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Accepted Version

Kuhnle, G. and Hobbs, D. (2017) Red wine and pomegranate extracts suppress cured meat promotion of colonic mucin-depleted foci in carcinogen-induced rats. Nutrition and Cancer, 69 (2). pp. 289-298. ISSN 1532-7914 doi: https://doi.org/10.1080/01635581.2017.1263745 Available at http://centaur.reading.ac.uk/68425/

To link to this article DOI: http://dx.doi.org/10.1080/01635581.2017.1263745

Publisher: Taylor & Francis

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Journal:	*Nutrition and Cancer: An International Journal*
Manuscript ID	N&C-01-16-2624.R2
Manuscript Type:	Original Article
Date Submitted by the Author:	03-Oct-2016
Complete List of Authors:	Bastide, Nadia; INRA-ENVT, TOXALIM (Research Center in Food Toxicology) Naud, Nathalie; INRA-ENVT, TOXALIM (Research Center in Food Toxicology) Nassy, Gilles; IFIP-Institut du Porc Vendeuvre, Jean-Luc; IFIP-Institut du Porc Taché, Sylviane; INRA-ENVT, TOXALIM (Research Center in Food Toxicology) Guéraud, Francoise; INRA-ENVT, TOXALIM (Research Center in Food Toxicology) Hobbs, Ditte; University of Reading, Department of Food and Nutritional Sciences Kuhnle, Gunter; University of Reading, Department of Food and Nutritional Sciences Corpet, Denis; Université de Toulouse, INPT-ENVT, TOXALIM Pierre, Fabrice; INRA-ENVT, TOXALIM (Research Center in Food Toxicology)
Keywords:	Cancer Prevention, Colorectal cancer, Polyphenols, Crypt foci

Red wine and pomegranate extracts suppress cured meat promotion of colonic mucin-depleted foci in carcinogen-induced rats

Nadia M. Bastide[*1], Nathalie Naud[*1], Gilles Nassy[2], Jean-Luc Vendeuvre[2,¤], Sylviane Taché[1], Françoise Guéraud[1], Ditte A. Hobbs[3], Gunter G. Kuhnle[3], Denis E. Corpet[1,4], and Fabrice H.F. Pierre[1]

[1]INRA UMR1331; TOXALIM (Research Center in Food Toxicology); Université de Toulouse; ENVT; INP; 180 chemin de Tournefeuille, F-31027 Toulouse, France

[2]IFIP-Institut du Porc, Paris, France

[3]Department of Food and Nutritional Sciences, University of Reading, Whiteknights, PO Box 226, Reading, RG6 6AP, UK

[4]Corresponding author: D. Corpet; E-mail: prof.corpet@gmail.com

Phone: +33 6953 62223; Fax: +33 561 285 244;

[*]NMB and NN contributed equally to this work

Count: <7000 words (references included), abstract 150 words, 1 figure, 2 tables, 50 refs.

One supplementary file with 2 tables submitted

Running title: Plant extracts reduce promotion by cured meat

Abstract

Processed meat intake is carcinogenic to humans. We have shown that intake of a workshop-made cured meat with erythorbate promotes colon carcinogenesis in rats. We speculated that polyphenols could inhibit this effect by limitation of endogenous lipid peroxidation and nitrosation.

Polyphenol-rich plant extracts were added to the workshop-made cured meat and given for 14-days to rats and 100-days to azoxymethane-induced rats to evaluate the inhibition of preneoplastic lesions. Colons of 100d study were scored for precancerous lesions (mucin-depleted foci, MDF) and biochemical endpoints of peroxidation and nitrosation were measured in urinary and faecal samples.

In comparison with cured meat-fed rats, dried red wine, pomegranate extract, α-tocopherol added at one dose to cured meat and withdrawal of erythorbate significantly decreased the number of MDF per colon (but white grape and rosemary extracts did not). This protection was associated with the full suppression of faecal excretion of nitrosyl iron, suggesting this nitroso-compound might be a promoter of carcinogenesis.

At optimised concentrations, the incorporation of these plant extracts in cured meat might reduce the risk of colorectal cancer associated with processed meat consumption.

Keywords: Colorectal Cancer, Cancer Prevention, Polyphenols, Processed Meat, Mucin Depleted Foci

Introduction

Colorectal cancer (CRC) is the third most common type of cancer worldwide, and the second cause of cancer death in affluent countries (1). Epidemiological studies show that processed meat intake is linked to the risk of CRC (2). The World Cancer Research Fund panel considers this risk as convincing and recommends avoiding processed meat consumption (3, 4). The World Health Organization classified consumption of processed meat as “carcinogenic to humans” (IARC Group 1) based on sufficient evidence for colorectal cancer (5). Making safer meat products could be an alternative to banning cured meat (6, 7). In carcinogen-initiated rats given a low-calcium diet freeze-dried cooked ham and moist hot-dog increase significantly the number of mucin depleted foci (MDF) (8, 9). The intake of an experimental cured pork meat, similar to an air-exposed cooked shoulder-ham (DCNO for dark cooked meat with nitrite, oxidized, described below), also promotes carcinogenesis in rats (10). In human volunteers, cured meat intake increases endogenous nitrosation and fat peroxidation and faecal water-induced oxidative DNA damage (7, 11).

We have speculated that haem iron could explain in part the promoting effect of processed meat (12, 13), and added experimental support to this hypothesis: Dietary hemin (free haem stabilized by a chloride ion) promotes azoxymethane-induced aberrant crypt foci (ACF) in the colon of rats (14). Hemin, but not haemoglobin, mimics the effect of ham on biomarkers associated with carcinogenesis (8).

Haem iron catalyses the formation of apparent total nitroso compounds (ATNC) (15) and of lipid peroxidation endproducts, e.g., 4-hydroxynonenal and other alkenals (16). 4-hydroxynonenal is cytotoxic and genotoxic to the intestinal epithelial cells (17, 18). Potentially carcinogenic ATNC are formed in the gastrointestinal tract by N-nitrosation of peptides derived amines or amides. Nitrosylated haem iron present in processed red meat also represents a significant part of measured ATNC (13, 19, 20). ATNC and alkenals could explain tumour promotion by dietary haem and by cured meat (6, 21, 22).

Our starting hypothesis here was that lipid peroxidation endproducts would promote carcinogenesis (23), and that polyphenols would decrease haem-induced luminal peroxidation (24) and hence carcinogenesis. There is ample evidence that polyphenols and plant extract can block haem-induced fat peroxidation: For instance, quercetin, red

wine and α-tocopherol suppress myoglobin-induced peroxidation in a fat/water emulsion that mimics the gastric environment and block the accumulation of conjugated dienes (25, 26). In human volunteers, red wine polyphenols strongly decrease postprandial plasma malondialdehyde after a red meat meal, probably by suppressing haem-induced peroxidation in the stomach (27-29). In rats, a mix of rutin and butylated hydroxyanisole inhibits hemin-induced lipid peroxidation in the gut, and suppress carcinogenesis promotion (14). In addition, polyphenols such as punicalagin and ellagic acid from pomegranate can chelate iron through catechol groups (30, 31), while propyl gallate, tannic acid, thymol, vanillin, and ascorbate and α-tocopherol can inhibit nitrosation and ATNC formation (32, 33).

The present study was designed to test the hypothesis that polyphenols can prevent the promotion of colon tumorigenesis by processed meat, by suppressing lipid peroxidation in the gut. In a short-term screening study, several agents were added to DCNO cured meat during the manufacturing process. Such diets were given to rats for 14 days. Early lipid peroxidation endpoints were measured in faeces and urine. Most promising agents selected during these screening studies were added to DCNO and tested for chemoprevention in a 100-day carcinogenesis study in rats. Tumorigenesis end points were azoxymethane–induced preneoplastic lesions (ACF and MDF) in rats. The results showed that dried red wine, pomegranate extract and α-tocopherol prevented meat-associated formation of fecal nitrosyl iron and promotion of preneoplastic lesions.

Materials and methods

Animal study design

Two sequential studies were performed on male Fisher 344 rats purchased at 4-5 weeks of age from Charles River (St Germain l'Arbresle, France): A 14-day study investigated the effect of plant extracts added to an experimental cured meat on early faecal and urinary biomarkers in rats. A 100-day study measured the anti-promoting effect of four plant extracts added to the same cured meat, on preneoplastic lesions in carcinogen-initiated rats. Animal care was in accordance with the guidelines of the European Council on animals used in experimental studies. Study was done in an accredited animal colony (French A 31504) by approved staff (e.g., P.I. Corpet: Certificat d'autorisation d'expérimenter sur animaux vertébrés vivants #31-121).

Short-term study design (14 days-long)

Forty three rats were housed individually in metabolic cages. They were kept at 22°C and 12h-12h light-dark cycle. After 3 days of acclimatization to the animal colony and to a standard AIN76 diet, rats were randomly allocated to eight groups. There were five rats in each experimental group given DCNO cured meat with plant extracts (described below), and eight rats in the control group fed DCNO. Rats were fed the experimental diets described below during 14 days, and allowed free access to tap water. Body weight was monitored every week. Food and water intakes were measured at days 13. Faeces and urine were collected at days 11 and 12 and frozen at −20°C. Animals were terminated by CO_2 asphyxiation on day 14. Faecal water samples (preparation described below) were analyzed for haem, cytotoxicity and thiobarbituric acid reactive substances (TBARS). Urine samples were analyzed for 1,4-dihydroxynonane mercapturic acid (DHN-MA).

Carcinogenesis study (100 day-long): Animals and design.

Eighty six rats were housed individually in stainless steel, wire-bottomed cage (same animal colony as above). After 7 days of acclimatization each rat received a single i.p. injection of azoxymethane (20 mg/kg i.p.; Sigma Chemical) in NaCl (9 g/L). Seven days later, they were randomly allocated to seven groups (N=10 rats per group, except control group, N=26) and fed the experimental DCNO-based diets described below. Body weights were monitored every week for four weeks, then every two-week. Food and water intakes were measured at days 20 and 80. Faeces were collected daily between days 18 and 21, and 80 and 91 and frozen at −20°C. Between days 74 and 76 each rat was put in a metabolic cage and urine was collected and frozen at −20°C. Rats were killed by CO_2 asphyxiation in a random order at day 96 to 98. Colons were removed and fixed in 10% buffered formalin (Sigma Chemical) between two sheets of filter paper with a blinding code. ACF and MDF were scored. Faecal water samples were analyzed for haem, TBARS, cytotoxicity and ATNC. Urine samples were analyzed for DHN-MA.

Animal diets

The type of meat and the additives that were given to groups of rats during the short-term and carcinogenesis studies are listed in the first column of Tables 1 and 2, respectively.

Pork meat was cured in a specialized workshop by IFIP-*Institut du Porc* (14-day study) and in a ham factory by Fleury Michon (Pouzauges-France) (100-day study). Meat was given as such (moist piece) to the rats, because freeze-drying boosts peroxidation of fat in meat (34). 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 (10). DCNO was made from *Musculus vastus intermedius*, cured with 2.19 g salt with 0.6% sodium nitrite (131 ppm NaNO2), and 1.4 g sodium erythorbate (an ascorbate isomer) per 100 g meat. DCNO was then heated at 70°C for 3 hour in vacuum-sealed plastic bags in a water bath. The final product contained 12 mg haem iron/kg, 71 mg sodium nitrite/kg, and 500 mg ascorbate/kg. One group of rats was given an erythorbate-free DCNO. The processed meat was divided into 1.3 cm thick slices of 300g, that were stored separately at - 20°C in air-tight plastic bags with low-oxygen permeability (14 day study) or under CO_2/N_2 50/50 atmosphere to avoid further fat oxidation (100 day study). Before being given to rats, each slice was exposed to air for five days in a dark refrigerator (4°C), then cut in ten 30 g portions that were given to rats at 5:00 p.m. for 14 or 100 days. A low-calcium powdered diet (35) was given in a separated feeder, 7.6 g/d/rat, so that each rat would eat roughly half meat / half powder (dry matter). This modified AIN76 diet was prepared by UPAE (INRA, Jouy, France) as follows (g/100g): sucrose, 59.5; corn starch, 15.0; cellulose, 12.5; AIN76 mineral mix without calcium, 8.7; AIN76 vitamin mix, 2.5; methionine, 0.75; calcium phosphate, 0.52; choline bitartrate, 0.5. Safflower oil (5g) was mixed with 100g powder to provide polyunsaturated fatty acids (MP Biomedicals, Illkirch, France).

Six polyphenols-rich plant extracts were added to DCNO during the curing process, at a concentration recommended by the supplier: white grape extract (NutriPhy® white grape 100, 72% of total polyphenols, CHR Hansen, Hoersholm, Denmark; 0.055% w/w in DCNO), carnosic acid (Stabilenhance® OSR5 extracted from rosemary leaves, 10% carnosic acid, Naturex, Avignon, France; 1% w/w in DCNO), and a water soluble rosemary extract, containing 7% of rosmarinic acid (Stabilenhance® WSR6, Naturex; 0.66% w/w in DCNO), red wine concentrate, 10% of total polyphenols (Avvinr9005®, Diana Naturals, Antrain, France; 2% w/w in DCNO), pomegranate extract, 12 % ellagic acid (Naturex, Ultimate Botanical Benefits; 0.6% w/w in DCNO), green tea extract, 98% of total polyphenols (Naturex; 0.08% w/w in DCNO). Polyphenol data were given by the suppliers and the composition of extracts was not determined more precisely in this pilot study. Another group was given DCNO supplemented with α-tocopherol (Covitol®, Nutrition & Health, Cognis, BASF; 0.045%): this fat-soluble antioxidant agent suppresses MDF in carcinogen-induced rats and was used as a positive control for protection (7). A last group of rat given DCNO without sodium erythorbate was added to the carcinogenesis study.

Meat composition

Processed meat was analyzed by Lareal (Vannes, France, laboratory specialized in physico-chemical and microbiological analyzes) for total iron, total pigments, nitrosylated pigments (36). Hexanal, a marker of secondary products of lipid peroxidation was analyzed by Lareal by gas chromatography of the headspace of the sample dispersed in phosphate buffer at 37°C, with solid-phase micro-extraction fiber. Trolox equivalent antioxidative capacity (TEAC-1), malondialdehyde (MDA by HPLC), and TBARS (after acidic extraction) were measured by ADIV (Clermont-Ferrand, France). The Oxygen radical absorbance capacity (ORAC) was measured by Naturex (Avignon, France). Two measures per processed meat batch were done.

Fecal and urinary measures

Analysis of haem, thiobarbituric acid reactive substances in, and cytotoxicity of faecal water, and 1,4-dihydroxynonane mercapturic acid in urine

Faecal pellets were collected under each cage for 24h, at day 11 of the short-term study and days 88-91 of the carcinogenesis study. TBARS value was used as a global measure of lipid peroxidation endproducts. Faecal water was prepared, and haem and TBARS were measured in faecal water exactly as previously described (7) except that 1 mL of distilled water was added to 0.42g of crushed fresh faeces, but not to 0.3g of dried faeces. 1,4-Dihydroxynonane mercapturic acid (DHN-MA) is the main urinary metabolite of 4-hydroxynonenal, which is a major toxic end product of endogenous fat peroxidation (16). The 24-hour urine was collected under each metabolic cage, at day 11 of the short-term study and days 74 to 76 of the carcinogenesis study. DHN-MA assay was done (n= 5 to 8 for the 14-day study and n=10 to 26 for the 100-day study) as previously described (7). To determine cytotoxicity of fecal water (n=6), the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used on a cancerous mouse colonic epithelial cell line, CMT93 (European Collection of Animal Cultures), as previously described (7).

ATNC analysis

ATNC were analyzed using a modification of the method previously used (37), using a CLD88 Exhalyzer (Ecomedics, Duernten, Switzerland). Sulfamic acid solution (500 µl, 5%) was added to 100 µl of faecal water to remove nitrite and samples were injected into a purged vessel kept at 60°C and filled with a standard tri-iodide reagent (38 mg I_2 was added to a solution of 108 mg KI in 1 ml water; to this mixture, 13.5 ml glacial acetic acid was added) to determine total ATNC. To determine mercury(II) stable compounds, 100 µl

10 mM aqueous HgCl2 was added prior to analysis; to determine mercury(II) and ferricyanide stable compounds, 100 µl each of 10 mM aqueous $HgCl_2$ and 10 mM aqueous $K_3Fe(CN)_6$ solution were added prior to analysis. Nitrosothiols were determined as the difference between total ATNC and mercury(II) stable ATNC; Nitrosyl iron was determined as difference between mercury(II) stable ATNC and mercury(II) and $K_3Fe(CN)6$ stable compounds. Data are concentrations (in µM), measured in triplicate in 100 µL of each sample.

ACF and MDF assays

ACF and MDF were scored by a single observer blinded for the origin of the colon, exactly as described previously (7). Number of lesions and number of crypts per lesions (i.e. size of ACF and MDF) were numbered.

Statistical methods

Results were analyzed using Systat 10 software for Windows, and all data were reported as mean ± SD (except Fig.1B). Values were considered firstly using one-way ANOVA. If a significant difference was found between all groups ($P < 0.05$), comparison of each experimental group with the control group was made using Dunnett's test. For ORAC analysis, data show results of two measures per processed meat batch, but Student t test statistics could be done because the within-pair correlation was high, however, P values should be taken cautiously (38).

Results

Fourteen-day study: Effect of plant extracts on peroxidation biomarkers

Fecal and urinary fat peroxidation biomarkers

Dietary DCNO cured meat increases the number of carcinogen-induced precancerous lesions, and urinary and faecal water peroxidation biomarkers in rats (10, 14). These early peroxidation biomarkers were thus measured here, because they correlate with haem-induced promotion of colon carcinogenesis (8, 39). Extracts of pomegranate, red wine, white grape, green tea, rosemary and carnosic acid and α-tocopherol were added to DCNO before being fed to rats for 14 days. As shown in Table 1, faecal water from rats given DCNO added with pomegranate, red wine or white grape extracts contained half TBARS than control rats given DCNO. In contrast, faecal water from rats given DCNO plus carnosic acid contained surprisingly twice more TBARS than controls. All tested extracts

led to some reduction in urinary DHN-MA but only rosemary extract significantly decreased the excretion of this 4-hydroxynonenal metabolite. All the tested plant extracts reduced fecal haem excretion in DCNO-fed rats, except for α-tocopherol and rosemary extract. Lastly, addition of plant extracts in DCNO did not affect the cytotoxicity associated with DCNO consumption.

Choice of polyphenol additives for the carcinogenesis study

Pomegranate, red wine, and white grape extracts that decreased TBARS in fecal water of DCNO-fed rats (Table 1) were chosen to be tested in the carcinogenesis study, because our starting hypothesis was that polyphenols would exert their protective action by inhibiting lipid peroxidation (25, 40). We also chose to test carnosic acid, a common additive to brine in Europe, because it surprisingly increased TBARS in faecal water. In addition we tested α-tocopherol as a protection control because it suppresses cured-meat promotion in rats (7). Lastly, a special DCNO meat, cured without erythorbate, was given to a group of rat to test the effect of this common additive.

Carcinogenesis study: effect of plant extracts

General observation

All rats survived and were healthy, except rats given carnosic acid that had diarrhea. Moist meat and powdered diet were given to the rats in separated feeders: the relative intake of meat and of powder that was 48:52 (dry weight) on day 18 of the study slowly changed to 39:61 on day 82. The final body weight of rats was 343±19 g without significant difference between groups except rats fed cured meat plus carnosic acid (321±16 g, $P<0.05$). Rats in this group ate and drank less than the rats in others groups: their average food intake per day was 12±1 g compared with 13±1 g in other groups ($P<0.05$). Water intake was reduced in rat fed carnosic acid and increased in rat fed α-tocopherol or white grape extract, compared with the other groups (full data not shown, $P<0.0001$).

Quantification of ACF and MDF

A DCNO-based diet increases the number of MDF and ACF in the colon of carcinogen-injected rats, in comparison with a no-meat control diet (7, 10). The DCNO diet was thus chosen as a promoting control to test potentially protective plant extracts. At the doses tested, all plant extracts decreased the number of MDF per colon in comparison with DCNO diet, but only α-tocopherol, pomegranate and red wine extracts led to a significant protection (Fig.1B). Number of ACF was not different between groups, nor the MDF and

ACF multiplicity (Table 3). Surprisingly, the removal of erythorbate from DCNO curing brine led to a significant reduction in the number of colonic MDF. Mean number of large ACF or of large MDF, with 4 or more crypts per foci, was similar in all dietary groups. In an attempt to explain the observed protection, diets, fecal water, and urine were analyzed for lipoperoxydes and nitroso-compounds.

Meat analyses

Trolox, MDA, TBARS, ORAC, hexanal and nitrosylated haem were analysed but only ORAC, hexanal ant nitrosylated haem values were reported here, because they are no significantly affected by the modification of process of meats. ORAC is a measure of antioxidant power. As expected, all tested plant extracts increased cured meat ORAC value 2-3 times, and suppressed hexanal production, a measure of meat peroxidation (Table 4). Haem and NO from nitrite can form nitrosyl haem (19), that might be the promoting factor in cured meat (8). The tested plant extracts did not change much nitrosylated haem concentration in meat, except red wine that increased it (Table 4).

Fecal and urinary fat peroxidation biomarkers

Faecal water from rats fed DCNO added with pomegranate, red wine or white grape extracts, or α-tocopherol contained 1.5 to 2 times less TBARS, and about 1.5 times less haem than faecal water from rats fed DCNO alone (Table 2). Pomegranate extract, carnosic acid and α-tocopherol also decreased urinary DHN-MA, a metabolite of 4-hydroxy-nonenal. Carnosic acid that significantly increased faecal TBARS in first study (Table 1) tended to increase it in this second study (not significant). Addition of plant extracts had not modified the cytotoxicity of fecal water in DCNO group except for carnosic acid that induces a significant increase in faecal water cytotoxicity (Table 2). Surprisingly, the absence of erythorbate in DCNO significantly decreased faecal TBARS and haem compared with erythorbate-supplemented DCNO, without modification of cytotoxic activity of fecal water (Table 2).

Faecal Nitroso Compounds

Apparent total nitroso compounds (ATNC) concentration in faecal samples was reduced by the addition of a plant extract to the curing brine of DCNO (Fig. 1A). The reduction was more than a three-fold (except white grape). Significance could not be formally established since only one value was obtained per group because faeces from all rats in one dietary group had been pooled, so results were interpreted with caution. “ATNC” are a complex mixture of nitrite-derived products, and the ATNC composition was not identical in the

faeces from different groups. Faecal ATNC from rats fed DCNO cured meat plus carnosic acid or white grape extract were made of 100% nitrosyl iron (Fig.1A). In contrast, faecal ATNC from rats fed DCNO with pomegranate or red wine extracts were 100% nitrosothiols (data not shown). Tocopherol fully suppressed nitrosation, but the removal of erythorbate from DCNO curing brine led to a fifty percent increase in faecal ATNC, no nitrosyl iron being detected (Fig.1A).

Discussion

This study shows that polyphenol-rich plant extracts can inhibit the promotion of colonic mucin depleted foci by cured meat that had been demonstrated repeatedly in this model (7-10): dried red wine and pomegranate extract suppressed cured meat-induced colon tumorigenesis promotion as well as α-tocopherol, while white grape extract and carnosic acid extracted from rosemary did not. Promotion 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 (41). Like tumours, MDF harbour mutations in genes affecting colon carcinogenesis (Apc and K-ras) and show Wnt signalling activation (42), a dramatic reduction of MUC2 expression (43), and a strong activation of the inflammatory process (44), all features suggesting that MDF are precancerous. Several rodents studies suggest that MDF are better predictors of colorectal cancer than ACF are (45), and respond more consistently than ACF to promotion by red and processed meat and by dietary haem (9, 20, 39) this is why we focused on MDF data.

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) (10). Hence, we chose to use DNCO to identify prevention strategies aiming at normalizing fecal biomarkers. But the prediction of cancer-promoting properties in food by simple chemical analysis would be a great step toward cancer prevention. Unfortunately, no correlation was seen between cured meat composition and the number of MDF: neither hexanal, ORAC, nitrosylated haem, or any other meat component was associated with MDF promotion. This supports the hypothesis that CRC promotion by processed meat is not directly due to a factor present in food. Meat-induced endogenous factors would thus promote MDF, e.g., aldehydes or N-nitroso compounds (6). Our starting hypothesis was that lipid peroxidation endproducts would promote carcinogenesis, and that polyphenols would decrease luminal peroxidation. Polyphenols can scavenge oxygen radicals, preventing the damage towards

macromolecules and peroxidation of fatty acids, and they can bind iron, thus reducing catalytic properties of haem (46). The measurement of ORAC is commonly used to study the radical-scavenging ability of polyphenols. Here, the tested plant extracts doubled or tripled the ORAC of meat (Table 4). In addition, faecal excretion of haem iron was reduced in rats given polyphenol-supplemented meat (Table 2). However, neither the antioxidant effect nor the reduced fecal haem iron was linked with MDF reduction: for instance carnosic acid tripled ORAC value in meat but did not reduce MDF number in rats (Fig.1B).

Our previous studies strongly suggest that faecal aldehydes collectively measured by the TBARS assay would participate to carcinogenesis promotion in meat-fed rats (8, 39). Present data support this hypothesis, since all plant extracts that decreased MDF number significantly reduced faecal TBARS concentration. In this way, previous *in vitro* data of our team allowed to propose that a premalignant cell selection by heme-induced aldehydes explains the heme-induced promotion of MDF (20). Thus limitation of peroxydation and aldehydes formation by antioxydant could explain the protective effect by limitation of the selection of preneoplastic cells. In addition, carnosic acid that increased TBARS did not reduce MDF number (Tables 1 and 4, fig.1B). In contrast, white grape extract reduced faecal TBARS but had no effect on MDF number. This discrepant group may suggest that TBARS are not the only parameter involved in CRC promotion.

Although data on the NOCs were obtained on faecal pools, our results support the hypothesis of Cross *et al* on the role of endogenous ATNC in the promotion of CRC by processed meat. The presence of nitrosyl iron in faeces, but not the other types of ATNC, was associated with the promotion of CRC by cured meat (Fig.1). Several human studies strongly suggest that the formation of ATNC can explain the positive links between processed meat intake and CRC (10, 11, 47). Here, α-tocopherol fully inhibited the formation of faecal ATCN and suppressed MDF promotion in rats. Similarly, the reduction of MDF by red wine and pomegranate extracts was associated with reduced faecal ATNC and lack of nitrosyl iron in faeces (Fig.1). Nitrosyl iron was indeed the only faecal marker that was consistently associated with MDF promotion. However, no dose-response relationship was seen, since white grape and carnosic acid that boosted nitrosyl iron formation did not increased the MDF number over the control number (fig.1). We nevertheless suggest that nitrosyl iron might be used as a short-term biomarker to screen additives added to cured meat to reduce cancer risk.

To test the hypothesis that nitrosation can explain promotion, an artificial model of cured meat was made with no erythorbate. Currently all commercial processed meats contain erythorbate. Indeed, this additive is usually added to brine during the curing process to increase nitrosylation and to block nitrosation (19). As expected, faecal ATNC value was higher in rats given erythorbate-free DCNO than in control rats given DCNO with erythorbate (+55%, Fig.1A). Surprisingly, this ATNC increase was associated with a decrease in the number of MDF per colon, which shows that all ATNC types do not promote tumorigenesis. However, no nitrosyl iron was detected rats given erythorbate-free DCNO (Fig.1A), as already observed by Hotter, Zhou and Mirvish (Abstract B-111, Frontiers in Cancer prevention, Am. Ass. Cancer Res., Boston, Oct. 2011). We thus suggest a central role of luminal nitrosyl iron in the promotion of colorectal tumorigenesis by cured meat, associated with a minor role of luminal aldehydes. This contradicts Hogg's hypothesis that the sequestration of the "nitrosating potential" of diet as nitrosyl iron is a protective mechanism (48). In contrast, it supports Kuhnle's hypothesis that nitrosyl haem may cause the formation of DNA adduct O6-carboxymethyl guanine in colonic cells (49), which are found in stools of volunteers given red meat (37) with a stimulating effect of haem-iron on adduct production during in vitro fermentation of meat (50). The evidence presented here is weak however, since no statistics could be done on ATNC data, and because no dose-effect relation was seen between nitrosyl haem excretion and MDF numbers (Fig. 1).

Major weaknesses of this study are the pooling of faecal samples before ATNC analysis, and the lack of a no-meat arm. Hence no statistics could be done on ATNC data: significance of the three-fold reduction in total ATNC by plant extracts, and of full suppression of nitrosyl-iron by three of the extracts is unknown. Also, the present study was not designed to confirm MDF promotion by cured meat (DCNO). This promoting effect had repeatedly been shown in the same model (7-10), but could not be tested again in the present study.

The finding that it is possible to counteract the cancer promoting effect of processed meat by adding selected plant extracts into the meat should have consequences on public health and on dietary recommendations. The World Cancer Research Fund's advice to avoid processed meat may be updated with the advice that any cured meat meal should also include a polyphenol- or tocopherol-rich plant food. Despite recommendations, individuals, particularly those in low socio-economic groups, consume large amounts of processed meat. These people are at a higher risk of CRC, early disability and death. We suggest the meat industry should use specific protective plant-based additives during the

curing process, as this could reduce cancer risk in all consumers. Making safer meat products might be a better approach than banning meat (6, 7).

Conclusions

This study shows that the incorporation of polyphenol-rich plant extracts (pomegranate or red wine) or of α-tocopherol inhibited the promoting effect of cured meat on preneoplastic lesions in carcinogen-induced rats. If these results were confirmed in volunteers' study, these agents might be added to meat during the curing process to make functional processed meat. This study represents and informative starting point, however future research should address dose dependence and potential efficacy of modified meats that might induce effects ranging from protection, lack of protection to possible cancer-promoting effect at other doses. The use of the protective agents would reduce colorectal cancer risk compared with processed meat. This study also shows that faecal excretion of a specific class of nitroso-compounds, nitrosyl iron, was associated with tumorigenesis promotion by cured meat.

List of abbreviations: ACF: Aberrant Crypt Foci; ATNC: Apparent Total N-nitroso Compounds; CRC: colorectal cancer; DCNO: Dark meat, Cooked, cured with sodium Nitrite, Oxidized by air; DHN-MA: DiHydroxyNonane Mercapturic Acid; MDF: Mucin Depleted Foci; TBARs: Thiobarbituric Acid Reactive Substances; ORAC: Oxygen Radical Absorbance Capacity;

Competing interests

G. Nassy and J.L. Vendeuvre were employed by the *Institut Français du Porc* (IFIP).
N.Bastide, N.Naud, S.Taché, F.Guéraud, D.Hobbs, G.Kuhnle, D.Corpet, F. Pierre: No conflicts of interest.

Authors' contributions

NMB and NN contributed equally to this work; FHFP, DEC, GN and JLV designed research; NMB, NN, ST, FG, DAH, and GCK conducted research; NMB, DEC and FHFP analyzed data and wrote the paper; DEC and FHFP had primary responsibility for final content. All authors have read and approved the final manuscript.

Acknowledgments

This work was supported by French Institut National de la Recherche Agronomique.

Working costs of the fourteen-day DCNO study were paid by Institut Français du Porc (IFIP). We thank Florence Blas-Y-Estrada for animal care. This article is written "*in memoriam*" of J.L. Vendeuvre.

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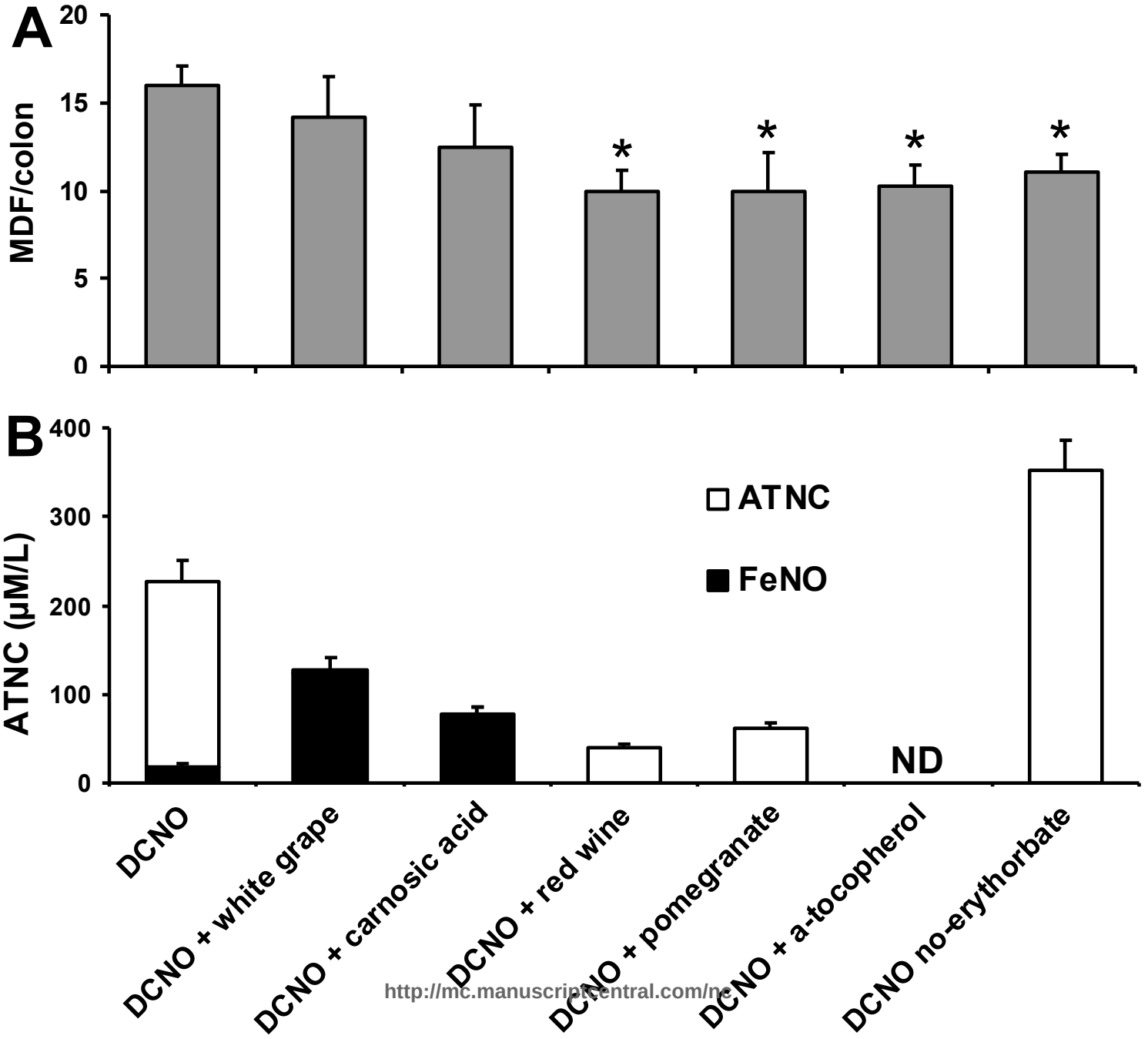
Figure 1: **Faecal excretion of nitroso compounds and promotion of preneoplastic lesions in the colon of rats.**

A: Mucin Depleted Foci (MDF) in the colon of azoxymethane-initiated rats given cured meat added with plant extracts for 100 days. Values are mean ± SEM (same data in Table 3). * Significantly different from DCNO by Dunnett's t test.

B: Apparent Total N-nitroso Compounds (ATNC) and nitrosyl iron (FeNO) values were obtained on pooled faecal samples from all rats in one group: error bars show analytical SD. ND: not detected.


A
MDF/colon
20
15
10
5
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*
*
*
*
B
ATNC (μM/L)
400
300
200
100
0
ATNC
FeNO
ND
DCNO
DCNO + white grape
DCNO + carnosic acid
DCNO + red wine
DCNO + pomegranate
DCNO + a-tocopherol
DCNO no-erythorbate

Table 1. Faecal and urinary biomarkers in rats given cured meat with plant extracts during the fourteen-day study

Diet	N. of rats	Haem in FW (nnmol/24h)	TBARs in FW (Eq. MDA nnmol/24h)	Urinary DHN-MA (ng/24h)	Cytotoxicity of FW (% of dead cells)
DCNO	8	279±90	138±46	410±210	22±8
DCNO + White grape	5	116±54**	77±23*	287±89	23±24
DCNO + Carnosic acid	5	140±72**	273±81***	258±83	29±13
DCNO + Rosemary	5	196±104	144±18	222±30*	20±19
DCNO + Red wine	5	174±70*	68±38**	262±53	35±11
DCNO + Pomegranate	5	97±38***	64±35**	307±225	37±5
DCNO + Green tea	5	150±56*	87±21	288±128	35±13
DCNO + α-tocopherol	5	193±130	98±56	370±194	12±16

Footnotes

FW: faecal water. TBARS: thiobarbituric acid reactive substances; MDA: malondialdehyde; DHN-MA: dihydroxynonene mercapturic acid; DCNO: dark meat, cooked, cured with sodium nitrite, oxidized by air.

Significantly different from DCNO by the Dunnett's t test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 2. Effect of cured meat diets on fecal and urinary biomarkers in carcinogen-initiated rats 80 days after an azoxymethane injection

Diet	N. of rats	Haem in FW (nnmol/24h)	TBARs in FW (Eq. MDA nnmol/24h)	Urinary DHN-MA (ng/24h)	Cytotoxicity of FW (% of dead cells)
DCNO	26	238±84	153±16	243±99	52±20
DCNO + White grape	10	145±40***	99±19***	235±80	57±32
DCNO + Carnosic acid	10	135±61***	164±39	122±34***	100±1***
DCNO + Rosemary	10	172±51**	76±11***	191±60	59±16
DCNO + Red wine	10	162±55**	85±11***	172±64**	66±17
DCNO + Pomegranate	10	135±41***	120±19**	124±44***	46±20
DCNO + Green tea	10	162±46**	132±14**	257±90	47±23

Footnotes

FW: faecal water. TBARS: thiobarbituric acid reactive substances; MDA: malondialdehyde; DHN-MA: dihydroxynonene mercapturic acid; DCNO: dark meat, cooked, cured with sodium nitrite, oxidized by air.

Significantly different from DCNO by the Dunnett's t test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 3 Preneoplastic lesions (ACF and MDF) in the colon of rats fed cured meat added with plant extracts for 98 d, 105 d after an azoxymethane injection[1]

Diet	No. of rats	MDF/Colon		Crypt/MDF		ACF/colon		Crypt/ACF	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
DCNO	26	16.0	5.8	2.6	0.8	106	22	3.4	0.2
DCNO + White grape	10	14.2	7.3	2.3	0.2	105	14	3.4	0.2
DCNO + Carnosic acid	10	12.5	7.4	2.6	0.7	95	17	3.5	0.2
DCNO + Red wine	10	10.0	3.5**	2.6	0.5	108	19	3.4	0.3
DCNO + Pomegranate	10	10.0	6.8**	2.3	0.4	108	28	3.6	0.2
DCNO + α-tocopherol	10	10.3	3.6**	2.4	0.4	99	21	3.4	0.3
DCNO without erythorbate	10	11.1	3.1*	2.6	0.5	103	24	3.5	0.3

[1] ACF: aberrant crypt foci. MDF: mucin depleted foci. Other notes: see Table 1.

Table 4 Processed meat analysis: Antioxidant activity, nitrosyl heme and hexanal concentrations after air exposure for five days at 4°C in cured meat added with plant extracts[1]

Processed meat	Oxygen radical absorbance capacity (Trolox eq.µmol/100g)	Nitrosylated Heme (mg/kg)	Hexanal (mg/kg)
DCNO	10.4 - 11.1 [2]	97 - 100	5-6
DCNO + White grape	25.8 - 27.3 **	97 - 101	1
DCNO + Carnosic acid	30.7 - 31.2 **	95 - 101	<1-1
DCNO + Red wine	22.0 - 27.0 *	123 – 138 *	<1-1
DCNO + Pomegranate	20.1 - 20.5 **	108 - 108	<1
DCNO + α-tocopherol	12.4 - 12.7 *	91 - 94	<1-1
DCNO without erythorbate	9.8 - 11.2	96 - 101	3-8

[1] Plant extracts were added during the curing process to DCNO (dark meat, cured with sodium nitrite, cooked and oxidized).

[2] Data show results of two measures per processed meat batch. Details: see Materials and Methods. * p=<0.05, p<0.003** compared with DCNO group.
Student t test statistics could be done because the within-pair correlation was high, however, P values should be taken cautiously (38)

Dr. Fabrice PIERRE
National Institute of Agronomic Research
UMR INRA 1331 ToxAlim
180 chemin de Tournefeuille
31027 Toulouse, FRANCE
email: fabrice.pierre@toulouse.inra.fr
Tel: 33 582 066 370

to L.A. Cohen
Editor of Nutrition and Cancer

Dear Editor,

Please find in the attached file a revised manuscript N&C-01-16-2624.R2, entitled " Red wine and pomegranate extracts suppress cured meat promotion of colonic mucin-depleted foci in carcinogen-induced rats" we resubmit for publication to Nutrition and Cancer: An International Journal.

All referee comments were considered, and the revised MS was changed accordingly, as detailed in the letter below, and highlighted "red" in the revised MS.

My co-authors and I hope that the revised manuscript will now be suitable for publication in Nutrition and Cancer. We thank you for your editorial services.

Yours sincerely

Fabrice Pierre

TOULOUSE, 10-02-2016

Please find below our propositions of modifications of the MS

Reviewer: 1
Administrative change made. Thank you.
=>We thank the reviewer for his review that has improved the MS

Reviewer: 2
Suggest a further minor revision to the last sentence of the Abstract:

At optimised concentrations, the incorporation of these plant extracts in cured meat might reduce the risk of colorectal cancer associated with processed meat consumption.
=> *We agree with the reviewer #2 and have modify the end of the abstract*
=>And we thank the reader for his review that has improved the MS

Reviewer: 3
No additional comments
=>We thank the reader for his review that has improved the MS