FAT, COOKING METHOD AND NITRITE-CURING OF MEAT INFLUENCES FORMATION OF MALONDIALDEHYDE AND NOC-SPECIFIC DNA ADDUCTS DURING *IN VITRO* DIGESTION

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Abstract – The formation of oxidation products and N-nitroso compounds (NOCs) during digestion of meat is considered the underlying cause of the association between red meat consumption and colorectal cancer (CRC). In the present study, the possible modulating effects of fat, cooking method and nitrite-curing of pork were investigated on the formation of these compounds. For this purpose, an *in vitro* model was used for digestion of pork varying in fat content (1, 5, 20%), cooking method (raw, cooked, overcooked), and whether or not nitrite-cured (120 mg nitrite/kg meat). Three fecal microbiota originating from three individuals were used to simulate colonic fermentation. Digestion of meat to which fat was added resulted in higher malondialdehyde (MDA) concentrations in duodenum and colon compared to the digestion of meat without added fat. Moreover, higher fat content favored the formation of the NOC-specific DNA adduct O^6-Carboxy-methylguanine (O^6-C-MeG) in the colon. Overcooking the meat resulted in significantly higher MDA and O^6-C-MeG concentrations compared to raw and cooked meat. Nitrite-curing resulted in significantly lower MDA concentrations, while the effect on the formation of O^6-C-MeG was not significant. In colon fermentation, very large differences between the different microbiota were observed for the formation of O^6-C-MeG.

Key Words – Colon, Health, Processed meat

I. INTRODUCTION

Different independent meta-analyses demonstrate a significantly increased CRC risk associated with a higher consumption of red meat and especially processed meat [1,2]. The biochemical mechanisms responsible for these epidemiologic associations are still not completely elucidated. The formation of cyto- and genotoxic oxidation products (such as MDA) and carcinogenic NOCs is considered the main factor responsible for this association [3].

Since especially processed meats are associated with a higher risk to develop CRC, the possible modulating effects of fat content, cooking method and nitrite-curing of meat on the formation of MDA and NOCs during digestion were investigated in the present study.

II. MATERIALS AND METHODS

Lean meat samples were collected from the *m. Longissimus dorsi* of pigs. In a first experiment, subcutaneous pork fat from one batch was added to the meat to obtain a total fat content of 1 (no fat added), 5 and 20%. After mincing, nitrite salt (0.6% nitrite) was added at 20 g/kg meat, corresponding to an amount 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. In experiment 2, uncured and nitrite-cured pork with 5% fat was used, either raw, cooked (core temperature of 65°C for 15 min) or overcooked (core temperature of 95°C for 30 min). After manufacturing, all meat samples were minced, vacuum packed and stored at -20°C until the start of the incubation.

For the *in vitro* digestion, the protocol described by Versantfoort *et al*. (2005) [4] was adapted by adding oxidants and antioxidants that are normally present in digestive juices. Hence, saliva also contained peroxidase [5] and $NaNO_2$ [6] while ascorbic acid [7], H_2O_2 [8] and $FeSO_4$ [8] were added to the gastric juice. During each incubation, 4 replicates of each meat sample (4.5g) were incubated 5 minutes with 6 ml saliva, 2 hours with 12 ml gastric juice, 2 hours with 2 ml bicarbonate buffer (1M), 12 ml duodenal juice and 6 ml bile. After duodenum digestion, 2 replicates of each meat sample were diluted with 44 ml H_2O to

obtain the same solid/liquid ratio as in colon (see further). Duodenum samples were stored at -20°C in aliquots after homogenizing with an ultraturrax. The 2 remaining replicates of each meat sample entered the colon digestion. SHIME medium (22 ml) [9] and human fecal microbiota (22 ml) were added to the digesta. Closed vessels were flushed with N_2 for 30 minutes to obtain an anaerobic environment. Subsequently, vessels were incubated for 72 hours while stirring at 37°C. Each incubation run was repeated 3 times with microbiota originating from 3 different individuals. Undigested control meat samples were obtained by homogenizing 4.5g meat in 82 ml H_2O, mimicking the liquid/solid ratio in duodenum and colon.

Concentrations of MDA in digesta were measured colorimetrically in accordance with Grotto *et al*. [10]. O^6-C-MeG was quantified by U-HPLC-MS/MS analysis after 182 µL of the filter sterilized sample was incubated for 18 hours at 37°C with 100 µg CT-DNA and internal standard (50 µL, 20 ng/ml O^6-D3-MeG) [11].
Data on MDA were analyzed per digestion stage using a linear mixed model with the fixed effects of fat, nitrite and fat × nitrite (experiment 1), or cooking, nitrite and cooking × nitrite (experiment 2), and the random effect of incubation run (SAS Enterprise Guide 5). Data on O^6-C-MeG were analyzed for each run (microbiota) separately due to the very large variation between different microbiota, using a linear model with the fixed effects of fat, nitrite and fat × nitrite (experiment 1), or cooking, nitrite and cooking × nitrite (experiment 2).

III. RESULTS AND DISCUSSION

In general, MDA concentrations after duodenum and colon digestion were clearly higher compared to before digestion (Table 1, Table 3). Standard deviations for MDA values after colon digestion were higher than for duodenum caused by the high and variable MDA concentrations in the applied microbiota originating from 3 different individuals (data not shown).
Uncured pork with added fat resulted in significantly higher MDA formation in the duodenum and colon digestion compared to pork without added fat (Table 1). Addition of nitrite to meat samples resulted in significantly lower MDA formation. Nitrite is a known antioxidant and may act as a precursor of the heat-stable nitrosomyoglobin by which the release of Fe^{2+} during heating is inhibited [12]. Consequently, less Fe^{2+} is available to catalyze the Fenton reaction, which is responsible for the initiation of oxidation processes. Intake of nitrite through drinking water (1 g/l) also significantly reduced haem-induced lipid peroxidation in the colon of rats by 25% [13].

Table 1 Malondialdehyde formation (nmol/ml digesta) in uncured and nitrite-cured pork with varying fat content during *in vitro* digestion (mean ± SD)

	Uncured		Nitrite-cured	
Undigested				
1% fat	4.0 ± 0.4	b,x	2.0 ± 0.0	y
5% fat	5.4 ± 0.0	a,x	2.0 ± 0.0	y
20% fat	5.3 ± 0.2	a,x	1.9 ± 0.1	y
Duodenum				
1% fat	9.1 ± 1.9	c,x	8.2 ± 1.7	y
5% fat	13.3 ± 1.8	a,x	8.4 ± 1.2	y
20% fat	12.6 ± 1.4	b,x	8.0 ± 1.7	y
Colon				
1% fat	15.5 ± 8.0	b	14.7 ± 8.0	a
5% fat	18.0 ± 6.9	a,x	14.3 ± 8.3	ab,y
20% fat	16.4 ± 6.9	b,x	13.4 ± 7.8	b,y

a,b,c = means for different fat contents with different superscripts are significantly different ($P < 0.05$); x,y = means for uncured and nitrite-cured with different superscripts are significantly different ($P < 0.05$)

The formation of the NOC-specific DNA adduct O^6-C-MeG was highly dependent of the microbial inoculum (Table 2, Table 4). Enzyme activity of microbiota affecting the mutagenicity of NOCs can greatly vary depending on the bacterial composition [14]. It is likely that the microbiota of subject 3 contained bacterial species with a much higher potency to activate NOCs to its carcinogenic derivates, resulting in much higher NOC-specific DNA-adducts.
Significantly higher O^6-C-MeG concentrations were found in the colon when meat samples contained 20% fat (Table 2). NO preferentially diffuses in a lipid environment where it reacts with O_2 to reform nitrosating species. The rate of this reaction was described to be 300 times faster in a lipid than in an aqueous environment [15].
Our results did not show significant differences in the formation of NOC-specific DNA adducts when meat samples were nitrite-cured. Hence, nitrite-curing was not perceived as a risk factor. Chenni

et al. [13] reported increased ATNC in the colon of rats when a haem diet was enriched by nitrite in drinking water. However, these authors concluded that this was probably not associated with an increased risk for colon cancer due to the level and the nature (iron-nitrosyl) of the compound that was formed.

Table 2 O^6-C-MeG formation (ng/ml digesta) in uncured and nitrite-cured pork with varying fat content after colon fermentation (mean ± SD)

	Uncured		Nitrite-cured	
Colon1	*nd*		*nd*	
Colon2				
1% fat	46.8 ± 0.9	c	48.9 ± 0.5	a
5% fat	50.6 ± 1.4	b,x	44.6 ± 1.1	b,y
20% fat	57.8 ± 0.8	a,x	50.8 ± 0.3	a,y
Colon3				
1% fat	441 ± 74	b	544 ± 7	b
5% fat	486 ± 73	b	541 ± 47	b
20% fat	697 ± 58	a	783 ± 44	a

SD = standard deviation; a,b,c = means for different fat contents with different superscripts are significantly different ($P < 0.05$); x,y = means for uncured and nitrite-cured with different superscripts are significantly different ($P < 0.05$)

Cooking of meat samples resulted in an increased formation of MDA (Table 3). Cleavage of the haem porphyrin during heating leads to release of Fe^{2+}, which becomes then available for catalyzing oxidation processes.

Table 3 MDA formation (nmol/ml digesta) in uncured and nitrite-cured pork upon different cooking methods during *in vitro* digestion (mean ± SD)

	Uncured		Nitrite-cured	
Undigested				
Raw	1.7 ± 0.1	b	1.9 ± 0.2	
Cooked	5.4 ± 0.0	a,x	2.0 ± 0.0	y
Overcooked	5.7 ± 0.3	a,x	2.0 ± 0.1	y
Duodenum				
Raw	9.3 ± 1.6	c,x	8.2 ± 1.6	b,y
Cooked	13.6 ± 1.9	b,x	8.2 ± 1.2	b,y
Overcooked	15.2 ± 1.3	a,x	9.4 ± 0.6	a,y
Colon				
Raw	11.7 ± 7.5	c	11.3 ± 8.4	
Cooked	15.1 ± 7.4	b,x	10.3 ± 7.3	y
Overcooked	16.4 ± 8.4	a,x	10.6 ± 7.1	y

a,b,c = means for different cooking methods with different superscripts are significantly different ($P < 0.05$); x,y = means for uncured and nitrite-cured with different superscripts are significantly different ($P < 0.05$)

Increased NOC-induced DNA adduct formation with increasing cooking time and temperature of meat was highly significant in the present study (Table 4). Previously, it has been described that cooking by deep-frying or pan-frying increases volatile N-nitrosamines in dry-cured sausages [16]. Several case control studies demonstrate an association between well-done red meat and CRC, usually explained by the formation of carcinogenic heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) [17,18,19]. However, Santarelli *et al*. [20] gave several arguments for a minor role of HCAs in this association. Since the present data indicates that NOC-induced DNA adduct formation is increased with increasing heating temperature, future studies investigating the association between cooking methods and CRC should also focus on NOC activity.

Table 4 O^6-C-MeG formation (ng/ml digesta) in uncured and nitrite-cured pork upon different cooking methods after colon fermentation (mean ± SD)

	Uncured		Nitrite-cured	
Colon1	*nd*		*nd*	
Colon2	*nd*		*nd*	
Colon3				
Raw	511 ± 212	b	408 ± 12	b
Cooked	632 ± 103	b	566 ± 14	b
Overcooked	783 ± 10	a	725 ± 113	a

a,b,c = means for different cooking methods with different superscripts are significantly different ($P < 0.05$)

IV. CONCLUSION

Our results suggest that the greater association of CRC with processed meat than with red meat, might be related to the higher fat contents and associated MDA and NOC formation during heating of processed meats. Based on our results, the hypothesized influence of nitrite could not be confirmed.

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