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Short communication

No mutagenicity and oral toxicity of winter mushroom powder treated with atmospheric non-thermal plasma

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ABSTRACT

This study aimed to evaluate the mutagenicity and oral acute toxicity of winter mushroom powder (PW) treated by atmospheric non-thermal plasma (ANP). Winter mushroom powder without plasma treatment (CW) containing an equivalent amount of sodium nitrite as PW was used as a control. The Ames test revealed that the number of revertant colonies did not significantly increase compared to that in the control. Acute toxicity was assessed in rats that were fed a single dose of winter mushroom powder (5000 mg/kg body weight). Results of the acute toxicity test revealed no remarkable clinical symptoms in any of the rats. No significant difference was observed in of the serum biochemical parameters between the treatments. Regardless of the ANP treatment, mild histological changes were observed in few rats in all groups. Therefore, it is concluded that ANP treatment did not cause any mutagenicity or acute toxicity in the winter mushroom.

1. Introduction

As consumer demand for health, food safety, and high-quality products increases, the food industry has been investigating a novel technology that can enhance the safety, shelf life, and quality of products. Recently, non-thermal technology has gained immense attention as a novel technology for application in the food industry because it can operate at relatively low temperatures to prevent degradation of food quality by heat treatment and improve safety (Pérez-Andrés, Álvarez, Cullen, & Tiwari, 2019).

Plasma, which is considered the fourth state of matter, is an ionized gas with increasing energy level (Liao et al., 2017). In non-thermal plasma, substantial electrical energy is applied to the electronic part, thereby increasing the temperature; however, other particles such as ions and materials are approximately at room temperature, so that the temperature of the overall plasma gas is kept relatively low (Nehra, Kumar, & Dwivedi, 2008). The potential use of atmospheric non-thermal plasma (ANP), which utilizes atmospheric air as the operational gas, in various industries such as medicine, textile, and biotechnology, has been suggested (Pérez-Andrés et al., 2019). Moreover, several recent studies have focused on ANP as a novel technology to be applied in the food industry.

Active species such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) present in the plasma easily react with numerous substances in food and have various effects. The most common effect of ANP is pasteurization by inducing oxidative stress in microorganisms present in food (Lee et al., 2016; Liao et al., 2017). Another effect involves improving food safety via degradation of residual pesticides or mycotoxins (Bourke, Ziuzina, Boehm, Cullen, & Keener, 2018). Furthermore, it has been reported that ANP technology can be used as a new curing method in manufacturing meat products as it generates nitrite via reaction of water molecules with RNS in the plasma (Jo et al., 2020a, 2020b; Lee, Lee, Yong, Lee, Jo, & Jung, 2017). However, the chemical changes in various compounds in food with plasma treatment are not fully understood, and whether the ingestion of plasma-treated food causes toxicity *in vivo* remains unclear. Therefore, sufficient toxicity studies on plasma-treated foods are required to reliably apply ANP to the food industry.

Winter mushrooms (*Flammulina velutipes*) are commonly cultivated around the world and possess various bioactivities such as antioxidant, anticancer, and antihypertensive activities (Yang, Lin, & Mau, 2002). These mushrooms have high pH; hence, they can be used as a natural nitrite source via stable plasma treatment because the pH does not drop easily during plasma treatment (Jo et al., 2020b). Therefore, the present

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study aimed to investigate the mutagenicity and acute oral toxicity of winter mushrooms with nitrite generated by plasma treatment.

2. Materials and methods

2.1. ANP treatment of winter mushrooms

Winter mushroom (*F. velutipes*) was purchased from a local market (Daejeon, Korea). After removing the inedible part, they were washed using tap water and then ground using a food blender (FPM250, De'Longhi-Kenwood Appliances Co., Italy).

The homogenate of winter mushroom was treated by ANP using a remote infusion system described in the study by Jo et al. (2020b). The ANP was infused for 130 ± 5 min until the pH of the winter mushroom homogenate reached 6. Plasma-treated winter mushroom homogenate was then lyophilized (Bondiro, Ilshin Co., Seoul, Korea). Winter mushroom powder was prepared thrice on independent days, and its nitrite content was analyzed according to the AOAC method 973.31 (AOAC, 1990). The nitrite content of plasma-treated winter mushroom powder (PW) was 4.87 ± 0.09 g/kg. The winter mushroom powder without ANP treatment was used as a control (CW), and the nitrite content of CW corresponded with that of PW by the addition of sodium nitrite.

2.2. Mutagenicity

A mutagenicity assay (Ames test) was performed according to the methods by Maron and Ames (1983) and Kim et al. (2016). The histidine-dependent *Salmonella enterica* Typhimurium strains TA98 (hisD3052/*rfa*/Δ*uvr*B/Δ*GalE503*/pAQ1) and TA100 (hisG46/*rfa*/Δ*uvr*B/Δ*GalE503*/pAQ1) were purchased from the American Type Culture Collection (Koram Biotech Corp., Seoul, Korea). PW and CW were dissolved in 70% ethanol, and the sample concentrations tested were 313, 1250, and 5000 μg/plate. Moreover, 70% ethanol was used as the negative control. 4-nitroquinoline-1-oxide (4-NQQ) and sodium azide (SA) were used as positive controls for TA98 and TA100, respectively, without metabolic activation. 2-aminoanthracene (2-AA) was used as a positive control for two strains with metabolic activation by the S9 mixture. A direct plate incorporation method was used. Mutagenicity was considered when the number of revertant colonies was more than two-fold that of the negative control and was dose-dependent.

2.3. Acute toxicity study

2.3.1. Experimental animals and design

The Institutional Animal Care and Use Committee of Orient bio Co. (Seoul, Korea) reviewed and approved all experimental protocols used in this study (Orient-IACUC-19098). Sprague-Dawley (SD) rats of both sexes with an age of 7 weeks were used for the experiment. All animals were acclimated for 7 days. They were kept at a temperature of 23.6 °C–25.9 °C and a relative humidity of 45.3%–59.9% with a 12 h light/dark cycle. Rats were allowed free access to water and feed (PMI LabDiet® 5053, PMI Nutrition International, USA). After a week of acclimation, the weights of ten male and ten female rats were 258.36–272.99 g and 193.10–205.48 g, respectively.

Ten rats (five males and five females) were randomly divided into two groups. PW and CW were dissolved in distilled water (100 mg/mL). After fasting for 16 h except for water, the sample was administered orally at a dose of 5000 mg/kg body weight of rats in two installments at intervals of 2 h. Clinical symptoms such as abnormalities in the skin, fur, and eyes, and abnormal behavior were observed at 0.5, 1, 2, and 4 h after administration, respectively. All rats were maintained for 15 days, and the clinical symptoms, dying conditions, and mortality were observed daily. Body weights of all rats were measured at 0, 1, 3, 7, and 14 days during the experimental period. On day 15, rats were sacrificed by inducing euthanasia with carbon dioxide.

2.3.2. Biochemical analysis of blood samples

Blood samples were collected from the carotid artery. The serum was isolated via centrifugation of blood at 1500 g for 5 min (5424R, Eppendorf, USA). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), total bilirubin (*T*-BIL), alkaline phosphate (ALP), and creatinine (CREA) contents were analyzed using a chemical analyzer (7180, HITACHI, Japan).

2.3.3. Histopathological study

The kidney and liver of all sacrificed animals were collected and fixed in 10% neutral buffered formalin overnight at room temperature. The tissue samples were embedded in paraffin wax using an automatic tissue processor (Lipshaw, Detroit, MI, USA). The paraffin block was cut to 4 μm using a rotary microtome (Leica RM2125 RT, Leica Biosystems, Germany). These sections were stained with hematoxylin-eosin stain, and thereafter, the histological lesions of the tissue samples were observed under a light microscope.

2.4. Statistical analysis

SAS software (version 9.3, SAS Institute Inc., Cary, NC, USA) was used for statistical analyses. The data of the Ames test were statistically analyzed using the GLM procedure. The statistical analysis of acute toxicity data was performed using *t*-test. A p-value of less than 0.05 was considered statistically significant.

3. Results and discussion

Nitrogen oxides, such as nitric oxide and nitrogen dioxide, present in the ANP gas, can generate nitrite when they react with water molecules in the plasma-treated sample (Jung et al., 2017a, 2017b; Jo, Lee, Yong, Choi, & Jung, 2020c; Lee et al., 2018). The nitrite content in the PW was 4.87 ± 0.09 g/kg (data not shown). We evaluated the toxicity induced by ANP treatment in the process of producing new natural nitrite sources using ANP technology. Therefore, in order to minimize the effect of the nitrite generated by ANP treatment, sodium nitrite was added to the CW so that the same amount of nitrite was present in both treatments.

3.1. Ames test

The Ames test analyzes the mutagenicity of chemical substances using the mutated *Salmonella* strain that cannot synthesize histidine. Toxic substances mutate *Salmonella* strains to allow histidine synthesis, and newly mutated bacteria can grow and form colonies in the absence of histidine (Mortelmans & Zeiger, 2000). *Salmonella* strain TA98 can detect mutations that cause DNA base frame shift by adding or deleting one or more bases, and TA100 can detect mutations that cause DNA base-pair substitution by single base changes (Wang et al., 2006). It is considered that the test substances are mutagenic when the revertant colony count is more than two-fold the negative control and is dose-dependent.

The results of Ames test revealed that regardless of the strain type and presence of S9 metabolic activation, the number of revertant colonies did not significantly increase upon any of the treatments compared to that in the control, and no dose-dependence was observed (Table 1). On the other hand, the number of revertant colonies in all positive controls increased more than two-fold compared to the negative control. Therefore, ANP treatment did not induce mutagenicity in winter mushrooms. Similar results have been found in previous studies with plasma treatment under various conditions. Lee et al. (2016) observed no mutagenicity via the Ames test in chicken breast treated with flexible thin-layer dielectric barrier discharge (DBD) plasma for 10 min, which was in accordance with the results observed by Kim et al. (2016)

Table 1
Revertant colonies in the *Salmonella enterica* Typhimurium reversion assay of the ANP-treated or untreated winter mushroom powder.

Treatment[1]	Dose (μg/plate)	Number of revertant colonies (His +) per plate			
		TA98 (−S9)	TA98 (+S9)	TA100 (−S9)	TA100 (+S9)
CW	313	26±8	26±23	225±52	272±44
	1250	53±9	82±26	239±32	334±14
	5000	45±26	53± 14	254±23	218±25
PW	313	41±26	84±49	309±56	309±47
	1250	39±22	67±33	280±99	285±48
	5000	50±11	51±15	270±48	306±16
Negative control	70% Ethanol	39±10	92±15	396±24	421±67
Positive control	4-NQO	356±118			
	2-AA		1,559±456		
	SA			1,058±347	
	2-AA				1,691±104

Abbreviations: 4-NQO, 4-nitroquinoline-1-oxide; SA, sodium azide; 2-AA, 2-aminoanthracene.
Values indicate the mean ± SD.
[1] CW: winter mushroom powder with sodium nitrite; PW: plasma-treated winter mushroom powder.

and Yong et al. (2018). In their studies, emulsion sausage and pork loin ham manufactured using water treated with DBD plasma for 4 and 2 h, respectively, did not show mutagenicity in the Ames test. Moreover, it was also reported that the mutagenicity of cooked egg white and yolk treated for 2 min with the plasma jets using nitrogen, helium, and oxygen gas was not observed during evaluation via the SOS chromotest (Lee et al., 2012).

3.2. Acute toxicity study

In the present study, the winter mushroom powder was administered to rats at 5,000 mg/kg body weight to evaluate the acute oral toxicity, wherein the nitrite level was about 24.35 mg/kg body weight.

In the oral acute toxicity test with SD rats, no sign of toxicity or death was observed during 14 days after feeding CW and PW (data not shown). The body weight of male and female SD rats increased normally for 14 days after administration (Table 2). No significant differences between the treatments were found in the female SD rats on all observation days. In the male SD rats, there was no significant difference of body weight between the treatments except for day 3. The changes in body weight indicate adverse effects of the substance, and in general, if the degree of weight loss is at least 10% lesser than the control value, it is considered to be a weight change due to toxicity (Lu et al., 2014). In this study, the difference in body weight between CW and PW was only found in male rats on day 3, and it was less than 10%. Therefore, it is difficult to explain that the change in body weight was due to toxicity.

The biochemical analysis of blood parameters did not reveal any significant difference between treatments, and no specific numerical change was observed by feeding the winter mushroom powder (Table 3). The liver and kidneys can be easily targeted by external toxins because they play a pivotal role in detoxification and toxin excretion (Ansari, Ali, Khan, & Mahmood, 2018). Damage to the liver and kidney leads to changes in blood biochemical parameters; therefore, these changes act as important data for the safety evaluation of substances in toxicity experiments. The levels of AST, ALT, *T*-BIL, and ALP in serum are used as indicators of hepatotoxicity, and BUN and CREA levels are used as indicators of nephrotoxicity (Han, Suh, Hong, Kim, & Min, 2016; Ozer, Ratner, Shaw, Bailey, & Schomaker, 2008). According to the control data of the SD rat reported by Han et al. (2010), the total bilirubin level was relatively low, but all blood biochemical parameters were within the normal range.

Histopathology of liver and kidneys revealed that inflammatory responses occurred in some male and female rats in all treatment groups (Table 4, Fig. S1). The level of inflammatory responses was mild. The inflammatory cells observed in kidneys were in the chronic stage and were not considered to be present due to the winter mushroom powder feeding. Also, since these histopathological changes occurred in all treatment groups they are not considered to be due to the ANP treatment.

ROS and RNS, which are key compounds in plasma activity, react easily with the food ingredients and lead to oxidation, and this reaction has various positive effects; however, it can simultaneously cause a negative chemical change in food (Bourke et al., 2018). Generation of nitrite sources via ANP requires longer treatment duration to create

Table 2
Body weight (g) of Sprague-Dawley (SD) rats subjected to oral administration of the ANP-treated or untreated winter mushroom powder to assess acute toxicity.

Sex	Treatment[1]	Day				
		0	1	3	7	14
Male	CW	263.9	287	325.9[a]	355	380.6
	PW	267.1	287.1	313.2[b]	344.3	367.2
	SEM[2]	1.19	1.88	2.83	3.22	4.69
Female	CW	198.8	214.6	231.2	235.1	232.5
	PW	202.9	211.9	233.2	237.3	240.3
	SEM	1.30	1.44	1.07	1.84	3.22

[a-b]Different small letters in the same column indicate significant differences between means ($P < 0.05$).
[1] CW: winter mushroom powder with sodium nitrite; PW: plasma-treated winter mushroom powder.
[2] Standard error of the least square mean ($n = 10$).

Table 3
Serum chemistry of SD rats subjected to oral administration of ANP-treated or untreated winter mushroom powder to assess acute toxicity.

Sex	Treatment[1]	AST (IU/L)	ALT (IU/L)	BUN (mg/dL)	*T*-BIL (mg/dL)	ALP (IU/L)	CREA (mg/dL)
Male	CW	93.2	42.8	17.16	0.02	226.4	0.62
	PW	90.4	39.6	17.04	0.01	162.4	0.63
	SEM[2]	2.25	3.62	0.61	0.004	17.81	0.013
Female	CW	97.4	29.4	17.36	0.03	111.4	0.61
	PW	86.8	37.8	18.72	0.02	146.2	0.61
	SEM	4.24	2.55	0.63	0.005	18.06	0.008

Abbreviations: aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), total bilirubin (*T*-BIL), alkaline phosphate (ALP), creatinine (CREA).
[1] CW: winter mushroom powder with sodium nitrite; PW: plasma-treated winter mushroom powder.
[2] Standard error of the least square mean ($n = 10$).

Table 4
Histopathology in SD rats subjected to oral administration of ANP-treated or untreated winter mushroom powder to assess acute toxicity.

Sex	Male		Female	
Treatment[1]	CW	PW	CW	PW
No abnormality	2	2	2	1
Kidneys				
Mineralization	0	0	1	2
Mononuclear inflammatory cell infiltration	1	0	1	1
Tubular basophilia	0	1	1	0
Liver				
Inflammatory cell infiltration	2	2	1	2

[1] CW: winter mushroom powder with sodium nitrite; PW: plasma-treated winter mushroom powder.

sufficient amount of nitrite in the materials. Reactive species concentration in the plasma-treated samples increases with an increase in the plasma input power and treatment time (Jo et al., 2020a; Liao et al., 2018). Therefore, ANP treatment for nitrite generation in food can lead to relatively more oxidative stress. Nevertheless, the acute oral toxicity test did not show any specific toxic effects caused by the ANP treatment. These results were similar to those reported in the study by Han et al. (2016). They observed the occurrence of acute toxicity and subacute toxicity by feeding cold plasma-treated edible film to rats and reported that cold plasma treatment did not cause toxicity to edible films (Han et al., 2016). Kim et al. (2016) observed immune toxicity in mice that were fed emulsion-type sausages cured with plasma-treated water. They reported no toxicity induced by the addition of plasma-treated water while observing the serum tumor necrosis factor (TNF)-α levels, small intestine length, and the number of Peyer's patches in the small intestine.

In the acute toxicity test conducted in this study, the winter mushroom powder containing nitrite generated by ANP treatment was fed to rats. Therefore, it is necessary to consider whether the observed toxicity symptoms were caused not only by the ANP treatment but also by the winter mushroom powder and nitrite.

Winter mushroom is one of the most commonly cultivated and consumed mushrooms. Although specific toxic symptoms did not occur when a relevant dose of winter mushroom was consumed, a previous study by Mustonen et al. (2018) observed that the myotoxic effect could occur in mice fed the 6 and 9 g/kg body weight/day of winter mushroom powder for 5 days. The equivalent dose in rats calculated by Nair and Jacob (2016) was 3 and 4.5 g/kg body weight/day. The dose of winter mushroom powder fed in our acute toxicity test was 5 g/kg body weight; however, no toxicity symptoms were observed. This may be because the winter mushroom powder was fed to rats only once and not continuously for several days.

Nitrite generated by ANP treatment exhibits toxicity when consumed at a high dose. An acute toxicity study with oral administration of 20 and 40 mg/kg body weight of sodium nitrite reported increased oxidative stress, decreased antioxidant capacity, and DNA damage in rat kidneys (Ansari et al., 2018). The nitrite content in 20 and 40 mg of sodium nitrite is 13.34 and 26.67 mg, respectively. In this study, the nitrite content of the winter mushroom powder fed in the acute toxicity test was 24.35 mg/kg body weight; however, no toxicity symptoms were observed. The toxicity caused by nitrite is mainly due to oxidative stress (Hassan, Hafez, & Zeghebar, 2010). Winter mushrooms contain various antioxidants (Yang et al., 2002); hence, the antioxidant ability of winter mushrooms may have relieved the oxidative stress caused by nitrite. Previous studies have reported that feeding natural substances with antioxidant activity inhibited the toxicity of nitrite in the toxicity experiments on rats (Hassan et al., 2010; Hassan & Yousef, 2010). Nevertheless, the nitrite fed to the rats in this study may not have been sufficient to induce toxicity. Furthermore, ANP treatment may have caused a change in the antioxidant ability of winter mushrooms; therefore, further research is needed to determine whether winter mushroom administration inhibits oxidative stress by nitrite.

4. Conclusion

The use of ANP technology for manufacturing natural nitrite sources requires longer treatment duration, which can result in more reactions between the reactive species in plasma and the food components. To confirm the safety of ANP treatment, the toxicity that could occur in winter mushrooms treated with ANP for a long time was investigated. The Ames test result revealed that winter mushroom with ANP treatment did not show mutagenicity. No significant toxic response was observed while feeding the rat with a winter mushroom powder of 5000 mg/kg body weight. In conclusion, the results of this study suggest that mutagenicity and toxicity did not occur in the winter mushroom treated with ANP for a long time for generating nitrite.

CRediT authorship contribution statement

Kyung Jo: Formal analysis, Data curation, Writing - original draft. **Seonmin Lee:** Investigation. **Hae In Yong:** Investigation. **Yun-Sang Choi:** Investigation. **Ki Ho Baek:** Investigation. **Cheorun Jo:** Resources, Methodology. **Samooel Jung:** Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127826.

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