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Changes in the mutagenicity of heterocyclic amines, nitrite, and *N*-nitroso compound in pork patties during *in vitro* human digestion

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ABSTRACT

The objective of this study was to determine the changes in mutagenicity caused by heterocyclic amines (HCAs), nitrite, and *N*-nitrosodiethylamine (NDEA) in pork patties during *in vitro* human digestion. The mutagenicity was higher in raw pork patties containing HCAs than in those containing nitrite or NDEA. In cooked pork patties too, the mutagenicity was higher in HCA-containing patties than that in nitrite- or NDEA-containing patties, both before and after *in vitro* human digestion. However, the mutagenicity of all pork patties decreased after *in vitro* digestion. In particular, their mutagenicity was drastically reduced during simulated large intestine digestion with enterobacteria, i.e., *Escherichia coli* and/or *Lactobacillus sakei*. These results indicate that high amounts of HCAs in pork patties show higher mutagenicity than nitrite and NDEA, although the mutagenicity decreases after *in vitro* human digestion.

1. Introduction

Cooking, specifically barbecuing or smoking of meat, generates high mutagenic potential. In early studies, heterocyclic amines (HCAs) such as 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) were isolated from cooked foods, and identified as potential mutagens (Kasai et al., 1980, 1981; Wakabayashi, Nagao, Esumi, & Sugimura, 1992). More than 20 HCAs have been identified in meat and shown to have mutagenicity toward *Salmonella typhimurium* (Nagao, 1999; Sugimura, 2000). *N*-nitroso compounds (NOCs) can react with the DNA of target tissues to form altered bases and, therefore, represent potential mutagens (Saffhill, Margison, & O'Connor, 1985). NOCs have been found in foods cooked by smoking, which uses heat to oxidize nitrogen to nitrogen oxides, which are able to nitrosate amines present in foods such as meat (Mirvish, 1986). NOCs are formed in meats containing nitrite (Cross & Sinha, 2004). When nitrite is present in acidic conditions such as those found in food processing operations, dinitrogen-, tri-, and tetraoxides can form, which are potent nitrosating agents (Cross & Sinha, 2004). The bacterial enzyme nitrite reductase is involved in bacterially catalyzed NOC formation: *Escherichia coli* showed a positive correlation between the activity of this enzyme and nitrosating ability (Calmels, Ohshima, Henry, & Bartsch, 1996). NOC formation or nitrosation could be involved the mutagenicity of foods.

The ingestion of these harmful substances can have adverse effects on the human body, thus the amount that the human body ingest must be taken into account. The daily intakes of the most common HCAs, which are MeIQx, 4,8-DiMeIQx, and PhIP were found to be 63–72 ng, 34–72 ng, and 2–16 ng, respectively (Rohrmann & Becker, 2002). Ingested and absorbed HCAs are converted to a genotoxic metabolite by *N*-acetyltransferase 1 (NAT1), *N*-acetyltransferase 2 (NAT2), hepatic cytochrome P-450 1A2 (CYP1A2) and sulfotransferases (Turesky, 2004). In addition, due to HCAs form HCA-DNA adducts in human colorectal tissue (Malfatti et al., 2006), they may increase the risk of colorectal cancer. Choi and Suh (2017) reported that estimated daily intake (EDI) of nitrite from processed meats in Korea was 1.5 μg kg^{-1} b.w. day^{-1} and this value was representing 2.5% of the ADI (60 μg kg^{-1} b.w. day^{-1}). However, nitrite is consumed not only in meat, but also in vegetables (Alexander et al., 2008) and even in drinking water (Binghui, Zhixiong, & Jing, 2006). It has been reported that cured meats and vegetables comprise 4.8% and 2.2% of daily nitrite intake, respectively (Archer, 2002). NDEA is one of the nitrosamines, and can cause hepatocarcinogenesis with strong toxicity (Bharati, Rishi, & Koul, 2012). Avasilcai, Nichifor, Bireescu, and Cuciureanu (2014) reported that NDEA intake through food consumption was 3.3 ng kg^{-1} b.w. day^{-1}. Due to their formation is closely related with concentrations of nitrate and nitrite (Mensinga, Speijers, & Meulenbelt, 2003), high nitrate and nitrite intake from meat products and vegetables may lead to the formation of NOCs.

Although HCAs, nitrite, and NOCs are considered as potential mutagens, the effect of these compounds on mutagenicity during *in vitro*

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Table 1
Formulation of pork patties containing heterocyclic amines (HCAs), nitrite, and *N*-nitrosodiethylamine (NDEA).

	Treatments[a]							
	CTL	T1	T2	T3	T4	T5	T6	T7
Raw meat (%)	88.5	88.5	88.5	88.5	88.5	88.5	88.5	88.5
Fat (%)	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Salt (%)	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
HCAs (ppm)		1.25			1.25	1.25		1.25
Nitrite (ppm)			150		150		150	150
NDEA (ppm)				100		100	100	100
Total (%)	100	100	100	100	100	100	100	100

[a] CTL: control patty; T1: pork patties containing 1.25 ppm of HCAs; T2: pork patties containing 150 ppm of nitrite; T3: pork patties containing 100 ppm of NDEA; T4; pork patties containing 1.25 ppm of HCAs and 150 ppm of nitrite; T5: pork patties containing 1.25 ppm of HCAs and 100 ppm of NDEA; T6: pork patties containing 150 ppm of nitrite and 100 ppm of NDEA; T7: pork patties containing 1.25 ppm of HCAs, 150 ppm of nitrite, and 100 ppm of NDEA.

human digestion of meat products is still largely unknown. Therefore, this study aimed to determine the effect of *in vitro* human digestion on the mutagenicity of five HCAs, nitrite, and *N*-nitrosodiethylamine (NDEA, an NOC) in pork patties before and after cooking.

2. Materials and methods

2.1. Manufacture of pork patties

Lean pork and back fat were purchased from a local meat market. Excess fat and connective tissue were trimmed from the meat, which was ground through a 4.5-mm plate. Before manufacture of pork patties, HCAs (50 μg of each 4,8-DiMeIQx, 7,8-DiMeIQx, PhIP, IQ, and MeIQx), nitrite, and NDEA (30 mg and 20 mg, respectively) were dissolved in 2 mL of distilled water by vortexing for 1 min. They were added to 198 g of pork mixture to adjust 1.25 ppm, 150 ppm, and 100 ppm of HCAs, nitrite, and NDEA, respectively. The exact concentrations of HCAs, nitrite, and NDEA in the pork patties were decided by a preliminary study, which found a minimum incidence level of mutagenicity in raw pork patties. The formulation and treatment of the pork patties are presented in Table 1. Ground pork meat (175 g), back fat (20 g), 1.5% sodium chloride (3 g), and single or mixed combinations of HCAs, nitrite, and NDEA solutions (2 mL) were mixed together for 1 min using a meat mixer (EF20, Crypto Peerless, Birmingham, UK) (Kim & Chin, 2017). About 70 g of the mixture was formed into one patty and cooked directly using pan-frying for 2 min on each side in a preheated 28-cm diameter pan. Heating temperature was adjusted to over 180 °C and center temperature of pork patty was 70 °C.

2.2. Methods

A human gastrointestinal digestion model that simulates the mouth, stomach, small intestine, and large intestine (with applied enterobacteria) was used in this study. This was a modified version of that described previously (Lee, Lee, Chung, & Hur, 2016). To simulate digestion by the large intestine, 35 mL of a solution containing enterobacteria, including *E. coli* and *Lactobacillus sakei*, was applied to the sample after digestion by the small intestine (35 mL). According to Bengmark (1998), *E. coli* and *L. sakei* occupy a high percentage of more than 400 species of human microflora. Due to their significant roles and representativeness, they were selected for the large intestinal digestion as previous study (Lee et al., 2016).

2.2.1. Digestive enzymes, and inorganic and organic solutions for in vitro human digestion

The digestive enzymes and inorganic and organic solutions used in this study were modified from those described previously (Hur, Lim, Decker, & McClements, 2011; Kim & Hur, 2017; Lee et al., 2016). The compositions of the simulated saliva, and gastric, duodenal, and bile juices are listed in Table 2.

2.2.2. Enterobacterial preparations used for digestion in the large intestine

Enterobacterial preparations were modified from those described previously (Kim & Hur, 2017; Lee et al., 2016). During *in vitro* human digestion, enterobacteria were applied to the samples during digestion in the large intestine. *E. coli* liquid agar was prepared using 2.5 g of Luria-Bertani (LB) Broth, Miller (Difco, MD, USA) with 100 mL of deionized-distilled water (DDW). *L. sakei* liquid agar was prepared using 5.5 g of lactobacilli and de Man, Rogosa and Sharpe (MRS) Broth (Difco) mixed with 100 mL of DDW. Each agar preparation was sterilized by autoclaving at 121 °C for 15 min, and cooled under tap water. Frozen (−80 °C) stocks of *E. coli* (American Type Culture Collection 25992) and *L. sakei* (Korean Collection for Type Cultures 3603) were thawed at room temperature (20–25 °C), and then warmed to 37 °C. *E. coli* and *L. sakei* stocks (1%) were added to 100 mL of the appropriate sterilized liquid agar. *E. coli* and *L. sakei* agar solutions were incubated at 37 °C for 12 h for activation. The activated *E. coli* and *L. sakei* were then applied to 100 mL of sterilized liquid agar for an additional 12 h at 37 °C. After incubation, the final numbers of *E. coli* and *L. sakei* were 8.43 and 8.55 log CFU mL^{-1}, respectively. For the large intestine digestion system, 35 mL each of the liquid-agar *E. coli* and *L. sakei* solutions was applied to the samples (after digestion in the small intestine) and incubated for 4 h at 37 °C. After the large intestine digestion, the numbers of *E. coli* and *L. sakei* were 9.23 and 9.31 log CFU mL^{-1}, respectively.

2.2.3. In vitro digestion procedure to analyze structural changes

(a) Initial system: 5 g of pork patties

Table 2
Constituents and concentrations of the various synthetic juices used in the *in vitro* human digestion model representing fed conditions.

	Saliva (Mouth step)	Gastric juice (Stomach step)	Duodenal juice (Small intestine step)	Bile juice (Small intestine step)
Organic and inorganic components	1.7 mL NaCl[a] (175.3 g/L)[b]	6.5 mL HCl (37 g/L)	6.3 mL KCl (89.6 g/L)	68.3 mL $NaHCO_3$ (84.7 g/L)
	8 mL urea (25 g/L)	18 mL $CaCl_2$ $2H_2O$ (22.2 g/L)	9 mL $CaCl_2$ $2H_2O$ (22.2 g/L)	10 mL $CaCl_2$ $2H_2O$ (22.2 g/L)
	15 mg uric acid	1 g bovine serum albumin	1 g bovine serum albumin	1.8 g bovine serum albumin
Enzymes	290 mg α-amylase	2.5 g pepsin	9 g pancreatin	30 g bile
	25 mg mucin	3 g mucin	1.5 g lipase	
pH	6.8 ± 0.2	1.50 ± 0.02	8.0 ± 0.2	7.0 ± 0.2

After mixing all the ingredients (inorganic components, organic components, and enzymes), the volume was increased to 500 mL with distilled water. If necessary, the pH of the juices was adjusted to the appropriate value.

[a] The numbers are the concentration of chemicals used to make the digestive juices.

[b] The numbers in parentheses are the concentrations of inorganic or organic components per 1 L distilled water.