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Calcium Inhibits Promotion by Hot Dog of 1,2-Dimethylhydrazine-Induced Mucin-Depleted Foci in Rat Colon

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+ This work is dedicated to the memory of Jean-Luc Vendeuvre, who brought his broad knowledge on meat chemistry and meat processing to this study, and died after the study was finished.

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Running Title: Promotion of colon carcinogenesis by hot dog is inhibited by calcium

Keywords: Processed meat, colorectal cancer, preneoplastic lesions, prevention, calcium

Abbreviations: ACF, aberrant crypt foci; MDF, mucin-depleted foci; ATNC, apparent total N-nitroso compounds; TBARS, Thiobarbituric acid reactive substances; DHN-MA,1,4-dihydroxynonane mercapturic acid

Brief description of the novelty and impact of the work (69 words < max 75 words)

This is the first experimental evidence that real store-bought cured meat can promote colon carcinogenesis, and that dietary prevention is possible. Hot dog increased the number and size of mucin depleted foci (precancerous lesions) in carcinogen-injected rats. The addition of calcium carbonate to hot dog diet fully suppressed the promoting effect of cured meat. Fecal nitroso-compounds correlated with promotion, and might be used as a biomarker in human volunteers.

Abstract:

Epidemiology suggests that processed meat is associated with colorectal cancer risk, but few experimental studies support this association. We have shown that a model of cured meat made in a pilot workshop promotes preneoplastic lesions, mucin-depleted foci (MDF) in the colon of rats. This study had two aims: to check if real store-bought processed meats also promote MDF, and to test if calcium carbonate, which suppresses heme-induced promotion, can suppress promotion by processed meat. A 14-day study was done to test the effect of nine purchased cured meats on fecal and urinary biomarkers associated with heme-induced carcinogenesis promotion. Fecal water from rats given hot dog or fermented raw dry sausage was particularly cytotoxic. These two cured meats were thus given to rats pretreated with 1,2-dimethylhydrazine, to evaluate their effect on colorectal carcinogenesis. After a 100-d feeding period, fecal apparent total N-nitroso compounds (ATNC) were assayed and colons were scored for MDF. Hot dog diet increased fecal ATNC and the number of MDF per colon compared with the no-meat control diet (3.0±1.7 vs. 1.2±1.4, $P<0.05$). In a third study, addition of calcium carbonate (150 µmol/g) to the hot dog diet decreased the number of MDF/colon and fecal ATNC compared with the hot dog diet without calcium carbonate (1.2 ± 1.1 vs. 2.3 ± 1.4, respectively, $P<0.05$). This is the first experimental evidence that a widely consumed processed meat promotes colon carcinogenesis in rats. It also shows that dietary prevention of this detrimental effect is possible.

Introduction

Colorectal cancer is one of the main causes of mortality in Western countries[1]. This cancer is strongly influenced by environmental factors, such as diet [2]. In its 2007 report the World Cancer Research Fund panel judges that "the evidence that red meat and processed meat are a cause of colorectal cancer is convincing" [3]. The panel thus recommends: “Limit intake of red meat and avoid processed meat”. Meta-analyses of cohort studies show that intake of red and processed meat increases the risk of colorectal cancer [4-7]. Consumption of white meat and fish is not associated with colorectal cancer risk, but heme iron intake is [8], and the association is stronger for processed meat than for red meat, per gram of meat [8, 9].

Four major hypotheses were proposed to explain the relationship between colorectal cancer and meat consumption [10]: (i) meat fat promotes carcinogenesis by raising bile acid levels in the gut; (ii) cooking meat at high temperature forms carcinogenic heterocyclic amines and polycyclic aromatic hydrocarbons; (iii) carcinogenic N-nitroso compounds are formed endogenously by nitrosation of amines and amides; (iv) heme iron in red meat can promote carcinogenesis because it increases cell proliferation in colonic mucosa, through lipid oxidation and/or cytotoxicity of fecal water. Hypotheses (iii) and (iv) could explain why cured meat seems more toxic than fresh red meat [9]. In a series of experimental studies we have shown that heme iron could partly explain the effect of red meat on carcinogenesis [11-13]. This promoting effect is associated with fat peroxidation and production of cytotoxic and genotoxic hydroxyl-alkenals like 4-hydroxy-nonenal in the gut. Inside fresh red meat, heme iron is bound to myoglobin, while in cooked cured meat, heme iron is mostly nitrosylated by sodium nitrite and freed up from myoglobin by heating, leading to the formation of nitrosylheme [14, 15]. We speculated that nitrosylheme in cured meat could be more potent to promote carcinogenesis than myoglobin in fresh meat, as suggested by epidemiology [9]. In support of this speculation, hemin (free heme stabilized by a chloride ion) is more toxic than hemoglobin [11, 16].

We have shown previously that a model of cured meat made in a pilot workshop promotes mucin-depleted foci (MDF), a surrogate endpoint biomarker for colon cancer, on the mucosa of DMH (dimethylhydrazine)-initiated rats, and raises fecal level of apparent total N-nitroso compounds (ATNC)[17]. We also showed that beef meat promotion of DMH-induced carcinogenesis is suppressed by dietary calcium [13]. The protection is likely due to chelation of

heme by calcium salts in the digestive tract, resulting in prevention of endogenous fat oxidation by heme iron.

The aims of the present study were (i) to check if some types of real cured meat that are bought in store can promote MDF like the already tested model of cured meat; (ii) to test if calcium carbonate, which suppresses heme-induced promotion, can suppress MDF promotion by cured meat. The results show that hot dog increased the number of MDF per colon and that calcium carbonate suppressed this promoting effect in a rodent model of carcinogenesis.

Financial disclosure, conflict of interest

This work was supported by French National Institute for Agricultural Research (INRA), the French National Research Agency [PNRA, HemeCancer Project, grant number ANR-05-PNRA-5.E14] and a National Institutes of Health [grant number, RO1-CA-143460].

R.L. Santarelli, as a PhD student, and J.L. Vendeuvre were paid by IFIP (Institut Français du Porc). This article is done “in memoriam” of J.L. Vendeuvre. The other authors disclosed no potential conflicts of interest.

Materials and Methods

General Design

Three sequential studies were performed: a 14-day study investigated the effect of nine types of cured meat bought in store on early fecal and urinary biomarkers in rats. A 100-day study was then performed to measure the promoting effect of two types of cured meat selected among the nine ones, on preneoplastic lesions in carcinogen-initiated rats. A third study was realized to test if dietary calcium carbonate would suppress cured-meat induced promotion of preneoplastic lesions.

Short-term study

Short-term study: animals

Fifty-five female Fischer 344 rats were purchased at 4 weeks of age from Charles River (St.Germain l'Arbresle, France). Animal care was in accordance with the guidelines of the European Council on animals used in experimental studies.

Rats were kept in an animal colony with a temperature of 22°C and a 12:12-hour light-dark cycle. Rats were housed individually into metabolic cages, and were allowed free access to tap water and to the standard AIN76 diet [18]. After 2 days of acclimatization, they were randomly allocated to 11 groups (5 rats per group) and fed experimental diets for 14 days. Body weights were monitored on days 2, 7 and 14. Food and water intakes were measured on days 6-7 and 12-13. Feces was collected during the last 2 days and frozen at -20°C. Urines were collected once at day 13 and were processed immediately.

Short-term study: experimental diets

Experimental diets were made in a specialized workshop by IFIP-Institut du Porc (Paris, France). Nine types of cured meat were bought in a store: a cooked city ham, a raw dry-cured country ham, a white chicken ham (steam cooked chicken breast treated with nitrite), a coarse pâté "*de campagne*", a smooth liverwurst liver pâté, a chipolata-type fresh breakfast sausage, a frankfurter hot dog, a Danish-type raw salami and a French "*saucisson*" (a fermented raw dry sausage). These products are stored at 4°C except country ham, salami and *saucisson* that are dry (<50% H2O) and salty (>3.5% NaCl) and stable at room temperature. These nine products were analyzed for fifteen components including moisture, fat and fatty acids profile, iron, heme, nitrosyl heme, nitrate and nitrite (composition and photo of the products shown on http://securiviande.free.fr/Santarelli-2013-supl.data.html). Because freeze-drying boosts the

formation of lipid oxidation products in meat [19], meat products were not dried before being added to a base powder (UPAE, INRA, Jouy, France). Each diet contained 55 g of processed meat (dry matter of moist meat), 40 g of modified AIN-76 powder and 5 g of safflower oil (MP Biomedicals, Illkirch, France) per 100 g (Table 1). Because some products were lean and other were very fatty, two control diets were made by UPAE to match protein, fat and iron content of experimental diets: cooked ham, raw dry ham and chicken ham were compared with a control diet containing 10 % fat, while pâté, liver pâté, breakfast sausage, hot dog, salami and *saucisson* were compared with a 25 % fat control (Table 1). Because calcium inhibits meat-induced promotion, all diets were low-calcium (0.27 g calcium phosphate/100 g diet) [13]. Daily portions of diets (25 g) were vacuum-packaged to avoid further lipid oxidation and stored at -20°C. They were given to rats every day at 5:00 p.m. for 14 days.

Promotion study

Promotion study: animals and design

Thirty rats (same strain, gender, and age as above) were housed in stainless steel, wire-bottomed cages with two rats per cage. After 5 days of acclimatization, each rat received a single i.p. injection of 1,2-dimethylhydrazine (180 mg/kg i.p. in NaCl 9 g/L; Sigma Chemical). Seven days later, they were randomly allocated to three groups of 10 rats and fed the experimental diets described below. Body weight was monitored every week during the first 4 weeks, and then every 2 weeks. Food and water intake was measured at day 20, 60 and 80. Feces was collected between days 90 and 95 and frozen at -20°C. Urines were collected between days 84 and 88 and frozen at -20°C: each rat was placed single in a metabolic cage to collect the urine. Animals were euthanized on day 98 and 99, and colons were removed and fixed in 10% buffered formalin (Sigma Chemical) between two sheets of filter paper with a blinding code. As described below, aberrant crypt foci (ACF) and MDF were then scored. Fecal water samples were analyzed for heme, thiobarbituric acid reactive substances (TBARS), ATNC and cytotoxicity. Urine samples were analyzed for 1,4-dihydroxynonane mercapturic acid (DHN-MA).

Promotion study: diets

Based on results from the short-term study, two products were selected amongst the nine tested cured meats: hot dog and *saucisson* (a fermented raw dry sausage). Diets were made by IFIP by mixing moist cured meat with a low-calcium modified AIN76 powdered diet prepared by UPAE. Composition of each diet was designed so that it contained 30% fat and

20% protein. Proportions of hot dog and *saucisson* thus differed in the diets to take in account their fat and protein content (Table 1). Daily portions of diets were packaged and given to rats as in the short-term study.

Protection Study

Twenty rats were managed exactly as reported above (same strain, gender, age, caging, animal colony, carcinogen initiation, monitoring, feeding period, colon harvest and measured endpoints). They were randomly allocated to two groups of 10 rats and fed a hot dog diet without or with calcium carbonate. Food and water intakes were measured at days 13, 58 and 93. Feces was collected on days 10-14, 57-60, 85-95 of the study and frozen at -20°C. Hot dog diet was made as in the promotion study described above, except for minor variations in composition due to minor difference found in hot dog analysis (Table 1). We tested the protecting effect of calcium by adding 1.5 g/100 g of calcium carbonate into the hot dog diet.

Biomarkers

Preparation of fecal water

Fecal pellets were collected under each cage for 24h, thus leading to five samples per group (five rats housed singly in short-term study; ten rats housed with two rats per cage in long-term studies). Preparation of fecal water was achieved by adding 1 ml of water to 0.3 g of freeze-dried feces. Samples were then incubated at 37˚C for 1 hour, stirred thoroughly every 20 minutes, and then centrifuged at 20,000 x g for 15 minutes. The supernatant (fecal water) was collected and kept at –20˚C until use.

Heme and TBARS in fecal water

Fecal water was analyzed because, according to bile acids studies, the soluble fraction of colon content would interact more strongly with the mucosa than the insoluble fraction [20]. Heme concentration of fecal water was measured by fluorescence according to Van den Berg et al. [21] as described by Pierre et al [11]. We supposed that processed meat would induce lipid oxidation in fecal water as already shown with red meat, and lipid oxidation products present in fecal water are cytotoxic against colon cells [22]. We thus measured TBARS in fecal water as a global measure of fecal lipid oxidation products. TBARS were measured in fecal water as previously described [13], and results are given as malondialdehyde equivalent.

Cytotoxicity of fecal water

The Apc mutation is detected in the majority of MDF in rats and of human colorectal cancers. Premalignant Apc mutated cells resist to cytotoxic aldehydes found in the gut of meat-fed rats: this resistance leads to the selection of premalignant cells, and would explain cancer promotion by red meat[22]. To know if the same mechanism may explain the promotion of carcinogenesis by cured meat, cytotoxicity of fecal water was quantified on three murine cell lines: (i) a cancerous mouse colonic epithelial cell line, CMT93 (European Collection of Animal Cultures); (ii) a colon epithelial cell lines derived from C57BL/6J mice (*Apc* +/+) and (iii) Min mice *Apc* +/- [23, 24]. Use of this triple cellular model could contribute to our understanding of the biological effects of fecal water on normal cells (*Apc* +/+), premalignant cells (*Apc* +/-) and cancerous cells (CMT93) [22]. CMT93 cell line was obtained from a rectal polypoid carcinoma in a C57BL/1CRF male mouse which had received injections of methylazoxymethanol acetate: these cells are stable in culture, and produce large tumours in nude mice after 1 month. Cytotoxicity of fecal water was quantified by tetrazolium dye reduction to formazan as previously described [13].

Urinary DHN-MA

The 24-hour urine was collected under each individual metabolic rat cage. Urinary 1,4-dihydroxynonane mercapturic acid (DHN-MA) reflects fat oxidation and formation of 4-hydroxynonenal in vivo and in the diet, as DHN-MA is its main urinary metabolite. DHN-MA assay was done by competitive enzyme immunoassay as previously described [25], using DHN-MA-linked acetylcholinesterase enzyme [26]. Each urine sample was assayed in duplicate that always agreed with each other.

Apparent total N-nitroso compounds

To determine ATNC in fecal water of rats 425 µL of fecal water was mixed with 25 µL of 2 N HCl and 50 µL of a freshly prepared saturated solution of sulfamic acid in water (SA reagent) to destroy any nitrite present. After storage for 15 min at room temperature and <4 h in ice, 100 or 200 µL samples of the mixture were injected into the reaction vessel. ATNCs were decomposed to NO by an HCl/HBr/HOAc/EtOAc mixture refluxing at <0.5 mm Hg and 20°C. The NO was swept by an argon stream through four wash bottles containing NaOH and Na_2SO_4 at room temperature and two empty wash bottles kept at −30 °C to remove water vapor and acids and was then determined in a Thermal Energy Analyzer (Advanced Chromatographic Systems, Charleston, SC, USA). At intervals, the tops of the wash bottles,

which were connected with Nalgene tubing, were removed without disconnecting them, rinsed, dried in an oven and reinserted into the wash bottles.

ACF and MDF Assays

Rats were killed by CO_2 asphyxiation in a random order at days 98 or 99. Colons were coded, fixed in formalin and scored for ACF incidence by Bird's procedure [27]. Briefly, numbers of ACF per colon and of crypts per ACF were counted after methylene blue staining, under light microscope at x 40 magnification, by a single trained observer blinded for the origin of the colon. ACF scoring criteria were as follows: focus often elevated above mucosa surface with a large pericryptal zone, bordering crypts larger and darker than normal crypts.

Colons were scored for MDF after staining with the high-iron diamine Alcian blue procedure [28]. Two trained observers, each one reading independently all the colons, blinded for the rat treatment, evaluated the number of MDF per colon and the number of crypts per MDF. MDF scoring criteria were as follows: focus with at least three crypts with no or very little apparent mucin.

Statistical Analysis

Results were analyzed using Systat 10 software for Windows. Values were considered firstly using one-way (ANOVA). If a significant difference was found between groups ($P < 0.05$), comparison of each experimental group with the control group was made using Dunnett's test. Results for lean cured meats in the short term study were compared with those of 10% fat control diet, while results for high fat meats were compared with those of the 25% fat control diet. A two-way ANOVA was used to analyze MDF data (groups and observers): the interaction group x observer was never found significant, thus data from the two observers were pooled. When total ANOVA was significant ($p<0.05$), pairwise differences between groups were analyzed using Fisher's least-significant-difference test.

Results

Short-term study

General observations

Final body weight of rats was 128 ± 14 g. At the end of the 14 day feeding period, body weight of rats given chicken ham was lighter (-10%), and given liver pâté heavier (+14%), than their respective controls. Rats given chicken ham, hot dog, or breakfast sausage ate less food than their respective controls (-23%, -13% and -20% respectively). Raw dry ham-fed rats drank more water per day (+69%) than controls (all $P<0.05$, full data not shown).

Fecal cytotoxicity and fat oxidation biomarkers (TBARS and DHN-MA)

Fecal water from cured meat–fed rats contained two to five times more fat oxidation products than control rats (Table 2), highest TBARS values being found in feces from rats given raw dry ham and *saucisson*. Fecal water from chicken ham- hot dog-, and *saucisson*-fed rats were more cytotoxic to the Apc +/+ and +/- cell lines than their respective controls (Table 2), and *saucisson* was the only food that enhanced fecal cytotoxicity against CMT93 cells. Urinary DHN-MA excretion was enhanced 7 to 15-fold in cured meat-fed rats compared with control rats, except in rats given hot dog or breakfast sausage (Table 2).

Choice of meats to be included into the promotion study

We could not afford a 100 day-carcinogenesis study with too many dietary groups, we thus decided to select two of them for inclusion in a long-term study. They were chosen according to three criteria: (i) strong modulation of biomarkers previously found to correlate with carcinogenesis, (ii) high level of intake in Western countries (iii) similar fat content so that only one control group be needed. *Saucisson* was selected because it strongly raised all the measured biomarkers in the short-term study (highest TBARS and cytotoxicity in Table 2). Hot dog was chosen because it also raised cytotoxicity, second only to *saucisson*, and is widely consumed by American and European subjects [29]. The fact that hot dog diet did not increase TBARS and DHN-MA (Table 2) was seen as an opportunity to check if fat oxidation is needed for carcinogenesis promotion by cured meat. Chemical analysis showed that major differences between these two products were moisture (55 and 30% respectively), total iron (67 and 23 mg/kg dry matter, respectively) and nitrate (67 and 19 mg/kg dry matter, respectively). Fat content in the dry matter was similar in hot dog and *saucisson* (66 and 51% respectively).

Promotion Study

Final body weight of rats was 211 ± 9 g without significant difference between groups. Diet intake was not significantly different between groups, but raw dry "*saucisson"*-fed rats drank more water than control rats (+28%, P<0.05, full data not shown).

Mucin Depleted Foci

Compared with control diet, hot dog-fed rats had more MDF per colon (P = 0.005, Fig. 1A) and more crypts per MDF (p = 0.002, and Table 3, study A). Raw dry "*saucisson"* marginally increased the number of MDF per colon (p = 0.06, Figure 1A). Cured meat diets did not increase the number of ACF or the number of crypt/ACF compared with the control diet (Table 3, study A, full data not shown).

Fecal and Urinary Biomarkers

As expected, no heme was detected in fecal water from control rats, but heme was present in feces from meat-fed rats (Table 3, study A). "*Saucisson"* increased urinary DHN-MA and TBARS values (p<0.05), but hot dog increased urinary DHN-MA value only (Table 3, study A). Fecal water from meat-fed rats was more toxic to CMT93 cells than fecal water from controls. The two cured meat diets enhanced the ATNC value three-fold compared with control diet (P<0.05, Fig. 1B).

Protection Study

We decided to study the protective effect of calcium carbonate against hot dog diet promotion of carcinogenesis. The final body weight of rats was 232 ± 5 g without significant difference between groups, and there was no difference in diet and water intake (data not shown)

Mucin Depleted Foci

Addition of calcium carbonate to a hot dog-diet significantly decreased he number of MDF per colon (P=0.01, Fig.1A) but did not change the mean number of crypts per MDF (Table 3, study B). Calcium carbonate did not change significantly the number of ACF per colon (P>0.05, Table 3, study B). A direct comparison between both studies is not statistically allowed because rats were not randomized from the same batch. However, Student's t test shows a significant difference between promotion study control rats and protection study rats

given the hot dog diet (one-sided P = 0.048). Also, rats given the (hot dog + calcium) diet had exactly the same number of MDF per colon than promotion study control rats (Table 3).

Fecal and Urinary Biomarkers

In contrast with results from the Promotion study, no heme could be detected in fecal water from hot dog diets fed rats (Table 3, study B). We have no explanation for this discrepancy between two sequential studies. Addition of calcium carbonate to hot dog-diet strongly reduced fecal TBARS values (Table 3, study B) and decreased ATNC value (Fig. 1B).

Discussion

This study shows that hot dog, a widely consumed cured meat, can increase the number of preneoplastic lesions in rats, and that addition of calcium carbonate to the diet can suppress this promoting effect. These results suggest that prevention of processed meat-promotion of colorectal carcinogenesis might be possible by dietary means, without banning sausage from the diet.

Promotion of colon carcinogenesis was evidenced on a surrogate endpoint biomarker, mucin depleted foci. MDF, formed by dysplastic crypts devoid of mucin, have been identified in the colon of humans at high risk for colon cancer [30]. Like tumors, MDF harbor mutations in genes affecting colon carcinogenesis (Apc and K-ras) and show Wnt signaling activation [31], a dramatic reduction of MUC2 expression [32], and a strong activation of the inflammatory process [33], all features suggesting that MDF are precancerous. Rodent studies suggest that MDF are better predictors of colorectal cancer than ACF are, this is why we focused on MDF data [28]. Cured meat did not increase the number of ACF in the present study, in contrast with previous studies [16, 17], and we have no explanation for this discrepancy.

Promotion by fresh red meat is consistently associated with increased fecal fat peroxides and cytotoxic activity of fecal water [11-13]. We speculated initially that red meat and cured meat would promote carcinogenesis by similar heme-induced mechanisms. This is why fecal TBARS, urinary DHN-MA, and cytotoxicity of fecal water were measured in rats given cured meat. Saucisson and hot dog were selected to be included in the long-term study because, in the short-term study, they strongly raised cytotoxicity of fecal water in rats (Table 2). In addition, fecal water from hot dog-fed rats was cytotoxic on normal and preneoplastic cells, but not on cancer cells which suggested it could select cancer cells as explained above (Table 2). However, as discussed below, data from promotion and protection studies (Table 3) suggested that fecal cytotoxicity was not associated with promotion by cured-meat associated promotion, which contrast with published fresh red meat effect [11-13].

Heated sausages and cooked ham are major contributors to cured meat intake in northern Europe and in North-America where colorectal cancer rates are high [29, 34]. We showed that a city ham from a store (added to diet after freeze-drying), and a model of cured meat similar to cooked picnic ham (dark-red muscle, nitrite-treated, cooked and exposed to air, given moist to rats) promote colon carcinogenesis in rats [16, 17]. However several other cured meat products do not promote chemically induced colorectal carcinogenesis [17, 35]. It

would thus be important to know why some cured meat types promote carcinogenesis while others do not.

Hot dog increased the number of MDF per colon, while *saucisson* only marginally did so (Table 3, study A), suggesting hot dog would be more toxic than *saucisson*. Chemical analysis showed that hot dog contains three times more iron and more nitrate per kg of dry matter than *saucisson* (see http://securiviande.free.fr/Santarelli-2013-supl.data.html). This might explain, at least in part, toxicity differences. In addition, major process differences between these two sausages are grinding, fermentation, shelf life, and heating: could these four differences change sausage toxicity? (i) A first difference between the products is grinding: *saucisson* content is coarse-cut while hot dogs are finely comminuted "emulsion" sausages [36]. This latter type of grinding disrupts cells and enables close molecular interactions: pro-oxidant heme has access to fat. However, Table 3 shows that formation of fat peroxides was higher in the gut of *saucisson*-fed rats than in hot dog-fed rats, which disqualify this explanation. (ii) A second difference lies in fermentation of *saucisson* by *Lactobacillus sakei*. This bacteria needs heme to grow and reduces heme availability in meat [37]. Here, heme concentration of fecal water from *saucisson*-fed rats was half that of hot dog-fed rats (Table 3). This would suggest that cured meat promotion is due to heme [8]. According to our fresh beef meat studies, heme promotes *Apc* mutated cells in the colon mucosa via cytotoxic peroxidation endproducts, estimated by TBARS, DHN-MA and cytotoxicity values [11-13, 22]. But no correlation was seen here between those biomarkers and MDF number (Table 3), which suggests they do not predict promotion by cured meat. Fresh meat and cured meat would thus not promote tumors through the same pathways [12, 13]. (iii) A third difference is that hot dogs are stored between 0 and 4°C less than 3 weeks, while *saucissons* are stored for months at room temperature. This long storage favors oxidation and food analysis showed eight times more TBARS in *saucisson* than in hot dog ($P<0.001$, full data not shown). As noted above, fat peroxides were higher also in the gut of *saucisson*-fed rats than in hot dog fed rats, but they do not correlate with MDF promotion (Table 3). This explanation again does not fit with data. (iv) Hot dog is cooked at 60-80°C while *saucisson* is not exposed to more than 30°C. In cooked cured meat, heme is partly nitrosylated by sodium nitrite and freed up from myoglobin [14, 15]: release of heme from myoglobin starts above 55°C and is complete at 75°C [36]. As a result, hot dog contains pink nitrosylheme, free from globin. *Saucisson* in contrast contains dark red nitrosylmyoglobin. Since free hemin is more toxic than hemoglobin [16], this could be a reason why hot dog was more potent than *saucisson* to promote MDF (Table 3).

Hot dog and *saucisson* significantly increased fecal ATNC values in rats (Table 3). Mirvish *et al*. have showed that hot dog diet increases fecal ATNC output in mice [38, 39], and that force-feeding mice with ATNC derived from hot dogs induces colonic ACF [40]. We have shown that a model of nitrite-cured meat increases the fecal level of ATNC and the number of MDF per colon, compared with a no-nitrite control processed meat [17, 39]. Production of ATNC in the gut or ingestion of preformed ATNC[41] could thus explain promotion of colorectal carcinogenesis by cured-meat. In human volunteers, dietary red meat and cured meat enhance the excretion of ATNC in ileal output and in stools [42-44]. Furthermore, dietary hemin increases fecal ATNC levels 2-4 times in mice given nitrite in water [45]. Both heme iron and nitrite thus seem needed to boost ATNC formation in the gut, and to explain cured-meat promotion of carcinogenesis in rats.

The addition of calcium to hot dog-based diet decreased the number of MDF per colon, and associated fecal biomarkers, TBARS and ATNC (Table 3). Calcium suppress hemin-induced proliferation in the gut [46] and ACF and MDF promotion [11, 13]. Calcium would suppress carcinogenesis in rodents because it precipitates irritating bile acids in the gut and reduces hyperproliferation[47] but the protection is not significant in rats given a diet with no meat and no heme[11]. Calcium carbonate was chosen because it is more potent than calcium phosphate to trap heme *in vitro* and to prevent heme-induced promotion [48]. This prevention by dietary calcium of cured-meat promotion may explain why Parnaud *et al*. failed to show that bacon promote carcinogenesis because the AIN76 diet is high in calcium [35]. Surprisingly no heme was detected in feces of hot dog-fed rats. However, the calcium diet reduced fecal levels of TBARS and ATNC, but not of DHN-MA and cytotoxicity (Table 3, study B). Table 3, study A results support that MDF promotion is associated with high fecal heme, cytotoxicity and ATNC and high urinary DHN-MA, while Table 3, study B results disqualify DHN-MA and cytotoxicity, but support only fecal TBARS and ATNC as promotion associated biomarkers. We thus think that fecal ATNC, and maybe fecal TBARS, are useful biomarkers to predict cured-meat associated promotion. They may also be the mechanistic links between cured-meat intake and cancer.

In conclusion, this study shows that store-bought hot dog promoted the formation of preneoplastic lesions in chemically induced rats, and that a high-calcium diet suppressed this promoting effect. Results support the hypothesis that nitroso-compounds are major pro-cancer

factors in the gut of processed-meat eaters. This study supports the concept that toxicity of specific foods can be counteracted by a nutrient, calcium [13]. WCRF recommends avoiding processed meat to decrease colorectal cancer risk [3]. This study suggests an alternative protective strategy against colorectal cancer[49]: processed meat meals should also include a calcium-rich food to counteract toxicity of cured meat.

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Table 1: Composition of diets (dry basis g / 100g of diet).

	Short-term study			Promotion study			Protection study	
	10% fat control diet	25% fat control diet	Cured meat diets	Control diet	Hot dog diet	Saucisson diet	Hot dog diet	Hot dog + calcium diet
Cured meat[a]	0	0	55	0	40	50	40	40
Base[b]	16.3	16.3	16.3	43	43	43	45	45
Casein	40	40	0	20	9.3	1.8	9.3	7.8
Lard	5	20	0	25	0	0	0	0
Safflower oil	5	5	5	5	5	5	5	5
Sucrose	33.7	18.7	23.7	7	2.7	0.2	0.7	0.7
Ferric citrate	0.012	0.012	0	0.03	0	0	0	0
Calcium carbonate	0	0	0	0	0	0	0	1.5

a- Dry matter. The amount of moist cured meat added to each diet depended on its water content. Short-term study (g of moist meat per 100 g of dry diet): 77 to 212. Promotion study: 71 or 90. Protection study: 90.

b- Base composition (g/100g total diet)

- Short-term study: corn starch, 6; cellulose, 5; AIN76 calcium-free mineral mix, 3.5; AIN76 vitamin mix, 1; methionin, 0.3; calcium phosphate, 0.27; choline bitartrate, 0.22.
- Promotion study: sucrose, 64.6; corn starch, 11.6; cellulose, 11.6, AIN76 calcium- free mineral mix, 8.1; AIN76 vitamin mix, 2.3; methionine, 0.7; calcium phosphate, 0.49; choline bitartrate, 0.47.
- Protection study: sucrose, 66.7; corn starch, 11.1; cellulose, 11.1, AIN76 calcium- free mineral mix, 7.8; AIN76 vitamin mix, 2.2; methionine, 0.7; calcium phosphate, 0.5; choline bitartrate, 0.4.

Table 2: Effect of nine cured meat products on lipid oxidation products and cytotoxicity of fecal water, and urinary DHN-MA, in rats given experimental diets for 14 d (values are means ± SD, n = 5)

Diet	No of rats	TBARS in fecal water (MDA equivalent, µmol/L)	Cytotoxicity of fecal water on cells			Urinary DHN-MA (µg/24h)
			Apc +/+ (%)	*Apc* +/- (%)	CMT93 (%)	
10% fat control	5	28± 7	19 ± 10	12 ± 7	63 ± 16	0.7 ± 0.5
Cooked ham	5	85 ± 34	37 ± 14	63 ± 6 [a]	52 ± 6	11.5 ± 0.4 [a]
Raw dry ham	5	166 ± 85 [a]	34 ± 33	16 ± 5	39 ± 2 [a]	11.2 ± 4.2 [a]
Chicken ham	5	63 ± 33	74 ± 5 [a]	74 ± 7 [a]	47 ± 6 [a]	5.2 ± 3.5 [a]
25% fat control	5	59 ± 16	23 ±20	56 ±10	74 ±3	0.7 ± 0.3
Pâté	5	110 ± 25 [b]	0 ± 19	51 ±2	44 ± 6 [b]	3.3 ± 0.7 [b]
Liver pâté	5	103 ± 40 [b]	16 ± 14	31 ± 8 [b]	45 ± 6 [b]	6.0 ± 1.1 [b]
Breakfast sausage	5	93 ± 67	27 ± 21	55 ± 11	58 ± 6 [b]	1.4 ± 0.6
Hot dog	5	98 ± 38	79 ± 3 [b]	74 ± 5 [b]	66 ± 8	1.1 ± 0.5
Salami	5	124 ± 40 [b]	10 ± 20	37 ± 1 [b]	29 ± 7 [b]	9.0 ± 0.1 [b]
Saucisson	5	179 ± 70 [b]	94 ± 2 [b]	97 ± 1 [b]	95 ± 7 [b]	4.4 ± 0.7 [b]

See Table 1 and Materials and Methods for more information about dietary groups and composition of diet.

MDA: malondialdehyde

a- significantly different from 10% fat control diet (P<0.05)

b- Significantly different from 25% fat control diet (P<0.05)

Table 3**:** Effect of processed meat diets on MDF formation in the colon of rats 99 d after the injection of 1,2-dimethylhydrazine, and fecal and urinary biomarkers after 80 d on experimental diets (values are means ± SD, n = 10 for each group)

Study	Diet	No. of Rats	Preneoplastic lesions: MDF /colon	Preneoplastic lesions: crypts /MDF	Preneoplastic lesions: ACF /colon	Fecal water concentrations: ATNC (nmol/g as NNO)	Fecal water concentrations: Heme (µmol/L/24h)	Fecal water concentrations: TBARS (µmol/L MDA eq.)	Fecal water cytotoxicity on CMT93 cells (% dead cells)	Urinary DHN-MA (µg/24h)
Study A Promotion	Control	10	1.2 ±1.4	2.6 ± 2.4	110 ± 17	10 ± 18	0 ± 0	52 ± 10	0 ± 4	0.4 ± 0.3
	Hot dog	10	3.0 ± 1.7[a]	4.7 ± 2.4 [a]	108 ± 32	34 ± 3 [a]	44 ± 50 [a]	70 ± 17	21 ± 16 [a]	2.7 ± 1.5 [a]
	Saucisson	10	2.4 ± 2.4	3.2 ± 2.2	102 ± 25	33 ± 18 [a]	20 ± 19	124 ± 23 [a]	79 ± 18 [a]	3.1 ± 1.6 [a]
Study B Protection	Hot dog	10	2.3 ± 1.4	3.5 ± 0.6	136 ± 25	48 ± 11	0 ± 0	74 ± 30	44 ± 12	0.2 ± 0.1
	Hot dog + calcium	10	1.2 ± 1.1[b]	3.3 ± 0.4	118 ± 19	34 ± 9 [b]	0 ± 0	6 ± 11 [b]	43 ± 13	0.2 ± 0.1

MDF, mucin depleted foci; ACF, aberrant crypt foci; FW, fecal water; MDA: malondialdehyde; Other abbreviations and composition of diets: see Materials and Methods and in Table 1

a: significantly different from control diet (P<0.05)

b: significantly different from hot dog diet (P<0.05)

Figure legend

Figure 1: (A) Effect of cured meat (hot dog, saucisson), and calcium carbonate (Ca), on mucin-depleted foci (MDF) in rat colon 99 d after the injection of 1,2-dimethylhydrazine, in two independent studies: Promotion study on the left, Protection study on the right. (B) Nitroso-compounds (ATNC) in feces of rats. Values are means and standard error of the mean (10 rats per group).

Diets contained 40% and 50% processed meat (dry matter), full composition shown in Table 1.

*: significantly different from control diet (left panel) or from hot dog diet (right panel)