

DOES DIGESTION OF RED MEAT CAUSE MORE PROCARCINOGENIC DNA ADDUCTS IN HEALTHY SUBJECTS AS OPPOSED TO WHITE MEAT?

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Abstract – Several studies have demonstrated that the consumption of red and processed meat can contribute to the risk of colorectal cancer. One of the hypotheses for the causal mechanism is that red and processed meat stimulate the formation of N-nitroso compounds (NOCs) in the gut. NOCs are able to alkylate and thus alter the composition of cellular DNA. It has already been reported that feces of volunteers on a high red meat diet contains high levels of NOCs. Furthermore, the occurrence of O⁶-CMG, a NOC-specific DNA adduct in colonic cells *in vivo*, has also been confirmed. In our previous work we demonstrated that the formation of O⁶-CMG specifically occurs in the colon and depends on the individual microbiota. In the present study we aimed to screen the colonic microbiota of 15 healthy volunteers for their ability to produce O⁶-CMG in *in vitro* digestions of meats. The production of O⁶-CMG was monitored by means of UHPLC-MS/MS. We found that O⁶-CMG adduct formation in digesta of different meats is indeed possible, but is very much reflected by the individual microbiota of volunteers. In general, a higher formation of O⁶-CMG was observed for red meat, which proved significant for individuals but not throughout the entire test survey.

Key Words – Colorectal cancer, N-nitroso compounds, Red meat consumption

I. INTRODUCTION

Both epidemiological and clinical studies have consistently demonstrated that diet and other lifestyle factors can contribute to the burden of human cancer. Colorectal cancer is the third most dominant cancer in the world and its prevalence shows a strong association with the consumption

of red and processed meat [1]. In literature, this correlation has been discussed extensively and several hypotheses have been suggested to explain the link between meat and colorectal cancer. The potential role of the known carcinogenic heterocyclic amines and polycyclic aromatic hydrocarbons, which are formed during cooking of all different sorts of meats and meat products, has been ruled out since only red and not white meat is associated with an increased risk of colorectal cancer [2]. Red and processed meat intake on the other hand, does show a dose-response relation with the endogenous formation and fecal excretion of N-nitroso compounds (NOCs), whereas there is no such relation for white meat [3,4]. Hence, N-nitroso compounds are currently considered as a plausible cause for the carcinogenicity of red and processed meat [5]. In volunteers, the red meat associated endogenous formation of NOCs has been correlated with the formation of NOC-specific DNA adducts which are promutagenic and genotoxic [4]. Both carboxymethyl (e.g. O⁶-carboxymethyl-2'-deoxyguanosine; O⁶-CMdG) and, to a lesser extent, methyl adducts (e.g. O⁶-methyl-2'-deoxyguanosine; O⁶-MdG) can originate from the alkylation of DNA by NOCs. Since O⁶-CMdG is not easily repaired by DNA-repair mechanisms *in vitro* and because of its prevalence in exfoliated colonic cells *in vivo*, this DNA adduct has great potential as a biological marker for DNA carboxymethylation [4].

II. MATERIALS AND METHODS

Reagents and Chemicals.

The chemical standard O⁶-methylguanine (O⁶-MeG) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and the internal standard O⁶-methyl-d³-guanine (O⁶-d³-MeG) was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). The O⁶-carboxymethyl guanine (O⁶-CMG) standard was derived by acidic hydrolysis of O⁶-carboxymethyl deoxyguanosine (O⁶-CMdG) with 0.1 M formic acid (80°C, 30 min) [6] and stored as a stock solution of 500 ng/μL. The stock solution of the chemical standards O⁶-MeG was prepared in ethanol at a concentration of 1 mg/mL. To obtain work solutions of the different concentrations needed, the stock solutions were diluted with ethanol or deionized water. The internal standard consisted of a work solution of O⁶-d³-MeG in deionized water at a concentration of 20 ng/mL. All solutions were stored in dark glass bottles at -20 °C.

Reagents for extraction and purification were of analytical grade (VWR International, Merck, Darmstadt, Germany). For MS application reagents of Optima® MS grade were used (Fisher Scientific UK, Loughborough, UK).

Digestion Protocol.

Human fecal samples were obtained from fifteen healthy volunteers (ages 20 to 60, 10 males and 5 females) who had not received antibiotics six months up to fecal sampling. The diet of these volunteers was unmonitored, and thus not taken into account. After sampling, the fresh fecal samples were homogenized (stomacher 400 Classic Laboratory Blender, Seward, West Sussex, UK) in phosphate-buffered saline (0.1 M, pH 7) to obtain fecal slurries (20% w/v). After centrifugation (2 min, 500 x g) of the fecal slurries, glycerol (Analar Normapur, Fontenay-sous-Bois, France) was added to the obtained supernatant (20 % v/v) for the purpose of cryoprotection during storage at -80 °C. For the promotion of anaerobe circumstances, sodium thiogluconate was added to the phosphate-buffered saline prior to use.

For the simulation of the digestion of meat, chicken or beef were prepared at 80 °C for 10 min in a hot water bath. Four grams of prepared meat

was mixed thoroughly with stomach juice (KHCO₃: 0.1 mol/L; NaCl: 0.1 mol/L; pH 1.5) (1/10 w/v) in triplicate to simulate chewing and stomach conditions. After transferring the mix of meat and stomach juice into an autoclaved penicillin flask, filter-sterilized pepsin was added (0.320 g/L). The flasks were sealed with butyl rubber lids and incubated at 37°C and 150 RPM. After 2 hours of incubation, 5 mL of enteric juice (NaHCO₃: 0.15 mol/L; Oxgall: 6 g/L; pancreatine: 0.9 g/L) was added per gram of meat and the flasks were incubated at the same conditions for another 4 hours. In order to simulate the digestion of meat in the large intestine 5 mL of boiled-up SHIME (Simulator of the Human Intestinal Microbial Ecosystem) broth ((arabinogalactan: 1 g/L; pectin: 2 g/L; xylan: 1 g/L; mucin: 4 g/L; potato starch: 4 g/L; glucose: 0.4 g/L; yeast cell extract: 3 g/L; peptone; 1 g/L; L-cystein: 0.5 g/L) [7]) and 5 mL of cultivated fecal inoculum was added per g of meat before incubating the flasks at 37°C and 150 RPM for 72 hours (colonic digestive simulation). To assure anaerobic conditions, the flasks were evacuated to vacuum, alternated with N₂-flushing for 1 hour at 1 bar. Prior to use, the fecal inoculum was cultivated in boiled-up BHI (Brain Heart Infusion, Oxoid Hampshire, England) broth with L-cysteine (0.5 g/L) for 24 hours (37°C, 150 RPM).

Extraction and Purification of DNA adducts.

After thawing, the digestive samples were filter-sterilized (0.22 μm) to remove all bacterial cells and excessive debris. Afterwards, 182 μL of the filter-sterilized sample was added to 100 μg of CT-DNA (Rockland Immunochemicals Inc., Gilbertsville, PA, USA) and 168 μL of TE-buffer (0.05 mol/L Tris; 0.010 mol/L EDTA; pH 8) before overnight incubation (18 hours, 37°C, 120 RPM). After this incubation step, O⁶-d³-MeG (50 μL of 20 ng/mL) was added as internal standard. In order to release the DNA bases, an acidic hydrolysis of the sample with 2 mL of 0.1 M formic acid at 80 °C for 30 min. was performed. Then, the samples were cooled down on ice and applied to a solid-phase extraction (SPE). The SPE-cartridge (Oasis HLB, 30 mg, 1cc) was conditioned with 2 mL of MeOH and 2 mL of deionized water beforehand. The sample was eluted from the SPE-cartridge with 2 mL of MeOH and evaporated to a drop (90 min, 20 °C,

SpeedVac® Plus (Savant, Holbrook, NY, USA)). Finally, the dried residue was redissolved in 100 µL of 0.05% acetic acid in deionised water.

UHPLC-MS/MS analysis.

Analysis of the NOC-specific DNA adducts O⁶-CMG, O⁶-d3-MeG and O⁶-MeG on a triple stage quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific, San José, USA) fitted with a heated electrospray ionisation (HESI II) source (in positive ion mode) was previously described by Vanden Bussche *et al.* [8].

Data Handling.

Processing of data was performed with Xcalibur 2.0.7. software (Thermo Fisher Scientific, San José, USA). Data on O⁶-CMG concentration in chicken and beef digests were analyzed with paired t-tests at a significance level of 5% (SPSS Statistics 20). The effect of the individual microbiota on O⁶-C-MeG formation was analyzed with ANOVA (SPSS Statistics 20).

III. RESULTS AND DISCUSSION

For all 15 volunteers the O⁶-CMG DNA adduct could not be detected in the sampled digestive fluids of stomach and small intestine. In samples taken at the end of the colonic digestion of different meats (e.g. after 72 hours of incubation during *in vitro* simulation of the colonic digestion (t72)), 7 out of 15 volunteers produced the O⁶-CMG DNA adduct (data shown in Table 1). Prior to the colonic digestive simulation (t0), 4 out of 7 digesta that were also positive at t72, already contained a low amount of O⁶-CMG. The O⁶-CMG concentrations in positive samples increased significantly ($p \leq 0.01$) from t0 to t72 for all 7 individuals. The O⁶-CMG positive volunteers were both females (2 out of 5) and males (5 out of 10), and belonged to different age categories.

Fecal inocula from volunteers A, B and F demonstrated a significantly higher formation of O⁶-CMG upon beef digestion compared to chicken ($p \leq 0.05$). O⁶-CMG concentration for volunteers C and G on the other hand, were found to be in a similar range (not significantly different, with $p > 0.05$) for chicken or beef digestions.

Fecal inocula of volunteers D and E generated a higher concentration of O⁶-CMG in digestions of chicken compared to beef, but this discrepancy did not prove to be significant ($p > 0.05$).

When all data from positive volunteers were taken into account (Figure 1), the promotion of DNA adduct formation was numerically higher for beef compared to chicken, but the difference was not statistically significant ($p < 0.05$).

Table 1 O⁶-CMG concentration (pmol/mL) in digesta (mean \pm standard error) after 0 (t0) and after 72 (t72) hours of the large intestinal digestion from positive samples/volunteers (ND: not detected). Significantly different ($p < 0.05$) O⁶-CMG concentrations for beef compared to chicken are marked with a *

ID	Time of sampling	Chicken	Beef
A	t0	9.7 \pm 0.5	14.00 \pm 0.4
	t72	174.0 \pm 10.9	231.45 \pm 14.2*
B	t0	ND	ND
	t72	548.0 \pm 33.2	1594.8 \pm 119.4*
C	t0	ND	ND
	t72	46.7 \pm 1.3	39.7 \pm 3.9
D	t0	29.9 \pm 1.2	32.3 \pm 2.1
	t72	619.5 \pm 21.8	492.3 \pm 25.6
E	t0	50.7 \pm 2.0	54.4 \pm 4.9
	t72	1324.5 \pm 103.5	1254.8 \pm 75.2
F	t0	33.7 \pm 1.2	41.08 \pm 4.6
	t72	226.4 \pm 6.5	539.32 \pm 77.6*
G	t0	ND	ND
	t72	37.7 \pm 0.8	39.8 \pm 1.7

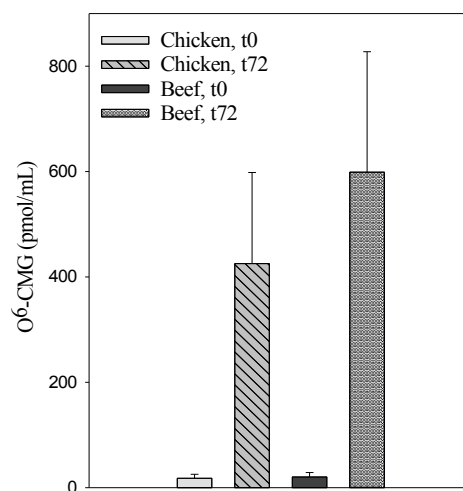


Figure 1. Mean O⁶-CMG concentration of digesta of all 7 O⁶-CMG forming fecal inocula (with standard error)

In order to verify the need for an active colonic microbiota for O⁶-CMG formation, BHI-cultivated and autoclaved fecal microbiota of a O⁶-CMG producing volunteer were used in a digestive simulation. It was shown that if the colonic microbiota were deactivated by autoclaving, production of O⁶-CMG did not take place.

IV. CONCLUSION

Since O⁶-CMG adducts could be detected in digestive samples after 72 hours of colonic incubation in 7 out of 15 volunteers and never in samples taken from stomach or small intestine simulations, it can be concluded that the production of O⁶-CMG depends on the addition of a cultivated and active colonic microbiota. The fact that autoclaving of microbiota inhibits the formation of O⁶-CMG, supports this hypothesis even further and shows that the formation of O⁶-CMG is highly dependent on the individual microbiota. In 4 out of 7 positive samples, the O⁶-CMG concentration was not significantly different in digesta of chicken or beef. On the other hand, for 3 positive fecal samples, the amount of O⁶-CMG in *in vitro* gastrointestinal digestions of chicken was significantly lower than beef, indicating that red meat may indeed exert procarcinogenic effects. When O⁶-CMG data from all 7 positive volunteers were taken into account, a higher, but not significantly higher O⁶-CMG formation was found in beef digesta compared to chicken. Further research is warranted to determine whether red meat indeed promotes the formation of O⁶-CMG and might play a role in the occurrence of colorectal cancer. It is not unlikely that other factors, such as levels of antioxidants and fat in different meats, play a significant role in this process.

ACKNOWLEDGEMENTS

The authors would like to thank the Research Foundation – Flanders (Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO-Vlaanderen) for their funding. Furthermore, the authors would also like to thank M. Naessens, L. Dossche, J. Goedgebeur, I. Opsteyn and D. Stockx for their practical assistance in the laboratory. Special thanks to all the volunteers for their kind cooperation.

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