STRUCTURAL CHANGES OF RESIDUAL NITRATE, NITRITE AND *N*-NITROSO COMPOUND AS CARCINOGENIC SUBSTANCE DURING *IN VITRO* HUMAN DIGESTION

Hyeong S. Kim[1], Seung Y. Lee[1], Sung Y. Yoon[1], Da Y. Lee[1], On Y. Kim[1], Myung S. Chung[2], and Sun J. Hur[1,*]

[1] Department of Animal Science and Technology, Chung-Ang University, Anseong-si, 456-756, Korea

[2]Department of Food Science and Technology, Chung-Ang University, Anseong-si, 456-756, Korea

[*]Corresponding author email: hursj@cau.ac.kr

Abstract – The high amounts of residual nitrate, nitrite and *N*-nitroso compounds (NOCs) in cured meats are harmful as carcinogenic substance for human. However, the effect of digestion on the structural changes of carcinogenic substances has not been studied. This study was performed to evaluate the structural changes of nitrite, nitrate, and *N*-nitrosodiethylamine (NDEA) during *in vitro* human digestion. Contents of nitrite and nitrate were significantly decreased during in vitro human digestion ($p<0.05$). In addition, no NOC was formed during the whole digestion steps. Digestive enzyme and juice shown to have influence the changes of nitrate and nitrite during *in vitro* human digestion. Therefore, harmfulness of nitrate, nitrite and NOC in meat products will be decreased during human digestion.

Key Words – carcinogenic substance, in vitro human digestion, *N*-nitroso compound.

I. INTRODUCTION

Sodium nitrate and nitrite positively affects the flavor, appearance, safety, and quality of cured meats [1]. Especially, addition of nitrite in cured meats gives the characteristic pink color [2] and flavor [3] by preventing rancidity by inhibiting lipid peroxidation [1, 4]. Moreover, nitrite improves safety of meat by inhibiting the growth of microorganisms, notably *Clostridium botulinum* [5, 6]. However, sodium nitrate and nitrite can produce carcinogenic substance such as *N*-Nitroso compounds (NOCs) with cooking, smoking or drying. Humans are exposed to NOCs from exogenous sources and of endogenous synthesis. Exogenous NOCs are directly derived from certain types of food, such as processed meat, salted or smoked fish, and pickled and dried vegetables [7]. Endogenous NOCs are formed from nitrosation of secondary amines or amides by N_2O_3 and $(H_2NO_2)^+$, both of which are nitrite-derived nitrosation agents. Although numerous studies have reported that NOCs in the foods are carcinogenic substance, the effect of human digestion on the structural changes of carcinogenic substances has not been studied. Therefore, the objectives of this study was to determine the structural changes of nitrate, nitrite and NOCs during *in vitro* human digestion.

II. MATERIALS AND METHODS

A. *In vitro* human digestion study

A human gastrointestinal digestion model that simulates the mouth, stomach and small intestine was used in this study. This was a modified version of that described previously [8]. Transit times were chosen on the basis of physiology as reported previously [9-11] although the transit times depend on the condition of each sample. In general, transit times were 5 min for the mouth step, 2 h for the stomach step and 2 h for the small intestine step. The pH values for the digestive juices and gastrointestinal tract were selected based on existing human anatomy and medical physiology literature [12-14] and our previous study [11]. In general, pH values were 6.8 ± 0.2 for the mouth step, 1.5 ± 0.2 for the stomach step, 8.0 ± 0.2 for the small intestine step, and 7.0 ± 0.2 for the large intestine step.

B. Digestive enzymes, inorganic and organic solutions of *in vitro* human digestion

Digestive enzymes, and inorganic and organic solutions used in this study were modified from those described previously [11-13].

C. *Enterobacter* bacterial preparations used for large intestine digestion

During *in vitro* human digestion, enterobacter bacteria were applied to samples during the large intestine digestion step. This is the first *in vitro* method to be developed that includes this step of human digestion.

E. coli liquid agar was prepared using 2.5 g LB Broth, Miller (Luria-Bertani) (Difco) with 100 mL deionized-distilled water (DDW). *L. casei* liquid agar was prepared using 5.5 g Lactobacilli MRS Broth (Difco) mixed with 100 mL DDW. Each agar preparaton was sterilized by autoclave at 121°C for 15 min and cooled in tap water. Frozen (-80°C) stock *E. coli* and *L. casei* were melted at room temperature then warmed to 37°C. One percent of *E. coli* and *L. casei* stocks were added to 100 mL of the appropriate sterilized liquid agar. *E. coli* and *L. casei* agar solutions were incubated at 37°C for 12 h for activation. The activated *E. coli* and *L. casei* were applied again to 100 mL of sterilized liquid agar for an additional 12 h at 37°C. After incubation, the final number of *E. coli* and *L. casei* colonies was log 10^8-10^{10}. For the large intestine digestion system, 38 mL of the liquid agar *E. coli* and *L. casei* solutions were applied to samples (after small intestine digestion) and incubated for 4 h at 37°C.

D. In vitro digestion procedure for the analysis of structural changes

1. Initial system: 5 g samples.
2. Mouth: 5 g samples were mixed with 5 mL of simulated saliva solution (pH 6.8) and then stirred for 5 min at 37°C.
3. Stomach: 10 mL of simulated gastric juice (pH 1.5) were added and the mixture was stirred for 2 h at 37°C.
4. Small intestine: 10 mL of duodenal juice and 5 mL of bile juice were added and the mixture was stirred for 2 h at 37°C.
5. Large intestine: after small intestine digestion, 38 mL of liquid agar containing *E. coli* and *L. casei* were applied to a sample previously digested in the small intestine step and incubated at for 4 h at 37°C (see above *Enterobacter* method).

E. Quantification of NOCs by high-performance liquid chromatography (HPLC)

Concentrations of sodium nitrate, nitrite and *N*-nitrosodimethylamine after digestion were analyzed using HPLC (HP agilent 1100, Hewlett Packard Co.) on a Fortis H_2O column (250 mm × 4.6 mm, 3 μm) using a water : methanol gradient (70 : 30, v/v) at a flow rate of 1 mL/min. The volume of sample injected for analysis was 10 uL, and the detection wavelength was set at 230 nm. All samples were passed through a 0.45-μm Whatman membrane filter before injection onto the HPLC column.

F. Statistical analysis

Statistical analyses were conducted for 3 batches of samples. Data for each batch were analyzed for structural changes of nitrate, nitrite and NOCs using the SAS software by the generalized linear model procedure. The Student-Newman-Keuls multiple range test was used to compare differences between means. Significant difference ($p < 0.05$) between mean values of quintuplicate samples were determined for structural changes of nitrate, nitrite and NOCs.

III. RESULTS AND DISCUSSION

Initial concentrations of both of sodium nitrate and nitrite, and NDEA were 150 ppm and 10 ppm, respectively. Then, the concentrations of sodium nitrite, nitrate, and NDEA were changed by *in vitro* human digestion. After digestion in the mouth, nitrite and nitrate contents was 120.1 and 117.2 ppm, respectively and then the values were gradually decreased by *in vitro* human digestion ($p<0.05$). In case of NDEA, more than 50% of reduction was occurred after mouth digestion step (4.60 ppm) and its concentration was gradually decreased during *in vitro* human digestion ($p<0.05$).

In the human body, nitrate is a stable, inert compound that cannot be metabolized by human enzymes. However, the nitrate-reducing activity of commensal bacteria may convert nitrate into nitrite and other bioactive nitrogen compounds that affect physiological processes and human health. After ingestion, nitrate is readily absorbed from the upper gastrointestinal tract. Up to 25% is actively excreted in saliva, where about 20% is converted to nitrite by bacteria in the mouth [15]. It has also been suggested that approximately 25% of ingested nitrate is recirculated into the saliva and

that 20% of salivary nitrate is reduced to nitrite, yielding a conversion of 5% of exogenous nitrate to endogenous nitrite [16-18]. According to this theory, most human nitrite exposure would be from the consumption of vegetables common in the diet.

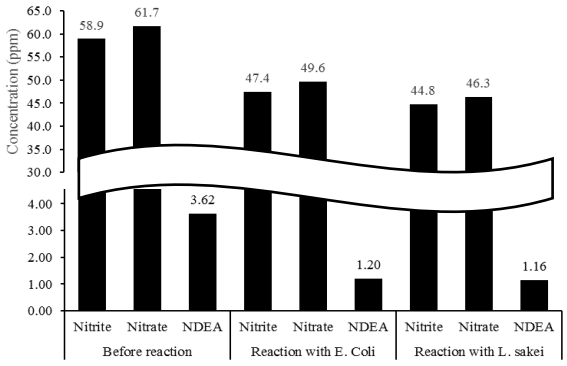
Table 1. Changes of sodium nitrite, sodium nitrate, and NDEA through *in vitro* human digestion (Unit: mg/kg)

Compound	Progress of digestion			
	Initial	Mouth digestion	Stomach digestion	Intestinal digestion
$NaNO_2$	150.0[a]	120.1[b]	82.7[b]	58.9[c]
$NaNO_3$	150.0[a]	117.2[b]	90.6[c]	61.7[d]
NDEA	10.0[a]	4.60[b]	3.58[c]	3.62[c]

[a-d] Means with having different superscripts within same row are different (p<0.05).

After small intestinal digestion, each compound was subsequently reacted with *E. coli* and *L. casei*, respectively. Two enterobacterium affected the amount of nitrite, nitrate and NDEA. Concentrations of all compound was decreased after reaction with enterobacterium (Figure 1) (p<0.05).

Figure 1. Changes of concentrations (ppm) of nitrite, nitrate and NDEA after reaction with *E. coli* and *L. sakei*.



Some microorganisms, i.e. *E. coli* and *Streptococcus aureus* accelerate nitrosation. Bacteria role in nitrosamine formation consists of nitrate reduction to nitrites, protein degradation to secondary amines, generating the enzyme catalysing nitrosation and formation of suitable environment for the reaction, i.e. acidification [19]. There have been many attempts to reduce the *N*-nitroso-compound concentrations in fermented foods. Lactic acid bacteria are found to contribute to the depletion of nitrite in many foods [20, 21]. According to Dodds and Collins-Thompson [22], two mechanisms, namely chemical depletion resulting from acid production during LAB growth and enzymatic depletion, lead to the depletion of nitrite in APT broth. It is shown that certain LAB possess a nitrite reductase enzyme system that reduces nitrite under anaerobic conditions, suggesting that LAB contribute to the depletion of nitrite in many foods [23]. *L. sakei* has nitrite reductase and heme-independent nitrite reductase that converts nitrite to NO, NO_2, or N_2O under anaerobic conditions, suggesting that *L. sakei* contributes to nitrite depletion [24, 25]. Faster nitrite depletion was also observed at lower pH values [26]. In this study, the production of lactic acid by *L. casei* which leads to a sharp pH drop may contribute the nitrite reduction and depletion.

IV. CONCLUSION

Structural changes of ingested nitrate, nitrite and NDEA as carcinogenic substance were occurred during *in vitro* human digestion progress. Their levels were significantly decreased during *in vitro* human digestion (p<0.05). In addition, none of other NOCs was formed during whole *in vitro* human digestion steps. Effect of *E. coli* and *L. casei* on the changes of nitrate and nitrite, and on the formation of NOCs was also evaluated. Both of *E. coli* and *L. casei* reduced levels of sodium nitrate, nitrite and NOC after *in vitro* human digestion (p<0.05). In conclusion, digestive enzymes, juice and enterobacteria can reduce the residual nitrate, nitrite and NOCs during *in vitro* human digestion. As a result of this study, we assume that potentially harmful substances in meat products will be decreased during human digestion. Basically, the amount of potentially harmful substances (residual nitrate, nitrite and NOCs) is small in meat products. Therefore, carcinogenicity of ingested meat products will be very low.

ACKNOWLEDGEMENTS

This work was carried out with the support of 'Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ010860032016)' Rural Development Administration, Republic of Korea.

REFERENCES

1. Pearson, A. M. & Gillett, T. A. (1996). Processed meats, third edition (Third Edition ed.). New York, NY: Chapman & Hall.
2. Fox Jr, J. B. (1966). Chemistry of meat pigments. Journal of Agricultural and Food Chemistry 14: 207-210.
3. Donald, B., Gray, J. I., Stanley, D. W. & Usborne, W. R. (1980). Role of nitrite in cured meat flavor: sensory analysis. Journal of Food Science 45: 885-904.
4. Richards, M. P. (2013). Redox reactions of myoglobin. Antioxidants and redox signaling 18: 2342-2351.
5. Duncan, C. L. & Foster, E. M. (1968). Effect of sodium nitrite, sodium chloride, and sodium nitrate on germination and outgrowth of anaerobic spores. Applied microbiology 16: 406-411.
6. Sofos, J. N., Busta, F. F. & Allen, C. E. (1979). Botulism control by nitrite and sorbate in cured meats: a review. Journal of Food Protection 42: 739-770.
7. Tricker, A. R. & Preussmann, R. (1991). Carcinogenic *N*-nitrosamines in the diet: occurrence, formation, mechanisms and carcinogenic potential. Mutation Research/Genetic Toxicology 259: 277-289.
8. Lee, S. J., Lee, S. Y. & Hur, S. J. (2015). Effect of *Escherichia coli* and *Lactobacillus casei* on luteolin found in simulated human digestion system. Journal of Food and Nutrition Research 3: 311-316.
9. Christensen, F. N., Davis, S. S., Hardy, J. G., Taylor, M. J., Whalley, D. R. & Wilson, C. G. (1985). The use of gamma scintigraphy to follow the gastrointestinal transit of pharmaceutical formulations. Journal of pharmacy and pharmacology 37: 91-95.
10. Degen, L. P. & Phillips, S. F. (1996). Variability of gastrointestinal transit in healthy women and men. Gut 39: 299-305.
11. Hur, S. J., Decker, E. A. & McClements, J. D. (2009). Influence of initial emulsifier type on microstructural changes occurring in emulsified lipids during in vitro digestion. Food Chemistry 114: 253-262.
12. Guyton, A. C. & Hall, J. E. (2006). Textbook of medical physiology (11th ed.). Philadelphia: Elsevier Saunders.
13. Marieb, E. N. & Hoehn, K. (2010). Human anatomy & physiology. San Francisco: Benjamin Cummings.
14. Evans, D. F., Pye, G., Bramley, R., Clark, A. G., Dyson, T. J. & Hardcastle, J. D. (1988). Measurement of gastrointestinal pH profiles in normal ambulant human subjects. Gut 29: 1035-1041.
15. Spiegelhalder, B., Eisenbrand, G., & Preussmann, R. (1976). Influence of dietary nitrate on nitrite content of human saliva: possible relevance to *in vivo* formation of *N*-nitroso compounds. Food and Cosmetics Toxicology 14: 545-548.
16. Miller, A. B., Choi, B. C., Howe, G. R., Burch, J. D., & Sherman, G. J. (1983). Epidemiological assessment of risk to humans from exposure to nitrosamines. IARC scientific publications, 57: 929-936.
17. Mirvish, S. S. (1983). The etiology of gastric cancer intragastric nitrosamide formation and other theories. Journal of the National Cancer Institute 71: 629-647.
18. Choi, B. C. (1985). *N*-Nitroso compounds and human cancer. A molecular epidemiologic approach. American Journal of Epidemiology 121: 737-743.
19. Rywotycki, R. (2003). The influence of environment, mode of nutrition and animal species on level of nitrosamine contamination in venison. Meat science 65: 1045-1053.
20. Oh, C. K., Oh, M. C., Kim, S. H., Hyon, J. S., Choi, W. J. & Lee, S. H. (1997). Depletion of nitrite by lactic acid bacteria isolated from kimchi (I). Korean Journal of Food Science and Nutrition 26: 549-555.
21. Park, K. Y. & Cheigh, H. S. (1992). Kimchi and nitrosamines. Journal of the Korean Society of Food Science and Nutrition 21: 109-116.
22. Dodds, K. L. & Collins-Thompson, D. L. (1984). Incidence of nitrite-depleting lactic acid bacteria in cured meats and in meat starter cultures. Journal of Food Protection 47: 7-10.
23. Yang, X. M., Liu, Q. M., Xi, L. F. & Tang, A. Z. (2004). Study on the control of nitrite content in the pickled of potherb mustard. Journal of Chinese Institute of Food Science and Technology 4: 48-51.
24. Collins-Thompson, D. L. & Rodriguez Lopez, G. (1981). Depletion of sodium nitrite by lactic acid bacteria isolated from vacuum-packed Bologna. Journal of Food Protection 44: 593-595.
25. Hammes, W. P., Bantleon, A. & Min, S. (1990). Lactic acid bacteria in meat fermentation. FEMS Microbiology Reviews 7: 165-173.
26. Theiler, R. F., Sato, K., Aspelund, T. G. & Miller, A. F. (1981). Model system studies on N-nitrosamine formation in cured meats: The effect of curing solution ingredients. Journal of Food Science 46: 996-998.