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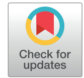
Original Article

Nutri-phenethyl Isothiocyanate Jelly Promotes Detoxification of a Tobacco-specific Oral Carcinogen in Male Active Cigarette Smokers



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Abstract

Background and objectives: Phenethyl isothiocyanate (PEITC), a phytochemical from cruciferous vegetables, is known to modulate detoxification enzymes. Fortification of PEITC into a complete nutrition gel, Nutri-PEITC jelly, has been shown to improve its bioavailability. This work aimed to study the effect of Nutri-PEITC jelly on active smokers' detoxification of tobacco-derived carcinogens.

Methods: This pre-post trial was conducted on 30 healthy, male, regular smokers. During the pre-intervention period, they smoked regularly for three days. During the postintervention period, they smoked regularly and consumed Nutri-PEITC jelly for three days (40 mg PEITC/day). The total amounts of *N*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol metabolites in 24-hour urine were measured by liquid chromatography-tandem mass spectrometry after deconjugation with β-glucuronidase and normalized to the urinary creatinine level. To ensure the consistency of smoking and the actual consumption of Nutri-PEITC jelly, the levels of urinary cotinine and PEITC were measured by using an enzyme-linked immunosorbent assay and liquid chromatography-tandem mass spectrometry, respectively.

Results: After consuming Nutri-PEITC jelly, the level of total urinary NNN metabolites (mainly glucuronide conjugates) was significantly increased by up to 4-fold ($p < 0.01$). In contrast, the level of total urinary 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol metabolites was not significantly altered ($p = 0.325$). The urinary cotinine level was similar ($p = 0.8832$), while the urinary PEITC level was greater than that of the pre-intervention period ($p < 0.001$).

Conclusions: The findings suggest that intake of Nutri-PEITC jelly may increase the detoxification of NNN, a tobacco-specific oral carcinogen, likely by promoting glucuronide conjugation. Future randomized controlled trials are warranted to confirm its potential for the primary prevention of smoking-related oral cancer.

Keywords: PEITC; Brassica vegetable; Functional food; Carcinogen; Active Smoking; Metabolites; Clinical trial; Primary Cancer Prevention.

Abbreviations: CYP, cytochrome P450; Gluc, glucuronide; GST, glutathione S-transferase; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNAL-d_3, methyl-deuterated 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*-nitrosonornicotine; NNN-d_4, pyridine-ring-deuterated *N*-nitrosonornicotine; PEITC, phenethyl isothiocyanate; TSNA, tobacco-specific nitrosamines.

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Introduction

Smoking is the top risk factor in many types of cancer, especially lung and oral cancer.[1,2] One of the major carcinogens in cigarettes is tobacco-specific nitrosamines (TSNA).[2,3] Among the seven types of TSNA, *N*-nitrosonornicotine (NNN) is known to induce oral cancer, while 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has been shown to induce lung cancer.[3,4] The human body can detoxify NNN and NNK and excrete the metabolites via the urine. NNK is first converted by a phase I enzyme (cytochrome P450) to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), the genotoxic metabolite, followed by glucuronide (Gluc) conjugation using a phase II enzyme, resulting in NNAL-Gluc, the non-

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toxic metabolite excreted in the urine.[5,6] NNN is mostly detoxified by Gluc conjugation using a phase II enzyme yielding mostly NNN-*N*-Gluc for urinary excretion.[5,6] Total NNAL and NNN metabolites in the urine are biomarkers of exposure and detoxification.[6] Smoking cessation has been promoted as the major approach for the primary prevention of tobacco-related cancer;[7] nevertheless, 1.3 billion people worldwide are still smoking.[8] Thus, novel approaches are needed to promote the detoxification of tobacco-specific carcinogens.

Phenethyl isothiocyanate (PEITC), a phytochemical derived from cruciferous vegetables such as watercress and Chinese cabbage, can inhibit phase I and activate phase II detoxification enzymes.[9] Intake of 40 mg of PEITC in oil per day and the daily consumption of watercress have been shown to reduce metabolic conversion from NNK to NNAL as well as increase the urinary excretion of total NNAL in regular smokers.[10,11] However, the reported effect was moderate. Previous animal studies have suggested that PEITC can help to detoxify the tobacco-specific oral carcinogen NNN. Nevertheless, the effect of PEITC on NNN metabolism in humans is unknown. Interestingly, PEITC fortified into Nutri-Jelly, a semi-solid, nutritious food gel,[12] has been shown to be better absorbed than PEITC in oil, suggesting improved bioavailability.[13] However, the effects of Nutri-PEITC jelly on the detoxification of NNN and NNK in smokers has never been studied. This pre-post trial aimed to test the effect of Nutri-PEITC jelly on the detoxification of tobacco-derived carcinogens in 30 healthy, male, active smokers. This work may pave the way for a new approach for the primary prevention of tobacco-related cancer.

Materials and methods

Ethical aspects and setting

The Mahidol University Central Institutional Review Board provided ethics approval for this study (project No. 2019/203.1911 and COA. No. 2020/043.2503), which was conducted according to the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice. All participants signed the informed written consent before this study. This study was registered at the Thai Clinical Trial Registry with No. TCTR20210519003. The protocol can be accessed at http://www.thaiclinicaltrials.org/#

Study design and participants

This project was a pre-post clinical trial performed with healthy, regular smokers. The inclusion criteria for screening participants were Thai men aged 20–60 years old who had no systemic diseases; smoked 5–45 cigarettes per day for 1–10 years; had no history of psychiatric disorders; used only cigarettes (no other narcotics); were taking no medications affecting cytochrome P450 1A2, 2A6, or 2A13[10] such as antidepressants, antihypertensive drugs, calcium channel blockers such as verapamil, beta-blockers such as propranolol, anticoagulants such as warfarin and ticlopidine, antibiotics such as ciprofloxacin and fluoroquinolones, and opioid analgesic drugs; had a body mass index of 18–30 kg/m^2, normal blood laboratory tests including kidney function (blood urea nitrogen, creatinine), liver function (aspartate transaminase, alanine transaminase, total bilirubin), blood lipid levels, normal blood sugar levels, and complete blood count; were able to communicate well; and provided written informed consent. They were able to consume Nutri-PEITC jelly; avoid eating cruciferous vegetables such as watercress, kale, cabbage, broccoli, Chinese cabbage, cauliflower, radishes, mustard, and wasabi; and avoid drinking alcoholic beverages for at least 24 h before enrolling in the study and throughout the study period. The exclusion criteria were as follows: having cancer or a history of cancer, liver, or kidney diseases; and taking paracetamol, chlorzoxazone, or *N*-acetylcysteine within 24 h before participating in the study and throughout the study period. Paracetamol and chlorosazone have the same metabolism as PEITC,[14] while *N*-acetylcysteine can conjugate to PEITC.[15]

Fig. 1. Nutri-phenethyl isothiocyanate jelly.

Sample size and power

The researcher calculated the sample size based on data from a previous study by Hecht *et al*.,[11] who studied the effects of PEITC in watercress on tobacco-derived carcinogens in smokers. Based on the mean difference of NNAL in the urine of 0.9 and the standard deviation of 1.12, the calculated effect size was 0.825. Using the G-power program with the principle of noncentrality parameter, the sample size for a paired t-test with an alpha value of 0.05 and a power of 0.95 was 23. Adding a 30% dropout rate, the total sample size was calculated to be 30 people.

Intervention

The Nutri-PEITC jelly product was obtained from the Dental Innovation Foundation under Royal Patronage. It is a semi-solid nutritious food gel (Fig. 1). Each cup contains 10 mg of PEITC and provides an energy of 110 kcal. The jelly was produced by using ultra-high temperature processing and aseptic filling into sterile cups at a Good Manufacturing Practices-certified plant. The participants were asked to consume four cups of Nutri-PEITC jelly per day to achieve a dose of 40 mg of PEITC per day. They were instructed to consume Nutri-PEITC jelly about 1.5 h before smoking according to the onset of PEITC action, the pharmacokinetics of PEITC in Nutri-jelly, and the metabolism of tobacco-derived carcinogens after smoking.[12,13,16]

Materials and equipment

A Supelco molecular imprinted polymer solid-phase extraction–TSNA bed (50 mg, 10 mL), β-glucuronidase (type IX-A, *Escherichia coli*), acetonitrile, formic acid, hydrochloric acid, dichloromethane, toluene, ammonium hydroxide (25% in water), disodium hydrogen phosphate (purity ≥ 99.5%), heptane (purity ≥ 99%), and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA), NNN, pyridine-ring-deuterated *N*-nitrosonornicotine (NNN-d_4), NNAL, and methyl-deuterated 4-(methylnitrosamino)-1-(3-pyridyl)-

1-butanol (NNAL-d_3) were purchased from Toronto Research Chemicals, Ontario, Canada. Oasis MCX 1 cc Vac cartridges (30 mg of sorbent per cartridge, 30 µm) were obtained from Waters GmbH, Darmstadt, Germany. Positive displacement pipette (Rainin, Mettler-Toledo S.A.E., Barcelona, Spain), benchtop centrifuge (Eppendorf, Hamburg, Germany), and liquid chromatography-tandem mass spectrometry (LC-MS/MS; Thermo Scientific, Waltham, MA, USA) machines were used at the laboratories of the Institute of Nutrition, Mahidol University.

Study procedure

This study was conducted at the Institute of Nutrition, Mahidol University. Thirty participants joined this project for seven days, divided into two periods, i.e., pre- and postintervention periods. For the preintervention period, each participant was asked to smoke regularly and to collect their 24-hour urine into three containers per day (1 L per container). Each container was prefilled with 5 mL of ammonium sulfamate preservative (100 mg in 5 mL of 1 N sulfuric acid). At the end of three days, the participants returned the urine-filled containers. Then, for the postintervention period, the participants were instructed to smoke regularly, consume four cups of Nutri-PEITC jelly per day for three consecutive days, and collect their 24-hour urine in a similar way as during the pre-intervention period. The urine samples of each day were randomly sampled into a 50-milliliter tube. All 24-hour urine samples (180 in total) from the 30 participants were stored at −20 °C until analysis.

Monitoring of smoking and PEITC consumption

Throughout the study, all participants recorded all meals, the brand of cigarettes smoked, the number of cigarettes smoked, the number of consumed Nutri-PEITC jelly products, and adverse events (if any).

Measurement of cotinine in the urine

To ensure the consistency of smoking throughout the study, the levels of cotinine in the urine during the pre-intervention and postintervention periods were compared. Cotinine was measured by a cotinine enzyme-linked immunosorbent assay kit (Cat. No. KA0930, Abnova, Taiwan), as described previously.[17] Ten microliters of each standard, control, or sample was pipetted into 96-well plates in duplicate by using a repeating pipette (Multipette, Eppendorf, Hamburg, Germany). Then, 100 µL of the enzyme conjugate was added to each well. The plate was shaken for 10–30 s with a microplate shaker (Fisher Scientific, Waltham, MA, USA) to ensure proper mixing. After incubating at room temperature (20–25 °C) in the dark for 60 min, the wells were washed three times with 300 µL of 1× washing buffer. Then, all liquid was aspirated with a multichannel pipette (8-channel pipettor, Axygen, Corning, NY, USA). To ensure that all residual moisture was removed, the plate was then inverted and vigorously slapped on an absorbent paper. This step is critical to ensure that the residual enzyme conjugate does not skew the results. After the plate was dried, 100 µL of substrate reagent was added to each well. The plate was incubated at room temperature in the dark for 30 min. Then, 100 µL of stop solution was added to each well, and the plate was shaken gently to mix the solution. The absorbance at 450 nm was read by using a plate reader (Epoch model, BioTek, Agilent, Santa Clara, CA, USA) within 15 min after addition of the stop solution.

Measurement of PEITC in the urine

To ensure that the participants did consume Nutri-PEITC jelly, the level of urinary PEITC was measured by using LC-MS/MS, as described previously.[18,19] Standard and urine samples were extracted with 600 µL of hexane using a positive displacement pipette and then mixed with a high-velocity vortex for 30 s. After centrifugation at 25 °C and 1,000*g* for 3 min, 500 µL of each of the top hexane layers containing PEITC was collected into a two-milliliter tube for all samples. Hexane extraction was performed once more, and the supernatant was combined with the first extract. Then, ammonia derivatization was performed by adding 400 µL of 2 M ammonia in methanol into the hexane extract. The samples were vortexed for 30 s and incubated on a microplate shaker (Fisher Scientific, USA) at room temperature for 4 h. Next, the samples were dried with a speed vacuum evaporator (CentriVap Benchtop Vacuum Concentrator, Labconco, Kansas City, MO, USA) at 55 °C for 40 min to remove the solvents. After drying completely, each sample was reconstituted in 250 µL of acetonitrile:water in a ratio of 3:2 (60% acetonitrile) and vortexed for 1 min. Then, all samples were filtered through a nylon filter (0.2 µm), placed in an Eppendorf tube, and stored at −20 °C before analysis. The ammonia-derivatized PEITC was measured by an LC-MS/MS system, consisting of an Ultimate 3000 ultra-high-performance liquid chromatograph (Thermo Scientific, USA) and a TSQ Quantis Triple Quadrupole Mass spectrometer (Thermo Scientific, USA). Five microliters of the eluate was injected through a Hypersil GOLD™ C18 column (100 × 2.1 mm, particle size: 1.9 µm) with a mobile phase of acetonitrile:5 mM formic acid (50:50), an isocratic flow rate of 0.3 mL/min, and a run time of 3.5 min. Positive ion electrospray ionization was used for mass spectrometric analysis with a spray voltage of 3,500 V, sheath gas of 50 arbitrary units, and auxiliary gas of 10.0 arbitrary units. The ion transfer tube and vaporizing temperatures were 325 °C and 350 °C, respectively.

The retention time of phenethyl thiourea was 1.6 min. The mass-to-charge ratios (m/z) of the phenethyl thiourea precursor and quantified product masses were 181 and 105.13, respectively. A collision energy of 18.18 V was used for the transition. The confirmation product mass for phenethyl thiourea was m/z 77.05, with a collision energy of 38.82 V for the transition.

Outcome measurement

The primary outcome measures were total NNN and total NNAL metabolites in the urine.

Measurement of total NNN and NNAL metabolites in the urine

The urine samples were prepared according to a previous study.[10] The amounts of total NNN and NNAL (including unchanged and Gluc-conjugated forms) were measured after deconjugation with β-glucuronidase by using LC-MS/MS. In brief, the stored urine samples from the pre- or postintervention periods of three days were pooled into one 50-milliliter tube. As shown in Figure S1, 6 mL of urine was treated with 40 µL of β-glucuronidase in phosphate buffer (pH 7.2; final concentration of 250 units/mL urine) at 37 °C overnight. This procedure freed the Gluc-conjugated metabolites, allowing the determination of total metabolites (including conjugated and unconjugated forms). The hydrolysate was applied to a TSNA molecular imprinted polymer cartridge, washed, and eluted with 3 mL of dichloromethane/toluene (1:1). The eluate dissolved in 1 mL of phosphate-buffered saline was applied to a cation-exchange cartridge (Oasis MCX), washed, and eluted with 1 mL of methanol/25% ammonium hydroxide (9:1). Ten microliters of the eluate dissolved in 0.1% ammonium acetate/0.1% formic acid (9:1) was injected into the LC-MS/MS system, consisting of an Ultimate 3000 ultra-high-performance liquid chromatograph (Thermo Scientific, USA) and a TSQ Quantis Triple Quadrupole Mass spectrometer (Thermo Scientific, USA). Chromatography was

performed on a Hypersil gold C18 column (100 × 2.1 mm, particle size: 1.9 µm). The mobile phase solvents included 15 mM ammonium acetate (A) and 0.1% formic acid in acetonitrile (B). The mobile phase solvents were run with a flow rate of 0.3 mL/min with the following gradients of A/B: 0–0.2 min, 70/30; 1–1.8 min, 5/95; 1.81–3.0 min, 70/30. For MS/MS, positive electrospray ionization was applied, and the MS/MS system was run in the multiple reaction monitoring mode. The MS/MS conditions are shown in Table S1. The precursor mass of NNN and the internal standard NNN-d_4 were 179 *m/z* and 182 *m/z*, respectively. The quantitative and confirmative fragment ions of NNN were 149 *m/z* and 120 *m/z*, respectively. The quantitative and confirmative fragment ions of the internal standard NNN-d_4 were 152.08 *m/z* and 123.92 *m/z*, respectively. The precursor masses of NNAL and the internal standard NNAL-d_3 were 210 and 213, respectively. The quantitative and confirmative fragment ions of NNAL were 93 *m/z* and 149 *m/z*, respectively. The quantitative fragment ion of NNAL-d_3 was 93. The confirmative fragment ions of NNAL-d_3 were 149 *m/z* and 121 *m/z*.

Standard curve generation and quantitation of urinary metabolites

Pure standard dissolved in methanol was used to generate a standard curve. The standard curve for the quantitation of NNN was generated between the concentration of NNN and the ratios between the area under the curve of NNN and the internal standard (NNN-d_4). Likewise, the standard curve for the quantitation of NNAL was created between the concentration of NNAL and the ratios between the area under the curve of NNAL and the internal standard (NNAL-d_3). The standard concentrations of NNN included 1.6, 3.2, 6.4, 12.8, 25.6, 51.2, 102.4, 204.8, and 409.6 ng/mL. While the standard concentrations of NNAL included 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2 ng/mL.

Statistical analyses

For the baseline characteristics of the participants, numerical data were presented as the mean ± standard deviation and categorical data were expressed as the number of participants. The concentrations of urinary NNN and NNAL metabolites (ng/mL) were calculated by linear regression from the standard curve. Then, the number of metabolites in 100 µL of the sample was obtained, followed by the calculation based on the fact that 100 µL of the sample came from 6 mL of urine. In addition, the urine sample of each person had a different content of water. Therefore, the weight of urine creatinine was used to adjust for the variation and yielded the level of metabolites per gram of creatinine.[20] Creatinine was measured by the Jaffe method by using Cobas 8000 (Roche Diagnostics, Indianapolis, IN, USA). The levels of NNN and NNK metabolites were divided by the values of urinary creatinine (mg/dL) of the corresponding participants, yielding the level of metabolites in ng/g creatinine. The level of urinary metabolites was compared between the pre-and postintervention periods by using the Wilcoxon matched-pairs signed-rank test due to a skewed distribution. All statistical tests were performed by using a two-tailed test. A p-value < 0.05 was considered statistically significant. Graph Pad Prism V.9.0.2 was used for graphing and statistical analysis. Power analysis was performed by using G-power V.3.1.9.2.

Results

Participant flow chart

As shown in Figure 2, 49 participants were recruited. After the screening, 17 participants were excluded; 11 participants had high liver enzymes and six participants were unable to be followed up. Thirty-two participants received the intervention. One participant withdrew from the study due to suffering from the lack of vegetable consumption. One participant was excluded after the analysis due to highly deviated data. Finally, data from 30 participants were included in the analysis.

Baseline characteristics

Table 1 shows that the average age of the participants was 33.72 ± 9.09 years old. The average amount of smoking was 15.03 ± 8.65 cigarettes for 15 years. The majority of participants rarely consumed vegetables (only once a month). Table 2 shows that all of the participants' hematological and blood biochemical data were within clinically acceptable limits.

Compliance with Nutri-PEITC jelly intake and avoidance of vegetable consumption

Table 3 shows that most of the participants consumed the required amount of jelly and were able to avoid consuming cruciferous vegetables throughout the study. Table 4 shows the number of participants refraining from vegetable consumption completely or still consuming a small amount of vegetables (less than 50 g per day), as specified.

Consistency of smoking

As shown in Figure 3a, there were no significant differences in the number of cigarettes smoked between the pre- and postintervention periods. Consistently, Figure 3b shows no significant difference in the average urinary cotinine levels between the pre- and postintervention periods.

Exposure to PEITC

Figure 3c, d shows that the amount of urinary PEITC in the postintervention period was significantly greater than that of the pre-intervention period ($p < 0.001$).

Adverse events

As shown in Table 5, nonserious adverse events, including nausea, dry mouth, and diarrhea, occurred on the first day of Nutri-PEITC jelly intake and disappeared without treatment. All participants were able to tolerate these minor events from Nutri-jelly intake.

Changes in total urinary NNN and NNAL after Nutri-PEITC jelly consumption

Figures 4a, b and 5a, b show chromatograms of NNN and the internal standards, respectively, and the standard curve. Figure 6a, b shows chromatograms of NNAL and the internal standards, respectively, and the standard curve. As shown in Figure 6c, the average level of total urinary NNN metabolites (normalized with the creatinine level) during the postintervention period (after consuming Nutri-PEITC jelly) was greater than that of the pre-intervention period (before consuming the jelly). Due to the large individual variation, the difference was not statistically significant. However, when analyzing the data as a percentage of baseline compared with one's data, Figure 6d shows a significant increase of up to 4-fold in urinary NNN metabolites after consuming Nutri-PEITC jelly ($p < 0.01$). In contrast, Figure 6e, f shows no significant difference in the total urinary NNAL metabolites between the pre- and postintervention periods ($p = 0.325$).

Discussion

Previous human studies have suggested that PEITC can help to de-

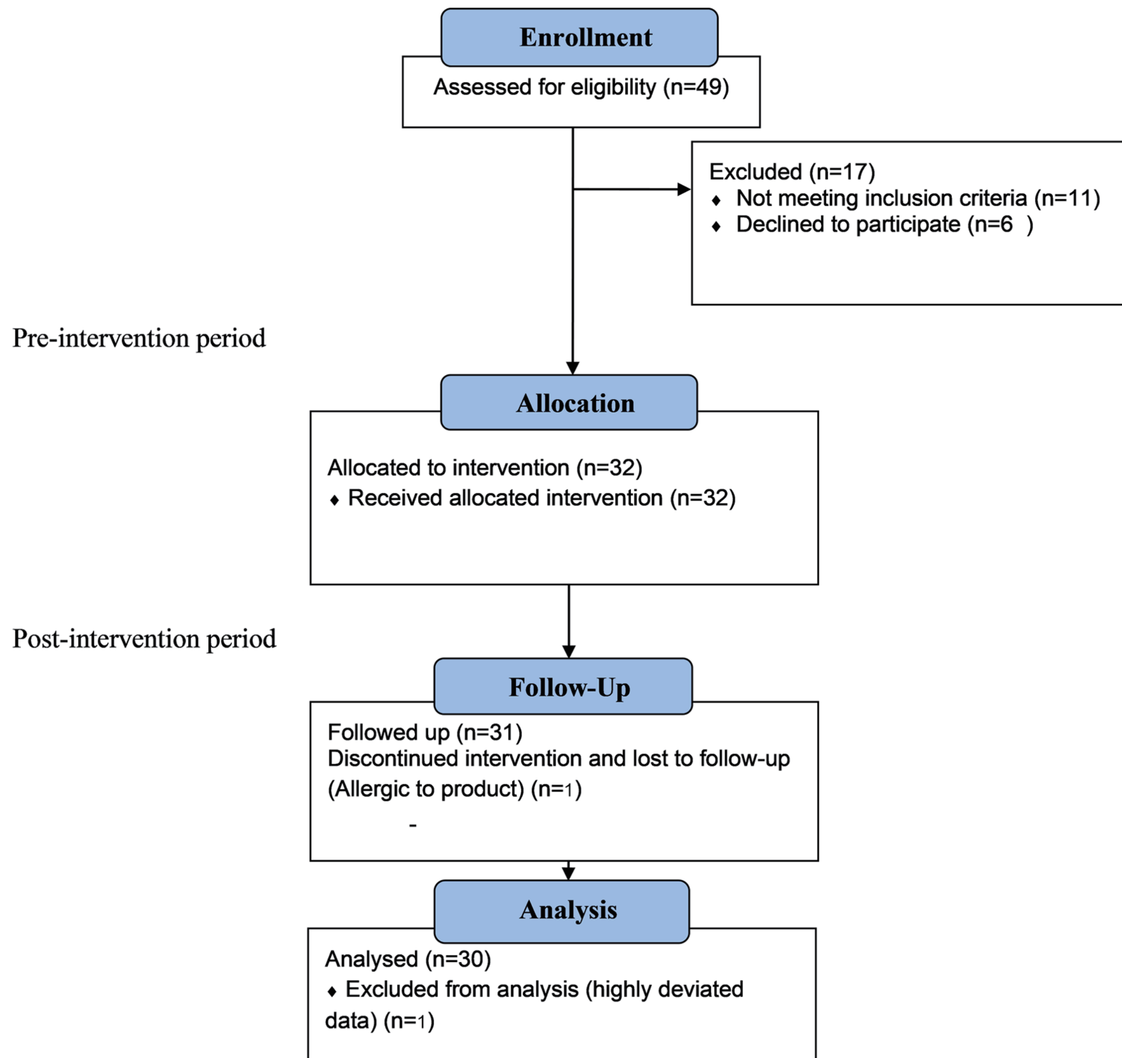

Fig. 2. Flow diagram of the participants.

toxify the smoking-derived carcinogen NNK.[10,11] However, such an effect of PEITC in Nutri-jelly is unknown. Similarly, previous animal studies have indicated that PEITC can help to detoxify the tobacco-specific oral carcinogen NNN.[9,21,22] Nevertheless, the effects of PEITC on NNN metabolism in humans are unknown. In this study, we found that consuming Nutri-PEITC jelly significantly increased the total urinary NNN metabolites, with no significant change in the total urinary NNAL metabolites. The major form of NNN metabolites in urine is the nontoxic NNN-*N*-Gluc, which is formed by the phase II enzyme UDP glucuronyltransferase.[5] Thus, the increase in total NNN after consuming the jelly suggests that Nutri-PEITC jelly likely promotes Gluc conjugation and excretion of NNN. Previous studies also have shown that an achievable dose of PEITC in humans can activate UDP glucuronyltransferase.[23,24] Thus, the finding of this study indicates that Nutri-PEITC jelly can help to detoxify smoking-derived carcinogens by promoting phase II detoxification of NNN. Since NNN is known to induce oral carcinogenesis,[4] Nutri-PEITC jelly may be a good candidate for the primary prevention of oral cancer in active smokers. Owing to its better absorption in the form of the Nutri-Jelly matrix than in oil,[13] future randomized controlled trials are warranted to study the effect of Nutri-PEITC jelly, compared with that of PEITC in oil, on biomarkers of oral carcinogenesis.

In 1995, Hecht *et al*. reported that the consumption of watercress (56.8 g/day,[11] providing approximately 14.2 mg of PEITC/day[25]) for three days significantly increased the urinary NNAL plus NNAL-Gluc by 35%, and the magnitude of increase was correlated to the amount of PEITC intake.[11] However, in this study, we found no changes in total urinary NNAL metabolites (NNAL plus NNAL-Gluc) after consuming 40 mg of PEITC/day in the form of Nutri-PEITC jelly. These seemingly contradictory results may be explained by the complex effect of PEITC on both phase I and II enzymes.[9] NNAL was the toxic byproduct of phase I metabolism of NNK, while the glucuronidated NNAL (NNAL-*N*-Gluc and NNAL-*O*-Gluc) were the nontoxic byproducts of phase II detoxification.[26] A previous study found that the levels of unconjugated NNAL and Gluc-conjugated NNAL in the urine of tobacco smokers were 41.4% and 58.6% of total NNAL metabolites, respectively.[26] Thus, the ratio of phase I and phase II byproducts of NNK metabolism in urine was about 40:60. Since PEITC can

Table 1. Characteristics of all participants (*n* = 32)

Characteristic	Range	Mean	Standard deviation
Age (years)	19–50	33.72	9.09
Weight (kg)	55.5–119	75.21	11.31
Height (cm)	160–181	170.66	4.85
Body mass index (kg/m^2)	20.52–38.85	25.85	3.74
Duration of smoking (years)	1–39	15.50	8.75
Number of cigarettes/day	5–30	15.15	8.08

Characteristic		N	%
Frequency of vegetable consumption	Daily	3	9.38
	Weekly	9	28.13
	Monthly	20	62.50
Systemic diseases	Yes[a]	6	18.75
	No	26	81.25
History of alcohol drinking	Never	6	18.75
	≤14 drinks	7	21.88
	>14 drinks	16	50.00
	Quit drinking	3	9.38

[a]Allergy (*n* = 3), migraine (*n* = 2), gout (*n* = 1)

both inhibit phase I enzymes and stimulate phase II enzymes,[25] treatment with PEITC likely reduces the formation of NNAL but promotes the formation of NNAL-Gluc, resulting in no changes of the total NNAL metabolites, as observed in this study. Considering the dose of PEITC in this study (40 mg/day) and the dose of PEITC in the study by Hecht *et al*. (14.2 mg/day), the results of these studies together suggest that a low dose of PEITC may primarily stimulate phase II enzyme activity; while at a higher dose, PEITC may also stimulate phase I enzyme activity. In 2016, Yuan *et al*. also found that the intake of 40 mg of PEITC in oil/day did not alter the total urinary NNAL metabolites but reduced the hydroxyl acid:total NNAL ratio, suggesting the main effect on phase I metabolism of NNK.[10]

The strengths of this research included multiple days of urine collection. The result of urinary metabolites in each participant came from the averaged pool sample of three days. Furthermore, the smoking habits were followed up throughout the study. The results showed that all participants smoked the same type and number of cigarettes throughout the trial period, supported by the constant urinary cotinine levels. In addition, this work was performed in healthy smokers, so the results may have application in primary cancer chemoprevention. Furthermore, all participants were asked to refrain from cruciferous vegetables throughout the study. Therefore, their baseline levels of urinary PEITC in the pre-intervention period were very low, allowing us to see a significant increase in the PEITC levels after consuming the Nutri-PEITC jelly. Nevertheless, there were some limitations to this study. First, it was a nonrandomized pre-post study. Therefore, some potential biases still may have occurred. In the future, a randomized controlled trial should be conducted to compare the effect of Nutri-PEITC jelly with that of Nutri-jelly as a placebo control to investigate its long-term effects on carcinogen detoxification and DNA adducts. Also, comparative studies for the effect of Nutri-PEITC on heavy and moderate smokers as well as long-term vs. recent smoker groups are warranted. Second, in this study, we did not directly measure the changes in phase I and II enzyme activities after Nutri-PEITC intake. Since the metabolism occurs mostly in the liver, it is challenging to measure the enzyme activities in humans. Thus, we discussed the possible mechanism of PEITC based on previous *in-vitro* and *in-vivo* studies.[9] The differential effect of Nutri-PEITC on NNN and NNAL is interesting and warrants future mechanistic studies. In addition, human liver microsomes may be used as a tool for further investigations of the molecular mechanism.[27] Future clinical trials may include assessing phase I enzyme activities through the metabolism of target drugs such as caffeine, dextromethorphan, losartan, and buspirone for cytochrome P450 (CYP)1A2, CYP2D6, CYP2C9, and CYP3A4 activities, respectively.[28] Phase II enzyme activities could be measured by blood lymphocyte glutathione S-transferase (GST) activity, GST-pi level, and serum total and direct bilirubin as surrogate markers for UDP glucuronosyltransferase 1A1 activity.[28] Although not a direct measurement of liver phase I–II enzyme activities, those assays can at least provide useful information. Third, the response to PEITC in jelly may also be dependent on other factors, such as the efficacy of liver enzymes and polymorphisms of the GST gene, which affect the metabolism of PEITC.[29] A previous study has shown that smokers with a polymorphism of GST M1 or T1 responded better to PEITC as a promoter of the detoxification of benzene, acrolein, and crotonaldehyde.[30] Therefore, future clinical trials of Nutri-PEITC jelly in smokers with different genetic backgrounds would be useful. Furthermore, a recent review suggests that local exposure to bioactive compounds can provide effective chemoprevention.[31] Since the intake of Nutri-PEITC jelly can deliver PEITC locally to the oral mucosa, future trials of Nutri-PEITC in smokers with potentially malignant disorders of the mouth would be worthwhile.

Table 2. Hematological and biochemical characteristics of all participants (*n* = 32)

Characteristic	Mean ± standard deviation	Reference range
Fasting plasma glucose, mg/dL	95.22 ± 5.94	70–110
Blood urea nitrogen, mg/dL	11.46 ± 2.94	6–20
Creatinine, mg/dL	1.40 ± 0.14	0.5–1.5
Estimated glomerular filtration rate by the Chronic Kidney Disease Epidemiology Collaboration, mL/min/1.73m^2	110.08 ± 26.98	>90
Cholesterol, mg/dL	200.28 ± 30.78	<200
Triglyceride, mg/dL	98.11 ± 30.73	<150
High-density lipoprotein cholesterol, mg/dL	56.22 ± 10.85	>45
Low-density lipoprotein cholesterol, mg/dL	121.64 ± 24.45	<130
Aspartate transaminase, U/L	21.54 ± 5.29	0–40
Alanine transaminase, U/L	26.79 ± 7.92	0–40
Total bilirubin, mg/dL	0.57 ± 0.21	0–1.2
White blood cell count, cells/mm^3	7.89 ± 1.70	4,000–10,000
Red blood cell count, million cells/mm^3	5.28 ± 0.39	M: 4.5–6.0, F: 4.0–5.5
Hemoglobin, g/dL	15.24 ± 1.07	M: 13.0–18.0, F: 12.0–16.0
Hematocrit, %	44.68 ± 3.06	M: 40–54%, F: 36–58%
Mean corpuscular volume, fL	84.04 ± 6.96	800.0–99.0
Mean corpuscular hemoglobin, pg	28.65 ± 2.30	27.0–31.0
Mean corpuscular hemoglobin concentration, g/dL	33.77 ± 1.15	33.0–37.0
Red blood cell distribution width, %	13.65 ± 1.21	11.0–14.5
Platelet count, cells/mm^3	266,107.10 ± 47,929.52	140,000–450,000
Neutrophils, %	51.79 ± 8.65	40–74
Lymphocytes, %	35.44 ± 7.49	19–48
Monocytes, %	7.84 ± 1.83	3–9
Eosinophils, %	4.76 ± 2.94	0–7
Basophils, %	0.80 ± 0.50	0–2

Table 3. Compliance of Nutri-PEITC jelly intake

Date of jelly consumption		1st day	2nd day	3rd day
Finish all	4 cups/day	19	19	20
Not finish all	1 cup remaining	4	5	4
	1 ½ cup remaining	3	3	3
	2 cups remaining	4	3	3

The table shows the number of participants consuming Nutri-PEITC jelly as specified.

Table 4. Monitoring of vegetable consumption before and after Nutri-PEITC intake

Day		Pre-intervention period			Postintervention period		
		1st day	2nd day	3rd day	4th day	5th day	6th day
Refrain completely		28	29	29	30	29	29
Consumed	Cabbage	0	0	1	0	1	1
	Cantonese lettuce	1	0	0	0	0	0
	Chinese kale	1	1	0	0	0	0

The table shows the number of participants with specified conditions.

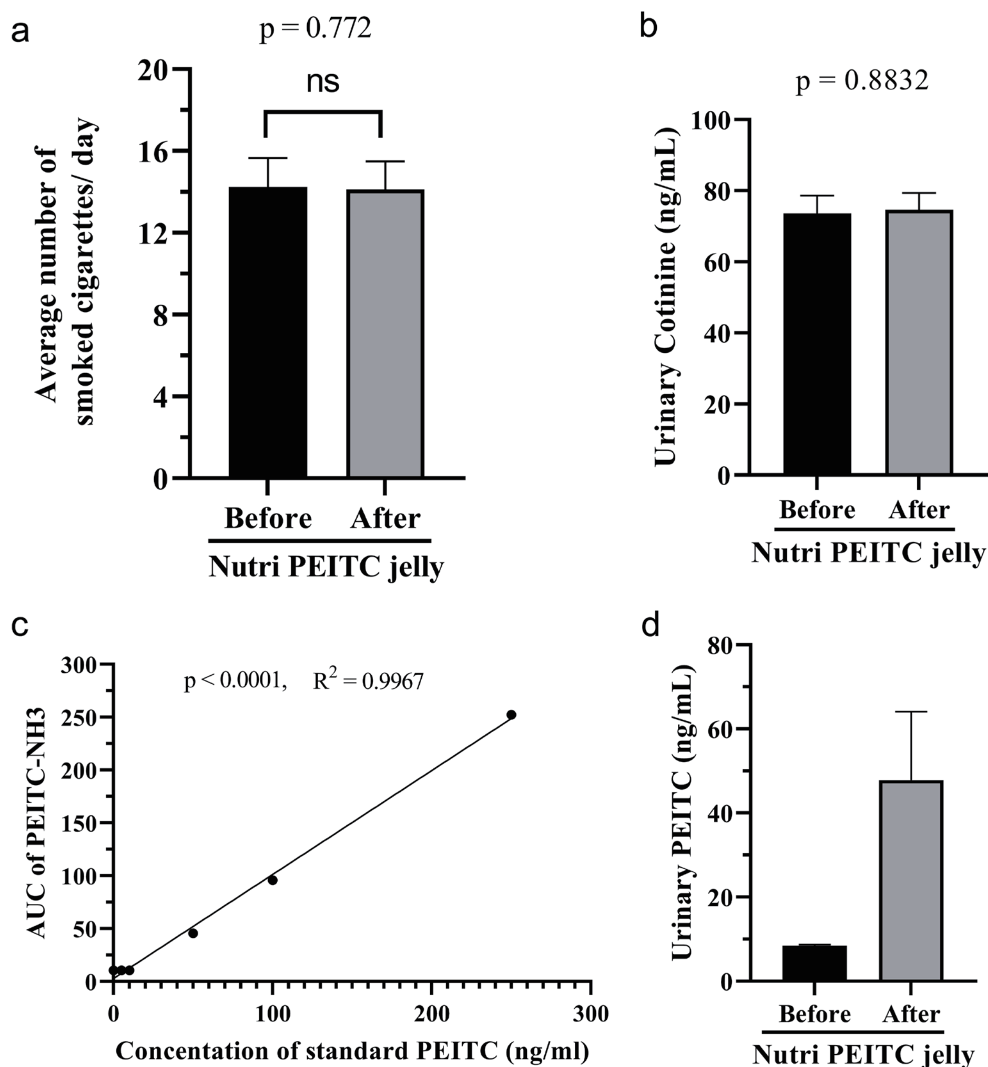

Fig. 3. Exposure to tobacco and exposure to phenethyl isothiocyanate (PEITC). The bar graphs show the mean ± standard deviation of the average number of cigarettes smoked/day (a) and the urinary levels of cotinine (b) and PEITC (d) in the pre-intervention (before) and postintervention (after) periods. *P*-values were obtained from Wilcoxon signed-rank tests. *** indicates $p < 0.001$. The line plot (c) shows the standard curve between the concentrations of standard PEITC and the area under the curves of phenethyl thiourea determined by liquid chromatography-tandem mass spectrometry. The R^2 and *p*-values were obtained by linear regression analysis.

Conclusions

This pre-post clinical trial of Nutri-jelly PEITC in smokers demonstrated that Nutri-PEITC jelly significantly increased the total urinary NNN, with no significant change in the total urinary NNAL. NNN-Gluc is the major form of urine metabolites. Therefore, these findings suggest that Nutri-PEITC jelly may increase the detoxification of smoking-derived carcinogens by promoting Gluc conjugation of NNN, the tobacco-specific oral carcinogen. To confirm its potential for the primary prevention of smoking-related oral cancer, future randomized controlled trials are warranted to study the effect of Nutri-PEITC jelly, compared with that of PEITC in oil, on biomarkers of oral carcinogenesis.

Supporting information

Supplementary material for this article is available at https://doi.

Table 5. Adverse events reported after intake of Nutri-PEITC jelly

Adverse event	Number of participants (%)	Treatment
Nausea	3 (10)	No (the symptom disappeared without treatment)
Dry mouth /throat	2 (6.7)	No (the symptom disappeared without treatment)
Diarrhea	1 (3.3)	No (the symptom disappeared without treatment)

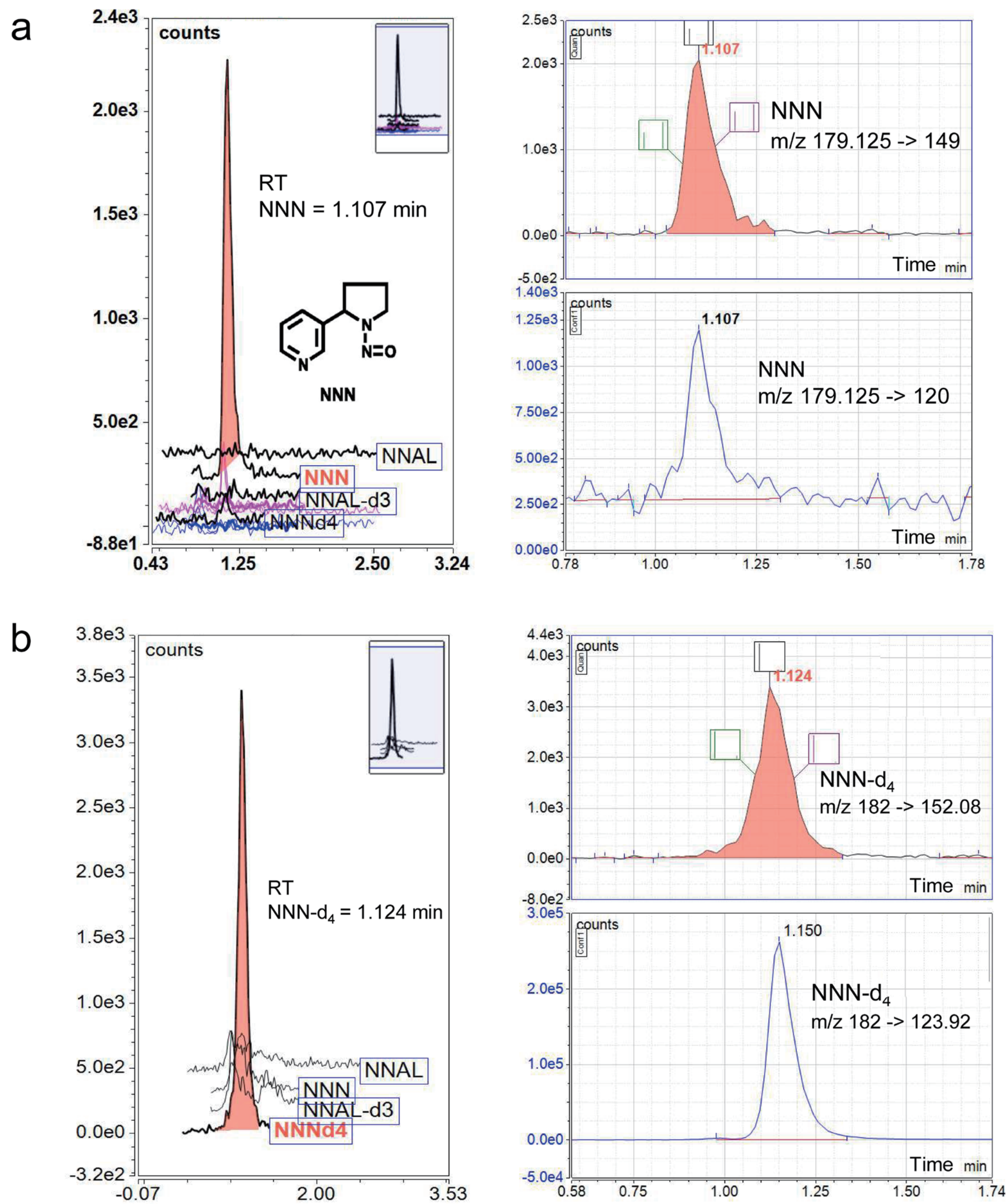

Fig. 4. Chromatograms and mass spectrometric quantitation of *N*-nitrosonornicotine (NNN) and pyridine-ring-deuterated *N*-nitrosonornicotine (NNN-d_4). Liquid chromatography-tandem mass spectrometry chromatograms of NNN (a) and the internal standard, NNN-d_4 (b). The left panel shows a liquid chromatography chromatogram with the specified retention time (RT). The right panel shows the mass spectrometry chromatograms of the quantitative (top panel with orange filled) and confirmative (lower panel without filling) fragment masses. The precursor mass-to-charge ratios (*m/z*) of NNN and NNN-d_4 were 179.125 and 182, respectively.

org/10.14218/CSP.2022.00019.

Fig. S1. Sample preparation for determination of urinary NNN and NNAL metabolites.

Table S1. Conditions of mass spectrometric analysis.

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Conflict of interest

DT and AL received a grant and the Nutri-jelly product for the

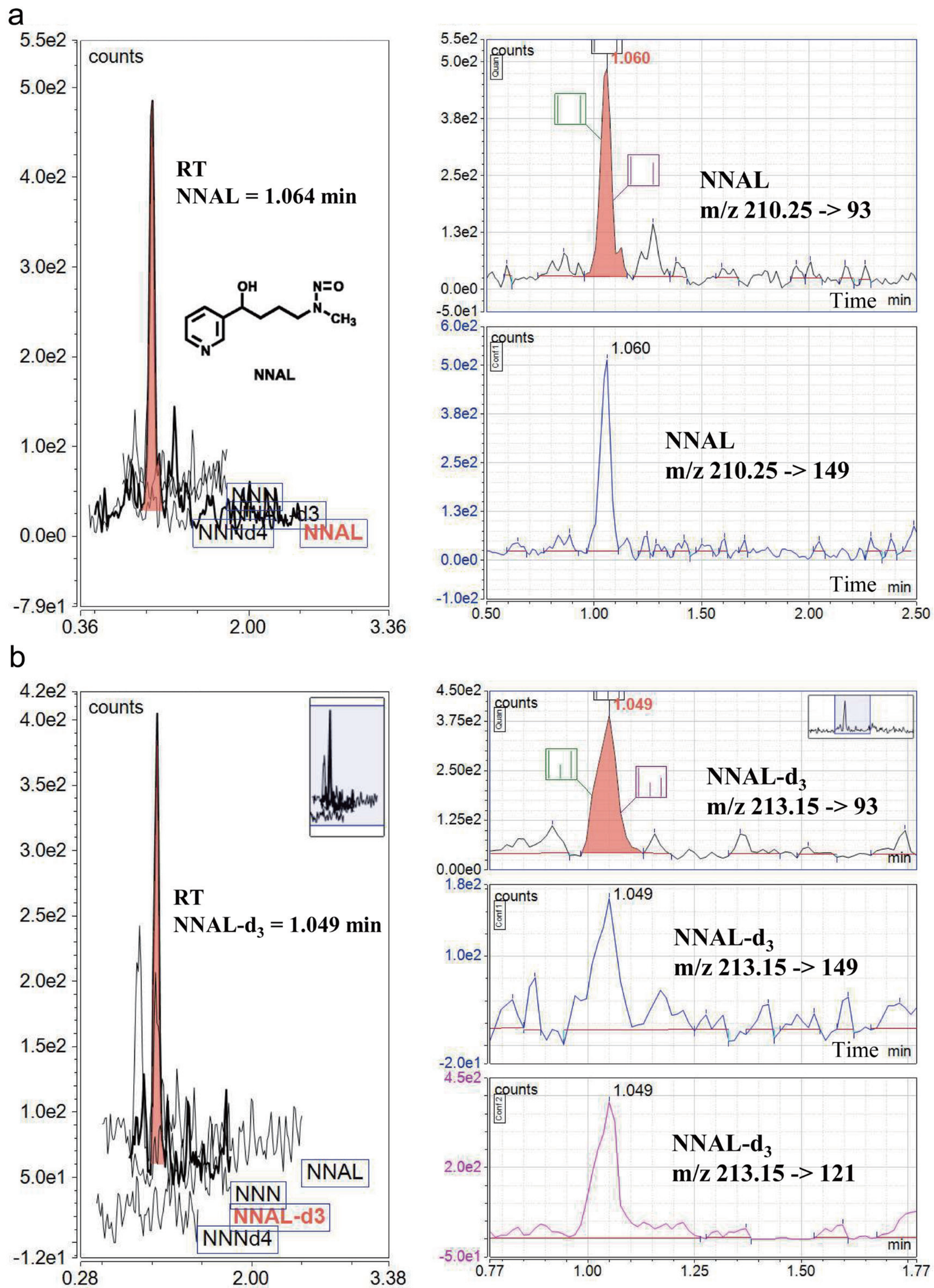

Fig. 5. Changes in the total *N*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) metabolites in the urine after consuming Nutri-phenethyl isothiocyanate jelly. The standard curves of NNN (a) and NNAL (b) show the mean ± standard deviation of triplicate ratios of the area under the curve between standard NNN and the internal standard (IS) pyridine-ring-deuterated NNN (a) or standard NNAL and the IS methyl-deuterated NNAL (b) at each concentration. The R^2 and *p*-values were obtained from linear regression analysis. The graphs show the mean ± standard deviation in ng/g creatinine of total urinary NNN (c) or NNAL (e) as well as the percentage of baseline total urinary NNN (d) or NNAL (f) in the pre-intervention (before) and postintervention (after) periods. *P*-values were obtained from Wilcoxon signed-rank tests. ** indicates $p < 0.01$.

trial from the nonprofit organization Dental Innovation Foundation under Royal Patronage, Thailand. However, the funder was not involved in the research design or operation. One of the authors, Prof. Dunyaporn Trachootham, has been an editorial board member of *Cancer Screening and Prevention* since July 2022. The authors have full control of the reported data. The other authors have no conflicts of interest.

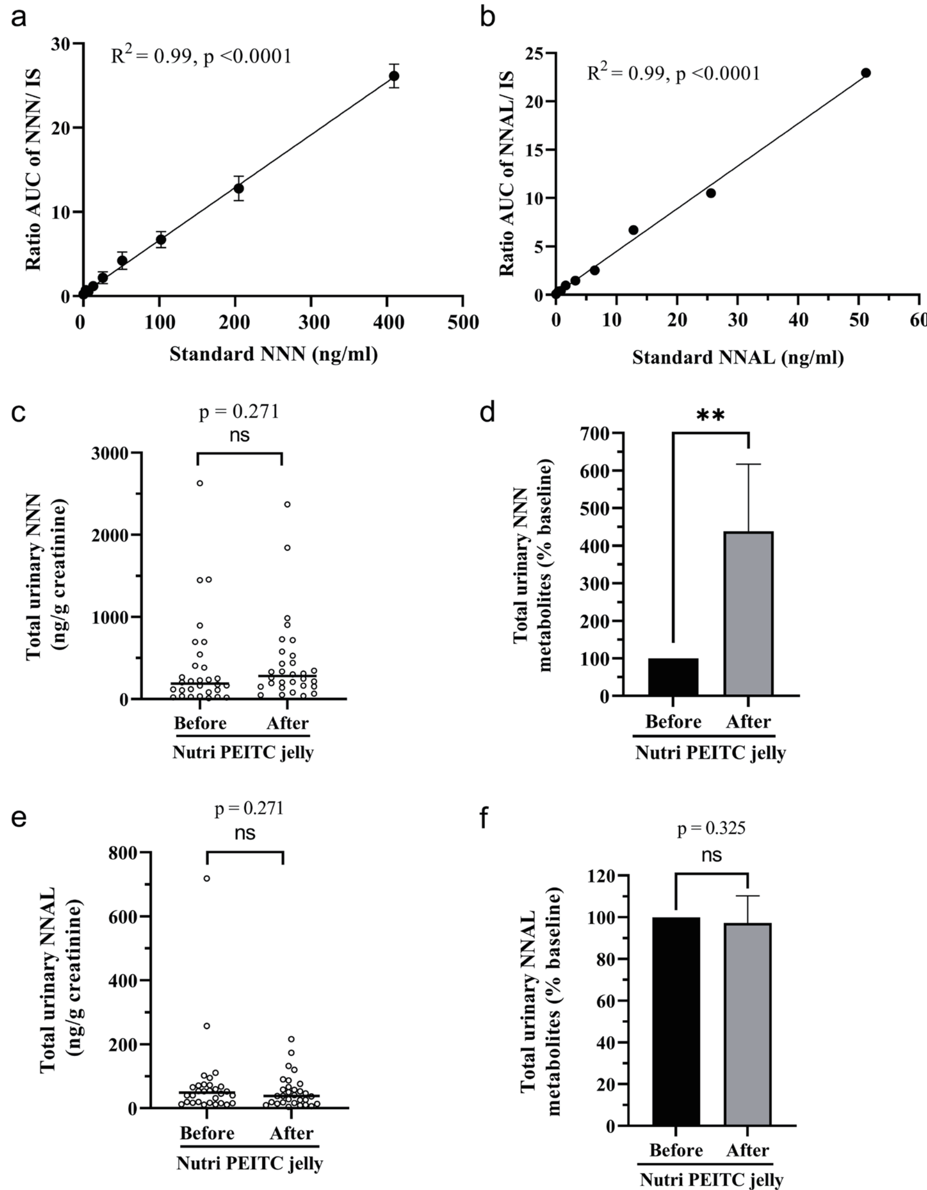

Fig. 6. Chromatograms and mass spectrometric quantitation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and methyl-deuterated 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL-d$_3$). Liquid chromatography-tandem mass spectrometry chromatograms of NNAL (a) and the internal standard, NNAL-d$_3$ (b). The left panel shows a liquid chromatography chromatogram with the specified retention time (RT). The right panel shows the mass spectrometry chromatograms of the quantitative (top panel with orange filled) and confirmative (lower panel without filling) fragment masses. The precursor mass-to-charge ratios (*m/z*) of NNAL and NNAL-d$_3$ were 210.25 and 213.15, respectively.

Author contributions

NP designed the study, performed the clinical trial and laboratory analysis, analyzed the data, and drafted the manuscript. PT and AL designed the study and provided scientific input for discussion. PT and AL designed the study and provided scientific input for discussion. DT obtained the grant, pursued ethics approval, designed the study, supervised the clinical trial and laboratory analysis, and edited the manuscript. All authors approved the final version of the submitted manuscript.

Ethical statement

Mahidol University Central Institutional Review Board (MU-

CIRB) provided ethical approval with project No. 2019/203.1911 and COA. No. 2020/043.2503. The study was conducted according to the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice (ICH-GCP). All participants signed the informed written consent before the study. This study was registered at the Thai Clinical Trial Registry with No. TCTR20210519003. The protocol can be accessed at http://www.thaiclinicaltrials.org/#.

Data sharing statement

No additional data are available.

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