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Global distribution of ustiloxins in rice and their male-biased hepatotoxicity☆

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ABSTRACT

Ustiloxins, a group of bioactive metabolites produced by the pathogen of rice false smut (RFS), have emerged as ubiquitous contaminants in RFS-occurred paddy fields and could accumulate in rice. Nevertheless, the prevalence of ustiloxins in rice and exposure risks of humans are limited. In this study, concentrations of ustiloxin A (UA) and ustiloxins B (UB), which are two predominant ustiloxins, were measured in 240 rice samples from China and 72 rice samples from 12 other counties. The detection rates (DRs) of UA and UB were 82.1% and 49.3%, respectively, and their concentrations in rice ranged from below detection limit (LOD: 0.22 μg/kg) to 85.96 μg/kg dw. Furthermore, for the first time, we reported the occurrence of UA (DR = 22.8%) in urine collected from residues of Enshi city, China. Urinary UA were significantly correlated with the activities of alanine aminotransferase in male, and this male-biased hepatotoxicity was further confirmed in mice exposure experiment. This study for the first time reported the widespread geographical distribution of ustiloxins in rice, as well as emphasized the occurrence of internal exposure and potential health risk in humans.

1. Introduction

Over the last decades, rice false smut (RFS) has been recognized as one of the most destructive fungal diseases and has been causing widespread concerns worldwide due to its ability to cause massive yield loss and discharge various bioactive metabolites (Sun et al., 2020; Qiu et al., 2019; Lu et al., 2015). Ustiloxins, chiefly including ustiloxin A (UA) and ustiloxin B (UB) (Kioso et al., 1992; Wang et al., 2016) (Table S1), are a major type of mycotoxins produced by the pathogen of RFS and mainly distribute in the powdery chlamydospores, which can be easily discharged into surrounding environments through air current and rain splash, causing new environmental challenges (Fan et al., 2016; Chen et al., 2020). Specifically, ustiloxins have been reported to be universally existed in surface waters (Cheng et al., 2019), rice straws (Miyazaki et al., 2009) and rice grains (Fu et al., 2017a, 2017b; Cao et al., 2016) in RFS-occurred paddy fields. More importantly, contamination of ustiloxins in peeled rice samples collected from RFS-uninfected panicles was founded (Fu et al., 2015), indicating that the uptake and translocation of ustiloxins in rice plants might occur. Globally, the outbreak of RFS has been reported in more than 40 countries (Ladhalakshmi et al., 2012), indicating the environment and human exposure to ustiloxins might be ubiquitous. Furthermore, ustiloxins have been identified as potent antimitotic agents in eukaryotic cells and can cause various adverse effects on animals (Koiso et al., 1994; Li et al., 2008). Toxicological studies have demonstrated that UA could cause malformation and necrosis in *Tetrahymena thermophila* (Cheng et al., 2019) and damage multiple organs and cause reproductive toxicity in aquatic organisms, poultry and rodents (Hu et al., 2019; Nakamura et al., 1994). However, hampered by the limited availability of pure UB, the toxicological studies of UB were serious lacking (Koiso et al., 1994). It was

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reported that the IC_{50} of UB towards human tumor cell lines and the inhibitory effect of UB on the polymerization of microtubule proteins were comparable with UA, suggesting that UB might have similar toxic effect *in vitro* as UA. Given the increasing infection frequency of RFS in rice growing areas worldwide, the exposure of ustiloxins and their risks to human health are of great concerns.

Rice ingestion has been considered as the most important source of ustiloxins to general population (Sun et al., 2020; Hu et al., 2018). Several studies have reported the occurrence of ustiloxins in brown rice from FRS-occurred paddy field (Cao et al., 2016; Fu et al., 2017a, 2017b; Hu et al., 2018). Likewise, high concentrations of UA were detected in peeled rice from local supermarket of Heilongjiang province (15–56 μg/kg) (Fu et al., 2015). These results demonstrate high dietary exposure risk of humans to ustiloxins. In addition, our recent work (Sun et al., 2021) has revealed the presence of UA in urine of mice after intragastric exposure, providing evidence that ustiloxins in food have the opportunity to pass through the gastrointestinal tract. However, the occurrence and distribution of ustiloxins in polished rice at large geographic scale is largely lacking and the magnitude of human burden remains unknown.

To fill this knowledge gap, we conducted the first worldwide survey to uncover the spatial distribution of ustiloxins in polished rice samples from 25 provinces/city across China and 12 other rice-planting countries. In addition, human exposure doses to ustiloxins were assessed by monitoring ustiloxins in serum and urine samples collected from residents of Xianfeng (Enshi City, Hubei Province, China), where was suffered serious RFS during the last years. Meanwhile, to explore potential health risks of ustiloxins exposure, the relationship of internal ustiloxin exposure with liver damage was evaluated, and a sub-chronic exposure experiment in mice was conducted to conformed our findings in humans.

2. Materials and methods

2.1. Reagents

Ustiloxin A (UA) and ustiloxin B (UB) were isolated and purified from rice false smut balls according to a previous work (Sun et al., 2021), and the purity was >95%. Methanol (MeOH), acetonitrile (ACN), dichloromethane (DCM), formic acid (FA), ammonium acetate (AA) and ammonium hydroxide (NH_4OH) (LC-MS grade) were obtained from Honeywell (Seelze, Germany). CCl_3COOH was purchased from Aladdin Corporation (Shanghai, China). Ultrapure water was from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Oasis HLB (60 mg, 3 cc) and CNW BOUD WAX (200 mg, 3 cc) SPE cartridges were obtained from Waters (Wexford, Ireland) and Anpel laboratory Technologies (Shanghai, China), respectively. The commercial assay kits for determining serum activities of ALT and AST were purchased from Solarbio, Life Sciences, China.

2.2. Rice survey

The nationwide survey of rice was conducted from October to December of 2019. Two hundred and forty polished rice samples with different origins were collected from 25 provinces and city, covering about 80% of rice producing areas in China. The rice samples were collected directly from paddy fields during August to October of 2019 with the help of the relatives of college students in Huazhong Agricultural University. For each province, at least 7 rice samples were collected from more than 3 different cities. The 25 provinces/city were located in six different rice-cultivating regions in China, i.e., South China, Southwest China, Central China, Northeast China, North China and Northwest China. Fujian, Guangdong, Guangxi and Hainan are representative provinces of the South China; Guizhou, Sichuan, Yunnan and Chongqing are representative provinces or city of the Southwest China; Anhui, Hunan, Hubei, Jiangsu, Jiangxi and Zhejiang are representative provinces of the Central China; Hebei, Henan, Shandong and Shanxi are representative provinces of the North China; Heilongjiang, Jilin and Liaoning are representative provinces of the Northeast China; Ningxia and Xinjiang Uygur Autonomous Region are representative provinces of the Northwest China. For rice produced oversea, seventy-two samples were purchased from E-commerce sites (Amazon and Taobao). Samples were stored in refrigerator (−20 °C) once arrived at our laboratory.

Table 1
The demographic characteristics of residents recruited in this study.

Group [a]	N	Age			BMI [b]		
		Range	Mean	SD	Range	Mean	SD
Male	41	40–78	62	10	18.4–27.5	23.5	2.2
Female	51	29–80	58	12	18.0–25.7	21.5	1.9

[a] All participants have no specific disease symptoms.
[b] Body Mass Index.

2.3. Sampling of serum and urine from residents

In this study, two villages named as Qingshan (29°43′ 1″ N, 109°11′ 57″ E) and Laozhai (29°36′ 5″ N, 109°8′ 43″ E) located in Enshi City (Hubei Province, China) were selected because serious ustiloxin contamination was found in the rice produced there (mean: 1.61 μg/kg) according to our previous work (unpublished data). The residents there were supposed to suffer from ustiloxin exposure with relatively high frequency. In the October of 2019, ninety-two volunteers (51 female and 41 male) were recruited from the two villages who habitually used rice produced and harvested by themselves as staple food. This study was carried out under the ethical approval from Human Ethics Committee of Huazhong Agriculture University (HZAUHU-2019-001), and each participant provided informed consent. Fasting blood (10 mL) and morning urine (5–10 mL) samples were collected at the People's Hospital of Xianfeng Country. Each blood sample was collected separately in two vacutainers, one for the detection of blood biochemical indexes in hospital and the other for ustiloxin analysis. The serum samples were transported with dry ice and stored in −80 °C once arrived at our laboratory. The urine samples were centrifuged (5000 rpm, 5 min) and transported with dry ice and stored in −20 °C once arrived at our laboratory. The demographic characteristics of the recruited residents are shown in Table 1.

2.4. Sub-chronic exposure experiment of mice

This study was approved by the Animal Ethics Committee of Huazhong Agriculture University (HZAUMO-2020-0056) and conducted in compliance with the guidelines for the care and use of research animals. One hundred and twenty C57 BL/6 J mice (20 days old, sixty males and sixty females) were obtained from the experimental animal center of Huazhong Agriculture University, China and 5 animals were housed in a cage under standard SPF conditions. The mice were supplied *ad libitum* with basic diet entirely free from UA from WQJX Bio-technology (Wuhan, China), and drinking water was provided *ad libitum*. In this study, mice were exposed to UA by drinking water since we found that UA could be easily released from rice samples with ustiloxin contamination in mimic oral, gastric and small intestinal phase (details for *in vitro* evaluation of bioaccessibility were described in the Supporting Information text) and was relatively stable in water (Fig. S1). After acclimatization for 5 days, mice were randomly divided into four groups with 15 males and 15 females per group, and exposed to UA (0, 0.8, 8, 80 μg/kg bw/d) via UA-containing water. The concentration of UA (0.8 μg/kg bw/d) used in this experiment was determined based on the contents of UA in polished rice nationwide. The drinking water was changed twice a week (Monday and Thursday), and the concentrations of UA in drinking water were adjusted based on the body weight and volume of drinking water measured during the exposure period. After exposure for 69 days, the urine samples were collected independently

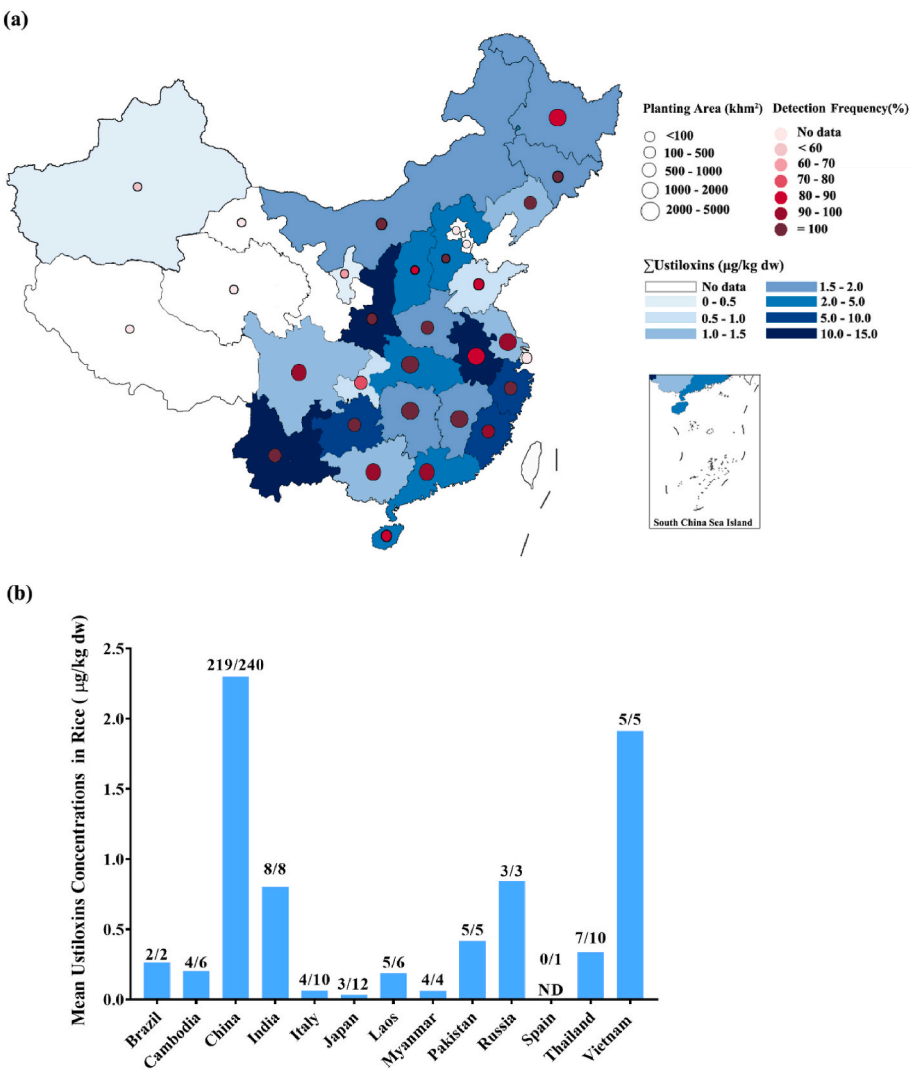

Fig. 1. Nation-wide survey of ustiloxins in rice. (a) Occurrence and distribution of ∑ustiloxins in rice collected from 25 provinces or city in China. Sizes of dots represent rice planting area of the province or city (collected from China national grain & oil information center), and the colors of dots represent detection frequency of UA in samples of rice. Colors of province or city show mean concentrations of UA in polished rice. (b) Mean concentrations and detection frequencies of ∑ustiloxins in rice collected from China and other 12 countries. ND means concentration below the detection limit. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

from each mouse over 12 h by using metabolism cage, and stored at −80 °C. In the second day, body weight and body length of each mouse were recorded, and the mice were killed by cervical dislocation and blood samples were collected. The blood samples were placed in refrigerator (4 °C) for 30 min and centrifuged to obtain serum. The serum samples were stored at −80 °C until analysis. The liver of mice was weighted, and the right median lobe of liver was collected for histological examination and quantification of UA. The histopathological samples were fixed in 2.5% glutaraldehyde solution.

2.5. Serum biochemical assays

The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine (CRE), glucose (GLU), triglyceride (TG) and total cholesterol (TCHO) in human serum were provided by the People's Hospital of Xianfeng Country. The levels of ALT and AST in mice serum were determined with commercial kits (Solarbio, Beijing).

2.6. Transmission electron microscope

To investigate liver damages of mice after exposure to UA, liver samples were immediately fixed in 2.5% glutaraldehyde after collection, and sent to Wuhan Regional Center of Life Science Instrument, Institute of Hydrobiology, Wuhan, China for transmission electron microscopy (TEM) analysis. In brief, liver samples were dehydrated and embedded after pre-fixed with 1% osmium tetroxide. After that, the samples were sliced and stained with uranyl acetate (Konjar et al., 2018). TEM images were performed with Hitachi HT7700 TEM.

2.7. Ustiloxin analysis

Ustiloxins were extracted following previously published method with some modifications (Sun et al., 2021). In brief, polished rice samples were lyophilized and grounded. After that, they were sieved and extracted with pure water, and then were further cleaned in WAX cartridge (CNW, 200 mg, 3 mL) before instrumental analysis. The lyophilized liver samples were extracted with MeOH/water (2:1), and purified by WAX cartridge (CNW, 200 mg, 3 mL). For serum, samples were diluted with 10% CCl_3COOH solution to remove proteins, and then were further purified with HLB cartridge (Waters, 60 mg, 3 mL). Urine samples were filtered, extracted with dichloromethane and further purified by coupling with two-step solid-phase extraction. Details for sample preparation were described in the Supporting Information text.

The separation and quantification of UA and UB were carried out by Waters ACQUITY UPLC® H-Plus Class system (UHPLC) coupled to Waters® XevosTM TQ-XS mass spectrometer (TQ-XS/MS) (Milford, MA, USA). More details of instrumental analysis and quality assurance/quality control were provided in Supporting Information text and Table S5.

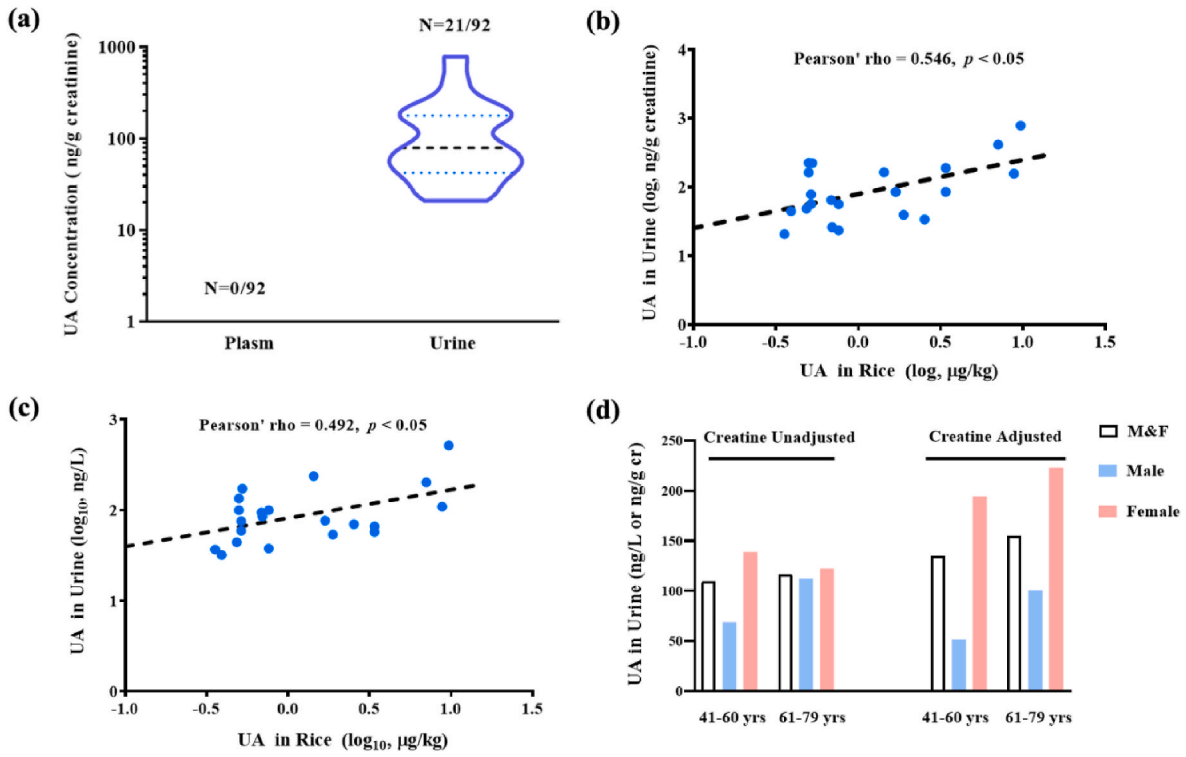

Fig. 2. (a) UA concentrations in serum and urine samples collected from volunteers (n = 92). The concentrations of UA in serum were all below detection limit, whereas UA was detected in 21 urine samples. (b) Pearson' relationship of urinary UA (n = 21, creatinine adjusted) with UA levels in rice. (c) Pearson' relationship of urinary UA (n = 21, creatinine unadjusted) with UA levels in rice. (d) Mean urinary UA concentrations (ng/L or ng/g creatine) for males, females and all volunteers from various age categories. M&F means males and females combined.

2.8. Statistics analyses

Data processing was performed using IBM SPSS statistics 22.0 (SPSS Inc., Chicago, IL, USA). The maxima, median and geometric mean concentrations (ng/mL) were used to describe the distribution of UA and UB in rice. For rice samples below the detection limit, a value equal to LOD divided square root of 2 was assigned. A normality test was performed using the Kolmogorov−Smirnov test. The UA concentrations in human urine samples were log-transformed for statistical analysis, and Pearson's correlation coefficients were used to describe linear relationships between blood biochemical indexes and urinary UA concentrations. Differences in liver weights and liver-somatic index (HIS) of mice between groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, with a statistical significance threshold of $p < 0.05$.

3. Results and discussion

3.1. Distribution of ustiloxin in rice of China and other 12 countries

Ustiloxins were detected in 219 (DR: 91.3%) polished rice samples collected from China, and the concentrations of ∑ustiloxins (total concentration of UA and UB) in those collected polished rice were in the range of LOD (<0.008) to 85.96 μg/kg of dry weight (μg/kg dw), with mean concentration of 2.27 μg/kg dw (a median value of 0.53 μg/kg dw) (Table S2). The ustiloxin levels in this study were comparable with those reported in the peeled rice collected from RFS infected paddy fields of Zhejiang (18.1–152.5 μg/kg for UA, 2.7–88.7 μg/kg for UB) (Cao et al., 2016) and Beijing (6.4–170 μg/kg for UA) (Fu et al., 2015b). With the exception of Xinjiang Uygur Autonomous Region (DR: 50%), Ningxia (66.7%) and Chongqing (77.8%), the detection rates of ustiloxins in all those selected provinces or city were higher than 80%, which was accordance with the wide occurrence of RFS across China (Lu et al., 2018a, 2018b; Zhang et al., 2006; Qi et al., 2021). For example, RFS occurred in approximately 2.40 million hectares per year in China between 2015 and 2017, which accounted for approximately one third of the annual production of rice-growing areas (Qiu et al., 2019). Among those samples collected in this study, the levels of UA (mean: 1.86 μg/kg dw, DR: 88.7%) were both significantly higher than those of UB (mean: 0.41 μg/kg dw, DR: 64.1%), and the ratios of UA/UB in most polished rice samples (about 70%) were in the ranges of 2.0–9.0, which was accordance with a previous study in which the ratios of UA/UB in rice false smut were in the range of 1.9–10.7 (Wang et al., 2016; Hu et al., 2018; Fu et al., 2017a, 2017b). The results demonstrated that ustiloxin contamination was ubiquitous in rice produced in China.

Just as shown in Fig. 1a, divergent spatial variations were found for ustiloxin contamination in rice. In particular, relatively serious ustiloxin contamination was observed in Yunnan (mean:10.56 μg/kg dw, DR: 100%), Guizhou (2.72, 100%), Anhui (11.56, 87.5%), Hubei (1.95, 100%) and Zhejiang (2.25, 87.5%), which is accordance with the particularly prevalent of RFS in the middle and lower reaches of the Yangtze River (Sun et al., 2020; Lu et al., 2018a, 2018b). In contrast, relatively lower levels were measured in Ningxia Province (mean: 0.08 μg/kg dw) and Xinjiang Uygur Autonomous Region (0.11), which were about 100-fold lower than Anhui. In general, relatively higher concentrations of ustiloxins were found in the Southwest China (mean: 3.55 μg/kg dw), following by North China (3.33) and Central China (2.63) (Fig. S2). This spatial distribution pattern of ustiloxin was consistent with previous research, in which the percentages of rice-cultivating area that suffered RFS disease in North China, Southwest China and Central China were around 10% and higher than those in Northeast China and South China during 2010–2020 (Yong et al., 2018; Qi et al., 2021).

Moreover, seventy-two polished rice samples collected from 12 other countries were analyzed to further characterize ustiloxin contamination in other rice-growing regions worldwide (Table S3). Although with relatively lower levels as compared with China, prevalence of ustiloxins

Table 2
Pearson correlation coefficients for log-transformed concentrations of urinary UA (creatine adjusted or unadjusted) and serum biochemistry indexes.

Serum Chemistry Index	Creatine Unadjusted			Creatine Adjusted		
	M&F	Male	Female	M&F	Male	Female
ALT	−0.136	0.633 *	−0.403	−0.165	0.724 *	−0.450
AST	−0.084	0.459	−0.403	−0.084	0.497	−0.353
BUN	−0.107	−0.492	−0.086	0.030	−0.380	0.190
CRE	−0.078	0.377	−0.176	−0.346	0.216	−0.392
GLU	−0.300	−0.324	−0.436	−0.263	−0.173	−0.294
TCHO	0.028	0.069	−0.019	0.240	−0.221	0.483
TG	0.124	−0.096	0.217	0.046	−0.055	0.157

kaM&F means males and females combined.
Bold text indicates significant ($p < 0.05$) correlations.
Correlation is significant with $0.1 < p < 0.5$.

was observed in most countries (except for Spain), with DRs ranging from 33.3% to 100%. Relatively higher concentrations ustiloxins were found in rice samples from Vietnam (mean: 1.91 μg/kg dw, median: 0.70 μg/kg dw), followed by Russia (0.84, 0.64) and India (0.80, 0.53), which were one order magnitudes higher than the concentrations measured in Japan (0.03), Myanmar (0.05) and Italy (0.06) (Fig. 1b). Differences in the concentrations of ustiloxins observed in rice among countries might be related to levels of severity of infection with RFS, genome and genetic diversity of pathogens, different progressing criterion of commercial rice or limited sampling sizes of this survey (Zhang et al., 2017; Pareja et al., 2012). Overall, these data demonstrated that ustiloxins in rice were nearly ubiquitous, which indicated that millions of people, particularly in South and South-East Asia, might be chronically exposed to ustiloxins through their daily diet.

3.2. Occurrence of ustiloxin in human serum and urine

Although the ubiquity of ustiloxin in rice was reported, no data on the occurrences of ustiloxins in humans speciments was available. To explore internal exposure doses of ustiloxins towards humans, occurrence and concentrations of ustiloxins in serum and urine samples from 92 volunteers were measured (Fig. 2a and Table S4). Only UA was detected in 23% of urine samples (11 of 51 women and 10 of 41 men), with mean and median concentrations of urinary UA were 112.6 ng/L (143.4 ng/g creatinine) and 76.4 ng/L (79.0 ng/g creatinine), respectively. This could particularly attribute to the relatively higher detection frequencies and concentrations of UA in rice (Table S2) compared with UB. Urinary UA concentrations were significantly and positively associated with the concentrations of UA in rice eaten by those individuals (Fig. 2b and c). Notably, the median and mean UA concentrations (1.55 μg/kg dw and 0.67 μg/kg dw, respectively) in rice collected from volunteers were comparable with those collected across China (1.89, 0.40) as mentioned above. The presentation of UA in urine of ordinary people suggested that general public have been, and will continue to be exposed to UA. To the best of our knowledge, this study was the first to validate the occurrence of internal exposure of UA in human.

Although no age-related accumulation pattern was found (Fig. S3), urinary UA concentrations showed difference between genders. Specifically, relatively higher urinary levels of UA in females (mean: 204.6 ng/g creatinine) were identified (76.2 ng/g creatinine) compared with males, though without statistically significant (Fig. 2d and Table S4). Further studies with larger sample sizes are needed to confirm the gender-related differences in internal burden of ustiloxins among people.

3.3. Associations between urinary UA levels and serum biochemical parameters

Limited toxicological studies have found that high dosage of ustiloxins exposure could cause pathological changes in liver and kidney of mammals (Nakamura et al., 1994; Fu et al., 2017a, 2017b). Therefore, the relationships of urinary UA with seven serum biochemical parameters were investigated and the results were summarized in Table 2. In male volunteers with detectable concentrations of UA in urine, the activities of serum ALT were moderately and significantly correlated with urinary creatinine-adjusted or unadjusted UA concentrations (Pearson's rank correlation coefficient: $r = 0.724$, $p < 0.05$ or $r = 0.633$, $p < 0.05$), although the activities of ALT were mostly within the normal reference range (Fig. 3a). Moreover, AST activities in male serum also presented an increasing trend with the increasing urinary UA ($r_p = 0.497$, $p < 0.2$ or $r = 0.459$, $p < 0.2$), although they were not statistically significant (Fig. 3b). However, no other significant associations were observed between urine UA levels and the other 5 blood chemistry parameters (Table 2). For females, no significant correlations were observed between concentrations of UA in urine and all the seven clinical blood chemistry parameters (Table 2). These data suggested that long-term UA exposure might be associated with potential impairment of liver function in male.

3.4. Sub-chronic exposure to UA caused a male-biased hepatoxicity in mice

To address the hypothesis that chronic exposure to UA by dietary consumption of rice caused liver damage in males, C57BL/6 J mice were exposed to UA (0.8, 8, 80 μg/kg bw/d) (Fig. S4). Occurrence of UA in liver of mice was observed fed with 80 μg UA/kg bw/d, demonstrated

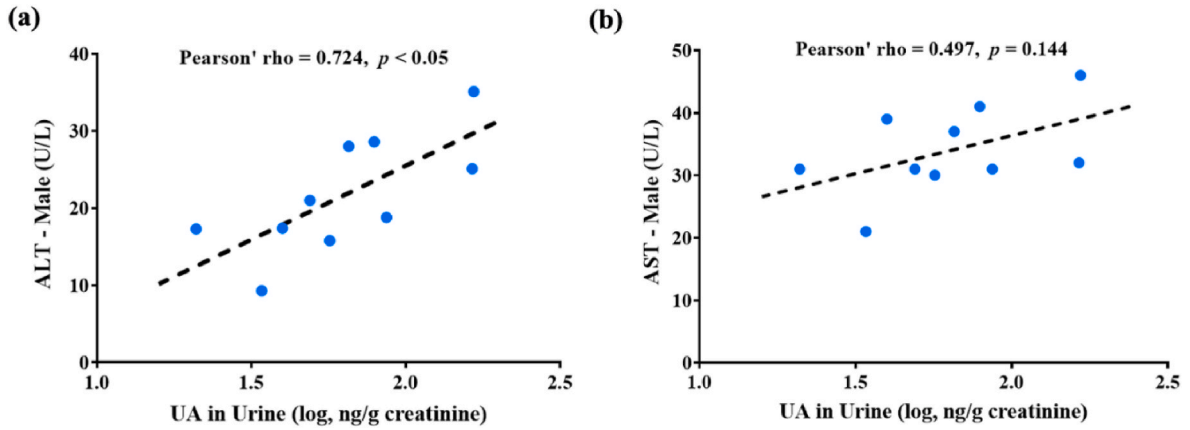

Fig. 3. Associations of urinary concentration of UA (creatine corrected) with serum ALT (a) and AST (b) in male. The data of male volunteers with detectable urine UA were used for analysis (n = 10). Log-transformed concentrations of UA in urine and rice were used for the analysis.

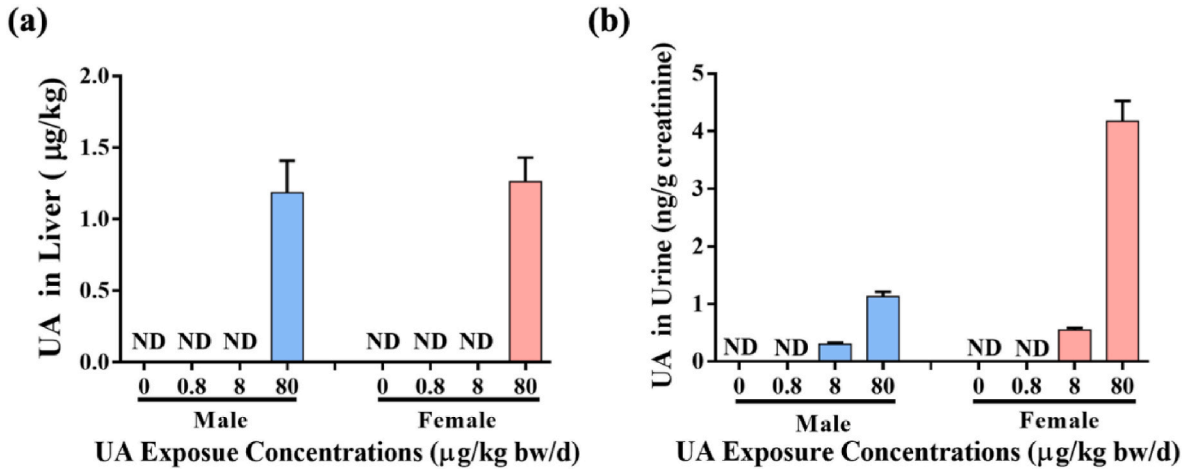

Fig. 4. Sub-chronic exposure to environmentally relevant concentrations of UA led to male-biased liver damage in C57 BL/6 J mice. Concentrations of UA in liver (a) and urine (b) in female and male mice after exposure of UA. Five biological replicates were used for males and females in each group, and ND means concentration of UA was blow the detection limit of method.

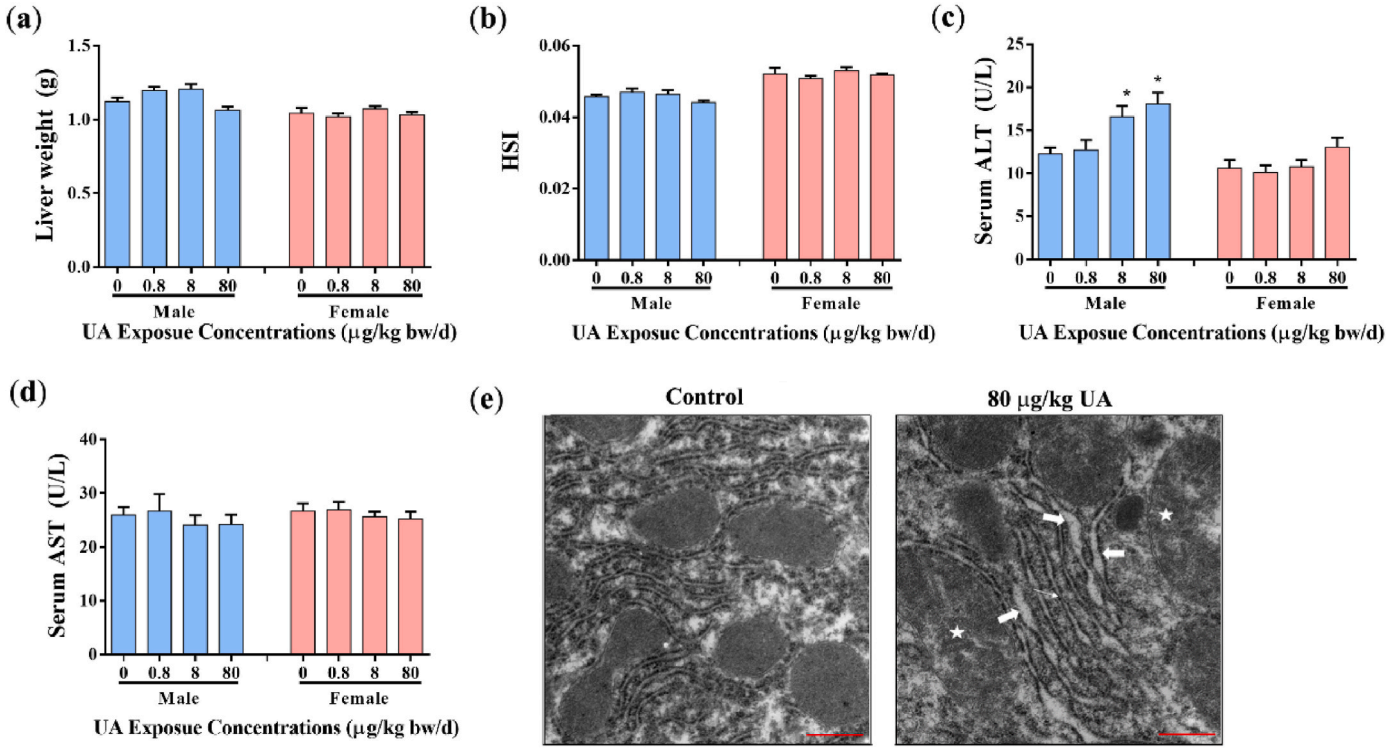

Fig. 5. Liver weight (a) and liver-somatic index (b) of male and female mice after exposure to different concentrations of UA for 10 weeks (n = 16–18). Activities of ALT (c) and AST (d) in blood sera of male or female mice fed diets containing various concentrations of UA for 10 weeks (n = 12). The error bars indicate SEM, and significant differences between exposure groups and control group were indicated by $*p < 0.05$. (e) Photomicrographs of sections of hepatocytes from control group (left) and high dose group (80 μg UA/kg bw/d, right). The pentacles indicate swollen mitochondria with relatively lesser electronic densities, and thick arrows show swollen rough endoplasmic, and narrow arrow indicates reduced density of member-bound ribosome. (Scar bar: 0.5 μm).

that UA could enter into the circulatory system after ingestion (Fig. 4a). Meanwhile, mice exposure to 8 or 80 μg/kg bw/d of UA exhibited comparable urinary UA concentrations with those observed in humans in this study (Fig. 4b), suggesting that those two doses could be considered as environmentally relevant exposures and could be used as a threshold for effects of UA on humans. Interestingly, corresponding to the phenomenon observed in human, urinary levels of UA in females were approximately 2–3 times higher than that of males, which might be due to the difference in gender-related metabolism.

UA exhibited negligible impact on absolute liver weights (Fig. 5a) and liver-somatic index (HSI, Fig. 5b) in males or females. However, similar to the trend observed in volunteers, UA significantly elevated serum ALT activities of male mice in a dose-dependent manner (Fig. 5c), and only marginal elevation without statistically difference was observed in females (Fig. 5c). Contrastingly, no significant changes in serum AST activities were observed in either male or female mice after exposure to any of the doses of UA (Fig. 5d). However, accumulation of UA in liver without no significant difference between genders was observed in mice exposure to the high dose of UA (Fig. 4a). Our results clearly demonstrated that male mice could be more effectively impacted

by UA than females. To further evaluate the effects of UA on liver, the ultrastructures of hepatocytes in male mice exposed to 80 μg UA/kg bw/d UA were examined. Consistent with a previous report (Nakamura et al., 1994), swelling mitochondria with relatively lesser density were observed after exposure to UA (Fig. 5e). Further, obviously swelling rough endoplasmic reticula with reduced numbers and densities of membrane-bound ribosomes were observed in male mice fed 80 μg UA/kg bw/d (Fig. 5e). Collectively, these data demonstrated that chronic exposure to UA caused liver injury of male mice. Further studies are needed to explore the complicated mechanisms of male-based hepatoxicity of ustiloxins.

4. Conclusion

In conclusion, our study uncovered the ubiquitous occurrence of ustiloxins in polished rice across China and other rice-growing countries, and for the first time demonstrated the presence of ustiloxins in human urine. The findings highlighted the toxicity concerns of male-biased hepatoxicity caused by ustiloxin exposure, which certainly warranted further studies. Further knowledge is urgently needed to assess potential long-term effects and explore the underlying mechanisms of ustiloxins exposure on human health.

CRediT authorship contribution statement

Qian Sun: Investigation, Data curation, Writing – original draft, Methodology, Software, Formal analysis. **Hao Liu:** Investigation, Validation, Formal analysis. **Yongkang Zhang:** Investigation, Data curation. **Xun'e Yi:** Investigation, Data curation. **Ren Kong:** Investigation, Data curation. **Shiyang Cheng:** Methodology. **Jianguo Man:** Writing – review & editing. **Lu Zheng:** Writing – review & editing. **Junbin Huang:** Writing – review & editing. **Guanyong Su:** Writing – review & editing. **Robert J. Letcher:** Writing – review & editing. **John P. Giesy:** Writing – review & editing. **Chunsheng Liu:** Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.118992.

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Supporting Information

Global distribution of ustiloxins in rice and their male-biased hepatotoxicity

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Text S1

Rice extraction and clean up. An amount of 500 mg of polished rice was weighted and grounded into power (150 μm), and then transferred into a 15-mL polypropylene centrifuge tube. After the addition of 10 mL pure water, the mixture was shaken for 10 min with a vertical oscillator, and ultrasonic for another 10 min. After centrifugation (7000 rpm, 10 min), the supernatant was transferred into a new tube and the remaining residues were extracted twice with 15 mL water. The supernatants were combined and extracted with 25 mL dichloromethane. The aqueous phase was collected and acidized with formic acid (pH = 5.0), and loaded on WAX cartridge (CNW, 200 mg, 3 mL), which has been preconditioned with 3 mL MeOH and 3 mL water (pH = 5.0). Then, the cartridge was subsequently washed with 2 mL water (pH = 5.0) and 2 mL MeOH, and the residues of UA and UB were eluted with 2 mL 1 % NH_4OH-MeOH. The fraction was evaporated to dryness under a flow of nitrogen and reconstituted with 200 μL 5 % MeOH-water for LC-MS/MS analysis.

Serum extraction and clean up. A volume of 500 μL of human serum was diluted with 500 μL ammonium acetate solution (100 mM) and 500 μL 10 % CCl_3COOH solution. Then, the mixed solution was vortexed and centrifuged (12000 rpm, 10 min, 4 °C) to remove precipitated proteins, and the supernatant was collected and extracted with 1.5 mL DCM. After centrifugation, the aquatic solution was collected and diluted with 150 μL ammonium acetate solution (500 mM, 5 % FA), and then purified with HLB cartridge. After washed with 2 mL 0.5 % FA solution, the fractions containing UA and UB were eluted with 2 mL 0.5 % NH_4OH-MeOH mixed solution

and evaporated to dryness under nitrogen. Finally, the residues of UA and UB were reconstituted in 200 μL of 5 % MeOH-water and analyzed by LC-MS/MS.

Urine extraction and clean up. In brief, 1 mL human urine or 200 μL mouse urine was extracted by using equal volume of DCM, and the supernatant were collected after vortex and centrifugation. After dilution with 3 mL ammonium acetate solution (50 mM) containing 0.5 % FA, the aquatic solution was loaded on preconditioned HLB cartridges (Waters, 3 mL, 60 mg). Then, the column was washed with 2 mL 0.5 % FA aquatic solution, and eluted with 2 mL of 25 % MeOH-Water solution after vacuumed for 20 min. The eluting solution was loaded on WAX cartridges (CNW, 3 mL, 200 mg) after being diluted with 6 mL ultrapure water and its pH was adjusted to 5.0 with ammonia. Then, the cartridge was eluted with 2 mL of 0.5 % NH_4OH-MeOH after being washed with 2 mL ultrapure water (pH = 5.0) and 2 mL of 0.2 % FA-MeOH. The elution solution was collected, concentrated and reconstituted in 50 μL 5% MeOH-water solution for further LC-MS/MS analysis.

Liver extraction and clean up. The mice liver was freeze-dried and grounded into powder (150 μm). After that, 50 mg of sample was weighted and transferred into a new tube, and extracted with 1 mL MeOH/water (2:1). After oscillation and ultrasonic, the mixture was centrifuged at 12000 rpm for 10 min and the supernatants were transferred into a new tube. The procedure was repeated once. The combined supernatants were diluted with 1 mL water and extracted with 2 mL dichloromethane. After vortex and centrifugation, the aqueous phase was decanted into a new 15-mL tube and diluted with 15 mL water (pH = 5.0). Finally, the extracts were extracted

through a WAX column according to the cleanup procedure descried above. The fraction of UA and UB was eluted with 2 mL 0.5 % NH_4OH-MeOH mixed solution and evaporated to dryness under nitrogen. The residue was reconstituted in 200 μL 5 % MeOH-water solution for further LC-MS/MS analysis.

Text S2

LC-MS/MS analysis. The separation and quantification of UA and UB in all samples were carried out by Waters ACQUITY UPLC® H-Plus Class system (UHPLC) coupled to Waters® XevosTM TQ-XS mass spectrometer (TQ-XS/MS) (Milford, MA, USA). Samples were separated on Acquity UPLC HSS T3 (100 mm × 2.1 mm, 1.8 μm, Waters) column. The column temperature maintained at 40 °C with a flow state of 0.3 mL/min, and the injection volume was set to 2 μL. Water containing 0.01% formic acid (A) and MeOH (B) were used as mobile phase for analyzing UA. Gradient condition for analysis of rice and paddy water samples was set as follows: 5 % B kept for 0.5 min, then increased linearly to 95 % B in 4.5 min and hold for 2 min, followed by a sharp decrease to 5 % B in 0.2 min and kept for 1.8 min. While, the gradient program for analyzing stalks, urine, serum and liver samples was adopted as follows: 5 % B kept for 0.5 min followed by a linear increase to 25 % B, then increased sharply to 100% B in 1 min and kept for 3 min, and then returned to initial state in 0.2 min and hold for 1.8 min before next injection. Mass analysis was performed in the multiple reaction monitoring (MRM) mode, using electrospray ionization source in positive model (ESI (+)). The capillary voltage was 1.0 KV. The

desolvation temperatures was 350 °C. The desolvation and cone gas flow rates were adjusted to 700 and 150 L/h, respectively. Transitions of *m/z* 674.30 > 209.00 and *m/z* 674.30 > 187.00 were used for the analysis of UA, with collision voltage of 30 V and 35 V, respectively. Transitions of *m/z* 646.13 > 187.00 and *m/z* 646.13 > 181.00 were used for the analysis of UB, with collision voltage of 30 V and 32 V, respectively. Given that the distinct matrix interference of sample, *m/z* 674.30 > 209.00 *m/z* 646.13 > 181.00 were used for quantitative analysis of UA and UB human urine, human serum, rice and mice liver, while *m/z* 674.30 > 187.00 *m/z* 646.13 > 187.00 were chosen for UA and UB in mice urine, respectively. Table S4 shows the extraction recovery, matrix effect and LOD (limit of detection) and LOQ (limit of quantification) of proposed methods in this study for the analysis of UA. Concentrations of creatinine in urine were measured with commercial kits (Solarbio, Beijing).

Text S3

Evaluation of Bioaccessibility of UA and UB *In Vitro*. The gastrointestinal digestion of ustiloxin-contaminated rice samples was carried out according to a previous study (Brodkorb et al., 2019) with slight alternations. In brief, rice sample (5 g) was added to 6.5 mL water, and equilibrated for 5 min. **Oral phase**: the mixture above was added into 5 mL simulated salivary fluid (15.1 mM KCl, 3.7 mM KH_2PO_4, 13.6 mM $NaHCO_3$, 0.15 mM $MgCl_2$, 0.05 mM $(NH4)_2CO_3$, 1.5 Mm $CaCl_2$ and 0.09 mM HCl, pH = 7.0) which contained 150 U/mL salivary amylase. Then, the mixture

was incubated for 2 min at 37 ℃. **Gastric phase**: 10 mL simulated gastric fluid (6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.12 mM $MgCl_2$, 0.5 mM $(NH4)_2CO_3$, 0.15 mM $CaCl_2$ and 15.6 mM HCl, pH = 3.0) containing 4000 U/mL pepsin was added into 10 mL residues from above oral phase. It was adjusted to pH = 3.0 with HCl solution, and then the mixture was incubated for 2 h at 37 ℃. **Intestinal phase**: Twenty mL residues from gastric phase was added into 20 mL simulated intestinal fluid (6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.12 mM $MgCl_2$, 0.15 mM $CaCl_2$ and 8.4 mM HCl, pH = 7.0) containing 200 U/mL trypsin and 20 mM bile salts, and the pH was adjusted to 7.0 with NaOH solution. Then, the mixture was incubated for 2 h at 37 ℃ in shaker. In each step, the digestion solution was weighted after centrifugation and corresponding concentrations of UA and UB were monitored. Finally, the bioaccessibility was calculated.

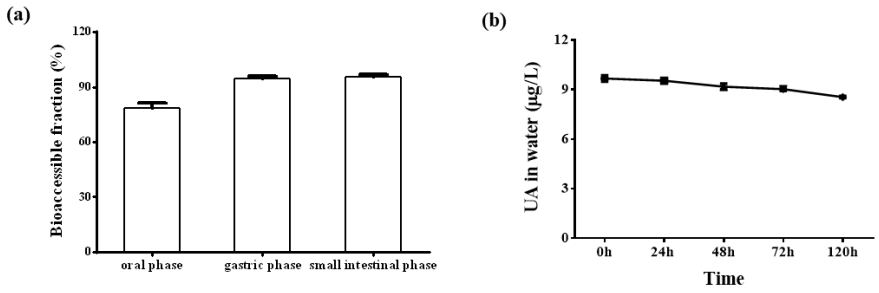


Fig. S1. (a) The bioaccessibility of UA in rice; (b) The stability of UA in drinking water.

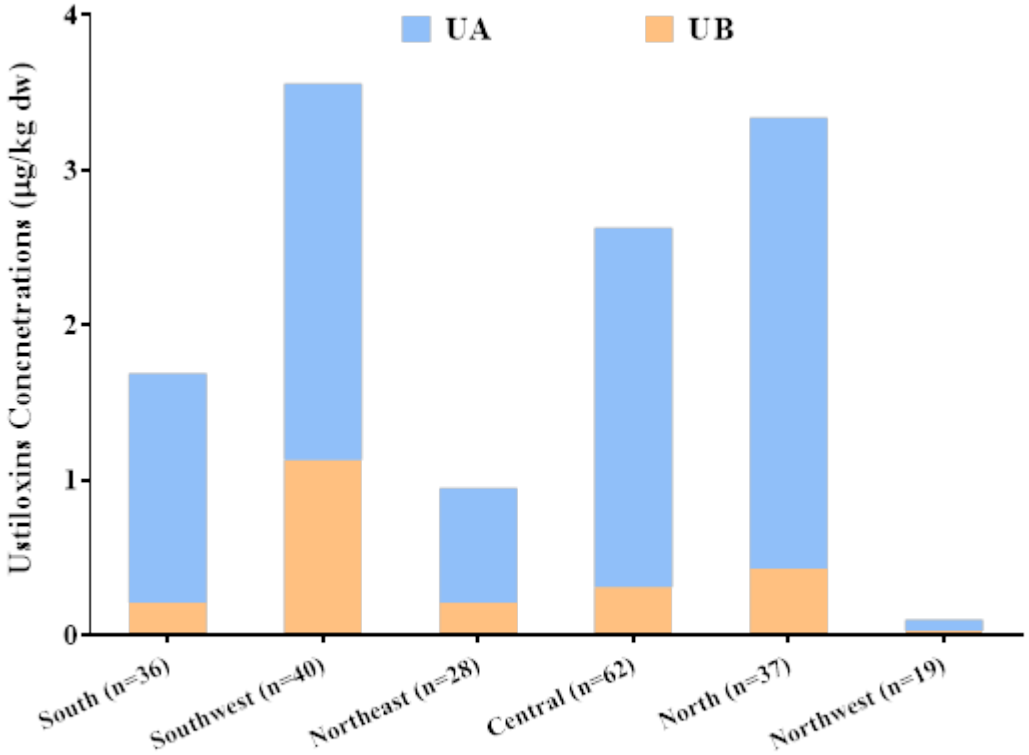


Fig. S2. Concentrations of ustioxins in rice from six geographical regions, China. The number in the bracket represents the total samples in each region.

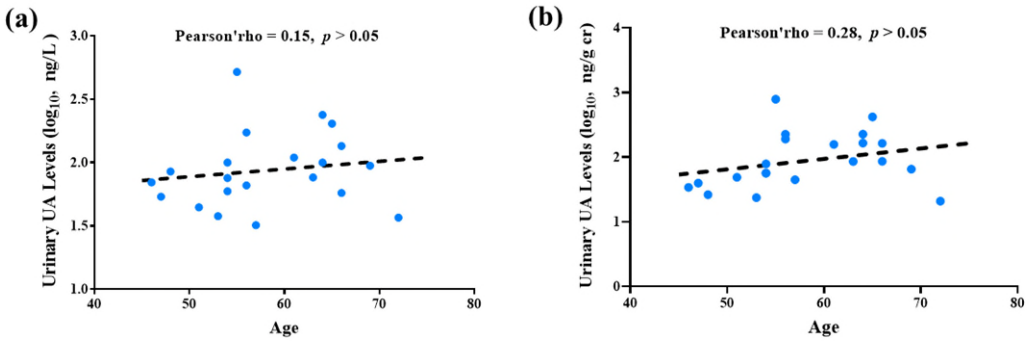


Figure S3. Pearson's relationship of urinary UA concentrations and age. (a) Creatine unadjusted urinary UA, (b) creatine adjusted urinary UA.

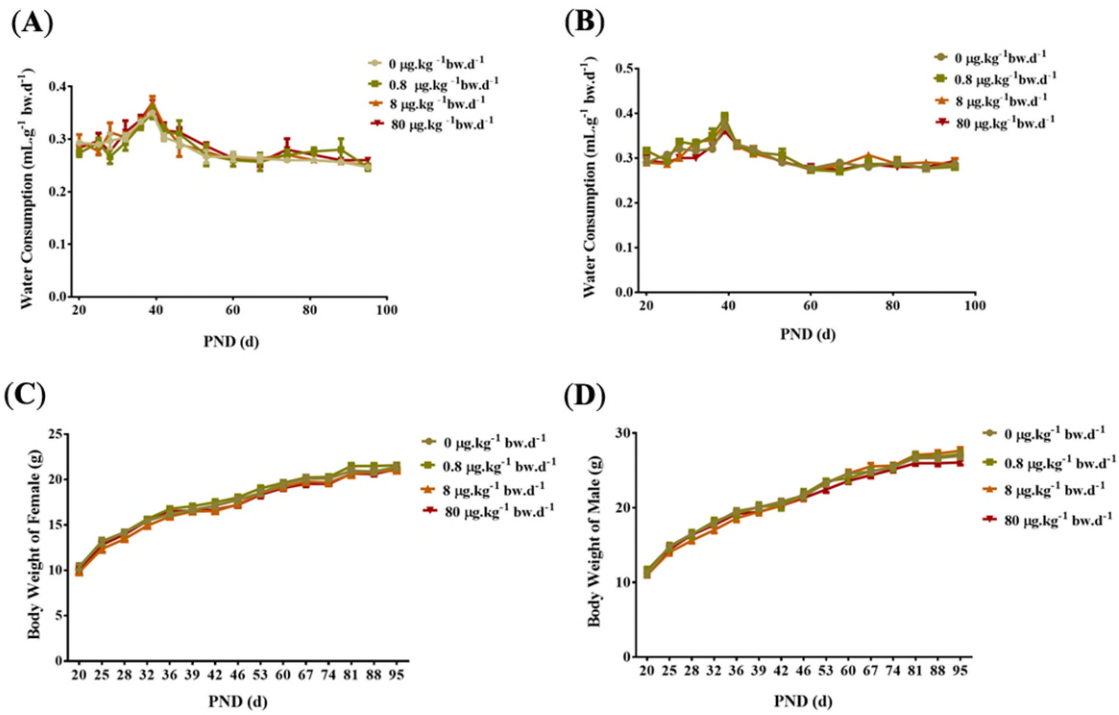


Figure S4. Water consumption of male (A) and female (B) mice per day during exposure period. Three biological replicates per group were used for males and females. Body weight of male (C) and female (D) mice were recorded until the end of the experiment (PND 95). Twelve biological replicates were used for males and females in each group. The error bars indicated SEM. PND: postnatal day.

Table S1. The chemical structures of UA and UB.

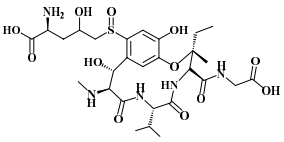
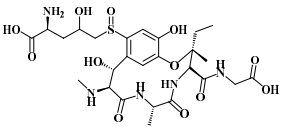
Compound	CAS NO.	Formular	Structure
Ustiloxin A	141044-51-1	$C_{28}H_{43}N_5O_{12}S$	
Ustiloxin B	151841-41-7	$C_{26}H_{39}N_5O_{12}S$	

Table S2. Descriptive statistics of ustiloxin residues (μg/kg dm) in 240 polished rice samples collected from 25 provinces and city in China.

Province/City	N	UA					UB					∑Ustiloxins			
		DF [a] (%)	Mean	GM[b]	Median	Max	DF (%)	Mean	GM	Median	Max	Mean	GM	Median	Max
Anhui	8	87.5	10.61	0.82	0.69	78.88	62.5	0.95	0.07	0.09	7.08	11.56	0.98	0.83	85.96
Fujian	10	90.0	2.22	0.86	1.04	6.99	80.0	0.33	0.13	0.13	1.07	2.55	1.08	1.20	7.00
Guangdong	10	90.0	1.53	0.60	0.73	6.42	70.0	0.25	0.08	0.11	1.12	1.77	0.77	0.95	7.54
Guangxi	9	77.8	0.48	0.16	0.18	1.42	55.6	0.09	0.04	0.04	0.37	0.58	0.28	0.19	1.79
Guizhou	11	100	2.14	1.05	1.42	6.59	90.9	0.58	0.25	0.48	2.01	2.72	1.50	2.03	7.69
Hainan	7	71.4	1.68	0.25	0.99	8.24	57.1	0.14	0.05	0.11	0.44	1.82	0.55	1.13	8.25
Heibei	9	100	1.35	0.90	1.46	3.15	66.7	0.23	0.08	0.22	0.56	1.58	1.23	1.68	3.65
Heilongjiang	9	88.9	0.84	0.54	0.31	3.77	55.6	0.31	0.06	0.08	0.63	1.16	0.48	0.63	3.78
Heinan	9	100	0.93	0.24	0.53	2.69	55.6	0.09	0.04	0.06	0.35	1.02	0.75	0.53	2.70
Hubei	16	100	1.62	0.88	0.62	8.66	81.3	0.33	0.11	0.15	1.23	1.95	1.06	0.75	9.92
Hunan	8	100	1.00	0.61	0.92	2.98	87.5	0.24	0.11	0.14	1.12	1.24	0.77	1.12	3.09
Inner Mongolia	10	100	1.04	0.53	0.23	3.53	70.0	0.22	0.08	0.16	0.67	1.27	0.46	0.52	4.08
Jiangsu	13	84.6	0.63	0.29	0.37	2.32	46.1	0.10	0.03	<LOD	0.53	0.42	0.23	0.37	2.85
Jiangxi	9	88.9	0.80	0.31	0.39	2.98	100	0.26	0.19	0.17	0.74	1.06	0.68	0.54	3.72
Jilin	9	100	0.92	0.28	0.51	2.40	77.8	0.19	0.09	0.17	0.44	1.11	0.63	0.67	2.69
Liaoning	10	100	0.38	0.29	0.25	1.20	80.0	0.10	0.06	0.07	0.34	0.48	0.34	0.33	1.54
Ningxia	9	66.7	0.06	0.03	0.03	0.31	22.2	0.02	0.01	<LOD	0.08	0.08	0.05	0.04	0.39

Continued table S2.

Province/City	N	UA					UB					∑Ustiloxins			
		DF [a] (%)	Mean	GM[b]	Median	Max	DF (%)	Mean	GM	Median	Max	Mean	GM	Median	Max
Shaanxi	9	100	9.68	2.20	2.22	59.00	77.8	1.41	0.31	0.46	7.10	11.09	2.55	2.51	66.10
Shandong	10	80.0	0.22	0.09	0.13	1.07	50.0	0.06	0.03	0.02	0.27	0.28	0.13	0.15	1.35
Shanxi	8	87.5	1.39	0.22	0.33	8.36	62.5	0.18	0.05	0.03	0.80	1.57	0.28	0.16	9.16
Sichuan	10	90.0	0.31	0.14	0.26	0.80	60.0	0.17	0.05	0.05	0.69	0.48	0.21	0.31	1.25
Xinjiang Uygur Autonomous Region	10	40.0	0.09	0.02	<LOD	0.53	30.0	0.03	0.01	<LOD	0.15	0.11	0.04	0.03	0.03
Yunnan	10	100	6.88	0.57	0.35	60.43	90.0	3.68	0.16	0.12	35.55	10.56	0.91	0.43	60.68
Zhejiang	8	87.5	2.06	0.73	1.22	7.95	50.0	0.19	0.05	0.07	0.57	2.25	1.11	1.30	7.95
Chongqing	9	77.8	0.19	0.09	0.13	0.68	28.6	0.02	0.01	<LOD	0.09	0.21	0.12	0.14	0.69
Total	240	88.8	1.89	0.33	0.40	78.88	64.1	0.41	0.06	0.08	35.55	2.30	0.48	0.54	85.96

[a] DF: detection frequency; [b] GM: geometric mean.

Table S3. Descriptive statistics for ustloxin in 72 polished rice samples collected from 12 countries.

Country	N	UA					UB					∑Ustiloxins			
		DF [a] (%)	Mean	GM[b]	Median	Max	DF (%)	Mean	GM	Median	Max	Mean	GM	Median	Max
Brazil	2	100	0.23	0.19	0.23	0.36	50.0	0.03	0.02	0.03	0.06	0.26	0.21	0.26	0.42
Cambodia	6	66.7	0.14	0.05	0.06	0.48	50.0	0.07	0.03	0.04	0.22	0.20	0.09	0.11	0.70
India	8	87.5	0.24	0.15	0.20	0.43	87.5	0.56	0.23	0.27	1.53	0.80	0.53	0.66	1.66
Italy	10	40.1	0.04	0.02	<LOD	0.17	20.0	0.03	0.01	<LOD	0.15	0.06	0.03	<LOD	0.22
Japan	12	25.0	0.03	0.01	<LOD	0.12	0	<LOD	<LOD	<LOD	<LOD	0.03	0.01	<LOD	0.12
Laos	6	66.7	0.06	0.04	0.06	0.16	83.3	0.12	0.06	0.06	0.37	0.18	0.12	0.18	0.43
Myanmar	4	100	0.05	0.04	0.04	0.12	0	<LOD	<LOD	<LOD	<LOD	0.05	0.04	0.04	0.12
Pakistan	5	40.0	0.25	0.03	<LOD	1.17	80.0	0.17	0.06	0.05	0.67	0.42	0.12	0.06	1.84
Russia	3	66.7	0.58	0.15	0.31	1.42	100	0.27	0.20	0.35	0.39	0.84	0.64	0.40	1.77
Spain	1	0	<LOD	<LOD	<LOD	<LOD	0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Thailand	10	60.0	0.26	0.05	0.05	2.02	60.0	0.08	0.04	0.04	0.48	0.34	0.08	0.07	2.50
Vietnam	5	100	1.53	0.56	0.79	5.33	80.0	0.38	0.13	0.16	1.28	1.91	0.70	0.96	6.61
Total	72	59.7	0.24	0.05	0.05	5.33	48.6	0.15	0.03	<LOD	1.53	0.39	0.10	0.08	6.61

Table S4. Urinary concentration of UA in volunteers above detection limit *.

Group	N	DF %	Creatinine Corrected (ng/g)			Uncorrected (ng/L)		
			Range	Mean	Median	Range	Mean	Median
All	21	22.8	23.8-789.7	143.4	79.0	32.1-518.9	112.6	74.6
Male	10	24.3	39.8-166.0	76.2	61.0	53.8-237.7	90.5	72.6
Female	11	21.6	23.8-789.7	204.6	157.9	32.1-518.9	132.7	84.9

* All participants have no specific disease symptoms. DF: detection frequency, GM: geometric mean.

Table S5. Method limits of quantification (LOQ), limit of detection (LOQ), extract recoveries and matrix effects of UA and UB in various matrixes (n = 3).

Samples	UA				UB			
	Recovery % [a]	**Matrix Effect %[a]**	**LOD [b]**	**LOQ [c]**	**Recovery (SD) %**	**Matrix Effect (SD) %**	**LOD [a]**	**LOQ [a]**
Rice	89.3 ± 8.6	101.8 ± 0.4	11.6	38.9	87.2 ± 6.2	96.8 ± 2.1	10.2	34.0
Human Serum	81.2 ± 7.6	104.2 ± 6.5	60.5	201.8	75.2 ± 5.6	94.3 ± 4.5	72.1	240.33
Human Urine	88.5 ± 1.3	112.8 ± 6.5	23.6	78.8	76.5 ± 2.3	105.3 ± 2.3	41.2	137.3
Mice Liver	85.3 ± 8.2	93.2 ± 1.3	174.4	581.2	79.3 ± 3.1	91.1 ± 3.2	156.7	522.3
Mice Urine	98.4 ± 9.0	103.7 ± 1.1	27.6	92.1	84.6 ± 7.2	96.4 ± 2.3	31.6	105.3

[a]: Data are expressed as mean ± SD; [b]: Calculated as 3S/N; [c]: Calculated as 10S/N

Reference

[1] Brodkorb A., Egger L., Alminger M., Alvito P., Assuncao R., et al., **2019**. INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nat. Protoc.*, 14: 991-1014. https://doi.org/10.1038/s41596-018-0119-1