Intragastric formation of N-nitrosodimethylamine after fish-with-vegetable meals using an in-vitro digestive model

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N-nitrosodimethylamine (NDMA) is an acute and chronic genotoxic carcinogen (Driver *et al.*, 1987; Peto *et al.*, 1991a,b). The human exposure to NDMA consists of direct intake from food (regular exposure) and endogenous synthesis after a meal of vegetables and fish (infrequent exposure)(Krul *et al.*, 2004).

The endogenous synthesis of NDMA starts with the intake of nitrate from vegetables and/or drinking water. The nitrate taken in quickly enters the blood from which is actively secreted to the saliva (Wagner *et al.*, 1983). In saliva nitrate is converted to nitrite by bacterial fermentation (Spiegelhalder *et al.*, 1976). In the stomach swallowed nitrite may react under acidic conditions with the fish component dimethylamine (DMA) to NDMA (Mirvish *et al.*, 1975).

Though *in vivo* experiments in human volunteers have indicated that NDMA can be formed in the stomach after a meal of nitrate rich vegetables and different types of amine-rich fish (Vermeer *et al.*, 1998; van Maanen *et al.*, 1998) only a crude estimate can be given of the actual amount. It can be argued that a more accurate estimate might be obtained by *in vivo* measurement of NDMA formation. However, for practical and ethical reasons a study of NDMA formation in the stomach of human volunteers is very difficult.

We therefore used an alternative way to estimate the formation of NDMA in the human stomach after a vegetable/fish meal, i.e. with a dynamic *in vitro* gastrointestinal model (Minekus *et al.,* 1995; Krul *et al.*, 2004). The (computer-controlled) *in vitro* system mimics the physiological processes in the human stomach, i.e. body temperature, pH profile after food intake, peristaltic movements, secretion of digestive enzymes, etc.

In the gastrointestinal model the nitrosation was investigated between nitrite and DMA and codfish (30 – 100 grams) under different gastric pH conditions (slow and rapid pH decrease). To simulate realistically the swallowing of nitrite-containing containing oral fluid the formation of nitrite was quantified with the aid of a toxicokinetic model for nitrate and nitrite in humans. With this model the flow of nitrite-containing oral fluid into the stomach was calculated for the intake of different levels of nitrate (0.1 – 10 times the ADI) and incorporated in the gastrointestinal model. The formation of NDMA was investigated for a total of 55 different nitrate/codfish combinations. The analysis of these data resulted in a quantitative relationship for NDMA formation as a function of nitrate intake from rich vegetables and concomitant codfish consumption (codfish calibration curve, Krul *et al.*, 2004).

Besides codfish the formation of NDMA was investigated for a variety of frequently consumed fish species such as herring, mackerel, plaice, pollack and salmon with the (concomitant) exposure to nitrate from vegetables. In these fish species the formation of NDMA relative to codfish was 0.39 (herring), 0.33 (tuna), 0.29 (schrimp), 0.44 (fish-fingers), 0.17 (pollack), 0.02 (mackerel) and 0.00 (salmon). Furthermore the (inhibitory) effect of adult-/children food matrices in the stomach on DMNA formation was determined. This resulted in a 75% inhibition (range: 53-89 %).

In order to estimate NDMA formation as occurring during actual meals the codfish calibration curve, its scaling to other fish species and the inhibitory effect of food in the stomach were combined with the concomitant consumption of fish and nitrate from vegetables as reported in the Dutch National Food Consumption Survey-3 (DNFCS-3, Kistenmaker *et al.*,1998). This resulted in 105 DNFCS-3 participants who consumed a meal with fish and nitrate-rich vegetables on at least 1 day.

The estimated amounts of NDMA (per kilogram body weight) for these participants of theDNFCS-3 were analyzed by the Statistical Exposure Model for Incidental Intakes

(STEM.II; Slob, 2006). In short, this model takes into account both the frequency of exposure days and the magnitude of the exposure on these days. This analysis resulted in the acute and long-term exposure to NDMA after a meal of nitrate rich vegetables and fish. The calculated exposures were compared with reference values for the acute and long-term carcinogenic potency of NDMA. Cancer reference values were obtained by analyzing acute and chronic carcinogenicity data ((Driver *et al.*, 1987; Peto *et al.*, 1991a,b) with the BenchMarkDose (BMD) approach. The BMD is the dose corresponding to a specified Benchmark Response (BMR), e.g., a 5 or 10% extra cancer risk, as calculated from a dose-response relationship which is fitted through the data. The lower limit of the

confidence interval around the BMD, i.e., the Benchmark Dose Lower bound (BMDL), represents the dose where the effect is smaller than the BMR with 95% confidence. For fitting of dose-response relationships, the software of PROAST (Slob, 2002; www.proast.nl) was used.

As measure for cancer potency the Margin Of Exposure(MOE) approach was applied. The MOE measures the distance (ratio) between human exposure and some toxicity measure, denoted as the PoD (Point of Departure) or RP (Reference Point). See Barlow et al. (2006) or O'Brien *et al*. (2006) for further

discussions of this approach. The current view is that the BMDL10 is the preferable PoD (Benford *et al.,* 2010; ILSI, 2009).

Using a BMDL10 for (total liver) chronic tumor incidence (0.029 mg/kg-bw) and the 95th percentile of the long-term NDMA exposure distribution (4.1 and 0.40 ng/kg-bw for children of 1 year of age and adults, respectively) resulted in MOEs of 7000 for children 1 year of age and 72,500 for adults.

Similarly, given the incidental high-peak exposure to endogenous NDMA formation in the human population, a risk characterization based on the results from the acute carcinogenicity study by Driver *et al.* (1987) is relevant. For this study a BMDL10 of 11 mg/kg-bw was calculated. The exposure analysis showed that most of the estimated amounts of NDMA in the participants of the food survey were below 0.10 µg/kg. Hence, the MOE for acute NDMA exposure after a fish and vegetable meal would be greater than 100,000.

Both the acute and the chronic MOEs indicate that the combined consumption of fish and nitrate-rich vegetables appears to lead to marginal increases of (additional) cancer risk.

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