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Tumour formation in multiple intestinal neoplasia ($Apc^{Min/+}$) mice fed with filtered or unfiltered coffee

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Abstract

Background: The aetiology of colorectal cancer has strong dietary links, and there may be an association between coffee and colorectal cancer risk.

Objective: To study the effects of filtered (low levels of kahweol/cafestol) and unfiltered (high levels of kahweol/cafestol) coffee on tumour formation in multiple intestinal neoplasia ($Apc^{Min/+}$) mice.

Design: $Apc^{Min/+}$ mice ($n=11$ per group) were fed for 9 weeks with 10% w/w of these two types of coffee. Coffee was served as a dietary ingredient mixed with a semi-synthetic AIN-93G-based diet. Plasma levels of caffeine and paraxanthine were used as compliance markers. At the end of the feeding period intestinal tumour number and size were determined. The levels of β-catenin and cyclin D1, two cell-signalling proteins important to the progression of neoplasia, were also analysed in the tumour tissue.

Results: Plasma caffeine and paraxanthine concentrations were 3.2 ± 1.4 and 1.7 ± 0.4 μmol l^{-1} in the filtered coffee group and 3.6 ± 2.3 and 1.6 ± 0.6 μmol l^{-1} in the unfiltered coffee group. The level of plasma xanthines was below detection in the control group. The total number of tumours was equal between the dietary groups: 29 for the control, 30 ($p=0.767$) for the filtered coffee and 29 ($p=0.430$) for the unfiltered coffee groups. The levels of β-catenin and cyclin D1 in the nuclear fraction of the tumour tissue were also the same between the groups.

Conclusions: Filtered or unfiltered coffee (10% w/w) does not exert antitumorigenic activity in $Apc^{Min/+}$ mice or change β-catenin and cyclin D1 signalling in the adenoma tissues. The results suggest that coffee does not change neoplasia progression in this animal model.

Keywords: *$Apc^{Min/+}$ mice*; *caffeine*; *coffee*; *colon cancer*; *paraxanthine*

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Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide (1). It occurs mainly sporadically and environmental factors, including diet, are strongly implicated in its aetiology (2). Diets high in vegetables, fruits, and perhaps fibre, or low in fat and red meat, have been suggested to be associated with a decreased risk of CRC. The association between coffee and CRC risk is a matter of controversy. The pooled results of 12 case–control studies showed a significant 28% reduction in CRC risk for a high versus low category of coffee consumption [pooled relative risk (RR) = 0.72, 95% confidence interval (CI) 0.61–0.84] (3). In addition, a meta-analysis, including five cohort and the above-mentioned 12 case–control studies, reported a significant pooled relative risk of 0.76 (95% CI 0.66–0.89) for a high versus low category of coffee consumption. However, a review of case–control studies showed inconsistent results between the coffee consumption and the risk of colon cancer (4). Cohort studies have not shown any reduction in CRC risk associated with coffee (3–6).

Animal studies on the effects of coffee constituents, such as caffeine, kahweol and cafestol, on colon tumorigenesis, have also shown conflicting results. Administration of caffeine at 0.1% level in drinking water enhanced heterocyclic amine-induced aberrant crypt number (7) and DNA adduct formation in the rat colon (8). Caffeine at a dose of 0.044% in drinking fluid had no inhibitory effect on

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tumour formation in the small intestine or colon of $Apc^{Min/+}$ mice (9). In contrast, coffee oil and its major constituents, kahweol and cafestol, decreased the adenocarcinoma frequency in the colon of 1,2-dimethylhydrazine-treated rats, and inhibited the formation of mutagen-DNA adducts in the colonic mucosa (10, 11). Data obtained from animal studies and human interventions indicate that some putative chemopreventive mechanisms of coffee and its components could be related to the induction of several phase II xenobiotic metabolizing enzymes such as glutathione *S*-transferase and *N*-acetyltransferase, and protection against DNA damage caused by mutagens (11–16).

Colon carcinogenesis can be studied either by inducing aberrant crypt foci or tumour formation with chemical carcinogens or by using mouse models with defects in the *Apc* gene. One such mouse model for human familial adenomatous polyposis (FAP) is multiple intestinal neoplasia ($Apc^{Min/+}$) mouse (17). $Apc^{Min/+}$ mice have a point mutation in the *Apc* allele, which in turn causes truncated Apc protein formation. Mutations in the *APC*/*Apc* gene lead to the accumulation of hypophosphorylated β-catenin protein in the cytosol and later in the nucleus, where β-catenin together with transcription factors (18) constitutively activate the expression of target genes such as *C-myc* and *cyclin-D1* (19, 20). Mutations in the *APC* gene predispose people to CRC, whereas in $Apc^{Min/+}$ mice, owing to their short lifespan, most of the tumours are benign adenomas. This experiment studied the possible chemopreventive effect of filtered or unfiltered coffee (10% w/w) on tumour formation in $Apc^{Min/+}$ mice. Both filtered and unfiltered coffees contain caffeine, but the concentration of kahweol and cafestol depends on brewing methods (21). Unfiltered coffee, which represents the traditional type of coffee consumed particularly in Scandinavia, contains cafestol and kahweol, whereas filtered coffee mostly lacks these compounds.

Materials and methods

Animal experiment

C57BL/6J–$Apc^{Min/+}$ mice were bred at the Laboratory Animal Centre of the University of Helsinki, Finland. Mice positive for the $Apc^{Min/+}$ genotype, as determined by polymerase chain reaction (PCR) amplification of weaning tail biopsy DNA (22), were assigned randomly to the experimental diets at a mean age of 34 ± 2 days (5 weeks) with 11 mice per group. Each diet group contained seven males and four females. Animals were housed in plastic cages in a temperature- and humidity-controlled animal facility, with a 12 h light/dark cycle. They had free access to the experimental semi-synthetic diets and tap water for the feeding period of 9 weeks. Fresh diets were provided four times per week. Body weights were recorded weekly. The experimental protocol was approved by the Laboratory Animal Ethics Committee of the University of Helsinki.

Diets

All the experimental diets were semi-synthetic AIN-93G-based (23) high-fat diets. The fat concentration (20 g per 100 g) and fat composition of the experimental diets were designed to approximate those in a typical Western-type diet; the diet provided intakes of saturated, monounsaturated and polyunsaturated fatty acids in an approximate ratio of 3:2:1 (24). Three diets were included: a control diet, a filtered coffee diet (10% w/w) and an unfiltered coffee diet (10% w/w). All diets were similar with respect to protein (20%), fat (40%) and carbohydrates (40%) on an energy basis (kJ). The filtered coffee was prepared in a regular procedure by adding 14 g of coffee per 1.25 dl (1 cup) of water and using a paper filter. The unfiltered coffee was prepared by boiling ground coffee beans with water (14 g of coffee per 1.25 dl). After cooling, the unfiltered coffee was decanted. The coffee diets were prepared by adding to the control diet 10% (w/w) of filtered or unfiltered coffee beverage. Coffee was served as a dietary ingredient and not a drink because it was found that coffee drink separated into layers in a drinking bottle. Food consumption per mouse was estimated to be 2.4 g per day. Therefore, the estimated amount of coffee consumed per body weight of the animals was approximately equivalent to 0.6 litres per day for humans. The composition of the experimental diets is shown in Table 1.

Intestinal tumour scoring and tissue samples

At the age of 14 weeks, the mice were killed by carbon dioxide asphyxiation. Blood was collected from the caudal vena cava into heparinized tubes and centrifuged at $6000 \times g$ for 1 min, after which plasma was stored at -70°C. The small intestine, caecum and colon were removed, opened along the longitudinal axis and rinsed with ice-cold saline.

Table 1. Composition of experimental diets (g kg^{-1} diet)[a]

Ingredient	Control	Filtered coffee	Unfiltered coffee
Casein	236.2	212.6	212.6
Dextrose	479	431.1	431.1
Butter	148.9	134.0	134.0
Sunflower oil	13.3	12.0	12.0
Rapeseed oil	62.2	56.0	56.0
Mineral mix AIN-93-MX	41.6	37.4	37.4
Vitamin mix AIN-93-VX	11.8	10.6	10.6
L-cystine	3.6	3.2	3.2
Choline chloride	3.6	3.2	3.2
Tert-butylhydroxyquinone	0.014	0.014	0.014
Coffee[b]		100	100

[a]Casein was obtained from Kainuun Osuusmeijeri (Sotkamo, Finland), dextrose from Six Oy (Helsinki, Finland), mineral and vitamin mix from Harlan Teklad (Madison, WI, USA), l-cystine, choline chloride and tertiary butylhydroxyquinone from Yliopiston Apteekki (Helsinki, Finland). Butter, sunflower oil, rapeseed oil and ground coffee beans for filtered and unfiltered coffee were from a local market.

[b]See Materials and methods.

The small intestine was divided into five sections of equal length. The caecum and colon were kept together. The small intestine and colon and caecum were then spread flat on a microscope slide. The number, diameter and location of intestinal tumours were determined with an inverse light microscope with a magnification of 67 × by two observers blind to the dietary treatment. The minimum detection limit of the adenoma diameter was 0.3 mm. Adenoma tissue was excised, and the normal-appearing mucosa tissue was scraped. Tissues were snap-frozen in liquid nitrogen and stored at −70°C for further analysis.

Plasma xanthine analysis

Plasma concentrations of caffeine, paraxanthine, theophylline and theobromine were determined to verify coffee intake. The analyses were performed by a new method based on liquid−liquid extraction and high-performance liquid chromatography (HPLC). Fifty microlitres of plasma was incubated with 5 µl of β-glucuronidase/sulfatase solution, 10 mM of ascorbic acid and 85 mM of sodium acetate for 18 h to cleave potential glucuronide and sulfate conjugates. Fifty microlitres of sodium acetate buffer (0.7 M, pH 2) and 1 ml of ethyl acetate were added, and xanthines were extracted into the upper phase during thorough mixing. The procedure was repeated three times, whereafter the upper phases were combined and dried down under nitrogen. The dried sample was dissolved into the HPLC mobile phase. Xanthines were then separated by HPLC. The equipment consisted of Agilent 1100 Series components including quaternary pump, autosampler, degasser and variable-wavelength ultraviolet (UV) detector (Palo Alto, CA, USA). Chromatographic separations were achieved using an Agilent Eclipse XDP-C18 (5 µM, 4.6 × 150 mm) (Palo Alto, CA, USA). The mobile phase consisted of 15% methanol in 10 mM sodium acetate buffer (adjusted to pH 5 with orthophosphoric acid). The flow rate was set at 1.5 ml min^{-1} and the wavelength at 274 nM. The recovery levels of the four xanthines were >90%. Intra-assay and interassay precision was below 4% and 9%.

Western blot analysis

The adenoma tissues of the small intestine were fractionated into nuclear, cytosolic and membranous fractions as described by Pajari et al. (24) for Western blot analysis. Normalized amounts of the nuclear proteins (5–10 µg), and constant amounts of rat brain or TMK (a human gastric cancer cell) homogenate (controls for interassay variation) were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and each sample was run twice, the duplicates being loaded on a different gel. The proteins were blotted onto a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech, UK) at 100 V for 1 h, and blots were blocked with 5% non-fat milk powder in Tris-buffered saline containing 0.1% Tween (TBS-Tween) overnight at +4°C. Antibodies against β-catenin (sc-7199) and a horseradish peroxidase (HRP)-conjugated secondary antibody (sc-2030) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cyclin D1 was from NeoMarkers (Lab Vision, Fremont, CA, USA). The blots were first probed with the primary antibodies for 2 h at room temperature, washed three times with TBS-Tween and incubated with the HRP-conjugated secondary antibody for 2 h. Signals were visualized by using the ECL reagents and film (Amersham Corporation, Arlington Heights, IL, USA) according to the manufacturer's instruction. Specificities of signals were verified either with incubations without appropriate primary antibody or by using a blocking peptide provided by a supplier (Lab Vision, Fremont, CA, USA). The specificity of the β-catenin bands was ensured earlier by using two other commercially available antibodies (24). Blots were scanned and analysed using a GS-800 Calibrating

Densitometer and the Quantity One program (BioRad Laboratories, Hercules, CA, USA). Results in duplicate were expressed as sample band intensity (optical density of the band multiplied by the band area) divided by control band intensity.

Statistical analysis

The Mann–Whitney non-parametric test was used to compare the two coffee groups with the control group. Tumour incidence in the colon was analysed by Fisher's exact test. The SPSS statistical program, version 12 (SPSS Inc., Chicago, IL, USA), was used for all statistical analysis. Differences were considered significant at $p < 0.05$.

Results

Weight gain of the $Apc^{Min/+}$ mice

The mice grew well during the feeding period and the weight gain and the final body weights of the $Apc^{Min/+}$ mice did not differ between the groups (Fig. 1). At a few time points, coffee-fed mice had slightly lower body weights than controls. The male mice in the filtered coffee group had lower body weights at the age of 11 weeks ($p < 0.05$), and the females in the unfiltered coffee group had lower body weights at the ages of 7, 8, 9 and 10 weeks ($p < 0.05$) compared with the control animals. The authors suggest that these small differences in body weights at certain time-points are not likely to be biologically significant.

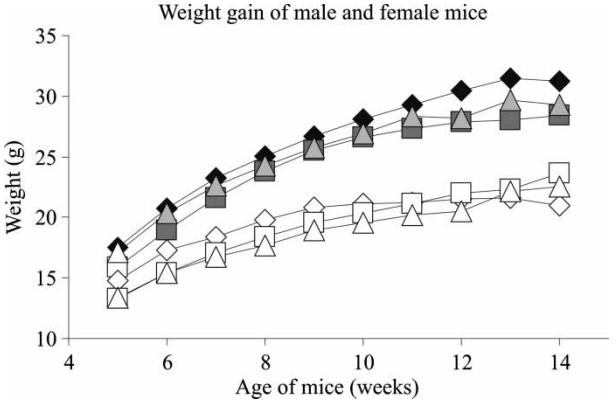

Fig. 1. Mean body weights of multiple intestinal neoplasia ($Apc^{Min/+}$) males and females consuming the control, filtered and unfiltered coffee diets for 9 weeks. ◇: Control female; ◆: control male; □: filtered female; ■: filtered male; △: unfiltered female; ▲: unfiltered male.

Adenoma results

The male and female $Apc^{Min/+}$ mice did not differ significantly in the number or diameter of adenomas and therefore the data from male and female mice were pooled. The range of adenoma number and adenoma distribution between the small intestine and the colon here were similar to those found in other studies (25).

The tumour number or size in the small intestine and the colon did not differ between dietary groups (Table 2). The total tumour number was 29 for the control group, 30 for the filtered coffee group and 29 for the unfiltered coffee group.

β-Catenin and cyclin D1 analysis

The subcellular localization, particularly nuclear β-catenin and cyclin D1, is considered to be an important determinant of their function. The effects of coffee on β-catenin and cyclin D1 in tumours were therefore analysed by a semi-quantitative Western blot method. As observed in a previous study (24), immunoblots of β-catenin gave one band at 98 kDa in rat brain homogenate (control), and one band (or two bands) at 92 kDa (full-length β-catenin) in the adenoma tissue of $Apc^{Min/+}$ mice (Fig. 2A). The β-catenin results are expressed as the intensity of the full-length band, which could be detected in almost all samples. Immunoblots of cyclin D1 gave one band at 35 kDa in the TMK (a human gastric cancer cell) homogenate (control), and two bands at 35 and 36 kDa, which probably represent full-length cyclin D1 with different levels of phosphorylation. The cyclin D1 results are expressed as the intensity of these two bands. The levels of nuclear β-catenin (Fig. 2B) and cyclin D1 (Fig. 2C) in the adenoma tissue did not differ between the dietary groups.

Plasma xanthine analysis

Caffeine and its metabolites paraxanthine, theobromine and theophylline were analysed from mouse plasma samples. Paraxanthine was clearly the dominant metabolite, and theophylline as well as theobromine was present in minute amounts only. Therefore, caffeine and paraxanthine were used as markers of compliance. Plasma caffeine and paraxanthine concentrations were 3.2 ± 1.4 and 1.7 ± 0.4 μmol l^{-1} in the filtered coffee group and 3.6 ± 2.3 and 1.6 ± 0.6 μmol l^{-1} in the unfiltered coffee group. Xanthine concentrations in the control group were below the limit of quantification

Table 2. Tumour number, and size in the small intestine and the colon of $Apc^{Min/+}$ mice fed with a control diet or diets supplemented with either filtered or unfiltered coffee for 9 weeks

	Control		Filtered coffee		Unfiltered coffee	
Diet	Median	Range	Median	Range	Median	Range
n[a]	11		11		11	
Small intestine						
Tumours per mouse	29	23–202	29	15–127	29	21–66
			$p=0.693$		$p=0.430$	
Tumours per mouse[b]	29	23–65	29	15–62	29	21–66
			$p=0.677$		$p=0.646$	
Tumour size (mm)	1.1	1.0–1.4	1.2	0.7–1.4	1.1	0.9–1.4
			$p=0.768$		$p=0.768$	
Colon						
Incidence	4/11		7/11		3/11	
Tumours per mouse	0	0–2	1	0–4	0	0–2
			$p=0.207$		$p=0.811$	
Tumour size (mm)	3.0	1.7–4.1	2.9	2.0–4.0	2.4	2.3–3.3
			$p=0.850$		$p=0.593$	
Total						
Tumours per mouse	29	25–202	30	15–131	29	21–68
			$p=0.767$		$p=0.430$	

Results are expressed as median and range (minimum–maximum). The Mann–Whitney non-parametric test was used to compare the filtered and unfiltered coffee groups with the control group. Differences were considered significant at $p<0.05$.

[a]The adenoma data from male ($n=7$) and female ($n=4$) mice were pooled.

[b]Exclusion of two extreme values (202 and 127) from the control and filtered coffee groups, respectively, did not change the statistical significance between the groups.

(0.9 and 0.6 μmol l^{-1} for caffeine and paraxanthine, respectively).

Discussion

To the authors' knowledge, this is the first study investigating the effects of filtered or unfiltered coffee on adenoma formation in the $Apc^{Min/+}$ mouse. The main difference between these two types of coffee is the levels of kahweol and cafestol, which are abundantly present only in unfiltered coffee. Kahweol and cafestol have some bioactivity in the human body, since unfiltered coffee has been shown to raise serum cholesterol in man (26). In the present study, filtered and unfiltered coffee was evaluated for possible antitumorigenic activities. Consumption of a coffee-free diet, or filtered or unfiltered coffee (10% w/w) for 9 weeks had a similar effect on tumour formation in $Apc^{Min/+}$ mice. Supplementation of the diet with coffee increased significantly the levels of plasma caffeine and paraxanthine in the plasma, indicating good compliance with coffee supplementation. The results show that filtered or unfiltered coffee, their constituents (caffeine, kahweol and cafestol) and the caffeine metabolite paraxanthine had no chemoprotective effect in the $Apc^{Min/+}$ mice. The caffeine result is in line with another study with $Apc^{Min/+}$, which showed that administration of caffeine at a dose of 0.044% in drinking fluid had no inhibitory effect on tumour formation in this animal model (9).

Even though the adenoma result did not differ between the groups, the authors wanted to determine whether coffee changes the subcellular localization of β-catenin and its target protein cyclin D1, since the improper regulation of cellular β-catenin pools is one of the driving forces in *Apc*-induced colonic neoplasia (18, 27). Dietary components have been shown to affect β-catenin localization in both the normal-appearing mucosa and the tumour tissue, thus diminishing the progression of neoplasia in $Apc^{Min/+}$ mice (9, 28, 29). In the present study no differences in the nuclear β-catenin and cyclin D1 levels were found between dietary groups, and therefore it may be concluded that coffee consumption does not affect cancer progression in the adenoma tissue. However, these results do not exclude the possibility that coffee consumption has a protective effect against other type of cancers in which the aetiology of the disease if different

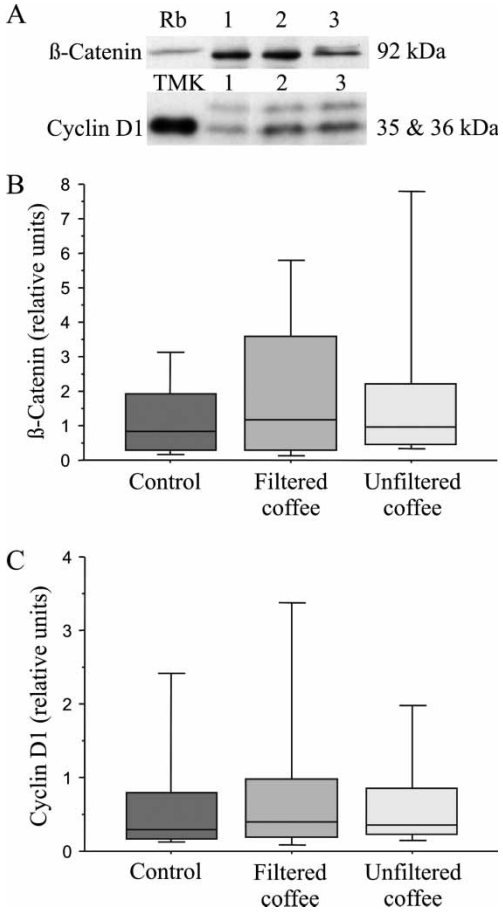

Fig. 2. (A) Representative immunoblots for β-catenin and cyclin D1 in the adenoma tissue of $Apc^{Min/+}$. Rat brain (Rb) and TMK homogenates were used as internal standards. Each of the following lines 1–3 contains nuclear samples from one mouse. (B) Nuclear β-catenin and (C) cyclin D1 levels in the adenoma tissue of $Apc^{Min/+}$ mice fed the control, filtered coffee and unfiltered coffee for 9 weeks. Results are represented as relative intensities. For each group, the box represents the interquartile range, which contains 50% of values. The whiskers extend from the box to the highest and lowest values. Medians are indicated by lines across the boxes. $n = 11$ for all groups.

from CRC. The results of three Japanese cohort studies showed that coffee consumption decreased the risk of liver cancer, although the exact mechanism of action remained unclear (30, 31).

In the present study, caffeine and paraxanthine were present in the plasma samples of all mice receiving coffee, but not the controls. Paraxanthine appeared to be a good compliance marker and, because of the smaller variation in paraxanthine results, a better marker than plasma caffeine. These findings are in line with those of Klebanoff et al. (32), who suggested that plasma paraxanthine reflects coffee consumption more accurately than plasma caffeine in humans, owing to its more favourable pharmacokinetic properties. To the authors' knowledge, this type of information is not available from animal studies, although the primary metabolism of caffeine, i.e. demethylation by CYP1A2, has been extensively studied in many species (33). The caffeine and paraxanthine concentrations were in the same range as those found among the Finnish population (I. Erlund, unpublished results). As the intake of coffee in the present experiment was similar to the mean daily consumption in Finnish adults, it may be concluded that caffeine bioavailability is not dramatically different in humans and in mice. Since weight gain was also similar in the three different groups, non-compliance cannot explain the results of the study.

In conclusion, this study found that filtered or unfiltered coffee (10% w/w) does not exert antitumorigenic activity in $Apc^{Min/+}$ mice or change β-catenin and cyclin D1 signalling in the adenoma tissues.

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