FOOD CHEMICAL CONTAMINANTS

N-Nitroso Compounds and Mutagens in Chinese Fermented (Sour) Corn Pancakes

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Stomach cancer rates in rural Linqu County, Shandong Province, China, are exceptionally high. A previous case-control study revealed that the risk of stomach cancer was 30% higher among those who consumed sour (fermented) corn pancakes at least daily. A previous study of the sour pancakes reported volatile nitrosamines in most specimens, and almost half reportedly showed mutagenic activity. Few households currently consume sour pancakes, and the duration of fermentation has been shortened. We tested specimens of pancake batter and sour pancakes from Linqu County for mutagenic activity using the Ames test; for N-nitroso compounds (NOC) we used the Nitrolite–thermal energy analysis (TEA) method. Results of the Ames test were inconclusive: only 1 out of 15 cooked pancakes showed a positive mutagenic response, and all 15 batter specimens were negative; however, several batter specimens showed a weakly positive trend of mutagenicity with extract concentration. Our assay for total nitroso compounds was weakly positive in only 1 out of 15 specimens of sour pancake batter. That specimen was also tested by gas chromatography–TEA for nitrosaminoacids and volatile nitrosamines, but none were detected. It seems unlikely that the Chinese sour pancakes are significantly contaminated by NOC or other mutagens.

Stomach cancer rates in Linqu County, Shandong Province, China, are unusually high (1). A case-control study revealed that the risk of stomach cancer in this rural area was 30% higher among those who consumed sour (fermented) corn pancakes, a local delicacy, at least daily (2). Corn is commonly contaminated by *Fusarium moniliforme*, a fungus which produces mycotoxins, including fumonisins, fusarin-C, and trichothecenes. Chinese corn has been shown to contain fumonisins and trichothecenes, and isolates of *F. moniliforme* from contaminated specimens have been shown to produce nitrosamines in vitro (3). Sour pancake extract was previously reported to be mutagenic and to contain nitrosamines as well as mycotoxin-producing species of fungi (4). Extensive background information on contamination of corn and other foodstuffs by fungi and nitrosamines is available in a review article on the epidemiology of esophageal cancer in China (5). Subsequent analyses detected various mycotoxins in Chinese corn and corn products, although not in excess of the levels deemed safe for human consumption in the United States (6). In the present study, we tested specimens of pancake batter and sour pancakes collected in Linqu County for mutagenic activity and for nitrosamines.

Experimental

Specimen Collection and Handling

Seven villages were selected at random, representing the 5 townships of Linqu County. In each village, up to 3 households were randomly selected from among those known to be preparing sour pancakes. At each household, specimens of pancake batter and cooked pancakes were collected in plastic tubes and protected from light. Specimens were frozen on dry ice and shipped to the American Health Foundation (Valhalla, NY) for the Ames test, and then to the

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Table 1. Precision[a] and accuracy[b] data for total N-nitroso compounds (NOC) measured in extracts of pooled samples fortified with 4 different NOC at 5 nmol/g

Compound[c]	Mean[d], nmol/g	SD, nmol/g	RSD, %	Recovery, %
		Recovery from pancake		
NINA	3.8	0.5	12	75
NPRO	3.3	0.2	5.2	65
MNU	1.8	0.2	9.2	36
NPYR	1.5	0.1	7.3	29
		Recovery from batter		
NINA	4.2	0.6	13	85
NPRO	4.5	0.2	5.3	91
MNU	2.8	0.3	8.7	57
NPYR	3.2	0.4	13	64

[a] Expressed as relative standard deviation (%; $n = 6$), calculated as (standard deviation/mean measured concentration) × 100.
[b] Expressed as recovery (%; $n = 6$), calculated as (mean measured concentration/spiked concentration) × 100.
[c] NINA = N-nitrosoisonipecotic acid; NPRO = N-nitrosoproline; MNU = N-methyl-N-nitrosourea; NPYR = N-nitrosopyrolidine.
[d] Mean concentration ($n = 6$), determined from the linear least-squares regression standard line using 6 concentrations of NINA as calibration standards.

Science Applications International Corp. (Frederick, MD) for the N-nitroso compounds (NOC) assay.

Ames Test

A dimethyl sulfoxide (DMSO) extraction was performed on all samples. To extract the pancake batter, 5 mL DMSO was added to 5 mL of each batter sample and the mixtures were vortexed at the maximum setting for 10 min. They were then centrifuged at 14 000 × *g* for 20 min at 4°C. Pancakes were extracted by adding 5 mL 50% aqueous DMSO to 1 g pancake. The samples were homogenized in a Polytron homogenizer and then centrifuged at 14 000 × *g* for 20 min at 4°C. The supernatants were clear and almost colorless. All samples and extracts were stored at –20°C until analyzed. Because the DMSO extracts may have contained histidine or histidine precursors, which the bacteria could have used and which could result in possible false-positive assays, the histidine concentrations were measured in the extracts by a published method (7).

The procedure, based on the methods of Maron and Ames (8) and Ames et al. (9) was performed under amber light and in a laminar-flow exhaust hood. For each specimen of pancake or batter, 4 concentrations of DMSO extract (7, 22, 67, and 200 µL per plate) were prepared for testing. After the nutrient broth had been inoculated and incubated overnight with *Salmonella typhimurium* TA98 or TA100, dilutions were prepared and incubated for 30 min with the bacteria [either with 0.5 mL phenobarbital/β-naphthoflavone-induced (10) male Sprague- Dawley rat liver S-9, or in 0.2M sodium phosphate buffer, without S-9], and then plated in triplicate. The number of revertants per plate was counted by using the Artek Counter Model 880 (BioLogics, Inc., Gainesville, VA) or manually, and averaged over the 3 plates. An assay was deemed positive if more than twice as many revertants were detected per plate, on average, as were detected with DMSO alone (11).

Two negative solvent controls (DMSO, 200 µL per plate, with or without histidine) were used for each strain. Three different positive chemical controls were used, each at a concentration of 5 µg per plate: sodium azide for strain TA-100 without rat S9, 2-nitrofluorene for strain TA-98 without rat S9, and 2-aminoacridine for both strains with rat S9. Sterility controls were tested with the Ames assay, using top agar and solvent control; these were prepared by pipetting 0.1 mL sample onto a nutrient agar plate. The plates were allowed to stand for a few minutes, incubated for 24–48 h at 37 ± 2°C, and checked for any growth of bacteria or fungi.

Total NOC Analysis

Each sample (1 g pancake or 2 g batter) was mixed with 2.5 mL 20% (w/v) aqueous solution of sulfamic acid (sufficient to reduce the pH below 2.0 in every case) to remove nitrite ion; then finely dispersed in an Omni homogenizer. A 2.5 mL aliquot of 100% DMSO was added, and each sample solution was dispersed a second time. The extracts were centrifuged for 15 min at 3000 × *g*, and the supernatant was removed for storage at –50°C until analysis. At the time of analysis, a 1 mL aliquot of extract was centrifuged again at 8000 × *g* for 10 min, followed by filtration through a 0.2 µm nylon membrane. The analytical method and apparatus used to measure total NOC were the same as described by Janini et al. (12). The standard nitrosamine chosen was

Table 2. Controls for *Salmonella* mutagenicity assay[a]

Specimen	Dose	TA98 without rat S9	TA98 with rat S9	TA100 without rat S9	TA100 with rat S9
Controls for pancake batter assays					
DMSO (negative control)	200 µL/plate	33 ± 5	31 ± 3	135 ± 21	131 ± 9
DMSO + histidine (histidine control)	200 µL/plate	28 ± 2	584 ± 127	200 ± 31	165 ± 16
2-Aminoacridine (positive control)	5 µg/plate	787 ± 69	1213 ± 11	912 ± 6	932 ± 2
Controls for cooked pancake assays					
DMSO (negative control)	200 µL/plate	73 ± 5	26 ± 6	131 ± 7	170 ± 19
DMSO + histidine (histidine control)	200 µL/plate	92 ± 4	51 ± 5	187 ± 8	186 ± 19
2-Aminoacridine (positive control)	5 µg/plate	817 ± 35	698 ± 14	1027 ± 35	968 ± 86

[a] Revertants per plate, average of 3 plates, mean ± standard deviation.

N-nitrosoisonipecotic acid (NINA, FW=158.1566); 20 mg solid NINA was diluted to 50 mL in deionized, filtered water. This stock solution was then diluted (1:500) to yield a high standard concentration of 5.06 µmol/L, and then serially diluted to make additional calibration standards of 2.53, 1.27, 0.63, 0.32, and 0.16 µmol/L. The limits of quantitation (LOQs) in the sample matrix were 1.3 nmol/g for pancakes and 0.7 nmol/g for batter.

It was previously demonstrated that the apparatus used in this study produced an equimolar response for several different NOC (12); therefore, a single compound was used to generate a calibration curve to estimate total NOC as a group. The utility of the analytical method to the sample matrix was evaluated by comparing extraction recovery data of several structurally different NOC in pancake and batter. Samples of pancake and batter which had no detectable NOC were pooled, aliquoted, and fortified separately at 5 nmol/g with the following compounds: N-methyl-N-nitrosourea (MNU), NINA, N-nitrosoproline (NPRO), and N-nitrosopyrolidine (NPYR). Samples were spiked in triplicate and analyzed in duplicate to generate 6 data points for each compound in each sample matrix.

The precision of the assay, as measured by percent relative standard deviation (RSD%), ranged from 7.26 to 11.92% and 5.30 to 13.69% for pancake and batter, respectively, and the accuracy as measured by percent recovery of the individual spiked NOC ranged from 29 to 75% and 57 to 91% for pancake and batter, respectively (Table 1). Overall recovery of NOC as a group was 51 ± 20% (n = 24) for pancake and 74 ± 16% (n = 24) for batter. For all NOC tested, recovery from batter was markedly higher than from pancake, most likely because of the higher water content of batter. The recoveries of NINA and NPRO were also enhanced in both matrixes possibly because of higher stability at low pH. This method is only intended to provide an estimate of the total NOC, and the precision and recovery data are sufficient to measure this.

Volatile Nitrosamine Assay

For the volatile nitrosamine assay, a 0.5 mL aliquot of the nitrite-free DMSO–water extract from the batter was transferred to a 4 mL vial containing 0.5 mL methylene chloride, and shaken vigorously for several minutes. The phases were allowed to separate, and the methylene chloride layer was removed to another vial containing a small amount of anhydrous sodium sulfate and mixed on a Vortex mixer for 1 min. A 2 µL aliquot of the methylene chloride extract was injected into an HP 5890 gas chromatograph (Agilent Technologies, Palo Alto, CA) coupled to the thermal energy analysis (TEA) detector through a pyrolyzer interface. The 6 volatile nitrosamines were separated in a 50 m × 0.53 mm DB-Wax capillary column (J&W Scientific, Folsom, CA) with helium carrier gas at a flow rate of 10 mL/min. The column oven of the gas chromatograph was held at 75°C for 5 min, then programmed to 200°C at a rate of 7°C/min and held for 5 min. The injector, pyrolyzer, and interface temperatures were set at 250, 550, and 225°C, respectively. A –120°C cold trap was maintained between the pyrolyzer and the TEA detector by cooling a mixture of methanol–ethanol solution (1 + 1) with liquid nitrogen.

The volatile nitrosamines N-nitrosodimethylamine (NDMA), N-nitroso-methylethylamine (NMEA), N-nitrosodiethylamine (NDEA), N-nitroso-methylbenzylamine (NMBzA), N-nitrospiperidine (NPIP), and NPYR were measured down to a limit of detection (LOD) of 0.25 nmol/g in the batter sample. A composite sample of batter was fortified with 2.5 nmol/g of each of the volatile nitrosamines and extracted as before with DMSO–water followed by methylene chloride. The recoveries from triplicate extractions ranged from 21 to 74% overall.

Nitrosamino Acid Assay

Sample preparation and instrument conditions used for the nitrosamino acid assay were similar to those of Ohshima et al. (13). The nitrosamino acids N-nitrososarcosine (NSAR), NPRO, N-nitrosopipecolinic acid (NPIC), *cis* and *trans* N-nitroso-2-methylthiazolidine-4-carboxylic acid (NMTCA), and N-nitrosothiazolidine-4-carboxylic acid (NTCA) were measured as their methyl esters down to a LOD of 0.1 nmol/g.

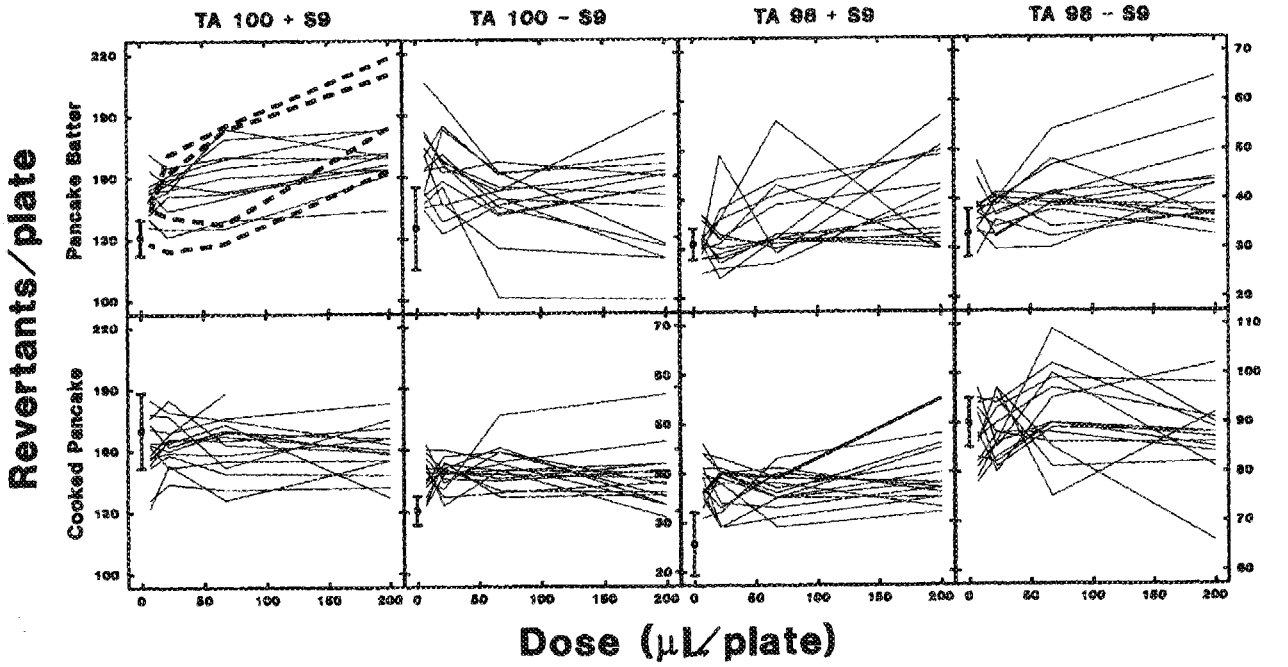

Figure 1. Mutagenicity of pancake batter and cooked pancake extracts in strains TA100 and TA98, with and without S9. The darker broken lines indicate results in which the correlation coefficients of data were >0.9 and the slope >0.2. The 0 mL/plate values are shown with standard deviations, which were omitted for other samples to improve clarity. The background levels of mutations for cooked pancake in strain TA98 with rat S9 were higher than the other TA98 values and were plotted on a different scale (bottom left). Sample No. 13 from cooked pancake in strain TA100 with rat S9 showed toxicity at the highest dose (47 ± 3.5 revertants per plate, mean ± SD), and this value was not plotted.

Results

In general, the specimens appeared to be actively fermenting, yielding copious quantities of gas whenever they were thawed for analysis.

Ames Test

The positive controls all gave positive responses in keeping with historical values (Table 2), validating the mutagenicity assay. The histidine growth control experiments indicated that background levels of histidine and histidine precursor compounds in the batter and pancakes would not be responsible for any increase in the mutation frequency observed (Table 2). Samples showed little evidence of toxicity at the doses tested, except for cooked pancake sample No. 13 tested with TA100 in the presence of rat S9, in which only 47 ± 4 mutants were seen at the highest dose, which is not plotted in Figure 1 for that reason.

The mutagenicity assay was weakly positive (based on the criterion of doubling the value for the negative control; 11) for only one specimen of cooked pancake (No. 7, solid line, Figure 1), and then only for strain TA-98 with rat S9. Several samples were suggestive of a positive response based upon the trends of increasing values with dose (Figure 1). The samples that showed the most consistent positive correlation of the mutation frequency with dose (correlation >0.9 with slope >0.2) were pancake batter samples Nos. 4, 5, 12, and 14 (Figure 1).

NOC Assays

Four of the 15 cooked pancake specimens (Nos. 2, 3, 8, and 9) were entirely used up in the Ames test before the NOC assay; total NOC levels were below the LOQs (<1.3 nmol/g) in the remaining 11 cooked pancake specimens. All 15 batter specimens were tested for total NOC, but only specimen No. 3 was positive, with a concentration in duplicate assays of 1.4 nmol/g (twice the LOQ in batter). That specimen was then tested for nitrosamino acids (LOQ 0.1 nmol/g) and volatile nitrosamines (LOQ 0.25 nmol/g); none were detected.

Discussion

The excess risk of stomach cancer associated with consumption of fermented (sour) corn pancakes in Shandong Province prompted a search for mutagenic or carcinogenic compounds in this dietary staple. Our study found no clear evidence of contamination by NOC or other mutagens. In contrast, a 1992 report (4) from the Beijing Institute for Cancer Research (Beijing, People's Republic of China) indicated that volatile nitrosamines were present in 92% of the sour pancakes, whereas 44% of the specimens showed activity in Ames tests, with most positive materials being direct mutagens (i.e., mutagenic without activation by rat S9). Three specific volatile nitrosamines (NDMA, NDEA, and NMEA) were reportedly present at concentrations of 0.4–3.8 ng/mL

(0.005–0.05 nmol/g), 0.2–8.1 ng/mL (0.002–0.08 nmol/g), and 0.3–1.4 ng/mL (0.003–0.02 nmol/g), respectively (4). These concentrations were well below the LOQs for our method; however, no validation data were provided, and it is possible that the earlier report was based on a false-positive result. Total nitrosamine levels in cured meat products tested by the U.S. Department of Agriculture reportedly ranged up to 24.8 parts per million (ppm), with the LOD being 0.1 ppm; because these values were based on the response of NPRO, this LOD is equivalent to 0.7 nmol/g, which is close to our LOQ (14). Nitrosamines and mutagens have been detected in Chinese salted fish and pickled vegetables after incubation with added nitrite at acidic pH, even when no nitrosamines or mutagens were detected in the same samples before incubation. However, we are not aware of any such experiments with corn products (15–17).

Changes in the preparation of sour pancakes may have indeed resulted in lower nitrosamine levels in recent years. Sour pancake consumption has fallen concomitantly with the decreasing consumption of salted and pickled vegetables, as fresh vegetables have become more widely available in rural China. Although approximately 50% of the households in Linqu County reported sour pancake intake in 1980, only 2–3% were still consuming the sour pancakes after 1992. In recent years, the duration of fermentation of sour pancake batter has been shortened, allowing fewer opportunities for contamination by nitrosamine-forming fungi, which may have accounted for our largely negative results. The specimens were freshly prepared for our experiments, without addition of active cultures traditionally consumed in earlier years.

With regard to the Ames assay, certain samples may have contained low levels of mutagens, but the results were not conclusive. Only 1 of the 15 specimens of cooked pancakes showed a positive response in the *Salmonella* mutagenicity assay, and all 15 batter specimens were negative. However, several batter specimens showed a weakly positive trend of mutagenicity with extract concentration. Such a trend was not observed for any of the cooked pancake specimens, perhaps because of the thermal lability of the mutagen, or superior extraction of the batter compared with that of the pancakes (although the pancakes had one positive result). On the whole, it seems unlikely that the Chinese fermented (sour) pancakes, as they are currently prepared, are significantly contaminated by nitrosamines or other mutagens. It is possible, however, that a decline in the mutagenic activity in certain foods has contributed to the downturn in stomach cancer mortality rates reported for men and women in rural areas of China during the 1990s (18).

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