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Nitrite Curing of Chicken, Pork, and Beef Inhibits Oxidation but Does Not Affect *N*-Nitroso Compound (NOC)-Specific DNA Adduct Formation during in Vitro Digestion

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ABSTRACT: Uncured and nitrite-cured chicken, pork, and beef were used as low, medium, and high sources of heme-Fe, respectively, and exposed to an in vitro digestion model simulating the mouth, stomach, duodenum, and colon. With increasing content of iron compounds, up to 25-fold higher concentrations of the toxic lipid oxidation products malondialdehyde, 4-hydroxy-2-nonenal, and other volatile aldehydes were formed during digestion, together with increased protein carbonyl compounds as measurement of protein oxidation. Nitrite curing of all meats lowered lipid and protein oxidation to the level of oxidation in uncured chicken. Strongly depending on the individual fecal inoculum, colonic digestion of beef resulted in significantly higher concentrations of the NOC-specific DNA adduct O^6-carboxymethyl-guanine compared to chicken and pork, whereas nitrite curing had no significant effect. This study confirms previously reported evidence that heme-Fe is involved in the epidemiological association between red meat consumption and colorectal cancer, but questions the role of nitrite curing in this association.

KEYWORDS: *heme-Fe, processed meat, colorectal cancer, health*

INTRODUCTION

Colorectal cancer (CRC) is the second most commonly diagnosed cancer in women and the third in men.[1] Different independent meta-analyses of epidemiologic studies demonstrate a significant increased CRC risk associated with a higher consumption of red meat and especially processed meat.[2,3] Consumption of poultry, which contains lower amounts of heme-Fe than pork and beef, has not been associated with CRC, and high consumption of fish is associated with a lower risk.[4] In 2007, the World Cancer Research Fund/American Institute for Cancer Research[1] made the recommendation to limit red meat consumption and avoid processed meat as much as possible.

The biochemical mechanisms responsible for these epidemiologic associations have not yet been completely elucidated. Corpet[5] suggested that heme-Fe in meat exerts a catalytic effect on the endogenous formation of geno- and cytotoxic oxidation products such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) and on the formation of genotoxic *N*-nitroso compounds (NOCs). Several mechanistic studies support a stimulating role for heme-Fe in endogenous oxidation[6,7] and nitrosation reactions.[8−10] Both in vivo and in vitro studies have demonstrated increasing lipid peroxidation during passage through the gastrointestinal system.[11−13] Subsequently, DNA can be damaged by binding with previously mentioned genotoxic compounds, for example, DNA adducts formed with MDA (pyrimido[1,2-α]purine-10(3*H*)-one-2′-deoxyribose), 4-HNE (e.g., 1,N^6-etheno-2′-deoxyadenosine), and NOCs (e.g., O^6-carboxymethyl-guanine).[10,14]

Unlike the role of heme-Fe, the importance of nitrite curing of meat in this context is not well studied. Nitrite salt is widely used as a curing agent in meat products to inhibit outgrowth of *Clostridium botulinum*, spoilage, and oxidative rancidity and to obtain a desired red meat color. The antioxidative mechanism of nitrite in meat was demonstrated by the group of Kanner[15] and involves the antioxidant activity of formed nitric oxide myoglobin, nitric oxide ferrous complexes, and *S*-nitrosocystein and inhibition of the Fenton reaction, which is responsible for the initiation of oxidation reactions. Furthermore, a stabilizing effect of nitrite was observed on the susceptibility of unsaturated lipids in the membranes to oxidation. In acidic conditions such as present in the stomach, nitrous acid generates dinitrogen trioxide (N_2O_3) and H_2O, which is in equilibrium with nitric oxide ($^{\bullet}NO$) and nitrogen dioxide ($^{\bullet}NO_2$).[16] A dual role of $^{\bullet}NO$ on lipid oxidation was described whereby a 1:1 ratio of $^{\bullet}NO$ to ROS enhances lipid peroxidation, whereas an excess of $^{\bullet}NO$ inhibits oxidation.[17] Corpet[5] suggested that nitrite curing of meat would be an important contributor to NOC formation. In aerobic conditions, $^{\bullet}NO$ reacts with O_2 to form nitrosating species (e.g., N_2O_3),[18] which induce the formation of genotoxic NOCs.

The aim of this study was to elucidate the likely mediating effect of nitrite curing on heme-Fe-induced oxidative and

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Table 1. Composition of Digestive Juices (1 L) Used for in Vitro Incubation of Meat Samples

digestion[a]													fermentation					
mouth			stomach			duodenum							colon					
saliva (pH 6.8)			gastric juice (pH 1.3)			duodenal juice (pH 8.1)			bile (pH 8.2)				SHIME medium[b] (pH 7.0)			cultured bacterial inoculum[c]		
Inorganic Solution																		
0.90	g	KCl	2.75	g	NaCl	7.01	g	NaCl	5.26	g	NaCl		6.8	g	KH_2PO_4	0.51	g	NaCl
0.2	g	KSCN	0.27	g	NaH_2PO_4	3.39	g	$NaHCO_3$	5.79	g	$NaHCO_3$		8.8	g	K_2HPO_4	0.01	g	KCl
0.9	g	NaH_2PO_4	0.82	g	KCl	0.08	g	KH_2PO_4	0.38	g	KCl					0.23	g	$Na_2HPO_4{\cdot}12H_2O$
0.57g	g	$NaSO_4$	0.40	g	$CaCl_2{\cdot}2H_2O$	0.56	g	KCl	0.15	mL	HCl 37%					0.02	g	KH_2PO_4
0.3	g	NaCl	0.31	g	NH_4Cl	0.05	g	$MgCl_2$										
1.6	g	$NaHCO_3$	6.50	mL	HCl 37%	0.18	mL	HCl 37%										
Organic Solution																		
0.20	g	urea	0.09	g	urea	0.10	g	urea	0.25	g	urea		1.0	g	arabinogalactan	0.06	g	$C_2H_3NaO_2S$
11.5	mg	uric acid	0.02	g	glucuronic acid	1.00	g	BSA					3.0	g	yeast extract	33.3	g	brain heart infusion
25.0	mg	mucin	0.65	g	glucose	9.00	g	pancreatin	1.80	g	BSA		0.4	g	glucose	0.45	g	L-cystein-HCl
2.50	IU	peroxidase[d]	0.33	g	glucosamine-HCl	1.50	g	lipase	30.0	g	bile		0.5	g	L-cystein-HCl	16.0	mL	fecal matter
			17.6	mg	ascorbic acid[f]								4.0	g	mucin	20.0	mL	glycerol
			1.00	g	BSA								2.0	g	pectin			
			2.50	g	pepsin								1.0	g	pepton			
			3.00	g	mucin								1.0	g	xylan			
													4.0	g	potato starch			
Add																		
6.90	mg	$NaNO_2$[e]	10.0	μL	H_2O_2 (30%)[g]	0.200	g	$CaCl_2{\cdot}2H_2O$	0.222	g	$CaCl_2{\cdot}2H_2O$							
			11.2	mg	$FeSO_4{\cdot}7H_2O$[h]													

[a]Digestion: based on Versantfoort et al.[20] unless otherwise indicated [b]SHIME medium: Molley et al.[26] [c]Bacterial inoculum was cultured under anaerobic conditions for 24 h at 37 °C and used immediately in the fermentation procedure. [d]Peroxidase: Güven et al.[21] [e]$NaNO_2$: Takahama et al.[22] [f]Ascorbic acid: Dabrowska-Ufniarz et al.[23] [g]H_2O_2: Nalini et al.[24] [h]$FeSO_4$: Nalini et al.[24]

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nitrosative stress during digestion of meat. For this purpose, uncured and nitrite-cured cooked chicken, pork, and beef as low, medium, and high sources of heme-Fe, respectively, were subjected to a multicompartment in vitro digestion model. In the digests, the major toxic lipid oxidation products MDA and 4-HNE and the less toxic simple aldehydes pentanal, hexanal, heptanal, and nonanal were determined. During the oxidation of proteins, carbonylation is one of the most prominent protein modifications[19] and, hence, measurement of protein carbonyl compounds (PCC) was used to estimate protein oxidation during digestion. The NOC-specific DNA adduct O^6-carboxymethyl-guanine (O^6-C-MeG) was determined in digesta to estimate DNA damage induced by nitrosative stress. O^6-C-MeG is a far more specific end point than the commonly used "apparent total nitroso-compounds" (ATNC) because (i) it demonstrates DNA damage caused by alkylating NOCs and (ii) ATNC also include noncarcinogenic nitrosyl-Fe and nitrosothiols along with the carcinogenic NOCs.

MATERIALS AND METHODS

Experimental Setup. Uncured and nitrite-cured cooked chicken, pork, and beef were compared before and after mimicked duodenal and colonic digestion. Each incubation run included all treatments in quadruplicate, from which two duplicates underwent digestion until the duodenum and two until the colon step. Each incubation run was performed three times with fecal inoculum originating from three different human volunteers.

Manufacturing of Meat Samples. Lean meat samples from the *m. Pectoralis profundus* (chicken), *m. Longissimus dorsi* (pork), and *m. Biceps femoris* (beef) were purchased in a local supermarket. The meat was manually chopped into cubes of approximately 1−2 cm^3. To exclude a possible confounding effect of differences in fatty acid composition, 4% subcutaneous pork fat was added to the different lean meat sources. Meat samples with added fat were first minced in a grinder (Omega T-12) equipped with a 10 mm plate, followed by grinding through a 3.5 mm plate. Thereafter, nitrite curing was applied by adding 20 g of 0.6% nitrite salt/kg meat, corresponding to an added concentration of 120 mg nitrite/kg meat. All meat samples were heated in a warm water bath for 15 min after the core temperature had reached 65 °C. After manufacturing, all meat samples were homogenized in three 5 s bursts using a food processor (Moulinex DP700), vacuum packed, and stored at −20 °C until the start of the incubation.

Incubations. The in vitro digestions consisted of an enzymatic digestion simulating the mouth, stomach, and duodenum gastrointestinal tract compartments, followed by a simulation of colonic fermentation. For the enzymatic digestion, the protocol described by Versantfoort et al.[18] was adapted by adding oxidants and antioxidants that are normally present in digestive juices (Table 1). Hence, peroxidase[21] and $NaNO_2$[22] were added to the saliva juice, and ascorbic acid,[23] H_2O_2,[24] and ferrous iron[24] were added to the gastric juice. Meat samples (4.5 g) were sequentially incubated for 5 min with 6 mL of saliva, for 2 h with 12 mL of gastric juice, and for 2 h with 2 mL of bicarbonate buffer (1 M, pH 8.0), 12 mL of duodenal juice, and 6 mL of bile juice. These enzymatic incubations were performed in quadruplicate. After completion, two replicates were diluted with 44 mL of H_2O to obtain the same solid/liquid ratio as in the colonic digestion (see below) and homogenized with an Ultraturrax (9500 rpm). While stirring on a magnetic field in the dark, samples were subdivided in 1.3 mL aliquots and stored at −20 and −80 °C pending analysis. The two remaining replicates underwent the additional colonic fermentation stage according to the method of Van de Wiele et al.[25] Simulation of the human intestinal microbial ecosystem (SHIME) medium (22 mL)[26] and a human fecal inoculum (22 mL) (for preparation, see Preparation of Human Fecal Inoculum) were added to the digesta. The vessels were flushed with N_2 for 30 min to obtain an anaerobic environment. Anaerobic conditions in flasks with digesta in the colon phase were confirmed, using resazurin-saturated test strips. Subsequently, the vessels were incubated for 72 h with stirring at 37 °C. To evaluate the rate of bacterial fermentation, total anaerobic bacteria were counted after 72 h of fermentation. One milliliter of digesta was serially diluted (10-fold) using a sterile peptone solution (1 g/L peptone, 0.4 g/L agar, 8.5 g/L NaCl, and 0.5 g/L cystein), after which 0.1 mL was added on an reinforced clostridial medium (RCM) plate. After 48 h of incubation at 37 °C, colony-forming units (CFU) were counted and expressed as $\log_{10}$ CFU/mL digesta. As for the duodenal samples, colonic digestion samples were homogenized by Ultraturrax at 9500 rpm. While stirring on a magnetic field in the dark, samples were subdivided in 1.3 mL aliquots and stored at −20 and −80 °C until analyses. Undigested control samples were obtained in duplicate by homogenizing 4.5 g of meat in 82 mL of H_2O, mimicking the liquid/solid ratio in the digested samples.

Preparation of Human Fecal Inoculum. Fresh fecal material was collected from three volunteers without known gastrointestinal diseases and without intake of antibiotics for at least 6 months. All volunteers were male, nonvegetarians on a Western diet, and aged 49, 26, and 38 years, respectively. Fresh fecal material was diluted in preheated PBS solution (1:4; w/v), to which sodium thioglycolate (1 g/L) was added as a reducing agent. The fecal slurry was filtered by a 1 mm metal sieve to remove particulate matter. Afterward, the inocula were stored at −80 °C on a glycerol stock (20%) in different aliquots. Before use in the colonic fermentation phase, the bacterial inoculum was cultured during 24 h at 37 °C to obtain the necessary microbiotic culture. For this purpose, fecal inoculum was diluted with brain heart infusion (BHI) broth (37 g/L BHI and 0.5 g/L cysteine) at a 1:9 ratio. Subsequently, anaerobic conditions in the flask were reached by flushing the headspace with N_2 during 1 h.

Chemical Composition of the Meat Samples. Meat samples were analyzed for dry matter, crude protein, and crude fat content according to ISO 1442-1973, ISO 937-1978, and ISO 1444-1973 methods, respectively. Lipids were extracted using chloroform/methanol (2:1; v/v)[27] and subsequently, fatty acids (FA) were analyzed as described by Raes et al.[28] Briefly, FA were methylated and analyzed by gas chromatography (HP6890, Brussels, Belgium) using a CP-Sil88 column for fatty acid methyl esters (FAME; 100 m × 0.25 mm × 0.25 μm; Chrompack, Middelburg, The Netherlands). Peaks were identified, on the basis of their retention times corresponding with standards (NuChek Prep. Inc., Sigma, Bornem, Belgium). Nonadecanoic acid (C19:0) was used as an internal standard to quantify the individual and total FA. The fatty acid profiles were expressed in grams per 100 g of FAME. Residual nitrite concentrations were measured colorimetrically at 538 nm after diazotization with sulfanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride (ISO 2918-1975). Nitrite concentrations were calculated on the basis of a standard curve obtained with sodium nitrite and expressed as milligrams of nitrite per kilogram of meat. Hematin was determined colorimetrically according to the method of Hornsey et al.[29] and converted to heme-Fe using the formula heme-Fe = hematin × atomic weight Fe/molecular weight hematin. Total Fe was determined by ICP-AES (Iris Intrepid II XSP, Thermo Electron Corp.) following destruction by Bunsen burner and dry incineration at 550 °C for 4 h, followed by dissolving in 3 mL of concentrated HNO_3, diluting to 10 mL of HNO_3, and filtration. Total Fe was calculated on the basis of a standard curve and expressed as milligrams per 100 g of meat.

Oxidation Products. MDA concentrations in digesta (−20 °C) were measured according to a modified method in accordance with Grotto et al.[30] TBARS were formed from the reaction of MDA with 2-thiobarbituric acid in an acid environment. After extraction in 1-butanol, the absorbance of the colored complex was measured colorimetrically at 532 nm. A standard curve with 1,1,3,3-tetramethoxypropane was used, and the concentration was expressed as nanomoles of MDA per milliliter of solution.

Levels of 4-HNE, pentanal, hexanal, heptanal, octanal, and nonanal were analyzed in digesta (−80 °C) through HPLC (Agilent 1200 series, provided with a degasser, autosampler, quaternary pump, column oven, fluorescence detector) using an adapted method of Holley et al.[31] All solutions were purified with activated carbon and

Table 2. Composition of the Meat Model Products Used in the in Vitro Digestion Experiment[a]

		uncured			nitrite-cured				*P* values		
	unit	chicken	pork	beef	chicken	pork	beef	RMSE	H	NC	H × NC
dry matter	%	30.1	31.9	30.6	31.3	31.9	30.0	0.45	**0.027**	**0.727**	0.040
protein	%	23.8	21.5	21.4	22.9	22.2	21.3	0.95	0.055	0.829	0.526
fat	%	4.7b	5.6a	5.3ab	4.1b	5.4a	4.6b	0.22	**<0.001**	**0.008**	0.414
SFA	g/100 g FAME	37.8	38.0	36.6	37.1	38.3	37.5	1.21	0.466	0.804	0.686
MUFA	g/100 g FAME	41.3ab	42.2a	39.6b	40.3ab	42.4a	38.2c	0.85	**0.004**	0.178	0.474
PUFA	g/100 g FAME	17.7a	16.0c	16.9b	17.5a	15.9b	16.3b*	0.20	**<0.001**	**0.021**	0.301
ALA	g/100 g FAME	0.80b	0.69c	0.90a	0.79b	0.65c	0.89a	0.015	**<0.001**	0.140	0.504
LC n-3 PUFA	g/100 g FAME	0.50b	0.29c	0.90a	0.53b	0.27c	0.91a	0.037	**<0.001**	0.919	0.568
LA	g/100 g FAME	14.1a	12.9b	12.7b	13.8a	12.8b	12.1c*	0.210	**<0.001**	**0.023**	0.252
LC n-6 PUFA	g/100 g FAME	1.43b	1.36b	1.75a	1.47b	1.38b	1.76a	0.055	**<0.001**	0.491	0.917
residual nitrite	mg/100 g				60.0a	36.4b	18.5c	0.58	**<0.001**		
total Fe	mg/100 g	0.43b	0.55b	1.38a	0.41b	0.49b	1.44a	0.079	**<0.001**	0.894	0.549
heme-Fe	mg/100 g	0.14b	0.25b	1.33a	0.15b	0.31b	1.34a	0.044	**<0.001**	0.433	0.844

[a]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ALA, α-linolenic acid (C18:3, n-3); LC n-3 PUFA, long-chain omega-3 polyunsaturated fatty acids (C20:5, n-3; C22:5, n-3; C22:6, n-3); LA, linoleic acid (C18:2, n-6); LC n-6 PUFA, long chain omega-6 polyunsaturated fatty acids (C20:4, n-6; C22:4, n-6; C22:5, n-6); FAME, fatty acid methyl esters; RMSE, root-mean-square error; H, heme-Fe content; NC, nitrite-curing. Means for different heme-Fe content (within curing treatment) with different letters (a–c) are significantly different ($P < 0.05$); * , significantly different from uncured equivalent ($P < 0.05$).

filtered. For preparation of the cyclohexanedione (CHD) reagent, 10 g of ammonium sulfate and 0.29 mL of acetic acid were dissolved in 100 mL, adjusted to pH 5, and purified with activated carbon. After filtration, 0.25 g of CHD, dissolved in 2 mL of activated carbon purified MeOH, was added to the mixture. All glassware was cleaned with ethanol and H_2O and dried in a 100 °C oven to remove contaminating aldehydes. One milliliter of digest was mixed with 4 mL of $CHCl_3$/MeOH (2:1, v/v) and 0.4 mL of NaCl (9%). After a 30 s vortex, the mixture was centrifuged (5 min, 1100*g*) and the $CHCl_3$ phase was collected. The remaining aqueous solution was mixed again with 2 mL of $CHCl_3$/MeOH, and the $CHCl_3$ phase was collected as described before. The combined $CHCl_3$ phases were dried under a gentle N_2 stream. The dried residue was resolved in 0.1 mL of MeOH, 0.4 mL of H_2O, and 1 mL of CHD reagent. After 1 h of heating at 60 °C in a warm water bath, samples were filter sterilized (0.2 μm cellulose membrane filter) in dark HPLC vials. The injection volume was 80 μL and the flow rate, 1 mL/min. Separation was done on a Supelcosil LC-18 column (25 cm × 4.6 mm, 5 μm, catalog no. 58295, Supelco), using stepwise elution (50% tetrahydrofuran from 0 to 40 min). The derivatized aldehydes were detected by a fluorescence detector at an excitation wavelength of 380 nm and an emission wavelength of 446 nm. Aldehydes were quantified using a standard curve and expressed as picomoles of aldehyde per milliliter of solution.

The measurement of PCC following their covalent reaction with 2,4-dinitrophenylhydrazine (DNPH) was done according to the method of Ganhão et al.[32] This reaction leads to the formation of a stable 2,4-dinitrophenyl hydrazone product. Total carbonyl content was quantified colorimetrically at 370 nm, using a molar absorption coefficient of 21.0/(mM·cm) and expressed as nanomoles of DNPH per milligram of protein. Protein concentrations were measured at 280 nm after reaction with 2 M HCl instead of DNPH, quantified using a standard curve with BSA, and expressed as milligrams per milliliter of solution. Both PCC and protein concentrations were reported separately.

***O*^{6}-Carboxy-methylguanine (O^6-C-MeG).** The internal standard O^6-methyl-d_3-guanine (O^6-d3-MeG) was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). *O*^{6}-Carboxymethyl-deoxyguanosine (O^6-C-MedG) was a gift from S.A. Moore (Liverpool John Moores University, UK). The NOC-specific DNA adduct O^6-C-MeG was quantified using U-HPLC-MS/MS.[33] In brief, 182 μL of filter-sterilized sample was incubated for 18 h at 37 °C with 100 μg of calf thymus (CT)-DNA. After addition of the internal standard (50 μL, 20 ng/mL O^6-d3-MeG), the mixture was dissolved in 2 mL of 0.1 M formic acid and hydrolyzed by heating (80 °C for 30 min). The hydrolysate was cooled on ice and then applied to an Oasis HLB cartridge (SPE) (30 mg, 1 mL), which was conditioned with 2 mL of 100% MeOH and equilibrated with 2 mL of deionized water. After loading the hydrolysate, a vacuum suction was applied on the SPE cartridge, followed by the elution step with 2 mL of 100% MeOH. The collected fraction was evaporated to dryness (90 min, 20 °C) using a SpeedVac Plus (Savant, Holbrook, NY, USA). Finally, the dried residue was dissolved in a total volume of 100 μL of mobile phase consisting of 95:5 0.05% aqueous acetic acid/H_2O. Chromatographic separation of the DNA adducts was achieved by reversed phase chromatography and gradient elution. Separation of the DNA adducts was carried out on a Nucleodur C18 ISIS column (5 μm, 250 × 4 mm, Machery Nagel, Düren, Germany), kept at 30 °C. Analysis was performed on a triple-quadrupole mass analyzer (TSQ Vantage, Thermo Electron, San Jose, CA, USA), fitted with a heated electrospray ionization (HESI-II) source operating in the positive ion mode. A standard curve of O^6-C-MeG was made by derivatization of O^6-C-MedG with 0.1 M formic acid at 70 °C for 1 h.[34] O^6-C-MeG was quantified using the standard curve and the area ratio of internal standard O^6-D3-MeG and expressed as nanograms of O^6-C-MeG per milliliter of digesta.

Statistical Analysis. For the meat characteristics, a linear model ANOVA procedure (SAS enterprise guide 5) was used with the fixed effects of heme-Fe, nitrite, and heme-Fe × nitrite. Data on MDA, 4-HNE, simple aldehydes, PCC, and protein content were analyzed for the undigested meat, duodenum step, and colon step, separately. For the undigested meat, the same linear model as for the meat characteristics was used, whereas for the duodenum and colon phases a mixed model ANOVA procedure was used with the fixed effects heme-Fe, nitrite, and heme-Fe × nitrite and the random effect incubation run. Data on O^6-C-MeG were analyzed for each run (fecal inoculum) separately due to very high variation between the different applied inocula, using a linear model with the fixed effects heme-Fe, nitrite, and heme-Fe × nitrite. Tukey-adjusted post hoc tests were performed for all pairwise comparisons. $P < 0.05$ was considered significant.

RESULTS

Meat Characteristics. Characteristics of the used meats are shown in Table 2. Pork contained a marginally higher fat content compared to the other meats. The fatty acid profile showed no significant differences in SFA between the different meats, whereas minor differences were observed for total MUFA and PUFA. The beef sample contained significantly

Table 3. Lipid Oxidation in Uncured and Nitrite-Cured Chicken, Pork, and Beef before and after in Vitro Digestion[a]

		uncured			nitrite-cured				*P* values		
	step	chicken	pork	beef	chicken	pork	beef	RMSE	H	NC	H × NC
MDA	BD	5.7b	5.4b	15.1a	2.8b*	2.0b*	7.4a*	0.41	**<0.001**	**<0.001**	**<0.001**
(nmol/mL)	D	9.8c	14.8b	28.1a	8.7b	9.2b*	11.0a*	0.96	**<0.001**	**<0.001**	**<0.001**
	C	13.9c	17.7b	26.3a	12.8b	13.0b*	15.3a*	1.21	**<0.001**	**<0.001**	**<0.001**
4-HNE	BD	48.5c	221.2b	428.6a	8.8	nd	nd	48.08	**0.004**		
(pmol/mL)	D	20.4c	211.6b	521.2a	12.2	19.4*	19.8*	69.57	**<0.001**	**<0.001**	**<0.001**
	C	16.4b	20.2ab	23.6a	15.8	17.1	13.3*	2.90	**0.088**	**<0.001**	**<0.001**
pentanal	BD	65c	172b	331a	84a	32b*	37b*	10.8	**<0.001**	**<0.001**	**<0.001**
(pmol/mL)	D	130c	314b	697a	103	124*	107*	66.2	**<0.001**	**<0.001**	**<0.001**
	C	108	126	125	101	106	87*	14.03	**0.098**	**<0.001**	**<0.037**
hexanal	BD	287c	1162b	1391a	107*	77*	77*	32.4	**<0.001**	**<0.001**	**<0.001**
(pmol/mL)	D	299c	1017b	2652a	214	212*	232*	178.5	**< 0.001**	**< 0.001**	**< 0.001**
	C	178b	209ab	241a	176ab	211a	138b*	25.0	**0.011**	**<0.001**	**<0.001**
heptanal	BD	3.0c	18.4b	56.0a	1.9	nd	nd	2.24	**<0.001**		
(pmol/mL)	D	23.2bc	65.0b	147.8a	20.8	16.1	23.9*	34.22	**<0.001**	**<0.001**	**<0.001**
	C	84.2a	67.5a*	38.9b	99.1a	132.0a	69.5b	25.35	**<0.001**	**<0.001**	0.065
nonanal	BD	10.1	14.9	16.9	12.7	10.3	6.8	3.03	0.866	0.060	0.067
(pmol/mL)	D	21.6c	28.9b	52.2a	16.0b	16.9b*	24.3a*	3.12	**<0.001**	**<0.001**	**<0.001**
	C	8.5	7.8	14.1	10.0	8.8	13.3	3.75	**0.005**	0.682	0.754

[a]MDA, malondialdehyde; 4-HNE, 4-hydroxy-2-nonenal; BD, before digestion; D, duodenal stage; C, colonic stage; RMSE, root-mean-square error; H, heme-Fe content; NC, nitrite-curing. Means for different heme-Fe contents (within curing treatment) with different letters (a−c) are significantly different ($P < 0.05$); *, significantly different from uncured equivalent ($P < 0.05$); nd, not detected.

Table 4. Protein Oxidation in Uncured and Nitrite-Cured Chicken, Pork, and Beef before and after in Vitro Digestion[a]

		uncured			nitrite-cured				*P* values		
	step	chicken	pork	beef	chicken	pork	beef	RMSE	H	NC	H × NC
PCC	BD	1.57	1.85	2.17	0.65*	0.76*	0.88*	0.279	0.082	**<0.001**	0.562
(nmol DNPH/mg	D	2.47b	2.81b	4.87a	1.78	1.79*	1.97*	0.445	**<0.001**	**<0.001**	**<0.001**
protein)	C	3.11b	3.54ab	4.19a	2.93	3.10	3.71	0.517	**<0.001**	**0.043**	0.742
protein	BD	6.44	6.44	6.05	6.40	6.09	6.20	0.580	0.851	0.865	0.875
(mg/mL)	D	2.22	2.28	2.41	2.24	1.98	2.05	0.207	0.497	**0.017**	0.174
	C	1.54	1.36	1.38	1.32*	1.26	1.25	0.109	**0.020**	**<0.001**	0.332

[a]PCC, protein carbonyl compounds; BD, before digestion; D, duodenal stage; C, colonic stage; RMSE, root-mean-square error; H, heme-Fe content; NC, nitrite-curing. Means for different heme-Fe contents (within curing treatment) with different letters (a−c) are significantly different ($P < 0.05$); *, significantly different from uncured equivalent ($P < 0.05$).

more ALA, LC n-3 PUFA, and LC n-6 PUFA and less LA compared to the other meats, whereas the chicken sample contained significantly more ALA and LC n-3 PUFA compared to pork. A clearly lower amount of residual nitrite in cured meat samples was found, compared to the added amount (120 mg/kg meat). The lowest amount of residual nitrite was found in beef, followed by pork and chicken. Beef had significantly higher heme-Fe and total Fe compared to chicken and pork.

Lipid Oxidation. Table 3 shows the concentrations of lipid aldehydes before the digestion and after duodenal and colonic digestion of the meats. Uncured beef showed significantly higher amounts of all lipid oxidation products before digestion, except for nonanal, compared to pork, followed by chicken. Concentrations of lipid oxidation products were significantly lower when meats were nitrite-cured to even undetected for 4-HNE and heptanal. Compared to uncured chicken, nitrite-cured chicken had significantly lower MDA and hexanal concentrations, whereas 4-HNE and heptanal concentrations were hardly detectable. An increase in all oxidation products was observed in the duodenum step; however, this was less distinctive for 4-HNE. Also, after duodenal digestion, uncured beef resulted in significantly higher concentrations of lipid aldehydes compared to pork, followed by chicken. Hexanal concentrations in duodenal digested uncured beef increased 2-fold during digestion while the hexanal concentrations in the uncured chicken and pork remained similar. Nonanal increased 3-fold during digestion of uncured beef, but only 2-fold in uncured chicken and pork. After duodenal digestion of nitrite-cured meats, 4-HNE and heptanal were detected, but in accordance with the other lipid oxidation products in much lower concentrations compared to their uncured equivalents. Except for MDA, the concentration of lipid oxidation products was clearly lower after 72 h of colonic digestion compared to the duodenum step or before digestion. Similar significant

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Table 5. NOC-Induced DNA Damage by Uncured and Nitrite-Cured Chicken, Pork, and Beef before and after in Vitro Digestion[a]

		uncured			nitrite-cured				*P* values		
	step	chicken	pork	beef	chicken	pork	beef	RMSE	H	NC	H × NC
O^6-C-MeG	BD	nd	nd	nd	nd	nd	nd				
(ng/mL)	D	nd	nd	nd	nd	nd	nd				
	C1	nd	nd	nd	nd	nd	nd				
	C2	10.8b	13.3ab	14.4a	11.9	11.9	11.9	0.89	0.071	0.121	0.066
	C3	539b	504b	762a	558b	620b	760a	45.4	**0.001**	0.143	0.225

[a] O^6-C-MeG, O^6-carboxy-methylguanine; BD, before digestion; D, duodenal stage; C1, C2, C3, colonic stage incubated with microbiota 1, 2, and 3; RMSE, root-mean-square error; H, heme-Fe content; NC, nitrite curing. Means for different heme-Fe contents (within curing treatment) with different letters (a–c) are significantly different ($P < 0.05$); nd, not detected.

effects of nitrite curing and heme-Fe content on most aldehyde concentrations were observed in the colon digestive samples. In contrast to other aldehydes in the colonic digestive fluids, heptanal was significantly lower in beef compared to chicken and pork and significantly higher in nitrite-cured meats compared to the uncured equivalents. Nitrite curing had no significant effect on nonanal in the colonic digestion samples. Octanal was not detected or close to no detection and hence was not included in the statistical analysis.

Protein Oxidation. Undigested nitrite-cured meats had significantly lower PCC concentrations than their uncured equivalents (Table 4). Higher PCC concentrations were observed after duodenal and colonic digestion with significantly higher PCC levels in uncured beef digests compared to chicken and pork after duodenum digestion and compared to chicken after colon digestion. Nitrite curing of the meat also resulted in lower protein oxidation in simulated duodenum and in marginally lower values in the colonic step.

A clear decrease in protein levels was observed starting from the nondigested meat to the duodenal and colonic digestive fluids (Table 4). No significant differences were observed between different meat samples before digestion. Cured meats had significantly lower protein levels in duodenum and colon compared to uncured equivalents. A marginal effect of heme-Fe was also observed in the colonic phase.

***O^6*-Carboxy-methylguanine.** No O^6-C-MeG was detected in meats before digestion or at the end of the duodenal phase (Table 5). In contrast to the other parameters, detection of O^6-C-MeG after 72 h of colonic digestion was highly dependent on the individual bacterial inoculum. Inoculum originating from the first test subject did not allow detection of O^6-C-MeG in any sample. When fermented using inoculum originating from the second and third test persons, O^6-C-MeG levels were low or very high, respectively. After 72 h of fermentation, a significant effect of heme-Fe concentration was observed. When fermented by inoculum 2, uncured beef had significantly more O^6-C-MeG than chicken, whereas no difference was seen in nitrite-cured meats. Fermentation by the third inoculum resulted in higher O^6-C-MeG formation in both uncured and nitrite-cured beef compared to chicken and pork. No significant effect of nitrite curing was found.

DISCUSSION

The objective of the present study was to test the involvement of heme-Fe and nitrite curing on the formation of toxic compounds during the digestion of meat. In uncured meats, the promoting effects of heme-Fe on oxidation and NOC-induced DNA damage during digestion were distinct. Our data confirm the importance of heme-Fe as catalyst of endogenous oxidation and nitrosation reactions, and hence it is likely to be involved in the association between red meat consumption and CRC. Curing with nitrite drastically inhibited the formation of the major toxic oxidation products MDA, 4-HNE, the minor toxic simple aldehydes, and PCC with no stimulating effect on the formation of NOC-specific DNA adducts. Consequently, nitrite curing of meat was not perceived as a risk factor in the presented study.

In accordance with our results, Hur et al.[11] already demonstrated a rise in TBARS during in vitro digestion of beef patties until duodenal digestion. Increased oxidation during digestion of meat under simulated duodenal conditions was confirmed in our study using more specific markers. Changes in 4-HNE concentrations before and after duodenal digestion were less distinct compared to other lipid oxidation products. However, measurable levels of 4-HNE were noted in nitrite-cured meat samples under simulated duodenal and colonic conditions, whereas it was not detected before digestion, indicating the formation of 4-HNE during digestion. Gorelik et al.[35] demonstrated absorption and accumulation of MDA in human plasma and urine after consumption of turkey cutlets. Increased plasma 4-hydroxy-2-hexenal (4-HHE) in mice after consumption of oxidized n-3 fatty acids was associated with oxidative stress and inflammation in the upper intestine.[36] Through systemic circulation, lipid oxidation products can reach sensitive tissues where MDA and 4-HNE can form adducts with DNA, increasing cancer risk.[37] In this way, higher plasma and tissue MDA in rats fed beef compared to chicken were associated with higher colonic DNA strand breaks.[38] In our experiment, the clearly lower concentrations of lipid oxidation products after colonic digestion, with an exception for MDA, could be attributed to degradation into volatile compounds and adduct formation with proteins or bacterial DNA. Oxidation of aldehydes by previously demonstrated aldehyde dehydrogenase activity of colonic bacteria[39] could also explain the observed decrease throughout colonic digestion. It should be noted that the fecal inocula likely also contained aldehydes. The net disappearance of the aldehydes during colonic digestion was thus probably underestimated.

The antioxidative effect of nitrite was clearly demonstrated in meats before and after digestion. Whereas high concentrations of the major cytotoxic 4-HNE were found in uncured beef, undetected to very low traces were observed for nitrite-cured beef. Nitrite curing of meat samples was performed by mixing with 0.6% nitrite salt. In contrast, adding salt without nitrite to meat increased TBARS and peroxides in meat.[40] Adding nitrite (1 g/L) in drinking water significantly reduced lipid oxidation in the colon of rats on a heme diet.[41] These results should be

interpreted with care because a dual role of •NO on oxidative stress has been described. Because a 1:1 ratio of •NO to ROS enhances lipid peroxidation whereas an excess of •NO inhibits oxidation,[17] consumption of oxidized nitrite-cured meat could result in a reversed effect. In contrast to other aldehydes in the colonic digests, heptanal was surprisingly lower in beef compared to chicken and pork and higher in nitrite-cured meats compared to their uncured equivalents. This unexpected result should be clarified in future experiments.

Because lipid oxidation products arise from PUFA, we aimed to exclude a possible confounding effect of different fatty acid profiles by adding 4% subcutaneous pork fat to the lean chicken, pork, and beef meats (approximately 1% intramuscular fat) of the present study. Despite this, there were some minor differences in the PUFA subgroups. Total PUFA was very similar across the different samples. 4-HNE and hexanal arise specifically from n-6 PUFA.[42] The small differences in n-6 PUFA content among the different meat samples in the present study are not expected to explain the large differences in 4-HNE and hexanal formation. LA, which belongs to the n-6 PUFA subgroup, was even higher in the chicken meat, but was associated with the lowest 4-HNE and hexanal formation.

Measurement of PCC as a marker for protein oxidation compared well with our observations on lipid oxidation. Higher heme-Fe concentrations in meats induced more protein oxidation, whereas nitrite curing prevented oxidation before and during in vitro digestion. It was previously demonstrated that CRC patients had higher plasma PCC levels than healthy controls.[43] The formation of PCC affects the nature and function of the protein, but the importance of dietary PCC to human health is still not completely clear. As protein oxidation is believed to be a free radical chain reaction,[44] it could be hypothesized that oxidized dietary proteins catalyze the oxidation of cellular proteins and hence induce cell damage.

The significantly lower PCC and protein levels in nitrite-cured meats compared to uncured meats during simulation of duodenum and colon digestion agree with Santé-Lhoutellier et al.,[45] who found a highly significant negative correlation between PCC concentrations and proteolytic susceptibility to pepsin. MDA and 4-HNE can also react with proteins, leading to the formation of Schiff bases, which are known to play a role in protein aggregation. Previously, it was shown that a higher amount of protein reaching the colon fermentation resulted in a higher formation of potentially toxic protein fermentation products such as ammonia, phenol, *p*-cresol, and indole. However, higher formation of these products was not associated with enhanced colon cancer promotion in rats.[46]

The increased O^6-C-MeG concentrations in colon samples when meats contained higher heme-Fe contents were in accordance with Bingham et al.,[8] who showed a dose-responsive increase in ATNC with consumption of red meat, whereas consumption of large amounts of white meat did not influence fecal ATNC. Cross et al.[9] showed that heme-Fe was responsible for endogenous intestinal N-nitrosation arising from red meat. Elevated O^6-C-MeG was also observed in colonic exfoliated cells of humans on a high red meat diet.[10] These observations are in agreement with extensive epidemiologic studies that showed a moderate but significantly increased CRC risk with high red meat consumption.[2,3] IJssennagger et al.[47] provided evidence for selective susceptibility of Gram-positive bacteria to heme cytotoxic fecal water, which was not observed for Gram-negative bacteria. Furthermore, heme-Fe increased colonic *Enterobacteria* and *Bacteroidetes* spp. and decreased *Lactobacilli* and *Firmicutes* spp.[47,48] Possibly, a bacterial shift induced by heme-Fe toxicity or heme-induced oxidative stress could alter the bacterial metabolic capacity and contribute to higher O^6-C-MeG formation in beef digesta. The pronounced differences in the detection of the NOC-specific DNA adduct O^6-C-MeG among the individual fecal inocula are most likely the result of variation in the gut bacterial composition of the fecal donor or varying concentrations of compounds in the fecal inoculum that are involved in formation of the DNA adduct O^6-C-MeG. More research is needed to clarify how and which bacterial species or compounds are responsible for the formation of O^6-C-MeG.

A higher formation of O^6-C-MeG when meats were nitrite-cured was not observed and, hence, our results do not support an earlier suggestion about the importance of nitrite curing on NOC formation.[5] Rats on a high heme-Fe diet displayed 3-fold higher fecal ATNC when drinking water contained sodium nitrate and nitrite (0.17 g/L $NaNO_2$ and 0.23 g/L $NaNO_3$).[41] However, because the increase in ATNC was mainly attributed to an increase of the nongenotoxic iron-nitrosyl, the authors concluded this was probably not associated with an increased colon cancer risk. Nitrite-cured meats also contain far less residual nitrite than many vegetables, which are not associated with increased risk in epidemiologic studies. Furthermore, nitrite is formed endogenously and recycled with the saliva. Santarelli et al.[49] showed clearly higher fecal ATNC in 1,2-dimethylhydrazin injected rats on a diet of dark, nitrite-cured, cooked, and oxidized meat. In contrast, no increase was observed in rats on a diet of dark, nitrite-cured, cooked, and anaerobically stored meat. Meats used in our experiment were stored anaerobically at −20 °C to prevent oxidation during storage. Our results did not show an increased O^6-C-MeG formation when nitrite-cured meats were stored properly.

In conclusion, our study presents a valuable in vitro tool to study oxidation and nitrosation processes during digestion of meat. Our results confirm an earlier-described catalyzing effect of heme-Fe in meat on oxidative stress and demonstrate NOC-induced DNA damage during digestion. Nitrite curing of meat resulted in a lower formation of toxic oxidation products, whereas no effect was observed on the formation of the NOC-specific DNA adduct O^6-C-MeG.

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ABBREVATIONS USED

ALA, α-linolenic acid; ATNC, apparent total nitroso-compounds; CRC, colorectal cancer; FA, fatty acids; 4-HNE, 4-hydroxy-2-nonenal; LA, linoleic acid; MDA, malondialdehyde; MUFA, monounsaturated fatty acids; LC n-3 PUFA, long-chain n-3 polyunsaturated fatty acids; LC n-6 PUFA, long-chain n-6 polyunsaturated fatty acids; ·NO, nitric oxide; NOCs, *N*-nitroso-compounds; O^6-C-MeG, O^6-carboxy-methylguanine; O^6-MeG, O^6-methylguanine; PCC, protein carbonyl compounds; ROS, reactive oxygen species; SFA, saturated fatty acids; TBARS, thiobarbituric acid reactive substances

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