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Incorporating Exogenous and Endogenous Exposures into Dietary Risk Assessment of Nitrates and Nitrites in Vegetables: A Probabilistic Integrated Toxicokinetic Modeling Approach

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ABSTRACT: This study aimed to estimate the dietary risk of nitrates and nitrites in vegetables based on internal dose in a probabilistic manner by integrating exogenous exposure based on measured concentrations in vegetables with endogenous exposure using a toxicokinetic (TK) model. We optimized and validated a previous TK model and incorporated Monte Carlo simulations to account for variability across different age populations for predicting internal dose. High levels of nitrates were detected in leafy vegetables (from 545 ± 274 to 1641 ± 873 mg/kg). Nitrite contents of vegetables were generally low (from 1.26 ± 1.40 to 8.20 ± 14.1 mg/kg). The dietary risk was found to be different based on internal versus external dose, suggesting that it is critical to include endogenous nitrite formation into risk assessment. Nitrate and nitrite exposure from vegetables is unlikely to result in appreciable risks for most populations but may be a potential risk for preschoolers.

KEYWORDS: *nitrate, nitrite, vegetables, risk assessment, toxicokinetic modeling*

INTRODUCTION

Nitrates and nitrites are ubiquitous in the environment and commonly found in human diets. Nitrates are widely used as fertilizers in agriculture, resulting in high levels of nitrate accumulation in a variety of vegetables. Vegetables are the primary source of exposure to ingested nitrates, comprising nearly 80% of the total nitrate intake in a typical human diet.[1] In contrast to nitrates, human exposure to nitrites is mainly endogenous through nitrate metabolism, with nearly 5−8% of the total nitrate intake being converted into nitrites.[2]

Over the last several decades, concerns have been raised regarding the elevated levels of endogenously formed nitrites and higher intake of dietary nitrites that may increase the endogenous formation of N-nitroso compounds and thus individuals' risks for cancer.[3] According to a report from the International Agency for Research on Cancer (IARC), the ingested nitrate or nitrite under conditions that result in endogenous nitrosation is probably carcinogenic to humans.[4] For this reason, many regulatory bodies and researchers have conducted monitoring programs for or investigated the contents of nitrates/nitrites in vegetables and assessed the dietary risk related to nitrate/nitrite exposure through vegetables based on the acceptable daily intake (ADI) for human populations from different places, such as Hong Kong,[5] European countries,[6−8] Australia,[9] and the United States (U.S.).[10] In addition, review articles have discussed the safety of dietary nitrates/nitrites from vegetables by reviewing data on concentration, consumption, or dietary intake (DI) and literature on the health-related effects of nitrates/nitrites.[11−13] However, there is still a lack of a comprehensive assessment of the concentration levels and the potential health risk of nitrates and nitrites in vegetables in Taiwan.

The current ADI values for nitrates (3.7 mg/kg bw per day) and nitrites (0.07 mg/kg bw per day) deduced by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) were determined based on the evaluation of toxicity in rats.[14] In humans, nitrates are reduced to nitrites by oral bacteria present in saliva, but this process is absent in rats.[1,15] Nitrate-to-nitrite conversion is therefore recognized to be a crucial factor in the assessment of nitrate and nitrite exposure, and this should be incorporated into the human risk assessment.[15] However, the majority of existing dietary nitrate/nitrite risk assessment studies have not fully considered the endogenous conversion of nitrates into nitrites in the body.[5,7,8,16] Currently, there are only a few studies using a specific percentage of nitrate-to-nitrite conversion to account for the total nitrite intake through exogenous nitrite exposure from the diet and the endogenous conversion from nitrates into nitrites in the saliva. A study in New Zealand adults found that the dietary risk of nitrites exceeds the ADI when using the nitrate-to-nitrite conversion factor of 5 or 20%.[17] The intake of nitrites in Swedish children may be of concern for young age groups when endogenous nitrite conversion is included in the intake estimates.[18]

However, the formation of nitrites in humans is a complex process, involving the uptake of nitrates and/or nitrites from food, the endogenous synthesis of nitrates, the secretion of nitrates from blood into saliva, the conversion of nitrates to nitrites by bacteria in saliva, and the reconversion of nitrites

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into nitrates in blood.[15] Therefore, a combined physiologically motivated toxicokinetic (TK) model to quantify the kinetics of internal doses of nitrates and nitrites in humans is recommended to achieve more reliable exposure estimates.[15] These above-mentioned complex processes have been described with a multicompartment TK model by Zeilmaker et al.[19] This model is adequate to simulate the kinetics of nitrates and nitrites after oral intake of nitrates in adult humans, but it does not consider the variability across age populations. To conduct better and more scientific risk assessment, an increasing number of studies have started using internal dose derived from either a biologically realistic TK or a physiologically based pharmacokinetic (PBPK) model to assess the health risk of chemicals, which has been shown to result in appropriate risk estimates.[20,21] The integration of internal dose metric derived from a TK or PBPK model with a probabilistic method has been recommended as a scientific approach for risk assessment by both European Food Safety Authority[22] and U.S. Environmental Protection Agency.[23]

To address the above-mentioned data gaps and study limitations, this study aimed to estimate the potential dietary risk of nitrates and nitrites due to vegetable consumption based on internal dose in a probabilistic manner by integrating exogenous exposure with the endogenous formation of nitrites using human TK modeling. To conduct such an internal-dose-based risk assessment, we optimized the previously established TK model,[19] validated our new model, and incorporated Monte Carlo (MC) simulations to account for variability across human populations for different age groups. The validated model was applied to conduct dietary risk assessments for different human populations from different countries/regions.

MATERIALS AND METHODS

Chemicals. Sodium carbonate (99%, reagent grade) and sodium bicarbonate (>99%, reagent grade) were purchased from Sigma-Aldrich (St. Louis, MO). Deionized water of 15 MΩ cm quality was prepared with a Pall Cascada Laboratory water system. A standard solution containing 1000 μg/mL nitrates and a standard solution containing 1000 μg/mL nitrites were purchased from AccuStandard (New Haven, CT). A multi-ion standard containing bromides, chlorides, fluorides, nitrates, nitrites, phosphates, and sulfates purchased from High-Purity Standards (North Charleston, SC) was used for identification of other analytes in the sample matrix.

Study Framework. Figure 1 illustrates a framework of the overall process of this study including dietary exposure investigation (Figure 1A), model optimization and validation (Figure 1B), the derivation of the internal dose equivalent to the ADI (Figure 1C), and dietary risk characterization (Figure 1D). Each process is described in detail below.

Core Food (CF) List for Nitrates and Nitrites. The food consumption data available from the Nutrition and Health Survey in Taiwan (NAHSIT) covering all ages (1 to 65+ years old) were used to establish the core food (CF) list for nitrates and nitrites in vegetables. In the NAHSIT, data on dietary intake and personal information of each participant were obtained through a face-to-face interview with a 24 h recall questionnaire.[24] In total, 44 567 consumption records of vegetables from 7556 respondents were aggregated into 45 CFs in nine categories [Table S1 in the Supporting Information (SI)] following the first CF methodology in Taiwan.[25] The mean consumption rate (CR) was calculated by the summation of all of the CRs of the surveyed vegetables in each CF divided by all respondents. A shortened CF list was created from the entire 45 CFs through the following criteria: (1) mean CR > 5 g/day and consumers percentage (CP) > 5% or (2) CP > 10%. The CR coverage of the shortened CF list relative to the extensive list of 45 CFs was then determined (i.e., a percentage ratio of the CRs in the shortened list out of the extensive list). A coverage of 80% or greater was considered to be representative of the sample list.[25]

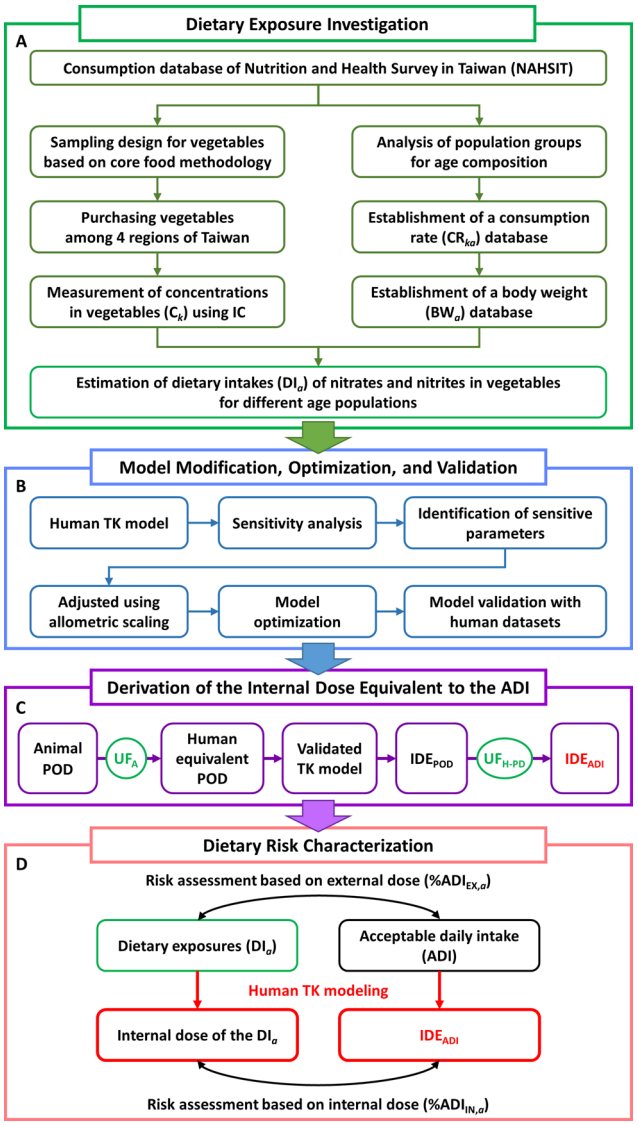

Figure 1. Schematic representation of the framework for chronic dietary risk assessment of nitrates and nitrites in vegetables. Abbreviations: TK, toxicokinetic; ADI, acceptable daily intake; POD, point of departure; UF_A, interspecies uncertainty factors; UF_{H-PD}, uncertainty factor for human variability in pharmacodynamic responses; IDE_{POD}, dose at the human equivalent POD; IDE_{ADI}, internal dose equivalent to the ADI.

Purchasing of Food Samples. Food samples were purchased randomly in four regions of Taiwan (north, central, south, and east) from September 2018 to February 2019. In each region, one major city with the largest population size was selected for sampling. A total of eight food samples of each CF were purchased from large retail stores, local supermarkets, traditional wet markets, night markets, and online stores. Purchased food samples were shipped at −15 °C to the analytical laboratory. All food samples were stored at −20 °C until analysis.

Sample Preparation and Chemical Analysis. All analyses were conducted in the Analytical Laboratory in the Super Micro Mass Research & Technology Center of the Cheng Shiu University in Taiwan, based on the official standard method (TFDAO0004.00) established by the Taiwan Food and Drug Administration. This center has been accredited by the Taiwan Accreditation Foundation under

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Table 1. Parameter Values after Model Optimization for the Human TK Model Used for Model Evaluation

parameter	symbol	unit	values
physiological parameters			
volume of the saliva compartment	VS	L	0.001[b]
volume of blood (fraction of BW)	Vb	unitless	0.079[c]
salivary flow rate	Qsal	L/h	normal (0.069, 0.0167)[b]
body weight	BW	kg	study specific
nitrate parameters			
dietary intake of nitrates	KuNO3	mmol/h	study specific
gastrointestinal absorption fraction	FaNO3	unitless	1[b]
gastrointestinal absorption rate constant	kaNO3	h^{-1}	5.35[b]
rate constant of endogenous nitrate synthesis	kend	mmol/h	0.198006[a]
overall elimination rate constant of nitrates from the central compartment	Skel	/(h $BW^{-0.25}$)	0.517331[a]
nitrate blood-to-saliva secretion rate	ksecNO3	h^{-1}	normal (0.045, 0.003)[b]
conversion rate of nitrates to nitrites in saliva	kconv	h^{-1}	normal (19.95, 1.5)[b]
volume of the central nitrate distribution compartment (fraction of BW)	FVNO3	unitless	normal (0.3, 0.0098)[b]
nitrite parameters			
dietary intake of nitrates	KuNO2	mmol/h	study specific
gastrointestinal absorption rate constant	SKaNO2	/(h $BW^{-0.25}$)	14.0004[a]
gastrointestinal absorption fraction	FaNO2	unitless	1[b]
nitrite blood-to-saliva secretion rate	ksecNO2	h^{-1}	normal (0.045, 0.003)[b]
rate constant of nitrite gastrointestinal decay to other products	Skdec	/(h $BW^{-0.25}$)	1.00242[a]
volume of the central nitrate distribution compartment (fraction of BW)	FVNO2	unitless	normal (0.65, 0.03)[b]
hemoglobin/methemoglobin parameters			
nitrite reaction rate constant with hemoglobin	kNO2	mM^{-1} h^{-1}	2.70321[a]
methemoglobin reductase maximum metabolic rate	SVmaxr	mM/(h $BW^{0.75}$)	123.326[a]
Michaelis–Menten constant of methemoglobin reductase activity	Kmr	mM	4589.93[a]
stoichiometric constant for regeneration of nitrates from methemoglobin	z	unitless	0.626123[a]
background concentration of hemoglobin oxidizing reactants in the blood	Cb	mM	8.04602×10^{-4} [a]
background concentration of hemoglobin in the blood	init CHB	mM	8.33752[a]
background concentration of methemoglobin in the blood	init CMetHg	mM	0.046[b]

[a]Sensitivity parameters. The values were calibrated by fitting the model to human data.[26] [b]Adopted from Zeilmaker et al.[19] [c]Adopted from Brown et al.[49]

the International Organization for Standardization (ISO) 17025 guidelines. All food samples examined in the study were prepared for a table-ready state before analysis, thus best representing the levels of nitrates and nitrites that would be consumed. The vegetables were boiled until cooked through. Each sample was homogenized for 3 min using the SCIENTZ-48 high-throughput tissue grinder with a frequency of 50 or 70 Hz. Approximately 5 g of the well-homogenized sample was mixed with 40 mL of deionized water in a 50 mL centrifuge tube and extracted in an ultrasonicator for 30 min. The centrifuge tube was incubated in a boiling water bath at 75 °C for 5 min, cooled down to room temperature, and then diluted to a final volume of 50 mL with deionized water. The diluted samples were filtered through a 0.45 μm membrane filter and analyzed by ion chromatography (Dionex DX-120 with Dionex IonPac AS9-HC 4 × 250 mm^2 column). The mobile phase was made up of sodium carbonate (6 mM) and sodium bicarbonate (2.5 mM) with a flow rate of 0.65 mL/min. The limit of quantification (LOQ) of both nitrates and nitrites was 1 mg/kg.

Dietary Exposure Assessment. Nitrates and nitrites occur naturally in the environment and can be distributed in food at very low concentrations. Thus, the nondetected samples were assumed to present a concentration equal to 1/2LOQ because it is not reasonable to assume that they are not present in the vegetables when the analytical results were less than the LOQ.[9] The data available from the NAHSIT was also used to establish the age-specific database including the CR of vegetables (Table S2 in the SI) and body weight (BW) (Table S3 in the SI) for preschoolers (2–6 years, n = 523), children (7–12 years, n = 1324), teenagers (13–18 years, n = 2546), adults (19–65 years, n = 2039), and elderly persons (>65 years, n = 893). Collectively, the dietary intake (DI) of nitrates or nitrites for a specific age population a (mg/kg bw per day) was estimated as follows

$$DI_a = \sum_{k=1}^{k} \frac{C_k \times CR_{ka} \times 10^{-3}}{BW_a} \quad (1)$$

where C_k is the concentration of nitrates or nitrites in a vegetable CF k (mg/kg), CR_{ka} is the consumption rate of a vegetable CF k for the specific age population a (g/day), BW_a is the body weight of the specific age population a (kg), and 10^{-3} is the unit conversion factor. To quantify the probabilistic distribution of DI_a, we implemented the random sampling Monte Carlo (MC) simulation. Lognormal distribution was assumed for C_k and CR_{ka}. The BW_a was assigned to be normally distributed. MC simulation was performed with 10 000 iterations via the Oracle Crystal Ball software (version 11.1, Oracle Corporation, Redwood Shores, CA) to ensure the stability of outcome distribution profiles. The simulation outcomes were presented as mean with a standard deviation (SD) or the 50th percentile with a 95% confidence interval (CI).

Human Toxicokinetic Model. The human TK model built upon the earlier model[19] simultaneously considered exogenous dietary exposures and endogenous formations of nitrates and nitrites to simulate their kinetics in the body (Figure S1 in the SI). A more detailed description of the TK model refers to Figure S1. Berkeley Madonna (version 8.3.9; University of California at Berkeley, Berkeley, CA) was used to implement the model and run all simulations. The model code is provided in the SI.

Model Sensitivity Analysis. To mitigate the uncertainty inherent in the estimation of model parameters from limited human data, we performed a sensitivity analysis to allow the limited human data to be used to optimize only the parameters that were most sensitive to the

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Table 2. Concentrations of Nitrates and Nitrites in Vegetables of the 21 Shortened Core Food List, Assuming Samples below LOQ Equal to 1/2LOQ[a]

	nitrates (mg/kg)					nitrites (mg/kg)				
vegetables (n = 8 each vegetable)	detection frequency (%)	mean	SD	GM	GSD	detection frequency (%)	mean	SD	GM	GSD
cabbage	100	312	164	273	1.76	75	2.83	1.03	2.49	1.94
Chinese cabbage	100	512	254	451	1.75	88	2.70	0.97	2.39	1.90
cauliflower and broccoli	100	118	94.2	68.9	3.90	88	2.82	1.98	1.88	3.01
sweet potato vines	100	545	274	488	1.67	63	8.20	14.1	3.88	3.33
Chinese mustard green	100	573	312	409	3.32	88	2.34	1.54	1.67	2.72
cruciferous leafy vegetables	100	1641	873	1347	2.22	63	1.48	1.39	0.77	4.47
asteraceae leafy vegetables	100	795	477	691	1.73	43	2.29	1.18	1.85	2.27
spinach	100	1153	874	814	2.81	75	2.35	1.21	1.88	2.29
celery	100	344	586	86.1	7.95	75	2.90	1.43	2.48	2.01
scallion and leek	100	212	213	107	4.61	88	2.70	1.49	2.09	2.47
tomato	100	17.7	9.25	15.4	1.81	75	1.44	1.01	1.09	2.30
bean sprouts	100	6.14	4.04	5.34	1.70	56	2.97	0.56	2.92	1.22
loofah	100	66.2	30.1	60.9	1.54	100	1.42	1.13	0.83	3.97
baby cucumber	100	138	86.1	113	2.02	50	2.28	0.77	2.05	1.79
carrot and daikon radish	100	311	461	97.4	5.41	88	1.26	1.40	0.81	2.47
bamboo shoot	100	62.1	79.3	37.4	2.82	13	1.55	1.46	1.02	2.66
onion	100	22.2	28.9	14.6	2.29	38	2.95	0.41	2.93	1.14
sweet potato	100	10.8	10.1	8.30	2.03	100	1.37	1.23	0.95	2.45
potato	100	71.8	41.5	60.6	1.91	38	1.27	1.08	0.92	2.32
shiitake mushrooms	100	7.52	3.22	6.90	1.58	38	1.65	1.05	1.29	2.25
Chinese fungus	100	3.27	0.63	3.21	1.21	63	1.44	0.99	1.14	2.13

[a]Detection frequency: the percent of results > limit of quantification (LOQ); SD: standard deviation; GM: geometric mean; GSD: geometric standard deviation.

available data in humans after exposure to a single oral dose of 324 mg of sodium nitrite.[26] Some of these parameters were adjusted based on BW using the standard allometric scaling[27] to account for the variability across age populations. Each of the original parameters available from Zeilmaker et al.[19] (Table S4 in the SI) was used to calculate normalized sensitivity coefficients (NSCs).[28] Parameters with |NSCs| ≥ 0.1 were considered sensitive and were therefore selected for model optimization. A more detailed description of the NSCs is provided in the SI (Section 1).

Model Optimization. Data used for model optimization were obtained from a human experimental study.[26] In this experiment, a single oral dose of sodium nitrite (324 mg) was administered to nine human volunteers (mean BW = 67 kg), and concentrations of nitrates and nitrites in plasma and levels of hemoglobin (Hb) and methemoglobin (MetHb) in the blood were measured for a period of 24 h. The parameter values listed in Table S4 were used as initial values in the model optimization. The sensitivity parameters (kel, kend, kaNO2, kdec, kNO2, Init CHB, z, Kmr, Cb, and Vmaxr), as shown in Table 1, were then calibrated using the Curve Fitting module in Berkeley Madonna and further optimized as needed by visually fitting model simulations to observed data.

Model Evaluation. The optimized model was used to generate the simulations of nitrate and nitrite levels in plasma, and then the simulation results were compared with the independent data sets using human subjects following dietary[29] and aqueous[30] exposures that have not been used in the model optimization. The parameter values used for model evaluation are given in Table 1, in which six parameters, including Qsal, ksecNO3, kconv, FVNO3, ksecNO2, and FVNO2, were assumed to be normally distributed. MC simulation was applied to estimate the effect of parameter uncertainty and variability in humans on model simulations. Each of the simulation was run for 1000 iterations in Berkeley Madonna to compute the mean plasma concentration and the SD. The 95% CI was then calculated based on the mean and SD. The mean absolute percentage error (MAPE) was used to evaluate the model performance. Additional details describing the human data[29,30] and MAPE are provided in the SI (Section 2).

IDE_{ADI} Derivation. On the basis of the no-observed-adverse-effect level (NOAEL) of 370 mg/kg bw per day for growth depression derived from a 2 year rat study and the uncertainty factors (UFs) of 100 (a factor of 10 for interspecies extrapolation and another factor of 10 to consider intraspecies variation), an ADI of 3.7 mg/kg bw per day for nitrates was determined.[14] An ADI of 0.07 mg/kg bw per day for nitrites was based on a NOAEL of 6.7 mg/kg bw per day for effects on the heart and lungs identified in a 2 year rat study as well as the UFs of 100. Following the methodology described in Hays et al.,[31] the internal plasma doses equivalent to the ADIs (i.e., IDE_{ADI}) for nitrates and nitrites were determined using the human TK model. In the derivation process, the point of departure (POD) (i.e., NOAEL) from a toxicological study in rodents was converted into human equivalent POD using interspecies uncertainty factors (UF_A) of 10. The validated human TK model was run by a chronic daily input of the dose at the human equivalent POD, and the resulting predicted steady-state plasma concentration (48 h) was defined as IDE_{POD}. During this simulation, age-population-specific BW was used and incorporated with MC simulation to compute the uncertainty of IDE_{POD_a} for a specific age population a. This value was then divided by the additional $UF_{H\text{-}PD}$ of 3.16 accounting for within-human variability in pharmacodynamic responses[23] to derive the IDE_{ADI_a} for different age populations.

Dietary Risk Characterization. Traditionally, the chronic dietary risks to the specific age population a of nitrates and nitrites in vegetables based on external dose metrics (expressed as the percent acceptable daily intake, $\%ADI_{EX_a}$) were calculated as follows

$$\%ADI_{EX_a} = \frac{DI_a}{ADI} \times 100 \quad (2)$$

where DI_a is the dietary intake of nitrates or nitrites for the specific age population a (mg/kg bw per day) and ADI values for nitrates and nitrites were 3.7 and 0.07 mg/kg bw per day, respectively.

For characterizing the internal-dose-based dietary risk, we input together lognormally distributed external DI_a estimates of nitrates and nitrites into the validated TK model to predict the plasma concentrations of nitrates and nitrites. There is not a built-in function

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in Berkeley Madonna to carry out MC simulations for lognormal distribution. Thus, the inverse natural logarithmic transformation of the "NORMAL" function was applied to produce lognormally distributed numbers.[32] Each MC simulation included 1000 iterations and was run for 48 h to achieve steady-state plasma concentrations. Then, the resulting distributed internal dose for the DI_a and the distributed IDE_{ADIa} were compared in terms of resulting internal-based risk assessment for a specific age population a ($\%ADI_{INa}$) as

$$\%ADI_{INa} = \frac{ID_{DIa}}{IDE_{ADIa}} \times 100 \quad (3)$$

where ID_{DIa} is the internal (plasma) dose in the ingestion route of nitrates and nitrites for the specific age population a (mmol/L) and IDE_{ADIa} is the plasma dose equivalent to the ADI for the specific age population a (mmol/L).

To perform the probabilistic risk assessment, we used a MC method with 10 000 simulations using Crystal Ball to quantify the uncertainty of the risk through the random sampling method from the probability distribution of each parameter, including C_k, CR_{ka}, BW_a, ID_{DIa}, and IDE_{ADIa}. Furthermore, to better understand the likely excessive dietary risk, we used a maximum probability of 1 to subtract the profile of cumulative density function, resulting in an exceedance risk profile, indicating the probability that the estimated %ADI exceeded a specified magnitude (e.g., the probability of %ADI > 100 or 200%).

Model Application for Different Human Populations. Model applications were conducted to estimate the internal-dose-based dietary risks of nitrates and nitrites for different populations from different countries/regions. Specifically, we incorporated the population-specific dietary intakes of nitrates and nitrites and age-specific BW available from France,[16] Australia,[9] Hong Kong,[5] and the U.S.[3] into the validated TK model to simulate the corresponding steady-state plasma concentrations at 48 h. The $\%ADI_{IN}$ and the probability of $\%ADI_{IN}$ > 100% can then be estimated using eq 3 by implementing the probabilistic risk assessment approach.

RESULTS

Concentrations of Nitrates and Nitrites in Vegetables. A total of 21 CFs (marked with grayscale) were selected from the entire 45 CFs as the shortened CF list (Table S1). The coverage of 81.4% based on mean CRs was calculated for the shortened CF list (184.8 g/day) compared to the extensive list with 45 CFs (227.1 g/day), indicating that the shortened CF list can represent the total diet of vegetables in Taiwan. A total of 168 vegetable samples in the shortened CF list were analyzed, and the values of nitrate and nitrite concentrations in 21 vegetable CFs are given in Table 2. Nitrates were detected in all vegetables with a detection frequency of 100%. Concentrations of nitrates in vegetables, in descending order, were cruciferous leafy vegetables (mean ± SD: 1641 ± 873 mg/kg), spinach (1153 ± 874 mg/kg), asteraceae leafy vegetables (795 ± 477 mg/kg), Chinese mustard green (573 ± 312 mg/kg), sweet potato vines (545 ± 274 mg/kg), and Chinese cabbage (512 ± 254 mg/kg). Vegetables containing lower levels of nitrates were shiitake mushrooms (7.52 ± 3.22 mg/kg), bean sprouts (6.14 ± 4.04 mg/kg), and Chinese fungus (3.27 ± 0.63 mg/kg). Nitrite levels in vegetables were generally low, with a detection frequency of 13−100%. Sweet potato vines had the highest nitrite level (8.20 ± 14.1 mg/kg), followed by bean sprouts (2.97 ± 0.56 mg/kg), onions (2.95 ± 0.41 mg/kg), and celery (2.90 ± 1.43 mg/kg), whereas potato (1.27 ± 1.08 mg/kg) and carrot and daikon radish (1.26 ± 1.40 mg/kg) had the lowest nitrite levels.

Dietary Exposures to Nitrates and Nitrites from Vegetables. The results showed that the order of DI estimates of nitrates was preschoolers ≥ elderly persons > children > adults > teenagers (Figure 2A). The DIs of nitrates for preschoolers and elderly persons were (median: 1.37, 95% CI: 0.29−11.94 mg/kg bw per day) and (1.49, 0.33−11.6 mg/kg bw per day), respectively. The higher DI estimates of nitrites were found in preschoolers (0.010, 0.003−0.056 mg/kg bw per day) and elderly persons (0.010, 0.003−0.062 mg/kg bw per day) (Figure 2B). The DI estimates for each age population are provided in Table S5 in the SI.

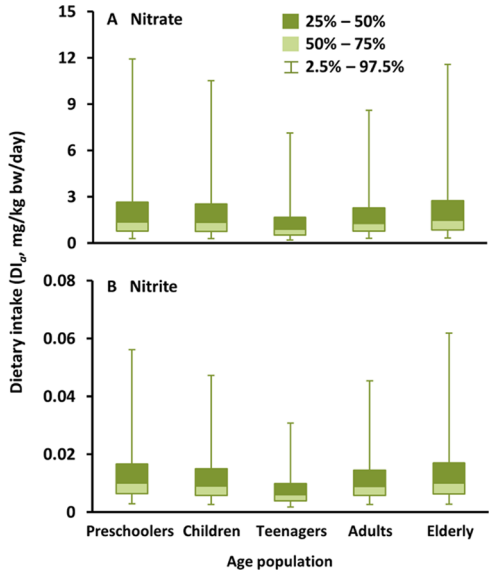

Figure 2. Estimated dietary intakes of (A) nitrates and (B) nitrites for different age populations.

To identify what kind of vegetables contributes the most to the total dietary exposure of nitrates and nitrites, we calculated the relative percent contribution of each vegetable species to the DI estimates, as shown in Figure 3. Cruciferous leafy vegetables were the main contributor to the nitrate intake for all age populations (32.95−47.29%) (Figure 3A). The other more significant contributors were cabbage for all age populations (9.63−13.77%), asteraceae leafy vegetables for adults (12.68%) and elderly persons (11.72%), and sweet potato vines (11.73%) and spinach (11.54%) for elderly persons (Figure 3A). As seen in Figure 3B, cabbage contributed the most to the nitrite intake for preschoolers (27.17%), children (24.39%), and teenagers (27.52%), followed by cruciferous leafy vegetables (11.58−13.62%) and sweet potato vines (8.12−11.47%). Figure 3B also shows that sweet potato vines were the major contributor to nitrite intake for adults (22.17%) and elderly persons (27.43%), followed by cabbage (17.26−21.01%).

Model Sensitivity Analysis. Only parameters with at least one |NSCs| ≥ 0.1 are shown in Figure S2 in the SI. A total of 10 parameters were identified as sensitive, including the overall elimination rate of nitrates from the central compartment (kel), the rate of endogenous nitrate synthesis (kend), GI absorption rate (kaNO2), the rate of nitrite GI decay to other products (kdec), nitrite reaction rate with Hb (kNO2), background concentration of Hb in the blood (Init CHB), stoichiometric constant for regeneration of nitrates from MetHb (z), Michaelis−Menten constant of MetHb reductase activity (Kmr), background concentration of Hb oxidizing reactants in the blood (Cb), and MetHb reductase maximum (Vmaxr).

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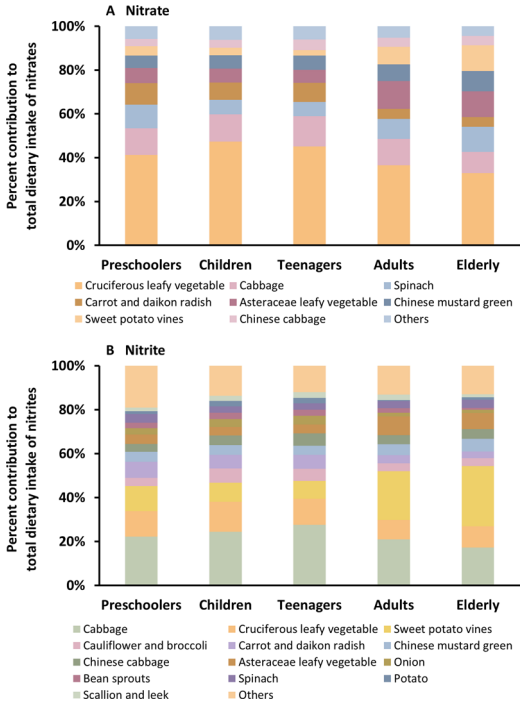

Figure 3. Percent contribution of each vegetable to the average dietary exposure of (A) nitrates and (B) nitrites.

Model Optimization. Among these 10 sensitive parameters, kel, kdec, kaNO2, and Vmaxr were set to be a function of BW using allometric scaling. The first-order rate constants kel, kdec, and kaNO2 were scaled using an allometric function of $BW^{-0.25}$, and the Vmaxr was scaled to $BW^{0.75}$. The BW-scaled four parameters and the remaining six parameters were then optimized using human data on nitrate and nitrite concentrations in plasma and MetHb and Hb levels in the blood.[26] The results of the model optimization for these 10 sensitive parameters in comparison to observed data are shown in Figure S3 in the SI. The optimized values of these 10 parameters are listed in Table 1.

Model Evaluation. The final parameters (Table 1) were used to predict nitrate and nitrite concentrations in plasma and then to compare the predictions with independent data sets. Figure 4A–C illustrates the comparisons of plasma nitrate concentrations between predictions and observed data in human volunteers exposed to various doses of nitrates in spinach (654 mg, Figure 4A), beetroot (643 mg, Figure 4B), and lettuce (1013 mg, Figure 4C).[29] In addition, the simulation results of nitrates (Figure 4D) and nitrites (Figure 4E) in plasma were compared with the measured concentration data from human volunteers exposed to an aqueous nitrite solution.[30] The MAPE values for panels A, B, C, D, and E of Figure 4 were 21.37, 24.26, 34.16, 18.31, and 19.87%, respectively, demonstrating that the present TK model was able to predict the available independent data following oral exposure with acceptable accuracies.

Dietary Risks of Nitrates and Nitrites from Vegetables. The plasma concentrations associated with external DIs (ID_{DIa}) and ADIs (IDE_{ADIa}) by age populations simulated from the TK model are given in Table S6 in the SI. The results based on external dose showed that 50th percentile $\%ADI_{EXa}$ estimates of nitrates (Figure 5A) and nitrites (Figure 5B) were all lower than 100%, even at the 97.5th percentile for nitrites. In contrast, the results based on internal dose ($\%ADI_{INa}$) presented a higher risk at the 50th percentile than the $\%ADI_{EXa}$ for all age groups for both nitrates and nitrites. In particular, the 50th percentile $\%ADI_{IN}$ of nitrates in preschoolers had the potential to exceed 100% (Figure 5A). We also found that the 97.5th percentile $\%ADI_{INa}$ of nitrites had the potential to exceed 100% in preschoolers, children, and elderly persons (Figure 5B).

Figure 6 and Table S7 in the SI present the exceedance risks assessed by both external dose and internal dose metrics, indicating that preschoolers had higher dietary risks of nitrates and nitrites than the other age populations. For preschoolers, the probabilities of $\%ADI_{EX} > 100\%$ were 0.179 for nitrates (Figure 6A) and 0.034 for nitrites (Figure 6B), whereas the probabilities of $\%ADI_{IN} > 100\%$ were 0.553 for nitrates (Figure 6C) and 0.228 for nitrites (Figure 6D). Although the 97.5th percentile $\%ADI_{EXa}$ of nitrates in all age populations had the

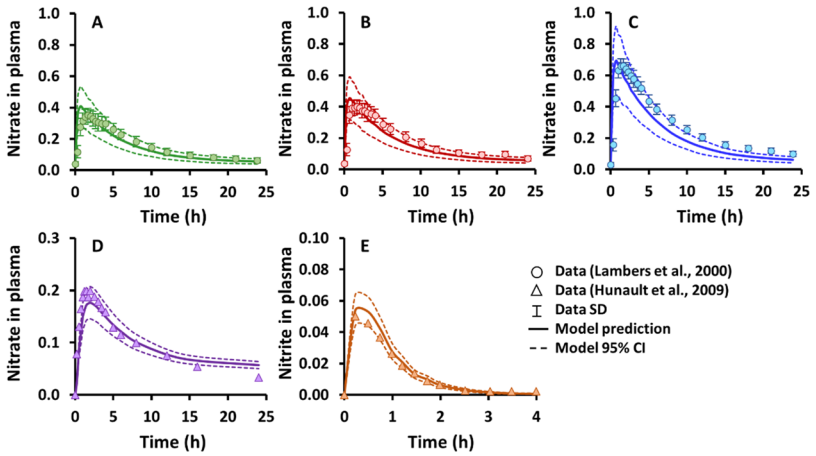

Figure 4. Comparison of nitrate and nitrite concentrations in plasma (mM) between model simulations and experimental data in human volunteers exposed to (A–C) sodium nitrate in vegetables and (D, E) sodium nitrite in an aqueous solution. For the vegetable exposure experiment, the nitrate concentration in plasma was analyzed from 12 human volunteers given 300 g of (A) spinach with 564 mg of nitrates, (B) beetroot with 643 mg of nitrates, and (C) lettuce with 1013 mg of nitrates.[29] For the solution experiment, (D) nitrate and (E) nitrite concentrations in plasma were analyzed from nine human volunteers given an aqueous nitrite solution with dose ranging from 290 to 380 mg.[30]

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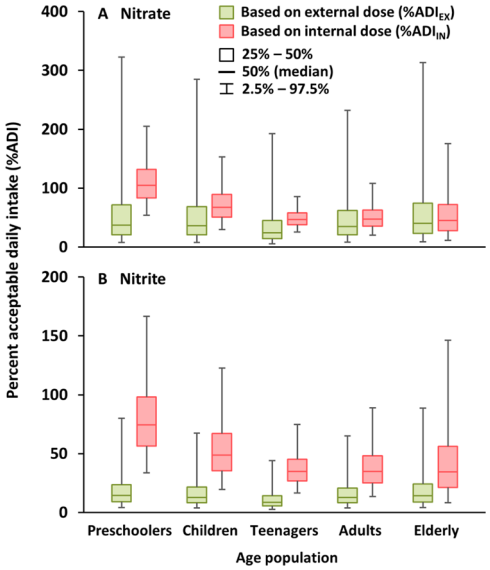

Figure 5. Estimated chronic dietary risks (%ADI) of (A) nitrates and (B) nitrites because of vegetable consumption for different age populations based on external and internal dose metrics.

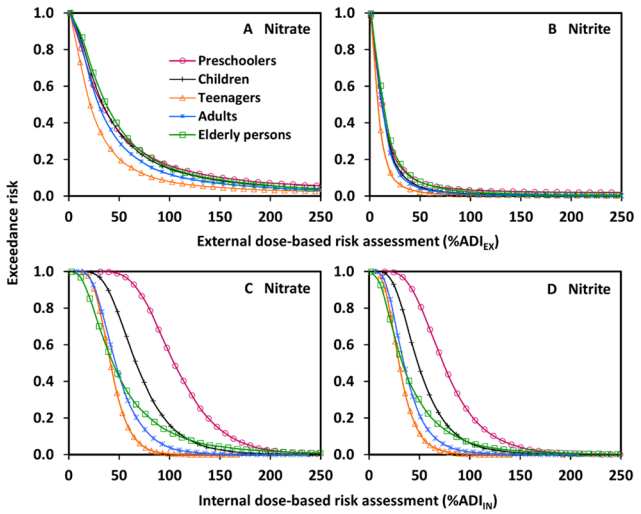

Figure 6. Exceedance risk profiles of (A) external-dose-based risk assessment (%ADI_{EX}) and (B) internal-dose-based risk assessment (%ADI_{IN}) for different age populations.

potential to be >200% (Figure 5A), the probabilities of %ADI_{EXa} of nitrates >200% (range: 0.033−0.072) were all less than 0.1 (Figure 6A), indicating that the high-end risk was very unlikely.[33]

Model Application To Estimate Dietary Risks for Different Human Populations. Table 3 demonstrated that Australian preschoolers were the high-risk population and had approximately 0.490 and 0.237 probabilities of %ADI_{IN} > 100% for nitrates and nitrites, respectively. Moreover, the probabilities of %ADI_{IN} > 100% of nitrates and nitrites for adults in France, Australia, and the U.S. (range: <0.001−0.012) were extremely unlikely (<0.05),[33] except for the Hong Kong adults who had a slightly higher probability of 0.113.

DISCUSSION

This study provides the first dietary exposure estimation of nitrates and nitrites from vegetables for different age populations in Taiwan, based on a comprehensive investigation of concentrations in vegetables and on the long-term massive consumption database from NAHSIT. This is also the first study integrating the exogenous dietary exposure, an endogenous formation simulation using a physiologically motivated TK model, and the probabilistic approach to predict the internal dose for estimating the potential dietary risk caused by nitrates and nitrites in vegetables. The present model can be applied in risk assessment to obtain more reliable exposure estimates (i.e., internal doses) and to improve the accuracy of dietary risk assessment of nitrates and nitrites. The model code is provided in the SI to allow other researchers to reproduce our results and to facilitate the application of this model to other studies.

Generally, vegetables can be divided into three groups based on their nitrate concentrations: low nitrate (<100 mg/kg), medium nitrate (100−1000 mg/kg), and high nitrate (>1000 mg/kg).[4] In this study, medium and high levels of nitrates were detected in leafy vegetables including cruciferous leafy vegetables, asteraceae leafy vegetables, spinach, Chinese mustard green, and sweet potato vines (range of means: 545−1641 mg/kg). Our results are in agreement with those reported in most studies.[5,9,16,34,35] We also found that Chinese cabbage and cabbage had concentrations ranging between 100 and 1000 mg/kg, which is similar to the finding in Australia[9] and New Zealand.[17] In contrast, nitrate levels in tomato, loofah, bamboo shoot, onion, sweet potato, and potato were lower (10.8−66.2 mg/kg), especially in mushrooms/fungi and bean sprouts (<10 mg/kg). This finding is also consistent with recent research, which showed that tomatoes, onions, sweet potatoes, potatoes, mushrooms, and bean sprouts contained relatively low levels of nitrates among the 66 vegetable species.[34] Our investigation found that nitrite concentrations were generally low with higher levels (>5 mg/kg) detected in sweet potato vines (42.9 mg/kg, n = 1), celery (5.79 mg/kg, n = 1), and broccoli (5.23 mg/kg, n = 1). Nitrite concentrations generally tend to be low in vegetables in China [not detected (ND)—35.0 mg/kg][36] and New Zealand (ND—27 mg/kg).[17]

In this study, the concentrations of nitrates and nitrites in vegetables did not show significant regional differences in Taiwan (Table S8 in the SI); however, a significant difference in nitrate levels of vegetables across geographic regions was observed in the U.S.[10] Moreover, in the U.S. study, it was found that organic vegetables were numerically lower in nitrate content than their conventional counterparts.[10] Multiple factors may influence nitrate levels in vegetables, including the natural nitrate content of soil, geographic conditions, cultivar type, level of nitrogen fertilizers applied, season, and light intensity.[10,34] We thus suggest that future studies are needed to explore whether regional differences and product types affect the dietary risk of nitrates and nitrites.

When comparing the results of dietary risks in different human populations (Table 3), one should be cautious about the representativeness of the samples that is dependent on the sampling methods, as there are large variations in concentrations among vegetables, and the consumption patterns across populations should also be noted. Based on the measured concentrations in 21 CFs and the use of external dose, our estimated dietary risks to nitrates (only the 97.5th percentile value was twice higher than the ADI) and nitrites (all percentile values were below 100%) through vegetables for adults are similar to the findings in Hong Kong adults[5] (Table 3), which may be due to similar consumption patterns between

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Table 3. Model Application To Estimate the Dietary Risk of Nitrates and Nitrites in Vegetables by Age Groups from Different Populations[a]

			nitrates				nitrites				
country	population	BW (kg)	dietary intake (mg/kg bw per day)	$\%ADI_{EX}$[b]	$\%ADI_{IN}$[c]	$P(\%ADI_{IN}) > 100\%$[d]	dietary intake (mg/kg bw per day)	$\%ADI_{EX}$[b]	$\%ADI_{IN}$[c]	$P(\%ADI_{IN}) > 100\%$[d]	method of dietary survey
France[16]	children (3−14 years)	30	mean: 1.4	mean: 37.8	mean: 73.3	0.007	mean: 0.00003	mean: 0.05	mean: 54.2	0.014	total diet study
			P97.5: 4.9	P97.5: 132	P97.5: 94.3		P97.5: 0.11	P97.5: 183	P97.5: 92.8		
	adults (>15 years)	60	mean: 1.1	mean: 29.7	mean: 47.5	<0.001	mean: 0.00003	mean: 0.05	mean: 34.5	0.001	
			P97.5: 3.1	P97.5: 83.8	P97.5: 60.6		P97.5: 0.06	P97.5: 100	P97.5: 66.7		
Australia[9]	preschoolers (2−5 years)	18	mean: 1.14	mean: 30.9	mean: 103	0.490	mean: 0.05	mean: 75.9	mean: 80.3	0.237	food samples were selected based on the Australian total diet study
			P90: 2.00	P90: 54.0	P97.5: 156		P90: 0.09	P90: 122	P97.5: 166		
	children (6−12 years)	36	mean: 0.91	mean: 24.6	mean: 63.0	0.099	mean: 0.04	mean: 51.0	mean: 50.8	0.072	
			P90: 1.69	P90: 45.6	P97.5: 132		P90: 0.06	P90: 88.0	P97.5: 133		
	teenagers (13−16 years)	61	mean: 0.70	mean: 19.0	mean: 40.4	0.001	mean: 0.02	mean: 34.5	mean: 31.6	<0.001	
			P90: 1.34	P90: 36.1	P97.5: 64.2		P90: 0.04	P90: 54.2	P97.5: 59.7		
	adults (≥17 yeas)	74	mean: 0.78	mean: 21.0	mean: 35.5	0.012	mean: 0.02	mean: 30.6	mean: 28.1	<0.001	
			P90: 1.55	P90: 42.0	P97.5: 84.5		P90: 0.04	P90: 56.8	P97.5: 69.3		
Hong Kong[5]	adults	60	mean: 3.5	mean: 94.6	mean: 60.4	0.113	mean: 0.0038	mean: 5.4	mean: 44.6	0.043	total diet study
			P95: 10	P95: 270	P97.5: 148		P95: 0.015	P95: 21.7	P97.5: 112		
United States[3]	adult men (50−71 years)	85.5 (19.0)[e]	median: 0.48	median: 13.1	mean: 34.5	<0.001	median: 0.009	median: 13.4	mean: 27.2	<0.001	food frequency questionnaire
			P75: 0.75	P75: 20.3	P97.5: 47.6		P75: 0.013	P75: 18.4	P97.5: 45.5		
	adult women (50−71 years)	74.6 (19.3)[e]	median: 0.73	median: 19.8	mean: 35.7	<0.001	median: 0.009	median: 13.4	mean: 27.8	<0.001	
			P75: 1.15	P75: 31.1	P97.5: 54.7		P75: 0.012	P75: 17.3	P97.5: 50.6		

[a]P97.5: 97.5th percentile; P90: 90th percentile; P95: 95th percentile; P75: 75th percentile. [b]Estimated based on the point value of dietary intake divided by ADI. [c]Estimated based on internal dose in a probabilistic manner. [d]$P(\%ADI_{IN})$ > 100% is the probability of $\%ADI_{IN}$ greater than 100%. [e]Mean (SD) adopted from the Exposure Factors Handbook.[50]

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Hong Kong and Taiwan. Our estimated $\%ADI_{EX}$ for adults was higher than that of American adults[3] (Table 3), which may be due to higher consumption of vegetables by Taiwan adults, resulting in higher dietary exposure of Taiwanese (P75: 2.29 mg/kg bw per day for nitrates and 0.015 mg/kg bw per day for nitrites) than that of Americans (P75: 0.75−1.15 mg/kg bw per day for nitrates; 0.012−0.013 mg/kg bw per day for nitrites).

Our results revealed that preschoolers were the high-risk population and had the potential of $\%ADI_{EX}$ exceeding 100%. Young children consume more food compared to adults when expressed as per kg BW, resulting in relatively higher exposures to nitrates and nitrites than adults. Although age stratification makes result comparison difficult, the dietary risks of nitrates and nitrites for French children aged 3−14 years with high dietary exposures (97.5th percentile) were 132 and 183% of the ADI, respectively (Table 3).[16] This study also demonstrated that the dietary risks of nitrates and nitrites based on internal dose were obviously different from that based on external dose. The risks estimated using the internal dose were increased, especially for preschoolers, mainly due to the simultaneous inclusion of both exogenous dietary exposures and endogenous formations of nitrates and nitrites and the consideration of the variability of sensitive model parameters across age populations using allometric scaling. The present approach was also applied to different human populations, and the results showed that the probabilities of $\%ADI_{IN}$ of nitrates and nitrites >100% for preschoolers were similar to those in Australia (Table 3).

The health outcomes of dietary nitrate and nitrite exposure have been a matter of debate for many years because their metabolite nitric oxide (NO) is considered to be beneficial to human health. A rapidly growing body of scientific evidence supports the benefits of physiology, nutrition, and therapeutics associated with dietary intake of nitrates/nitrites.[2,37−39] The most well-studied benefit of dietary nitrates/nitrites is associated with the cardiovascular protective effects due to the improvement of myocardial function in heart failure and the reduced blood pressure.[38,40] The mechanism for the protective effects is mainly mediated by the NO generation via two pathways: (1) the classical L-arginine−NO pathway and (2) the nitrate−nitrite−NO pathway (Figure 7).[2,38,39] The NO produced from the dietary intake of nitrates/nitrites via the nitrate−nitrite−NO pathway has been considered to be an important role in maintaining NO homeostasis when the NO production from the L-arginine−NO pathway is insufficient.[2,38,39] In view of this benefit, the need to consider nitrates and nitrites as potential nutrients has been suggested, as they play a critical dietary role in compensating for the inability of the endothelium to convert L-arginine into NO due to aging.[38]

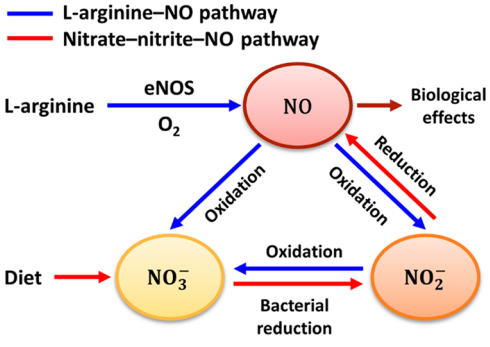

Figure 7. Human nitrogen cycle. The L-arginine−nitric oxide (NO) pathway: the endothelial nitric oxide synthase (eNOS) catalyzes the formation of NO from L-arginine. Once formed, NO can undergo oxidation to reform both nitrites and nitrates where the cycle continues. The nitrate−nitrite−NO pathway: the ingested nitrate from a diet can be reduced to nitrites by oral bacteria and subsequently to NO. This human nitrogen cycle was adapted from Bryan and Ivy[38] and Bryan and Loscalzo.[39]

It is important to study whether the potential health risks of dietary nitrates/nitrites outweigh the discovered health benefits. Based on a review of the available epidemiological data, Milkowski et al.[41] concluded that the inconclusive data on the cancer risk of nitrates/nitrites are far outweighed by the potential benefits of restoring NO homeostasis; thus, the authors suggest that the risk−benefit balance should be a strong consideration for new regulatory or public health guidelines. In recent years, the findings from the original animal studies used as the basis of the JECFA ADI have been questioned and discounted.[38,42] Applying the Benefit−Risk Analysis for Foods framework and based on a POD value of 1721 mg/kg bw per day for BW changes from a 2 year rat study, Wikoff et al.[42] derived an alternative ADI of 17.2 mg/kg bw per day, which would allow benefits to be realized while still protecting public health. This POD value was then used instead of the JECFA value into the TK model to obtain the IDE_{POD} for estimating the dietary risk following eq 3, resulting in the probabilities of $\%ADI_{IN}$ of nitrates and nitrites for preschoolers to be less than 0.001, indicating that the current exposure levels of nitrates and nitrites from vegetables for preschoolers do not present an appreciable safety risk. This brings clear issues related to the current ADI, which can be used to derive the suggestions of what and how much of specific vegetables a particular age population should consume.

This study has several limitations. Due to the lack of data, model application to children primarily relied on using allometric BW scaling. Allometry is a reasonable method for children >2 years of age[43] and has been used to scale a validated adult model to children.[44,45] In this study, model optimization was performed only for sensitive parameters. This approach is beneficial in terms of avoiding the uncertainty inherent in estimating many model parameters from limited human data. Due to the lack of age-specific data, model optimization for sensitive parameters and model validation were conducted using only adult human data. Although the validated model well predicted the plasma concentrations of nitrates and nitrites, and can be scaled to children, internal dose data in children, once available, could be used to improve the present model. Currently, there are no experimental data from humans that are granular enough to be used for the development and validation of a NO compartment to assess the impacts of different factors on the endogenous NO production and its bioavailability, such as the sulfate level in water,[46] the consumption of thiocyanate-containing vegetables[47] or high-fat meal,[48] age, fitness status, smoking, dietary supplement use, dietary macronutrient and micronutrient composition, abundance of bacterial nitrate reductases on the tongue, stomach acidity, the use of antiseptic mouthwash or antibiotics, and patients suffering from atherosclerosis, diabetes, and obesity.[2,38,39] When relevant data are available, these factors can be considered to improve this model.

In conclusion, the assessment results are different based on external versus internal dose, suggesting that it is critical to

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include the kinetic process of the endogenous formation of nitrites into the risk assessment of nitrates and nitrites. The results using current ADIs and internal dose suggest that nitrate and nitrite exposure from vegetables is unlikely to result in appreciable safety risk for most age populations but may be a potential concern for preschoolers. Dietary intake of nitrates and nitrites has been associated with potential risks and health benefits. Thus, there is a need for risk−benefit analysis of dietary exposure to nitrates and nitrites in the future food safety assessment and in the determination of new public health regulatory limits for ADI.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.9b06720.

Model sensitivity analysis; model evaluation; the TK model code; structure of the human TK model of nitrates and nitrites; normalized sensitivity coefficients of parameters; model optimization results; list of core foods, mean consumption rate, and consumer percentage for nitrates and nitrites in vegetables in Taiwan; consumption rate of 21 vegetable core foods by age populations; body weight of age populations in Taiwan; original parameter values for the human TK model; dietary intake estimates of nitrates and nitrites for different age populations; predicted values of internal plasma dose equivalent to ADI and plasma dose of dietary intake for nitrates and nitrites; exceedance risks assessed by external dose and internal dose metrics; and nitrate and nitrite concentrations in vegetables in regions of Taiwan (PDF)

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Author Contributions

Y.-J.L. and Z.L. conceived and designed the study. C.-J.C. contributed to the NAHSIT database analysis, food purchasing, and sample preparation. J.-W.C. conducted the chemical analysis. Y.-J.L. contributed to data analysis, TK modeling, and risk assessment. Y.-J.L. drafted the manuscript. Z.L. mentored and coordinated the project, checked the model code, provided the facilities to conduct the computational analysis, and comprehensively revised the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

$\%ADI_{EX}$, external-dose-based percent acceptable daily intake; $\%ADI_{IN}$, internal-dose-based percent acceptable daily intake; ADI, acceptable daily intake; BW, body weight; CF, core food; CI, confidence interval; CP, consumers percentage; CR, consumption rate; DI, dietary intake; Hb, hemoglobin; ID_{DI}, internal dose at the dietary intake; IDE_{ADI}, internal doses equivalent to the ADI; IDE_{POD}, dose at the human equivalent POD; JECFA, Joint FAO/WHO Expert Committee on Food Additives; LOQ, limit of quantification; MAPE, mean absolute percentage error; MC, Monte Carlo; MetHb, methemoglobin; NAHSIT, Nutrition and Health Survey in Taiwan; ND, not detected; NOAEL, no-observed-adverse-effect level; NSCs, normalized sensitivity coefficients; PBPK, physiologically based pharmacokinetic; POD, point of departure; SD, standard deviation; SI, Supporting Information; TK, toxicokinetic; UF_A, interspecies uncertainty factors; $UF_{H\text{-}PD}$, uncertainty factor for human variability in pharmacodynamic responses; UFs, uncertainty factors; U.S., United States

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