune parameters (*17, 19*), and

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to reduce the oxidative DNA damage in lymphocytes in humans (*17*). In rats, a cloudy apple juice showed immunomodulatory activities and further antigenotoxic, antiproliferative properties in colonocytes and significantly reduced preneoplastic lesions in a model developed to study colon cancer (*20*).

Because of these observations of a strong in vivo cancer-preventive activity by the cloudy apple juice, the aim of the present study was to identify the fractions which contained the bioactive substances of the juice. As we proposed that additive or synergistic effects of apple juice constituents might be responsible for the observed anticancer activities (*20*), instead of using single polyphenolic or fiber substances, we fractionated the cloudy apple juice to yield two complex apple juice fractions: a total polyphenol (monomeric and polymeric polyphenols) fraction and the heterogeneous cloud fraction consisting of proteins, fatty acids, polyphenols, and cell wall polysaccharides. Besides the cloudy apple juice used as a positive control, these analytically characterized fractions were applied either separately or in combination in frame of a preinitiation design in a rat model initiated with 1,2-dimethylhydrazine (DMH). This animal model is mimicking some of the alterations encountered during colon carcinogenesis as colonocyte DNA damage, hyperproliferation, and the induction of preneoplastic lesions in the distal colon.

MATERIALS AND METHODS

Apple Juice Preparations. The apple juice was produced by the Geisenheim Research Center. The following apple varieties were used: cv. *Topaz* (25%), cv. *Bohnapfel* (17.5%), cv. *Winterrambour* (22.5%), cv. *Bittenfelder* (15%) and mixed table-apple varieties (20%). The fruits were further processed as described recently to yield the cloudy juice formulation (*20*). Bottled apple juice was stored at 4 °C until further use.

Polyphenol Extract. The extract was produced from the above juice using an adsorber resin technique subsequent to the clarification of the cloudy juice. One hundred liters of the apple juice was loaded onto a chromatographic column (100 × 10 cm, Pharmacia BPG 100, Freiburg, Germany) packed with 5 L of adsorber resin (XAD16 HP, Rohm and Haas, Arnsberg, Germany). The resulting juice effluate was colorless because of the adsorption of the polyphenols to the resin. Juice constituents like sugars, organic acids, and minerals were washed out with 6 bed volumes of distilled water. Polyphenols were eluted with 3 bed volumes of ethanol (96%). The ethanolic fraction was gently concentrated by evaporation, was transferred into the water phase, and was finally freeze-dried.

High-Performance Liquid Chromatography (HPLC) Analysis of Juice and Polyphenol Extract. 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 × 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 (*21*). Detection wavelengths were 280, 320, and 360 nm. The juice was injected directly after centrifugation and 0.45-μm filtration with cellulose acetate membranes. Quantification was carried out using peak areas from external calibration curves.

Table 1 shows the dosages of polyphenolic compounds in the polyphenol and the CloA group. The HPLC analysis revealed a similar mixture of characteristic apple polyphenols in the CloA and the PF extract with a comparable daily uptake of polyphenols between the two groups. Daily dosage of polyphenols in the PF−CF group was similar to the PF group since fluid uptake of 22.0 mL in the PF−CF group is approximately the same as in the PF group (21.8 mL) with the same polyphenol concentration.

Cloud Fraction. Cloud particles were isolated from 125 L of the above-described cloudy apple juice. The juice was concentrated in a Sartocon beta ultrafiltration system (Sartorius, Göttingen, Germany) using a molecular cutoff of 300 kD at a feed pressure of 3.6 bar and a retentate pressure of 0.5 bar. The effective membrane area was 1.2 m^2, and the retentate temperature was kept at 30 °C throughout the whole process. The concentration process was stopped at a retentate volume of 10 L. The resulting juice retentate was diafiltered with 200 L of distilled water. During this process, the water feed supply was adjusted equally to the permeate flux. After diafiltration, the retentate (13.7 L) was quantitatively removed from the system and was frozen at −22 °C. The retentate contained 6.8 g/L cloud particles (determined gravimetrically after freeze drying) or 0.75 g/L referring to the juice source.

Table 1. HPLC Analysis of Polyphenolic Compounds in the Polyphenol Fraction (PF) and the Cloudy Apple Juice (CloA)

	PF		CloA	
polyphenol	[mg/L][a]	(mg/kg bw)[b]	[mg/L][a]	(mg/kg bw)[b]
procyanidin B1	7.0	(0.64)	5.8	(0.61)
procyanidin B2	15.1	(1.39)	20.9	(2.21)
epicatechin	19.2	(1.77)	17.9	(1.90)
phloretin-2′-galactoside	9.0	(0.83)	6.3	(0.67)
phloretin-2′-xyloglucoside	69.5	(6.39)	59.8	(6.33)
phlorizine	27.9	(2.57)	19.7	(2.09)
chlorogenic acid	181.5	(16.69)	155.9	(16.51)
3-coumaroyl quinic acid	9.5	(0.87)	1.9	(0.20)
caffeeic acid	4.8	(0.44)	0.6	(0.06)
4-coumaroyl quinic acid	77.3	(7.11)	76.7	(8.12)
quercetin-3-rutinoside	2.6	(0.24)	0.5	(0.05)
quercetin-3-galactoside	0.8	(0.07)	1.7	(0.18)
quercetin-3-glucoside	1.4	(0.13)	1.0	(0.11)
quercetin-3-rhamnoside	4.1	(0.38)	2.5	(0.26)
sum	429.7	(39.53)	371.2	(39.31)
total phenols (Folin)	667.0		667.0	

[a] Data represent the mean of three independent HPLC measurements. [b] The mean daily polyphenol uptake during the experiment as detailed in parentheses is calculated with 237.9 g as the mean body weight and a mean daily fluid intake of 21.8 mL PF (667.0 mg/L) and 25.1 mL CloA during the intervention.

Analysis of the Cloud Fraction. The lipid fraction was extracted from the cloud material (100 mg) with 40 mL chloroform/methanol (2/1, v/v) for 24 h under magnetic stirring. The resulting suspension was filtered through a weighed solvent-resistant membrane filter (0.2 μm) and was rinsed two times with 4 mL of chloroform/methanol into a 50-mL graduated flask. The filter with the fat-free residue was dried under vacuum and was weighed. The weight loss to the source material corresponded to the lipid moiety. Fatty acids were identified after saponification and methylation to fatty acid methyl esters (FAME) with trimethylsulfoniumhydroxide (TMSH) in one step according to Matter (*22*). FAMEs were determined by GCMS after split injection onto a 30 m × 0.25 mm i.d. Zebron ZB-Wax column (film thickness 0.25 μm, Phenomenex, Aschaffenburg, Germany). Quantification was carried out using the internal standard nonadecanoic acid (Sigma-Aldrich, Taufkirchen, Germany).

The carbohydrate fraction was analyzed after 1 M sulfuric acid hydrolysis and separation of the released sugars by means of HPAEC/PAD (*23*).

Protein content was determined after hydrochloric acid hydrolysis and amino acid detection with HPAEC/PAD. About 10 mg of the material was hydrolyzed in 1 mL of 6 mol/L HCl at 120 °C over 24 h. Hydrochloric acid was removed under a stream of compressed air. The residue was taken up in 50 mL of water. An aliquot of the solution was filtered through 0.45-μm membranes into HPLC-vials. For HPAEC, a Dionex BioLC (Idstein, Germany) equipped with an AminoPac PA10 250 × 2 mm column and an AminoPac PA10 50 × 2 mm guard column was used. Separation was carried out with a sodium hydroxide/sodium acetate gradient (0.25 mL/min) after a 10-μL injection. Detection mode was pulsed amperometric, and quantification was based on external standard substances (amino acid standard kit, Sigma-Aldrich).

Because of the encapsulation of the polyphenols in the cloud particles, the attempt failed to liberate native polyphenols from the cloud material by mild extraction with organic solvents for subsequent HPLC identification and quantification. Hot potassium hydroxide turned out as the only solvent for a complete dissolution of the material. About 5

mg of the material was weighed into a sealable tube, was dissolved in 2 mL 0.5 mol/L KOH, and was kept in a boiling water bath for 10 min. An aliquot of 100 μL was analyzed for total polyphenol content with the Folin−Ciocalteu-reagent on the basis of a (+) catechin calibration (*24*).

Ash was determined gravimetrically after burning the cloud material in platinum dishes at 550 °C in a laboratory furnace. Cations were analyzed by means of graphite furnace atomic adsorption spectrometry (AAS Perkin-Elmer 4100, Freiburg, Germany).

The processed cloudy juice contains variable amounts of colloidally dissolved pectic substances with an estimated concentration of 0.75 g/L juice. The analysis of the cloud fraction revealed 48.6% lipids consisting of approximately 70% fatty acids (33% linoleic acid, 19% palmitate, 18% others) and 30% of unidentified lipid constituents (suggested phytosterols, phospholipids), 24% proteins consisting of 26% arginine and 20% lysine as the major aminoacids, 18% of unidentified polyphenols, and 7.4% of cell wall polysaccharides. The range of the cloud particle size is 1−5 μm.

Animals. Male Fischer 344 (F344) rats ($n = 72$; 100 g body weight on arrival) were obtained from a licensed animal supplier (Harlan Winkelmann, Borchen, Germany) and were housed in a temperature- and humidity-controlled animal unit under ambient temperature of 21 ± 2 °C and a 12 h−12 h light−dark cycle. The rats were allowed to acclimatize for 7 days during which they consumed ad libitum tap water. Because of the described effects of isoflavones on AOM-induced development of aberrant crypt foci (*25*), a standard rat cereal-based diet free of soy protein was used for the present study (R/M-H; Ssniff; Soest, Germany) and HPLC-analysis confirmed the absence of potential isoflavone contamination (data not shown). The cereal-based diet consisted of 56.7% carbohydrates, 19.0% protein, 3.3% fat, and 4.9% fiber (12.2 KJ/g metabolizable energy) (*20*).

Experimental Protocol. One week after arrival, the rats ($n = 72$) were randomly assigned to four treatment groups ($n = 12$/group) receiving either cloudy apple juice (CloA), the polyphenol (PF), or the cloudy fraction (CF) separately or PF and CF in combination (PF−CF) ad libitum until the end of the experiment. A control group (Cont; $n = 24$) received tap water. PF and the CF were freshly diluted in drinking water at concentrations according to the CloA total polyphenol concentration (667 mg/L) and cloud content (0.75 g/L). The juice and the diluted extracts were freshly provided daily and fluid consumption was recorded. Body weight was recorded twice per week. One week after starting the juice and extract intervention, half of the control group ($n = 12$ animals) and all intervention groups received intraperitoneal injections of DMH (20 mg/kg body weight) four times at one-week intervals whereas the other half of the control group ($n = 12$) received 0.9% NaCl intraperitoneally. Three weeks after the last injection, animals were sacrificed by decapitation and biological samples were prepared and processed as detailed below for the respective analytical methods.

The experimental protocol was performed in accordance with the guidelines of the Ethics Committee responsible for the administrative district of Karlsruhe.

Single-Cell Microgel Electrophoresis Assay (Comet Assay). Immediately after decapitation of the animals ($n = 6$/group), the entire colon was rapidly removed and the distal colon was dissected from the proximal colon; the limit of the proximal portion was defined by the mucosal fish bone pattern. Processing of one longitudinal half of the distal colon and comet assay procedure was in accordance with a previously published protocol (*20*). Fifty cells of each slide (150 cells per animal) were analyzed using the imaging software of Perceptive Instruments (Halstead, United Kingdom). The amount of damaged DNA was expressed as the percentage of DNA in the tail (tail intensity).

mRNA Analysis. Mucosal cells of a longitudinal half of the distal colon of $n = 6$ animals/group were collected in 1.8 mL of 1×PBS by carefully scraping the luminal colon mucosa with a glass slide. Total RNA was extracted and further processed by using reverse transcription (RT) polymerase chain reaction (PCR; PTC 200, MJ Research Inc., Waltham MA) technology according to recently published protocols (*20*). Sense and antisense primers specific for COX-2, COX-1, γ-GCS, GST-M2, GST-P, and GAPDH as the internal standard for normalization were used for PCR-based amplification. Primer sequences for amplification of the glutathione (GSH)-related enzyme cDNAs were taken from a previously published protocol (*26*).

Primers were tested for linearity over cycle number, and analyses were all carried out in the linear portion of the curve, therefore allowing semiquantitative analysis of mRNA amount. Ethidium bromide stained bands were quantified using an automated computer-based image analysis system (QuantityOne/FluorS Imager; Biorad, München, Germany). Quantities of each PCR product were normalized to the corresponding GAPDH amplicon.

Assays of Colonic Cell Proliferation and Aberrant Crypt Foci. To investigate the proliferative activity of epithelial cells, $n = 6$ animals/group received a 30 mg/kg intraperitoneal injection of 5-bromodeoxyuridine (BrdU) 1 h before sacrifice. After excision of the distal colon and carefully flushing with prewarmed 0.9% NaCl, the mucosa was pinned flat on a paraffin wax block in a Petri dish, mucosal side up, and was fixed in 4% PBS-buffered formaline solution for minimum 1 h (BrdU assay) or 16 h (aberrant crypt foci (ACF) assay).

The distal 5-mm strip of the colonic mucosa specimen was resected for BrdU assay and after a further overnight fixation the remaining colonic mucosa was rinsed in PBS, was stained with 0.2% methylene blue for 5 min, and was examined under an inverse microscope (Zeiss Axiovert 100) at 100× magnification. Crypts were considered aberrant if they were visibly enlarged and protruding when compared to 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 strips were paraffin-embedded, were sectioned, and were further processed for immunohistochemical visualization of incorporated BrdU as described recently (*20*). After immunostaining, 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-labeled and -unlabeled epithelial cells randomly selected from entire tissue sections.

Immunological Analysis. Immune cell suspensions from resected spleens ($n = 4$ animals/group) were prepared and splenocytes were isolated as previously described (*20*, *27*).

The expression of cell surface markers on the immune cells of spleen was investigated by immunofluorescence using phycoerythrin-conjugated mouse antirat monoclonal antibodies to CD4 (Caltag, Hamburg, Germany) and fluorescein-conjugated mouse antirat 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 leukaemia cell line, YAC-1, was used as target cell line (*27*).

Antioxidant Status. Total plasma antioxidant activity in rats was determined by using the FRAP assay with minor modifications as decribed before (*16*) and the improved decolorization TEAC assay (*28*). Inter- and intraassay variability was less than 5%, respectively. The plasma concentration of malondialdehyde, which is a marker for in vivo lipid peroxidation because of the formation of reactive species (e.g., reactive oxygen species), was analyzed using HPLC and fluorescence detection according to a previously published protocol (*29*).

Statistical Analysis. All data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was accomplished using ANOVA and Newman−Keuls multiple group comparison tests to identify significant differences between groups. P-values <0.05 were considered significant.

RESULTS

General Observations. Total energy intake and body weight gain did not differ between groups. During the experiment, body weight curves were similar in all groups independent of DMH-treatment. Without considering the available energy from the apple pectin (834 mg/L) (*20*), the mean daily caloric intake by the cloudy apple juice (CloA) intervention was 46.3 KJ (CloA

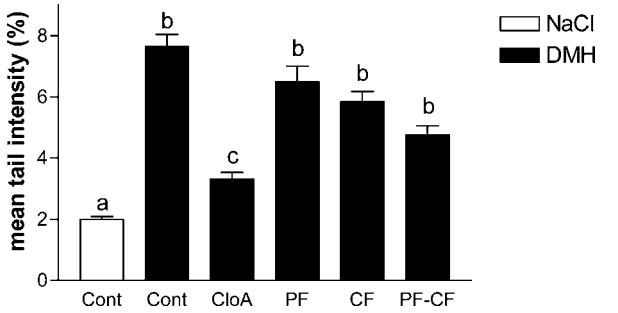

Figure 1. Comet assay results of genotoxic DNA damage expressed as the tail intensity (mean ± SEM; $n = 6$/group) analyzed in mucosal cells of the distal colon. Columns not sharing the same superscript differ significantly.

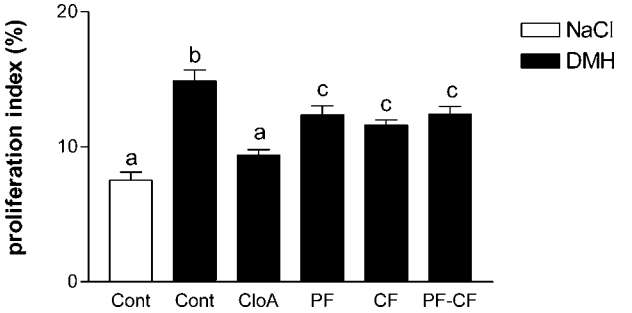

Figure 2. Proliferation index in the distal colon as the percentage of BrdU-positive cells (mean ± SEM; $n = 6$/group) determined within 25 randomly chosen crypts per animal. Columns not sharing the same superscript differ significantly.

consumption; 25.1 mL/animal/day; 1845.1 KJ/L) per animal. CloA rats compensated the increased juice-derived energy by a decreased solid food intake compared to controls (Cont) receiving tap water ($p < 0.01$, Cont: 19.9 ± 1.4 g; CloA: 16.2 ± 1.1 g; 12.2 KJ/g), which resulted in a comparable mean daily total energy intake (Cont: 242.8 KJ; CloA: 243.9 KJ) and growth rate independent of the treatment group.

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**). The treatment with DMH induced a significant increase in DNA-damage in Cont when compared with Cont/NaCl. This strong genotoxic effect of DMH was significantly reduced by the intervention with CloA ($p < 0.01$). None of the intervention groups receiving the apple juice fractions either separate or combined significantly reduced the genotoxic effect of DMH.

Gene Expression. The treatment with DMH did not significantly change mucosal COX-1 and COX-2 mRNA levels in the distal colon of control animals. Further, neither CloA nor the fractions used for intervention lead to significant gene expression changes in groups treated with DMH (data not shown). Moreover, besides mRNAs of cyclooxygenases, we also analyzed transcripts coding for the glutathione (GSH)-related enzymes γGCS, GST-P, and GST-M2, which are crucial factors in determining the sensitivity of cells to a broad spectrum of toxic chemicals and which have previously shown to be modulated by black tea and red wine polyphenols applied in an AOM postinitiation protocol (*26*). Of the GSH-related enzyme mRNAs analyzed, the gene expression of GST-M2, γGCS, and GST-P was not affected by DMH or by either of the interventions (data not shown).

Epithelial Proliferation. In all animal groups analyzed, BrdU was exclusively incorporated in crypt cell nuclei located at the bottom of the colon crypts. Compared to the Cont/NaCl group, DMH significantly increased the epithelial proliferation in the Cont group (**Figure 2**, $p < 0.001$). This observed hyperproliferative effect of DMH was significantly reduced by intervention with CloA ($p < 0.001$), the polyphenol fraction (PF; $p < 0.05$), the cloud fraction (CF; $p < 0.01$), or PF−CF ($p < 0.01$). However, when compared to the Cont group receiving NaCl, the proliferative activity in the colon epithelium of DMH-treated groups with either PF, CF, or PF−CF intervention was still significantly elevated ($p < 0.001$, PF/DMH vs Cont/NaCl; $p < 0.001$, CF/DMH vs Cont/NaCl; $p < 0.001$, PF−CF/DMH vs Cont/NaCl) while intervention with CloA reduced the proliferative activity to control levels ($p > 0.05$, CloA/DMH vs Cont/NaCl).

Aberrant Crypt Foci. There were no aberrant crypt foci (ACF) in the distal colon of the NaCl treated Cont/NaCl group.

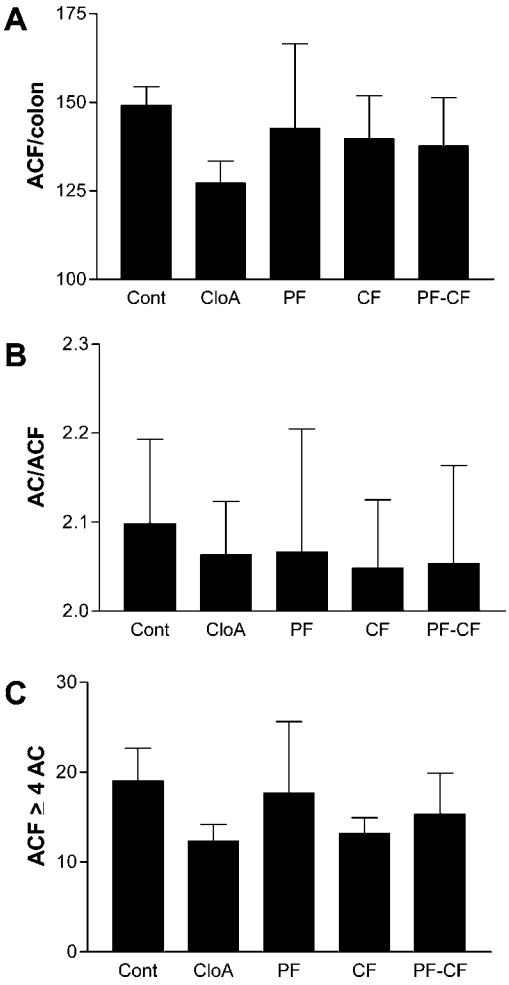

Figure 3. Analysis of aberrant crypt foci number (**A**, ACF) and size (**B**) and the number of large ACF with four or more aberrant crypts (**C**) in the distal colon mucosa of DMH-treated animals (mean ± SEM; $n = 6$/group).

The total number of large ACFs (≥4 crypts/foci per colon) was reduced by 35% in animals with CloA intervention when compared to the Cont/DMH group (**Figure 3A**). However, individual variability was considerable and a statistically significant reduction of ACF multiplicity (**Figure 3B**) and the number of large ACFs were not observed in any of the intervention groups (**Figure 3C**).

Immunological Parameters.To evaluate potential effects of the apple juice preparations on parameters of systemic immunity, we analyzed lytic activity of NK cells isolated from spleen and quantitated CD4- and cytotoxic CD8-positive subsets of splenocytes.

Table 2. CD4/CD8 Ratio and Natural Killer Cell (NK) Activity of Splenocytes[a]

	Cont/NaCl	Cont/DMH	CloA/DMH	PF/DMH	CF/DMH	PF-CF/DMH
CD4/CD8	2.7 ± 0.4	2.2 ± 0.3	2.4 ± 0.5	2.0 ± 0.2	2.0 ± 0.3	2.0 ± 0.3
NK activity	40 ± 6	44 ± 6	47 ± 3	39 ± 3	41 ± 6	45 ± 6

[a] Results are presented as the means ± SEM of $n = 4$ animals per group. CloA, cloudy apple juice; PF, polyphenol fraction; CF, cloud fraction; PF–CF, polyphenol and cloud fraction.

There was no statistically significant effect on NK cell activity and CD4:CD8 ratio of either the DMH-treatment or the intervention in any of the groups (**Table 2**).

Antioxidant Status. As determined with the FRAP and TEAC assays, the plasma total antioxidant capacity was not modified by DMH or the dietary treatment. The plasma level of malondialdehyde as a marker of lipid peroxidation did not change by any of the interventions (data not shown).

DISCUSSION

The present study is the first attempt to analytically and functionally characterize the heterogeneous cloud juice fraction (CF) which until now was not taken into consideration as a bioactive fraction of juices showing in vivo cancer-preventive properties.

Recent studies in our laboratory have demonstrated that a cloudy apple juice (CloA) reduced DNA damage, hyperproliferation, and the number of large aberrant crypt foci in the distal colon of DMH treated rats (*20*). The present study was designed to determine whether the apple juice polyphenol fraction (PF) or CF represent the bioactive fraction in the model of DMH-induced colon carcinogenesis.

Data of the present study confirm our previous findings of reduced DNA damage, hyperproliferation, and the number of large aberrant crypt foci in the distal colon of DMH treated rats because of cloudy apple juice intervention. Furthermore, we demonstrated inhibition of proliferation by separate or combined intervention with PF and CF but no modulation of genotoxicity and development of preneoplastic lesions. Thus, both fractions contain bioactive compounds which inhibited processes involved in DMH-induced carcinogenesis although none of the fractions were able to completely mimic the cancer-preventive effects of CloA.

The most promising candidates among bioactive apple constituents are the procyanidins which were shown to effectively modulate cancer-related processes in vitro and in vivo. Apple procyanidins but not the apple polyphenolic monomers were effective in inhibition of colonic cancer cell growth mediated by activated MAPK pathways and activation of apoptosis-related caspase-3. Further, the highly pure procyanidin extract significantly reduced AOM-induced aberrant crypt foci in the rat distal colon (*10*). However, our earlier results showed that a clear apple juice, which contained the same amount of procyanidins than the cloudy juice, did not reduce large ACFs and was less active regarding antiproliferative and antigenotoxic effects in the DMH treated rat (*20*). This indicates that cloudy apple juice contains cancer-preventive compounds in addition to the procyanidins.

The anticancer effects of apple procyanidins (*10*) or the apple polyphenol fraction as shown in the present study might indicate effects directed from (1) the systemic side by polyphenols/bioactive metabolites absorbed in the small intestine or (2) bioactive metabolites reaching the colon or (3) the bioactivity of polyphenols in modulating colonic bacterial contents as recently shown by Dolara et al. (*30*) for wine polyphenols. At present, it is not clear from our results by which mechanisms the complex apple juice, the polyphenols, or their metabolites affect colon carcinogenesis by modulating the colonocyte proliferation. Several activities of polyphenols or the cloud constituents should be considered in respect to the demonstrated antiproliferative activity as presented in this study. A polyphenol antioxidant scavenging of free radicals often reduce the oxidative DNA damage with subsequent consequences on epithelial proliferation as recently published for single apple polyphenols and complex apple extracts (*5*, *31*) for short-chain fatty acids as fermentation products of dietary fiber in vitro (*32*) and in vivo (*33*) or for wine polyphenols in vivo (*30*). Confirming our recent results also in the present study, CloA significantly reduced colonocytes DNA damage by DMH (*20*) while all the fractions, either separate or in combination, did not affect genotoxicity. This antigenotoxic effect of CloA was not paralleled by alterations in systemic antioxidant status since CloA, similar to the fractions, was ineffective in changing the plasma antioxidative capacity as analyzed by different assay systems. The chemical properties of polyphenols and dietary fiber may also give them detoxifying and antiinflammatory properties (*34*), and this should be considered with respect to mechanisms which modulate genes related to these processes. Indeed, recent in vitro studies have shown that mixtures of apple polyphenols upregulated different glutathione S-transferases genes concomitantly to growth inhibition in colon cancer cells (*9*). In contrast to these in vitro data, in the present in vivo study neither CloA nor the fractions altered the expression of colonic genes coding for gluthatione-associated enzymes and genes which are involved in inflammatory response pathways or cell cycle regulation such as the cyclooxygenases (*35*).

In several studies dealing with the cancer-preventive aspect of fruit or juice constituents, the main focus has been on the polyphenolic constituents, which are present at particulary high concentrations in apple (*36*) and apple juice (*37*). In the present study, our results revealed that the polyphenols alone were not responsible for the observed antiproliferative effect of the cloudy juice as also CF in a juice-equivalent dosage showed antiproliferative efficiacy. By our analytical results, we could show that the cloud particles are heterogeneously composed of lipids, proteins, polysaccharides, and polyphenols with a particle size ranging from 1 to 5 μm. These particles are hydrocolloids with an architectural structure composed of a positively charged nucleus of proteins which is surrounded and complexed by negatively charged polysaccharides such as pectins. As a whole, these colloids are encapsulated by a hydrate shell. Recent studies have shown that cell wall polysaccharides develop secondary structures forming hydrophobic pockets to complex polyphenols during juice processing (*38*) which might be the reason for the relatively high polyphenol content of approximately 20% as analyzed in CF. Because of the strong interaction of polyphenols and polysaccarides, we were not able to analytically characterize the polyphenolic constituents in the cloud. However, on the basis of recent data, it is most suggestible that the polyphenols derived from CF belong to the group of polymeric procyanidins since the affinity constants of polyphenols with apple pectin were highest with high molecular weight procyanidins (*38*). Monomeric apple polyphenols such as hydroxycinnamic acids and (−)-epicatechin did not bind to apple cell wall polysaccharides (*39*). As pectins are relatively stable throughout the gastric and small intestinal passage and are finally metabolized in the colon, we hypothesize that these cloud colloids might serve as vectors transferring the entrapped procyanidins into the colon under

protection from absorption in the small intestinum. On the basis of this hypothesis, future research giving a detailed functional evaluation of CF will show the relative importance of this juice fraction compared to the classical freely accessible polyphenols with regard to a health-promoting cancer-preventive bioactivity.

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