

Calcium and α -tocopherol suppress cured-meat promotion of chemically induced colon carcinogenesis in rats and reduce associated biomarkers in human volunteers^{1–3}

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ABSTRACT

Background: Processed meat intake has been associated with increased colorectal cancer risk. We have shown that cured meat promotes carcinogen-induced preneoplastic lesions and increases specific biomarkers in the colon of rats.

Objectives: We investigated whether cured meat modulates biomarkers of cancer risk in human volunteers and whether specific agents can suppress cured meat–induced preneoplastic lesions in rats and associated biomarkers in rats and humans.

Design: Six additives (calcium carbonate, inulin, rutin, carnosol, α -tocopherol, and trisodium pyrophosphate) were added to cured meat given to groups of rats for 14 d, and fecal biomarkers were measured. On the basis of these results, calcium and tocopherol were kept for the following additional experiments: cured meat, with or without calcium or tocopherol, was given to dimethylhydrazine-initiated rats (47% meat diet for 100 d) and to human volunteers in a crossover study (180 g/d for 4 d). Rat colons were scored for mucin-depleted foci, putative precancer lesions. Biomarkers of nitrosation, lipoperoxidation, and cytotoxicity were measured in the urine and feces of rats and volunteers.

Results: Cured meat increased nitroso compounds and lipoperoxidation in human stools (both $P < 0.05$). Calcium normalized both biomarkers in rats and human feces, whereas tocopherol only decreased nitro compounds in rats and lipoperoxidation in feces of volunteers (all $P < 0.05$). Last, calcium and tocopherol reduced the number of mucin-depleted foci per colon in rats compared with nonsupplemented cured meat ($P = 0.01$).

Conclusion: Data suggest that the addition of calcium carbonate to the diet or α -tocopherol to cured meat may reduce colorectal cancer risk associated with cured-meat intake. This trial was registered at clinicaltrials.gov as NCT00994526. *Am J Clin Nutr* 2013;98:1255–62.

INTRODUCTION

Colorectal cancer is the third most common type of cancer in the United States (1). Epidemiologic studies have suggested that there is an association between colorectal cancer and intake of meat (2), and risk associated with cured meat is higher than with fresh red meat (3). The World Cancer Research Fund panel stated that “the evidence that red meat and processed meat are a cause of colorectal cancer is convincing” and recommended to “limit the intake of red meat and avoid processed meat.” However, if these

recommendations were adhered to, iron and zinc supplies would decrease with a possible effect on women and elderly people (4). Experimental studies showed that a cooked ham and an experimental cured meat promoted colon carcinogenesis in rats (5, 6).

Four major hypotheses have been proposed to explain the effect of processed meat on colorectal cancer as follows: 1) fat present in meat promotes carcinogenesis by raising intestinal bile acids; 2) cooking meat at a high temperature forms carcinogenic heterocyclic amines; 3) endogenous nitrosation yields nitroso compounds that can be carcinogenic; and 4) heme iron in red meat promotes carcinogenesis because it increases cell proliferation in the mucosa through lipid oxidation and the cytotoxicity of fecal water. Nitrosation and heme iron hypotheses have received most experimental support (3). The consumption of fresh red meat or cured meat increases the concentration of nitroso compounds in human stools and mice and rat feces, and heme is responsible for this effect (6–10). The concentration of fecal nitroso compounds is associated with the nitric oxide–specific DNA adduct O⁶-carboxymethylguanine in the human colon (11)

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and mucin-depleted foci (MDF)⁴ (a putative precancer lesion) promotion in carcinogen-initiated rats given cured meat (6). Aberrant crypt foci (ACF) (another putative precancer lesion) are induced in mice by gavages with nitroso compound extracted from hot dogs (12). On the other hand, dietary beef, hemoglobin, and chlorine heme promote a dose-dependent formation of ACF and MDF in rats and raise biomarkers of intestinal fat oxidation and cytotoxicity of fecal water, which suggest that heme iron is a cancer promoter, and specific fecal biomarkers are associated with cancer promotion (13, 14).

Specific additives can suppress the promoting effect of heme; a calcium-rich diet abolishes most effects of dietary heme, including the beef meat-induced promotion of MDF (13–15). These data suggest that calcium could reduce colorectal cancer risk in meat eaters and support the concept that toxicity associated with excess intake of the specific food component heme iron may be prevented by another nutrient, calcium (13). Besides, antioxidant molecules added into meat can also suppress the heme-induced promotion of carcinogenesis (14).

In this study, we first investigated the effect in rats of 6 additives on fecal biomarkers induced by cured-meat eating. Two additives were chosen for inclusion in a carcinogenesis study in the chemically induced rat model of colon carcinogenesis. These 2 compounds were also evaluated in healthy volunteers to test their ability to counteract the modulation of fecal and urinary biomarkers by cured meat.

SUBJECTS AND METHODS

Animal studies

Two sequential studies were performed in rats. A 14-d study was conducted to test the effect of 6 agents added to the diet on fecal and urinary biomarkers in rats. Subsequently, a 100-d carcinogenesis study was conducted to test the protection afforded by 2 of 6 agents selected.

Fourteen-day study: animals, design, and diets

Female Fischer 344 rats ($n = 35$) were purchased at 5 wk of age from Charles River (Laboratories). The study was conducted in an accredited animal colony by approved staff, and animal care was in accordance with the guidelines of the European Council on Animals used in Experimental Studies. Rats were housed individually in metabolic cages. The rats were kept at 22°C and in a 12-h:12-h light:dark cycle and were allowed free access to a standard AIN76 semipurified diet and tap water (16). After 2 days of acclimatization, rats were randomly allocated to 7 groups of 5 rats. Each group was given an experimental cured meat for 14 d, which was added with 1 of 6 potential protective agents or none (control). Body weights were monitored on days 0, 7, and 14, and food and water intakes were monitored on days 6–7 and 12–13. Feces were collected during days 13 and 14 and were frozen at –20°C. Urine samples were collected on day 13 and processed immediately.

⁴Abbreviations used: ACF, aberrant crypt foci; Apc, adenomatous polyposis coli; ATNC, apparent total *N*-nitroso compound; DCNO, dark cooked meat with nitrite, oxidized; DHN-MA, 1,4-dihydroxynonane mercapturic acid; H2AX, histone; MDF, mucin-depleted foci; TBARS, thiobarbituric acid reactive substances.

The diet was made by the French Pork and Pig Institute workshop by mixing 55 g (dry weight) of an experimental cured meat (French Pork and Pig Institute) with 45 g of a modified AIN76-base powder made by Preparation Unit of Experimental Food (French National Institute For Agricultural Research) that contained (in g/100 g total diet) sucrose, 23.8; corn starch, 6; cellulose, 5; safflower oil, 5; AIN76 calcium-free AIN76 mineral mix, 3.5; AIN76 vitamin mix, 1.0; methionine, 0.3; calcium phosphate, 0.2; and choline bitartrate, 0.2. The diet contained 40% protein, 15% fat, and 0.27% calcium. Meat was not freeze dried but given moist to avoid fat oxidation (5, 17). The experimental cured meat, which was similar to air-exposed picnic ham and called dark cooked meat with nitrite, oxidized (DCNO), was chosen because it promotes carcinogenesis in rats (6). Dark-red *Supraspinatus* pig muscle (15–17 mg heme/100 g) (18) was cured with 2 g salt/100 g containing 0.6 g sodium nitrite/100 g, and 0.36 g sodium erythorbate. The muscle was heated inside a vacuum-sealed plastic bag immersed in a 70°C water bath for 1 h. This cooking denatured myoglobin and freed heme iron from the protein (19). The cured meat was exposed to air at 4°C in the dark for 5 d before being given to the rats. Several antioxidant agents and calcium salts can reduce heme-induced fat peroxidation in the gut and heme-induced carcinogenesis promotion in rats (13, 14). These agents (rutin, carnosol, α -tocopherol, and calcium carbonate) and 2 additives already in use in cured meat (the fat substitute inulin and the pH corrector trisodium pyrophosphate) were tested in DCNO at a dose already known to counteract heme toxicity. Three of the items were incorporated inside meat during curing process as follows: rutin (0.1% of the total diet; Sigma Aldrich), carnosol (0.07%; extracted from rosemary; Naturex), and α -tocopherol (0.05%; Sigma Aldrich). Two others were added to the diet powder as follows: calcium carbonate (1.5 g CaCO₃/100 g diet equivalent to 150 μ mol/g; Sigma) or inulin (4.5%; Orafit HP, Azelis). As a last treatment, meat pH was buffered with trisodium pyrophosphate (Na₃PO₄, 0.84%; La Bovida). Each diet was stored under vacuum at –20°C and dispensed daily at 1700.

One hundred-day study: animals, design, and diets

Rats ($n = 36$), the same kind as previously described, were housed in pairs in stainless steel wire-bottomed cages in the same animal colony as previously described. After acclimatization the rats received a single intraperitoneal injection of 1,2-dimethylhydrazine (180 mg/kg; Sigma) in NaCl (9g/L H₂O). Seven days later, the rats were randomly allocated to 3 groups ($n = 16, 10,$ and 10) and fed the experimental diets daily for 98–99 d before carbon dioxide euthanasia. Colons were removed, washed with cold Ringer solution, opened, coded, and fixed flat between 2 sheets of filter paper in 10% buffered formalin (Sigma) before ACF and MDF scoring. Body weight was monitored every week during the 4 first weeks and every 2 wk thereafter. Food and water intakes were measured at days 15, 59, and 94. Feces were collected on days 85–95 and kept at –20°C. Each rat was placed in a metabolic cage, and urine was collected on days 70–74 and kept at –20°C.

The diet was obtained from the same providers previously mentioned. The control-diet composition was (in g/100 g): moist cured-meat DCNO, 47 (dry weight), sucrose, 28.7; casein, 5; safflower oil, 5; corn starch, 4.8; cellulose, 4.8; AIN76 calcium-free mineral mix, 3.35; AIN76 vitamin mix, 0.95; methionin, 0.3; calcium phosphate, 0.21; and choline bitartrate, 0.17. The following

2 potential protective agents were tested: calcium carbonate was added to the control diet (1.5 g/100 g diet at the expense of casein). α -Tocopherol was added to DCNO during the curing process (0.05% of the diet). Each diet was stored at -20°C under vacuum and dispensed daily at 1700.

Human study: volunteers, crossover design, and diets

This study was performed in the Nutritional Investigation Unit of the Human Nutrition Research Center of Auvergne (Clermont-Ferrand, France). A single-blind randomized, controlled crossover trial was conducted in human volunteers who satisfied the following criteria: men aged 40–75 y, BMI (in kg/m^2) from 20 to 30, no history or clinical symptom of colonic disease, no history of family colon cancer, normal standard blood tests (blood cell counts, renal and liver function, C-reactive protein, serum glucose, and lipids), alcohol and tobacco consumption <30 g and <5 cigarettes/d, respectively, and no detectable microscopic bleeding in stools. Volunteers were recruited from the Nutritional Investigation Unit data file, which was declared to the National Commission on Informatics and Liberties. All participants gave freely their written informed consent before their selection in the study and after being given information on the objectives, benefits, risks, and nature of the study products. Volunteers with a calcium dietary intake ≥ 1500 mg/d and those treated with vitamins A, C, or E or calcium supplements were excluded. After telephone contact and first selection, volunteers had a medical visit, a dietetic evaluation, and biological measurements for inclusion-criteria checking. Finally, 18 volunteers were included after giving their written informed consent.

As shown in the study design (Figure 1), during the 1-wk run-in period, adaptation volunteers were asked to eat a diet without beef or pork meat and low in antioxidant products (the no-meat control period). Volunteers were randomly submitted to 3 alternated 4-d intervention periods as follows: experimental cured meat (DCNO), DCNO and calcium capsule (DCNO + CaCO_3) and tocopherol-enriched DCNO (DCNO + tocopherol) in a random order (doses are given in the next paragraph). Intervention periods were separated from each other by a washout period ≥ 3 d (same diet as in the run-in period). Urine and stool samples were collected during the last 3 d of each intervention period and at the end of each washout period. Each subject came to the Nutrition Investigation Unit 4 times in addition to the screening visit. Before the first intervention period (visit 1), volunteers were given cured meat needed for the whole study (3 packages of ham; one package per period). Volunteers were asked to keep the ham frozen at home and to open packages in their fridge at 4°C 4 d before consumption to match the previous rat study design and mimic the oxidation that occurs usually when ham is not vacuum packed. Placebo or calcium capsules for the first intervention period were also distributed at visit 1 depending on the random assignment. During visits 2–4, each volunteer brought back ham packaging and collected frozen urine and stools. At visits 2 and 3, volunteers were

given capsules that corresponded to intervention periods 2 and 3. The study was approved by a written decision of the Person Protection Committee (French CPP Sud-Est VI, 8 September 2009) and authorization of the French Ministry of Health (Afsaps, 19 May 2009). This trial was registered at clinicaltrials.gov as NCT00994526.

Volunteers were given the same experimental cooked ham (DCNO) previously tested in rats that was cured by Fleury-Michon Charcuterie. Volunteers were asked to eat 180 g DCNO/d (four 45-g slices) during each 4-d intervention period. One calcium carbonate capsule (500 mg Ca/capsule; Montalembert Pharmacy) was consumed 2 times/d during the 4-d DCNO + CaCO_3 period (1 g Ca/d). Placebo capsules taken during the 2 other intervention periods contained 500 mg crystalline cellulose. α -Tocopherol was incorporated during meat curing (0.05% wt:wt) to provide meat for the DCNO + tocopherol intervention period. Compliance to the diet and supplements was assessed after the collection of food and drug packages at the end of intervention periods.

Analytic techniques

Analysis of heme and thiobarbituric acid reactive substances in fecal water and 1,4-dihydroxynonane mercapturic acid in urine

Fecal values were measured in fecal water because, according to bile acid studies, the soluble fraction of colonic contents interacts more strongly with the mucosa than the insoluble fraction (20). For rats, fecal pellets were collected under each cage for 24 h. One milliliter of sterilized water was added to 0.3 g dried rat feces. Samples were incubated at 37°C for 1 h and stirring thoroughly every 20 min. After centrifugation at $20,000 \times g$ for 15 min, fecal water (supernatant fluid) was collected and kept at -20°C until use. For volunteers, 1 g human stool/5 mL DMEM was added before stirring vigorously for 30 s. The mix was submitted to centrifugation, and fecal water was harvested and kept as previously described. Heme was measured by fluorescence in fecal water according to Sesink et al (21) as already described (22). Thiobarbituric acid reactive substances (TBARS) were measured in fecal water according to Ohkawa et al (23) exactly as previously described. 1,4-Dihydroxynonane mercapturic acid (DHN-MA) is the main urinary metabolite of 4-hydroxynonanal, which is a major toxic end product of endogenous fat peroxidation. A DHN-MA assay was done by using a competitive enzyme immunoassay as previously described with the use of a DHN-MA-linked acetylcholinesterase enzyme (24). Each urine sample was assayed in duplicate.

Fecal water cytotoxicity

Fecal water cytotoxicity was quantified on 3 cell lines as previously described (13). The adenomatous polyposis coli (*Apc*) mutation has been detected in the majority of MDF in rats and of human colorectal cancers. *Apc*-mutated cells resist cytotoxic aldehydes in the gut of meat-fed rats; this resistance leads to the

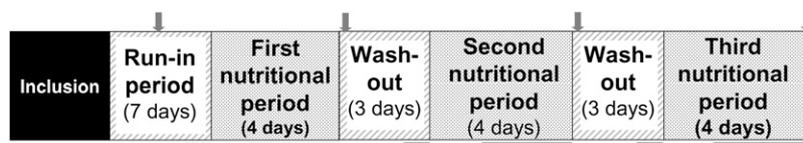


FIGURE 1. Clinical trial flowchart. The gray arrows correspond to medical visits. Bold lines correspond to periods of urine and feces collection.

selection of premalignant cells and explains cancer promotion by red meat (25). To investigate whether the same mechanism could explain the promotion of carcinogenesis by cured meat, the cytotoxicity of fecal water was quantified on the following 3 cell lines: 1) a cancerous mouse colonic epithelial cell line CMT93 (European Collection of Animal Cultures), 2) colon epithelial cell lines derived from C57BL/6J mice (*Apc*^{+/+}), and 3) from Min mice *Apc*^{+/-} (26). The use of this triple cellular model including wild-type cells (*Apc*^{+/+}), preneoplastic cells (*Apc*^{Min/+}), and cancerous cells (CMT93) could contribute to our understanding of the effects of digestive content on early steps of colon carcinogenesis.

CMT93 cells were seeded in 96-well plates at 37°C at 1.6×10^4 cells/well in 200 μ L DMEM. At confluence, cells were treated for 24 h with a fecal water sample diluted at 10% (vol:vol) in the culture medium. Cells were washed with phosphate-buffered saline. *Apc*^{+/+} and *Apc*^{Min/+} cells have a temperature-sensitive mutation of the simian virus 40 large-tumor antigen gene (tsA58), under the control of interferon- γ . These cells are immortalized because they express active simian virus 40 large-T antigen gene at the permissive temperature (33°C). Cells were cultured at a permissive temperature of 33°C in DMEM supplemented with 10% (vol:vol) fetal calf sera, 1% (vol:vol) penicillin/streptomycin, and 10 U interferon- γ /mL until subconfluence. The studies were performed at a nonpermissive temperature of 37°C without interferon- γ to inhibit the simian virus 40 large-T antigen gene transgene and limit proliferation. *Apc*^{+/+} and *Apc*^{Min/+} cells were seeded into 96-well culture plates at the seeding density of 10^4 cells in DMEM culture medium. Cells were grown at 33°C with interferon- γ for 72 h until subconfluence. Cells were transferred at 37°C without interferon- γ for 24 h. The cytotoxicity of fecal water was quantified by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide test (0.45 mg/mL in phosphate-buffered saline). The reaction product was solubilized in 100 μ L lysis buffer (10% sodium dodecyl sulfate and 0.01 mol NaOH/L) before the color of reaction product was quantified by using a plate reader at 570 and 690 nm. Fecal water genotoxicity was also assessed in the current study by measuring phosphorylation of histone (H2AX) on *Apc*^{+/+} and *Apc*^{Min/+} cell lines (see Supplemental Material under "Supplemental data" in the online issue).

Apparent total N-nitroso compound analysis in fecal samples from volunteers and rats

The apparent total N-nitroso compound (ATNC) was analyzed in the fecal water of rats by SSM by using a published method (27). Briefly, 425 μ L fecal water was mixed with 25 μ L 2 N HCl and 50 μ L freshly prepared saturated solution of sulfamic acid in water to destroy any nitrite present. After storage for 15 min at room temperature and <4 h in ice, 100- or 200- μ L samples of the mixture were injected into the reaction vessel. ATNCs were decomposed to nitric oxide by using an hydrochloric acid/hydrogen bromide/acetic acid/ethyl acetate mixture refluxing at <0.1 mm Hg and 28°C. Nitric oxide was swept by an argon stream through 4 wash bottles that contained NaOH and Na₂SO₄ at room temperature and 2 empty wash bottles kept at -30°C to remove water vapor and acids and was determined by using a Thermal Energy Analyzer (Advanced Chromatographic Systems).

ATNCs were analyzed in fecal water from volunteers by GCGK with an Ecomedics CLD 88 Exhalyzer (Ecomedics) by using a modification of a published method (28). Briefly, 100 μ L

fecal water were incubated with 500 μ L 5% (wt:vol) sulfamic acid solution to remove nitrite, and samples were injected into a purge vessel kept at 60°C and filled with a standard triiodide reagent (38 mg I₂ was added to a solution of 108 mg KI in 1 mL H₂O). To this mixture, 13.5 mL glacial acetic acid was added) to determine the total ATNC. Reported values are concentrations (in μ mol/L) measured in 100 μ L sample.

ACF and MDF assays

Rats were killed by CO₂ asphyxiation in a random order at days 98–99 of the experimental diet. Fixed colons were scored for ACF by using Bird's procedure (29) as follows: after methylene blue staining, numbers of ACF per colon crypts per ACF were counted under a light microscope at magnification $\times 40$ in duplicate by 2 independent readers who were blinded to the origin of the colon.

Colons were stained by using the high-iron diamine alcian blue procedure. Two blinded investigators evaluated the number of MDF per colon and the number of crypts per MDF. MDF scoring criteria were a focus containing ≥ 2 crypts with no or very little apparent mucin (30, 31).

Statistical analysis

Data were analyzed with Systat 10 software (Systat Software Inc) for Windows and reported as means (\pm SDs) (except in **Figure 2**). Biochemical values were first considered by using 1-factor ANOVA. If a significant difference was shown between all groups ($P < 0.05$), a comparison of each experimental group with the control group was made by using Dunnett's test. ACF and MDF scoring was done in duplicate, and thus, these variables were tested first by using 2-factor ANOVA (groups and readers). The group \times reader interaction was never significant, and when total ANOVA was significant ($P < 0.05$), pairwise differences between groups were analyzed by using Fisher's least-significant-difference test. The difference of fecal water cytotoxicity between *Apc*^{+/+} and *Apc*^{Min/+} cell lines was tested by using Student's *t* test. Human volunteer data were analyzed by using Wilcoxon's signed-rank test, with each volunteer acting as his or her own control. Bonferroni correction for 3 comparisons (ie, DCNO compared with no-meat control period, DCNO + CaCO₃ compared with DCNO, and DCNO + tocopherol compared with DCNO) was made for the multiple-comparison analysis.

RESULTS

Fourteen-day animal study

The mean body weight of rats was 145 ± 11 g on day 14. Rats that were given rutin- and tocopherol-supplemented DCNO cured meat gained more weight than did DCNO-fed control rats ($P < 0.05$; data not shown), but dietary and water intakes were similar in all groups (10 ± 2 and 25 ± 6 g/d, respectively). All tested additives decreased fecal water oxidation (TBARS) and urinary DHN-MA concentrations compared with in the DCNO-control group ($P < 0.05$; **Table 1**). Fecal water from rats given DCNO showed similar cytotoxicity on the 3 tested cell lines, but the addition of CaCO₃, rutin, or α -tocopherol to the diet resulted in a survival advantage of wild *Apc*^{+/+} cells compared with mutated *Apc*^{Min/+} cells. All tested additives, except inulin, decreased the fecal water cytotoxicity against CMT93 cells compared with

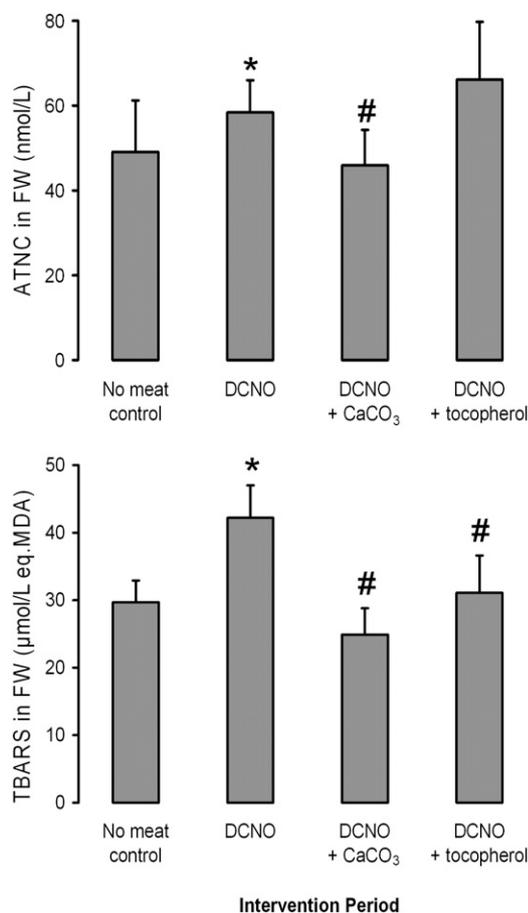


FIGURE 2. Effects (means \pm SEMs) of cured-meat diets on nitrosated compound (ATNC, top graph) and fat peroxide (TBARS, bottom panel) formations in the FW of human volunteers at day 4 of each nutritional period. Design, diet composition, and analytic methods are given in Subjects and Methods. The following dietary periods were run in a random order for each volunteer and separated by washout periods—control: no red-meat period; DCNO: 4-d period during which each volunteer was given 180 g cured meat/d; DCNO + CaCO₃: calcium carbonate capsules (1 g Ca/d) and 180 g cured meat/d; and DCNO + tocopherol: α -tocopherol-enriched cured meat (168 g/d, 0.05% tocopherol). $n = 17$. *Significantly different from the no-meat control period ($P < 0.017$; Wilcoxon's test); #significantly different from the DCNO period ($P < 0.017$; Wilcoxon's test). ATNC, apparent total *N*-nitroso compound; DCNO, dark cooked meat with nitrite, oxidized; FW, fecal water; eq.MDA, equivalent malondialdehyde; TBARS, thiobarbituric acid reactive substances.

unchanged DCNO. Fecal water from rats given DCNO plus inulin was highly cytotoxic to *Apc*^{+/+} and CMT93 cells but not at all mutated *Apc*^{Min/+} cells (Table 1).

One hundred-day animal study

The final body weight of rats was 217 ± 1 g and was similar in all 3 groups, and the food intake was not significantly different. Rats given DCNO + CaCO₃ or DCNO + tocopherol had less MDF per colon than did control rats given DCNO only (both $P = 0.01$; Table 2). In contrast, the 2 tested additives did not reduce the number of ACF per colon, and the size of MDF (crypts per MDF; Table 2) was not significantly reduced. The addition of CaCO₃ to the DCNO diet decreased the amount of all tested biomarkers previously associated with carcinogenesis (heme, TBARS, and cytotoxicity in fecal water; ATNC in feces and urinary DHN-MA; all $P < 0.05$; Table 2). In contrast, the ad-

dition of α -tocopherol to cured meat decreased only the concentration of heme in fecal water and DHN-MA in urine ($P < 0.05$; Table 2). The fecal ATNC concentration was significantly decreased by the 2 tested additives (Table 2). Furthermore, no difference was seen between the γ -H2AX induction by fecal water of the 3 experimental groups (see Supplemental Figure S1 under "Supplemental data" in the online issue).

Human study

General observations

The nutritional intervention was scheduled with 18 healthy volunteers, but one volunteer later declared that he was also participating in another trial. Thus, this volunteer was excluded, and 17 persons completed the study and were analyzed. Subjects were aged 56.0 ± 9.5 y, and their BMI was 24.9 ± 2.3 . Blood cell counts were normal in all subjects. The mean serum creatinine concentration was 79.8 ± 8.8 μ mol/L, glucose concentration was 5.4 ± 0.5 mmol/L, cholesterol concentration was 5.2 ± 0.5 mmol/L, triglycerides concentration was 0.9 ± 0.3 mmol/L, serum alanine amino transferase concentration was 24.6 ± 10.6 IU/L, γ -glutamyl transferase concentration was 29.1 ± 20.9 IU/L, prothrombin time was $100 \pm 9\%$, and C-reactive protein concentration was 1.2 mg/L (range: 0.7–8.5 mg/L). The assessment of compliance showed that diet guidelines were strictly followed, but 1 d, one volunteer ate 3 slices of ham instead of 4. All calcium and placebo supplements were taken without any detected fault.

TBARS, ATNC, cytotoxicity, genotoxicity, and urinary DHN-MA

Biomarker measurements showed a significant increase in ATNC and TBARS concentrations in the fecal water of human volunteers fed 180 g cured meat (DCNO) for 4 d compared with during control periods (Figure 2). The addition of calcium carbonate to the cured-meat diet significantly decreased fecal ATNC and TBARS. The addition of tocopherol into cured meat had no effect on fecal ATNC concentrations but decreased fecal water TBARS. The Urinary DHN-MA and fecal water cytotoxicity were not changed by dietary changes in volunteers (data not shown). Moreover, compared with the control group or period, the DCNO period tended to reduce γ -H2AX induction by fecal water from volunteers (NS; see Supplemental Figure S2 under "Supplemental data" in the online issue), and no difference was seen between nutritional periods with cured meats (see Supplemental Figure S2 under "Supplemental data" in the online issue).

DISCUSSION

To our knowledge, the current study was the first one to show that the same cured meat that increased carcinogenesis in rats also increased promotion-associated fecal biomarkers in rats and human volunteers. The study also showed that this increase and the promotion of carcinogenesis in rats can be suppressed by dietary calcium or α -tocopherol.

The promotion of colon carcinogenesis was shown with the surrogate endpoint biomarker, MDF. MDF, which are formed by dysplastic crypts devoid of mucin, have been identified in the colon of humans at high risk of colon cancer (32). Like tumors, MDF harbor mutations in genes that affect colon carcinogenesis (*Apc* and *K-ras*) and show Wnt-signaling activation (33), a dramatic reduction of MUC2 expression (34), and a strong

TABLE 1Effects of agents added to cured meat given to rats on lipoperoxidation markers in feces and urine and on the cytotoxicity of fecal water¹

Diet ²	TBARS in fecal water	DHN-MA in urine	Cytotoxicity of fecal water on cells		
			<i>Apc</i> ^{+/+}	<i>Apc</i> ^{Min/+}	CMT93
	$\mu\text{mol/L MDA.eq}$	$\text{ng}/24\text{ h}$		% of dead cells	
DCNO	93 ± 12	5317 ± 1906	49 ± 15	58 ± 9	50 ± 5
DCNO + CaCO ₃	46 ± 19 ³	668 ± 541 ³	30 ± 8	52 ± 5 ⁴	21 ± 8 ³
DCNO + inulin	52 ± 24 ³	237 ± 137 ³	66 ± 45	0 ± 11 ^{3,4}	87 ± 9 ³
DCNO + rutin	43 ± 9 ³	1634 ± 702 ³	37 ± 12	58 ± 10 ⁴	21 ± 6 ^{3,4}
DCNO + carnosol	49 ± 21 ³	195 ± 118 ³	43 ± 51	17 ± 21 ³	14 ± 9 ³
DCNO + α -tocopherol	39 ± 12 ³	183 ± 90 ³	13 ± 21 ³	43 ± 12 ⁴	12 ± 9 ³
DCNO + Na ₃ PO ₄	55 ± 9 ³	152 ± 39 ³	10 ± 10 ³	18 ± 10 ³	21 ± 12 ³

¹ All values are means ± SDs. *n* = 5. *Apc*, adenomatous polyposis coli; DCNO, experimental dark cooked cured pork meat with nitrite, oxidized; DHN-MA, 1,4-dihydroxynonane mercapturic acid; MDA.eq, malondialdehyde equivalent; TBARS, thiobarbituric acid reactive substances.

² Concentration of each additive is given in Subjects and Methods.

³ Significantly different from DCNO [column's stats; *P* < 0.05 (Dunnett's *t* test)].

⁴ Significantly different from cytotoxicity against *Apc*^{+/+} cells [row's stats; *P* < 0.05 (Student's *t* test)].

activation of the inflammatory process (35), all of which are features that suggest that MDF are precancerous. Rodents studies have suggested that MDF are better predictors of colorectal cancer than ACF are, which is why we focused on MDF data (30).

The promotion of colon carcinogenesis by fresh, moist cured meat (DCNO) in rats has been associated with increased fecal nitroso-compound (ATNC) concentrations and increased fecal biomarkers of fat peroxidation (TBARS) (6). Hence, we chose to use DCNO to test whether a cured meat could increase these early biomarkers in human volunteers and identify prevention strategies (6). Results of our crossover study showed that the consumption of DCNO for 4 d was enough to increase ATNC and TBARS concentrations in stools of volunteers compared with during the control period. A similar study in volunteers by Joosen et al (9) showed that the ingestion of 400 g processed meat/d for 14 d increased fecal ATNC from 3.5 nmol/g in controls given a vegetarian diet to 181 nmol/g. This increase in fecal ATNC was not associated with increased genotoxicity. In contrast, the cured-meat intervention compared with the vegetarian diet decreased fecal water-induced DNA strand breaks (9). In our study, the cured-meat diet (DCNO) tended to reduce γ -H2AX induction by fecal water volunteers compared with the control period (NS; see Supplemental Figure

S2 under "Supplemental data" in the online issue), and we observed no difference in γ -H2AX induction between the 3 cured-meat periods in rats or volunteers (see Supplemental Figures S1 and S2 under "Supplemental data" in the online issue). These results were in striking contrast with the observation by Hebel et al (36) that a beef-meat diet intervention did not change fecal ANTC but increase fecal water genotoxicity in volunteers, likely because beef meat contains more heme iron than does cured pork meat. Thus, the intake of cured meat can modulate biomarkers associated with the promotion of colon carcinogenesis in rats (TBARS, cytotoxicity but not genotoxicity), which gives experimental support to the epidemiology-based conclusion that processed meat could be a cause of colorectal cancer (37, 38).

Because nitrite and heme seem necessary to promote carcinogenesis in rats (6), the reduction of their concentrations in meat could reduce the toxicity of cured meat. This strategy is not easy to implement because it would increase microbiological risks and reduce sensory qualities of cured meat, which is why we looked for additives that could reduce the toxicity. This study showed that dietary calcium carbonate inhibited the cured-meat promotion of colon carcinogenesis in rats. A reduction of the MDF number was associated with the normalization of fecal TBARS and ATNC in rats (Table 2), and a parallel normalization was seen

TABLE 2Effects of dietary α -tocopherol and calcium carbonate on colon carcinogenesis biomarkers (MDF and ACF) and on fecal and urinary biomarkers associated with meat-induced promotion in rats previously injected with dimethylhydrazine and fed cured meat (DCNO) for 98–99 d¹

Diet ²	Rats	MDF/colon	Crypt /MDF	ACF/colon	Heme in FW	TBARS in FW	FW cytotoxicity on CMT93	ATNC in feces	DHN-MA in urine
DCNO	16	2.7 ± 2.1 ³	3.7 ± 1.3	126 ± 20	32 ± 16	70 ± 8	57 ± 7	53.5 ± 14.8	1.3 ± 0.6
DCNO + α -tocopherol	10	1.4 ± 1.5 ⁴	2.4 ± 2.1	125 ± 15	6 ± 8 ⁴	64 ± 10	51 ± 10	25.6 ± 2.7 ⁴	0.5 ± 0.2 ⁴
DCNO + CaCO ₃	10	1.3 ± 1.6 ⁴	2.5 ± 1.4	124 ± 24	<1.5	23 ± 11 ⁴	24 ± 17 ⁴	40.6 ± 5 ⁴	0.2 ± 0.1 ⁴

¹ ACF, aberrant crypt foci; ATNC, apparent total *N*-nitroso compound; DCNO, dark cooked meat treated with nitrite and oxidized by air; DHN-MA, 1,4-dihydroxynonane mercapturic acid; FW, fecal water; MDA.eq, malondialdehyde equivalent; MDF, mucin-depleted foci; TBARS, thiobarbituric acid reactive substances.

² Diet contained 47% DCNO. Tocopherol (0.05%) was added into DCNO (DCNO + α -tocopherol), and calcium carbonate (150 $\mu\text{mol/g}$) was added to the diet (DCNO + CaCO₃). Detailed compositions are shown in Subjects and Methods.

³ Mean ± SD (all such values).

⁴ Significantly different from DCNO, *P* < 0.05 (Dunnett's *t* test).

in stools of volunteers (Figure 2). A high-calcium diet consistently blocked the effects of red meat and dietary heme iron on the gut mucosa, including the proliferation, carcinogenesis promotion, and associated biomarkers (13–15). These results can explain why beef meat and bacon do not promote rodent carcinogenesis when added into a high-calcium diet (39). The current data suggest that the cured-meat effect could be neutralized by adding calcium to the meat or by consuming a calcium-rich food in the same meal. Indeed, calcium-carbonate supplements reduced risk of recurrent colorectal adenomas in volunteers (40); chemoprevention by calcium might be due in part to the binding of dietary heme iron. Thus, this beneficial effect of calcium has a drawback because it would increase risk of iron deficiency.

This study also showed that the addition of α -tocopherol into cured meat inhibited the promotion of colon carcinogenesis in rats. The MDF-number reduction by α -tocopherol was associated with the normalization of urinary DHN-MA in rats fed cured meat (Table 2). We proposed that the promotion by heme iron would have been a result of fat oxidation end products (13, 14, 22); the *Apc* mutation renders cells resistant to 4-hydroxy-2-nonenal, which is an end product of heme-induced fat oxidation (25), which we measured by its urinary metabolite DHN-MA. Thus, the selection of *Apc* mutated cells by cytotoxic peroxides would explain the heme-induced promotion of colon carcinogenesis (41). In the current trial, in the 14 d study, the reduction in fecal TBARS by α -tocopherol, calcium carbonate, and rutin (Table 1) was associated with reduced cytotoxicity against nonmutated *Apc*^{+/+} cells, which might have explained the reduced promotion. However, this reduction in fecal TBARS and cytotoxicity was not seen in the 100-d study (Table 2), which casted doubt that cytotoxic peroxides and the selection of *Apc* mutated cells would explain promotion by cured meat. In contrast, the protection by α -tocopherol was associated with reduced fecal ATNC in rats (Table 2). This association supports the hypothesis that *N*-nitroso compounds are the major pro-cancer molecules from cured meat, which is a hypothesis supported by the studies of Mirvish et al (8, 12, 27, 42, 43) in rodents, of Bingham et al (7, 10, 28, 44) in volunteers, and a previous carcinogenesis study from this team (6). The addition of α -tocopherol to cured meat halved fecal ATNC in rats (Table 2) but did not reduce significantly fecal ATNC in volunteers. These results suggested that vitamin E would not be sufficient to reduce cured-meat toxicity in humans. Our previous carcinogenesis studies suggested that fat peroxides such as 4-hydroxynonenal would explain the promotion by fresh red meat (13, 14, 22, 24), whereas this study and a previous one (6) suggested that nitrosation and ATNC would explain the promotion by cured meat.

Recommendations to avoid processed meat intake may reduce the colorectal cancer burden (37, 38). However, people of lower social status whose processed meat intake is high are not receptive to such nutritional messages and are less likely than affluent people to change risky behaviors (45, 46). The resulting consequences on the quality and length of life are dramatic (eg, the disability-free life expectancy is 70 y in affluent British neighborhoods but declines to 53 y in deprived ones) (47), and colorectal cancer incidence varies between socioeconomic classes and, therefore, contributes to health inequalities (48). Simply conveying information on risks and benefits has almost no effect on food choices in less-educated people and, thus, tends to enlarge the gap. Changing the food (eg, by adding protective additives to cured meat) might prevent the cancer promotion by meat and could be an answer to health inequalities.

In conclusion, the consumption of cured meat for a few days increased biomarkers associated with the heme-induced promotion of colon carcinogenesis. Dietary calcium and α -tocopherol counteracted this promoting effect in carcinogen-injected rats. This protection was associated with the normalization of fecal biomarkers in rats and humans. We previously proposed the concept that a nutrient (calcium carbonate) can inhibit the promoting effect of another nutrient (heme iron) (13). In the current study, we show that this concept stands true in humans at the biomarker level. This article also suggests that the curing process might be changed to reduce cancer promoting properties of cured meat. This effect could lead to protective strategies to decrease the colorectal cancer burden in individuals who are the most exposed by changing the food, not the consumer.

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