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Salivary nitrate/nitrite and acetaldehyde in humans: potential combination effects in the upper gastrointestinal tract and possible consequences for the in vivo formation of *N*-nitroso compounds—a hypothesis

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Abstract
Subsequent to the dietary uptake of nitrate/nitrite in combination with acetaldehyde/ethanol, combination effects resulting from the sustained endogenous exposure to nitrite and acetaldehyde may be expected. This may imply locoregional effects in the upper gastrointestinal tract as well as systemic effects, such as a potential influence on endogenous formation of *N*-nitroso compounds (NOC). Salivary concentrations of the individual components nitrate and nitrite and acetaldehyde are known to rise after ingestion, absorption and systemic distribution, thereby reflecting their respective plasma kinetics and parallel secretion through the salivary glands as well as the microbial/enzymatic metabolism in the oral cavity. Salivary excretion may also occur with certain drug molecules and food constituents and their metabolites. Therefore, putative combination effects in the oral cavity and the upper digestive tract may occur, but this has remained largely unexplored up to now. In this Guest Editorial, published evidence on exposure levels and biokinetics of nitrate/nitrite/NO_x, NOC and acetaldehyde in the organism is reviewed and knowledge gaps concerning combination effects are identified. Research is suggested to be initiated to study the related unresolved issues.

Keywords Nitrate · Nitrite · Acetaldehyde · *N*-nitroso compounds · Upper gastrointestinal tract · Combination effects

Introduction

The toxic effects of nitrate/nitrite and thereof derived *N*-nitroso compounds (NOC) as well as acetaldehyde and its precursor ethanol have been well studied after exposure to the individual compounds. We hypothesize that the combined exposure to nitrate/nitrite and acetaldehyde through food and alcohol consumption may potentially entail as yet unexplored local and/or systemic combination effects. The concomitant exposure of the epithelium of the aerodigestive tract to a combination of cytotoxic/genotoxic compounds may potentially lead to enhanced locoregional transforming events, for instance as a consequence of enhanced regenerative proliferation of initiated cells in comparison to exposure of the epithelium to the respective single agents (IARC 2010; MAK 2013).

Sustained presence of acetaldehyde and nitrite in saliva can be expected to occur subsequently to the intake of the respective precursors ethanol and nitrate (see below). It is up to now unclear whether effects on tissues in direct contact with acetaldehyde may be influenced by simultaneously enhanced salivary contents of nitrite and cognate nitrosating agents (NO_x) potentially originating from it. Reliable information on the dosimetry of acetaldehyde/

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nitrite/NO_x in association with such combination effects is not available in the scientific literature.

Systemic effects may be expected as well, especially regarding in vivo *N*-nitrosation. It is well known that aldehydes can act as *N*-nitrosation catalysts, accelerating the formation of NOC (Fig. 1). Certain aldehydes can also modify the pH dependency of *N*-nitrosation, thereby leading to NOC formation at pH values close to neutral (Keefer and Roller 1973). Moreover, and as discussed in more detail below, given the simultaneous presence in the upper gastrointestinal tract of dietary constituents like the amino acid cysteine, a ring closure reaction with acetaldehyde to methylthiazolidine carboxylic acid (MTCA) and its *N*-nitroso product, *N*-nitroso-2-methylthiazolidine-4-carboxylic acid (NMTCA), may take place (Fig. 2). Of note, NMTCA (and other such nitrosated amino acids) is neither mutagenic nor carcinogenic (Ohshima et al. 1982, 1984; Ohshima and Bartsch 1988). In consequence, this ring closure/*N*-nitrosation reaction may be conceived as a detoxification pathway for both, nitrite and acetaldehyde, when co-occurring with amino acids such as cysteine.

To substantiate potential research objectives, background information on exposure levels, biokinetics and potential effects of nitrate/nitrite, NOC and acetaldehyde in the organism is presented, with special reference to saliva and the oral cavity.

Nitrate/nitrite/NO_x

The total body burden of nitrate and nitrite for a long time was conceived to essentially reflect dietary intake and environmental exposure until the discovery of an additional and significant contribution accountable to endogenous nitrate biosynthesis. The latter was reported to be markedly increased after endotoxin treatment of experimental animals (Wagner et al. 1983). Later on, it was shown that the activation of mouse macrophages induces the formation of nitrite and nitrate and that L-arginine functioned as the precursor (Marletta et al. 1988). Subsequently, Hevel et al. (1991) discovered that the formation of nitrite and nitrate occurred through NO generation by the inducible nitrogen oxide synthase, similar to NO formation in endothelial cells, in which case NO was identified as the endothelium-derived relaxation factor that induces vascular smooth muscle relaxation (Palmer et al. 1988). The Senate Commission on Food Safety (SKLM) of the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) has extensively reviewed the complex metabolic network between nitrate, nitrite and NO_x. In its 2014 Opinion, the SKLM stated: "In the organism, nitrate and nitrite may function as an alternative source for nitrogen monoxide (NO), an important and multifaceted physiological signaling molecule, normally generated from arginine by NO synthases (NOS). Although NO is rather short-lived, it may react under oxidative

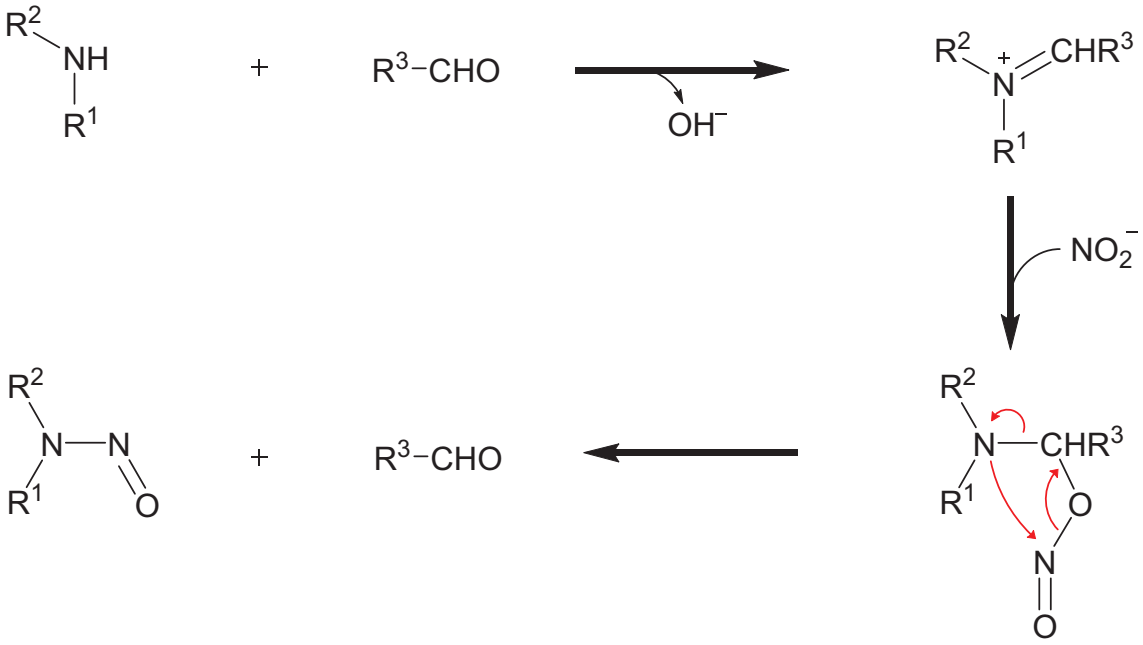

Fig. 1 *N*-Nitrosation catalysis by aldehydes (scheme modified from Keefer and Roller (1973)). Nonenzymatic nitrosation occurs smoothly under neutral and basic conditions in the presence of appropriate catalysts, e.g., formaldehyde

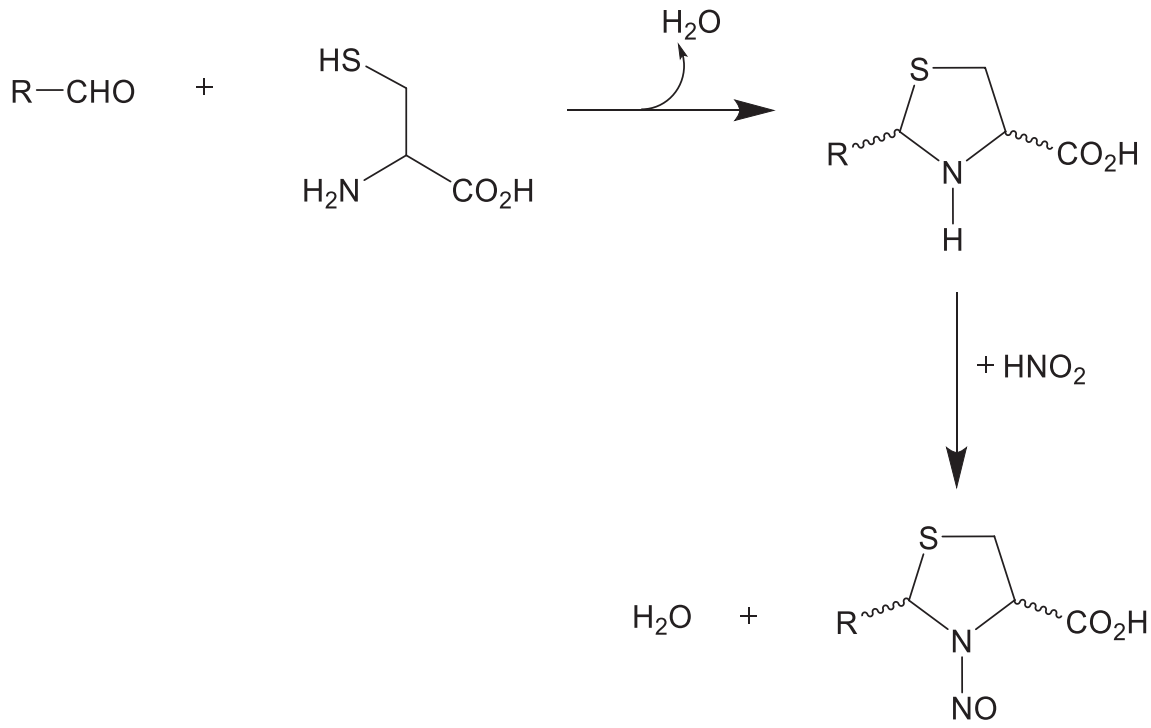

Fig. 2 Formation of *N*-nitroso-2-methylthiazolidine-4-carboxylic acid (NMTCA; R = CH_3; scheme modified from Ohshima et al. (1984)): a ring closure reaction with acetaldehyde to methylthiazolidine carboxylic acid (MTCA) and its *N*-nitroso product, *N*-nitroso-2-methylthiazolidine-4-carboxylic acid (NMTCA)

conditions, i.e., in the presence of oxygen and/or reactive oxygen species (ROS) to give rise to nitrite (NO_2^-), nitrate (NO_3^-) as well as nitrosyl peroxide (NOOH) and/or corresponding radical/ionic intermediates contributing to oxidative/nitrosative damage. These NO-derived species may lead to an array of reaction products under cellular or in vivo conditions, including *N*-, *S*- and *O*-nitroso compounds as well as nitro derivatives of amino acids, peptides, proteins and DNA bases. The biological activities of such NO-related secondary products have not been fully explored up to now. They may include pharmacological effects, e.g., on blood vessels and blood pressure, the induction of oxidative stress/inflammation and ultimately the endogenous formation of NOCs." (Habermeyer et al. 2015, and references therein).

In vitro effects of nitrite/NO/NO_x on cells of the human upper aerodigestive tract have been studied, for instance, in HA1 fibroblasts, thereby showing a reduction in clonogenic survival (40% at 1.7 mM NO in the medium), with a stronger efficacy in glutathione-depleted cells (Walker et al. 1995). Varying effects on cell proliferation such as a concentration- and time-dependent decrease in proliferation and an enhanced mRNA expression for several interleukins and tumour necrosis factor α (TNFα) were observed in the human gastric adenocarcinoma cell line AGS exposed to sodium nitrite up to a concentration of 25 mM for 72 h (Sun et al. 2006). Moreover, nitrite was reported to exert genotoxicity in some in vitro and in vivo genotoxicity assays (IARC 2010).

EFSA (2008) considered different scenarios to estimate nitrate exposure levels and calculated a mean dietary nitrate uptake for adults of 157 mg/day, equivalent to 2.6 mg/kg body weight/day, based on a body weight of 60 kg. However, a large interindividual variability of exposure levels was observed. In humans, nitrate excreted in urine has been reported to exceed the amount ingested. Thus, endogenous nitrate biosynthesis in humans has been shown to occur at a level of about 10 µmol/kg body weight/day, equivalent to about 0.7 mg/kg body weight/day or roughly 50 mg/day for a person weighing 70 kg (Tannenbaum et al. 1978; Green et al. 1981).

Exposure to exogenous nitrite is estimated to predominantly result from residual nitrite used as an additive in meat products. Based on the detailed individual food consumption data combined with average occurrence data on nitrites in foods, mainly for cured meat products, a mean dietary consumer exposure to nitrites of 5–30 µg/kg body weight/day and 9–60 µg/kg body weight/day was assessed for the adult population and children in the EU, respectively (EFSA 2010). Some minor additional contribution may result from nitrite present in vegetables but is deemed negligible, if good agricultural and household practices are adhered to.

Moreover, nitrite is formed from nitrate by chemical and/or microbiological reduction, a process that may occur in the environment, during food processing or (inadequate) food storage and also in the organism. Orally ingested nitrate is rapidly absorbed in the upper gastrointestinal tract and distributed via the blood circulation. Whenever it encounters appropriate reducing conditions in the mammalian host and/or its microbiome, it will undergo partial conversion into nitrite and/or related nitrosating agents.

Figure 3 illustrates the biokinetics of nitrite formation from ingested nitrate in the human oral cavity. Ingested nitrate rapidly distributes through the blood circulation after absorption from the upper gastrointestinal tract. When reaching the salivary glands, nitrate is secreted by active transport from blood into saliva, achieving salivary nitrate levels up to 20 times the plasma level. In the oral cavity, salivary nitrate is partially converted into nitrite by oral and commensal microbial reductases (Eisenbrand et al. 1980). Moreover, it has been shown that increased salivary nitrite production resulting from nitrate intake enhances oral nitric oxide production in humans (Duncan et al. 1995).

The oral cavity harbours the second largest and diverse microbiota after the gut with over 700 species of bacteria and other microorganisms, including fungi, viruses, archaea and protozoa (Deo and Deshmuk 2019). The principal bacteria genera detected in the healthy oral cavity are summarized in Table 1.

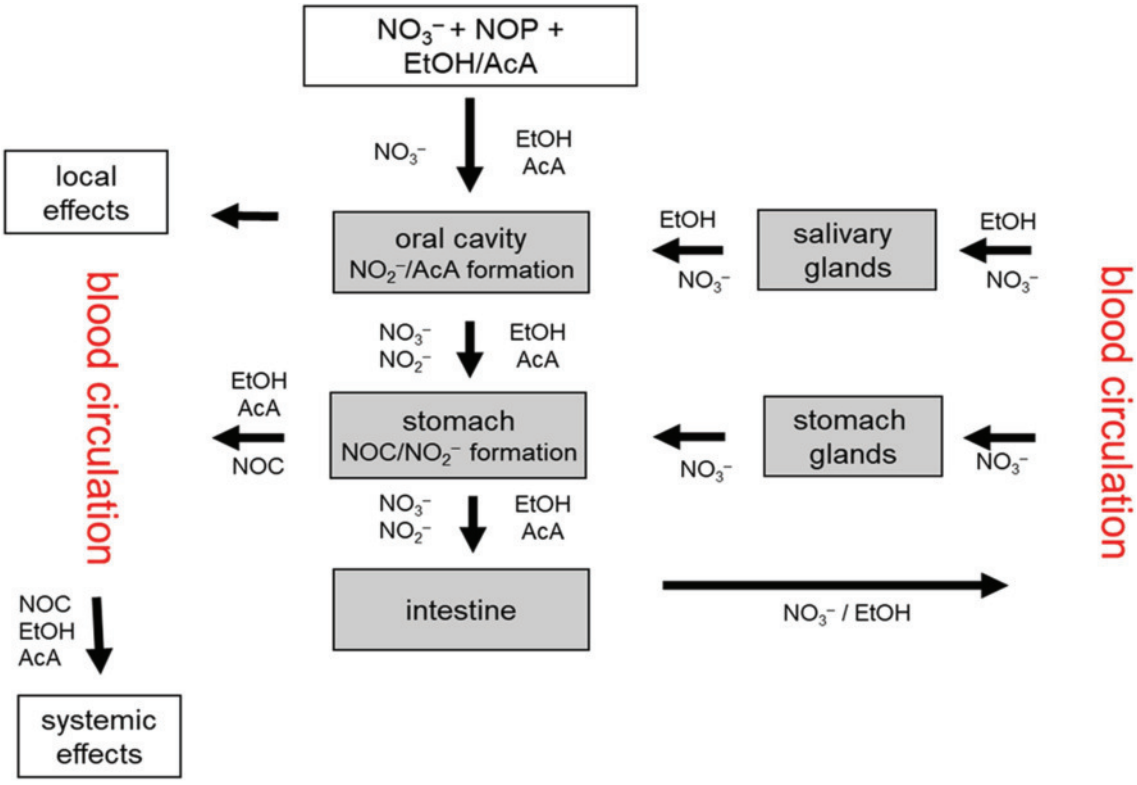

Fig. 3 Kinetics of the dietary components nitrate, nitrite, ethanol, acetaldehyde and nitrosatable precursors (scheme modified from Habermeyer et al. (2015)). *AcA* acetaldehyde, *EtOH* ethanol, NO_2^- nitrite, NO_3^- nitrate, *NOC N*-nitroso compounds, *NOP* nitrosatable precursors

Table 1 Principal bacterial genera in the healthy oral cavity (Marsh 2000)

Gram-positive bacteria	
Cocci	*Abiotrophia*, *Peptostreptococcus*, *Streptococcus*, *Stomatococcus*
Rods	*Actinomyces*, *Bifidobacterium*, *Corynebacterium*, *Eubacterium*, *Lactobacillus*, *Propionibacterium*, *Pseudoramibacter*, *Rothia*
Gram-negative bacteria	
Cocci	*Moraxella*, *Neisseria*, *Veillonella*
Rods	*Campylobacter*, *Capnocytophaga*, *Desulfobacter*, *Desulfovibrio*, *Eikenella*, *Fusobacterium*, *Hemophilus*, *Leptotrichia*, *Prevotella*, *Selemonas*, *Simonsiella*, *Treponema*, *Wolinella*

Approximately 25% of the orally ingested nitrate is secreted through the salivary glands and up to about 7% of the totally ingested nitrate becomes converted to nitrite in the oral cavity during enterosalivary circulation (Spiegelhalder et al. 1976; Tannenbaum et al. 1976). The average increase of nitrite concentration in human saliva was found to be 20 ppm nitrite per 100 mg nitrate ingested (Spiegelhalder et al. 1976). Saliva-derived nitrite may contribute to NOC formation in the stomach when *N*-nitrosatable compounds are present.

NO_x represent a further group of potentially relevant endogenous nitrosating agents. In contrast to nitrite, which requires an acidic environment to generate NOC, NO_x can give rise to NOC under neutral or even basic conditions. In the mammalian organism, nitrate, nitrite and NO_x are metabolically interconvertible. Amongst NO_x, nitrogen monoxide (NO) is preeminent because it works as a multifaceted physiological signalling agent that is consistently generated by NO synthases in the organism from the amino acid arginine. Nitrate and nitrite, whether endogenously synthesized or taken up from exogenous sources, are known as alternative sources for endogenous NO. NO itself is not a nitrosating agent, but may give rise to nitrosating intermediates under certain conditions. The physiological aspects of NO chemistry may be categorized into direct and indirect effects (Grisham et al. 1999). On the one hand, under normal physiological conditions and at relatively low rates of NO production, direct effects are conceived to be based on those reactions in which NO interacts directly with a biological molecule or target to exert regulatory and/or anti-inflammatory functions. On the other hand, indirect effects are conceived as reactions mediated by NO-derived intermediates such as reactive nitrogen oxide species derived from the reaction of NO with oxygen or superoxide when fluxes of NO are enhanced. The latter types of reactions may predominate during periods of active inflammation (Grisham et al. 1999). Such inflammatory conditions occur for instance during infections induced by bacteria, parasites or viruses and have been shown to favour the enhanced biosynthesis of NO, nitrite and nitrate (SKLM 2014 and references therein; Habermeyer et al. 2015).

The WHO International Agency of Research on Cancer (IARC) concluded that there is inadequate evidence in experimental animals for the carcinogenicity of nitrate and limited evidence for the carcinogenicity of nitrite (IARC 2010). However, IARC (2010) also stated that there is sufficient evidence in experimental animals for the carcinogenicity of nitrite in combination with amines or amides, through NOC formation. Moreover, IARC concluded that ingested nitrate or nitrite under conditions that result in the en-dogenous nitrosation of secondary amines and amides and the subsequent generation of NOC is probably carcinogenic to humans (Group 2A; IARC 2010).

It is well established that numerous drugs are also secreted into saliva after their absorption and distribution through the blood circulation, often to an extent that allows the monitoring of blood levels of the parent drug and/or its metabolite(s) through measurements in saliva. Following a simultaneous or prior nutritional nitrate uptake in amounts as brought about by the consumption of nitrate-rich vegetables, part of the ingested nitrate will be recirculated and secreted into saliva to become partially converted into nitrite in the oral cavity. This may entail a potential risk of *N*-nitrosation of a simultaneously excreted drug and/or its metabolites in the upper gastrointestinal tract. Drugs for which in vivo *N*-nitrosation in saliva and gastric juice has compellingly been documented are, amongst others, amidopyrine and piperazine (Bellander et al. 1985; Bellander 1990; Spiegelhalder 1990). Public and scientific interest in drugs as potential targets for *N*-nitrosation has been renewed by recent reports of the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA) on trace contaminations of carcinogenic nitrosamines in many drugs (EMA 2020; FDA 2021). It may be inferred that in addition to the generic risk of contamination, certain drug molecules may also carry a risk of endogenous nitrosation, as already reported many years ago (Spiegelhalder 1990; Bellander et al. 1985; Bellander 1990). Although progress in elucidating causes of contamination and in developing mitigation measures to avoid contamination of drugs has recently been achieved, the potential additional risk of in vivo nitrosation remains largely unexplored up to now. Of note, not only drugs and their metabolites, but also food constituents may share the described biokinetic properties, in other words to some extent they may undergo enterosalivary circulation after having been absorbed from the gastrointestinal tract. Furthermore, up to the present time, there are no data available that would allow to estimate the potential effect of acetaldehyde on the in vivo formation of NOC.

Beyond undergoing enterosalivary circulation, nitrosatable food constituents may be prone to endogenous *N*-nitrosation when encountering nitrosating agents anywhere in the body. Endogenous NOC formation has been exemplified especially for *N*-nitrosatable secondary amino acids like proline (Knight et al. 1991), hydroxyproline (Ohshima et al. 1982) as well as thiazolidine-4-carboxylic acid and congeners (Ohshima et al. 1984). The corresponding NOC, formed from the respective amino acids following nutritional uptake and/or during digestion, are excreted almost quantitatively with urine. In volunteers, ingestion of nitrate has been demonstrated to lead to enhanced urinary excretion of *N*-nitrosated amino acids (Ohshima et al. 1982; Ohshima and Bartsch 1988).

Of note, enhanced ingestion of cysteine entails enhanced urinary excretion of *N*-nitrosothiazolidine-4-carboxylic acid (NTCA) and *N*-nitroso-2-methylthiazolidine-4-carboxylic

acid (NMTCA), as reported by Ohshima et al. (1984). Formation of these thiazolidine-based biomarkers of endogenous nitrosation requires the presence of formaldehyde (NTCA) or acetaldehyde (NMTCA) to enable the formation of the respective thiazolidine ring structures. Thus, excretion of NTCA and NMTCA in urine not only reflects the in vivo presence of nitrosating agents but also that of formaldehyde and/or acetaldehyde, both of which are physiological intermediates of energy metabolism and/or may be taken up with food as aldehydes or their parent alcohols. Since the above-mentioned *N*-nitrosated amino acids are neither mutagenic nor carcinogenic, they are utilized as valuable surrogate biomarkers for the in vivo formation of potentially carcinogenic NOC.

Acetaldehyde

Some reports describe effects in human primary aerodigestive or gastrointestinal tract cells exposed to acetaldehyde at mM concentrations. This included reversible effects on the proliferation and adherence of human gingival fibroblasts (Cattaneo et al. 2000), the transient up-regulation of mRNAs for inflammatory mediators in human nasal respiratory epithelial cells (Gosepath et al. 2006) or the induction of oxidative stress and mitochondrial damage at µM concentrations in the human intestinal goblet-like cell line LS174T (Elamin et al. 2014). In a variety of different experimental test systems, acetaldehyde has been reported to exert cytotoxic and genotoxic effects (MAK 2013).

Following ingestion of ethanol with food and/or alcoholic beverages, the concentration of acetaldehyde in saliva increases (Homann et al. 1997). The rise of salivary acetaldehyde has been found to be primarily governed by microbial metabolism mediated by the microbiome of the oral cavity (Homann et al. 1997; Helminen et al. 2013). According to this model, the absorbed ethanol after systemic distribution circulates back to saliva from blood to become converted to acetaldehyde (Fig. 3). Kinetics are characterized by an instant increase of salivary acetaldehyde, followed by a long-term phase, lasting as long as ethanol is present in the circulation and excreted into saliva. In addition to microbial metabolism in the oral cavity, enzymatic conversion of ethanol by human alcohol dehydrogenases and other dehydrogenases (to a minor extent also by CYP 450s) contributes to ethanol metabolism and clearance in connection with aldehyde dehydrogenases (ALDHs). The latter mediate rapid acetaldehyde turnover by oxidation to acetic acid. The prime transient metabolite, acetaldehyde, is considered a genotoxic and carcinogenic agent that also may cause mucosal irritation at high concentrations (Hartwig et al. 2020). Epidemiological observations suggest dose-dependent higher risks (expressed as odds ratios, ORs) for oropharyngeal cancer in humans with deficiency of aldehyde dehydrogenases (ALDH2-deficients) as compared to ALDH2-proficient humans (Salaspuro 2020). This genetic defect is supposed to result in enhanced local (or even systemic) exposure in the upper digestive tract to acetaldehyde.

After administration of a standard dose of ethanol (0.5–0.6 g/kg body weight; observation period 30–180 min), salivary acetaldehyde concentrations (approx. 24–53 µM) were approx. tenfold higher than the blood acetaldehyde concentrations in ALDH2-active individuals (Yokoyama et al. 2008). Under these experimental conditions, substantially higher acetaldehyde concentrations were measured in blood (up to 25 µM), saliva (up to 76 µM) and gastric juice (up to 47 µM) of ALDH2-deficient individuals than those in blood, saliva and gastric juice of ALDH2-proficient individuals (Yokoyama et al. 2008; Maejima et al. 2015).

Additional exposure to acetaldehyde may occur via food, since acetaldehyde is not only naturally present in many foods, but is also used as a flavoring agent. In the latter context, it should be mentioned that acetaldehyde is included in the list of flavoring substances that may be used in or on foods in the European Union and has "Generally Recognized as Safe" (GRAS) status in USA. The available estimates of acetaldehyde exposure from food are based on limited data. Exposure to acetaldehyde resulting from its use as a flavouring agent was estimated, based on its production volume and the exposed population, by various consultative bodies in Europe, USA and Japan to range between 9.6 and 19.2 mg/person/day (0.14–0.27 mg/kg body weight/day; JECFA 1998; Burdock 2004; FSC 2005; BfR 2010). Total food-related exposure was estimated, based on limited literature data in combination with typical consumption data, to range between 2 and 112 mg/person/day (0.03–1.6 mg/kg body weight/day (FSC 2005; Lachenmeier et al. 2010).

Open research questions

The salivary microbiome is considered to play a major role regarding the locoregional biokinetics of acetaldehyde, nitrite and NOC in the upper digestive tract. However, mammalian metabolism within the host may also play an important role, contributing to endogenous ethanol/acetaldehyde exposure and to sustained formation of nitrate/nitrite and NOC. In this context, it remains to be established whether these agents in combination may elicit potentially adverse effects, not only in the upper digestive tract but also at other locations in the organism. Conversely, it may also be possible that endogenous formation of noncarcinogenic NOC may contribute to the reduction of the endogenous formation of carcinogenic NOC and/or of acetaldehyde (through formation of NMTCA), thereby representing a protective effect.

Taken together, saliva-mediated enhanced locoregional exposure of the upper gastrointestinal tract to potentially toxic/genotoxic agents in a combination comprising acetaldehyde (and/or ethanol), nitrite and NOC has not been taken into consideration up to now. It is, therefore, proposed to investigate potential local and systemic combination effects, based on physiologically based biokinetic (PBBK) modelling of nitrite/ acetaldehyde biokinetics, to uncover locoregional cell and tissue responses to the simultaneous exposure with nitrite and acetaldehyde and to monitor biomarkers for systemic effects, with special attention to the modified urinary output of NOC and relevant metabolites.

Suggested studies

To test the hypothesis and address these open questions, well-designed experimental research is required, based on adequate in vitro and model systems that allow to explore the influence of the three relevant constituents (nitrite, acetaldehyde and amino compounds) on the generation of genotoxic/mutagenic agents under various simulated physiological conditions.

Such research may be performed using dynamic in vitro test systems, simulating pH variation and peristaltic gut motion and modelling the passage of digestives through the human upper gastrointestinal tract, as, e.g., described by Krul et al. (2004). This human gastrointestinal tract model system has previously been applied to study endogenous nitrosation, as exemplified by NDMA formation from dimethylamine as a substance of chemical origin or released from foods such as fish (Krul et al. 2004). Another option may be to rely on the pig cecum model to study the variables influencing intestinal formation of NOC (Engemann et al. 2013).

As a complement to research based on in vitro models, human intervention studies with adequate, consumer-relevant intake levels of nitrate and/or ethanol/acetaldehyde may be conceived to study the influence of the three components on NOC formation kinetics by monitoring the urinary excretion of NOC and their metabolites, focusing on established biomarkers such as *N*-nitroso amino acids (nitrosoproline, NTCA and NMTCA) or others to be developed.

Based on the data generated from in vitro and human studies PBBK modelling may additionally be conducted to inform about internal dosimetry of nitrate/nitrite/NOx/NOC and acetaldehyde.

In addition, the potential scavenging effect of endogenous *N*-nitroso amino acid formation that may decrease or even protect from the generation of carcinogenic NOC may be studied in the above model systems. To achieve this goal, a representative biomarker for the dosimetry of carcinogenic NOC formation may be required. Dihydrouracil (DHU) is an intermediate of mammalian metabolism, consistently formed from uracil by dihydropyrimidine dehydrogenase and detected in human plasma and urine. Amounts of DHU excreted in urine of healthy humans have been reported to range from about 2 to 10 mg/day (Wang et al. 2013). The corresponding NOC, *N*-nitrosodihydrouracil (N-DHU), is a hepatocarcinogenic, DNA carboxyethylating agent (Bulay et al. 1979; Wang et al. 2013). Further marker molecules may be of use as *N*-nitrosation biomarkers as well, including certain drug molecules recently found to be prone to NOC contamination (EMA 2020).

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