Prevalence of Aflatoxin M₁ in Milk and Its Potential Liver Cancer Risk in Taiwan

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ABSTRACT

Pasteurized milk may contain the liver carcinogen aflatoxin M₁ (AFM₁) if the cows that produce the milk ingest feed contaminated with aflatoxin B₁. In this study, we collected 144 milk samples of three main brands in Taiwan twice a month over a 1-year period and purchased two samples each of eight domestic and imported brands of infant formula at two different times in 2005. Samples underwent solid-phase extraction, cleaning in immunoaffinity columns, and quantification by liquid chromatography–tandem mass spectrometry. We found autumn and winter levels of AFM₁ to be higher than those in the spring and the summer. We also found higher concentrations of AFM₁ in low-fat milk than in whole-fat milk. We were able to detect trace amounts of AFM₁ (1.17 to 54.7 ng/liter) in all of our milk samples, but there was only one sample in which the level of AFM₁ slightly exceeded the regulatory limit of the European Union (50 ng/liter). We were unable to detect AFM₁ in any of the infant formulae. Using a World Health Organization method of evaluating risk of liver cancer, the group we found to be at greatest risk was 6- to 9-year-old girls (average, 12.2 additional cases per billion); the group with the lowest risk was men of 45 to 64 years of age (average, 3.45 additional cases per billion), the latter consuming less milk than all other groups. Consequently, the risk for liver cancer due to ingestion of milk contaminated with AFM₁ was estimated to be low in Taiwan.

Aflatoxins are a group of several toxic secondary fungal metabolites produced by some Aspergillus spp., especially Aspergillus flavus and Aspergillus parasiticus (9). These molds are widely distributed in the environment and may contaminate crops and foods (16, 21). Aflatoxin M₁ (AFM₁), the major metabolite of aflatoxin B₁ (AFB₁), which is found in mammals, is produced when the hydrogen at the fourth carbon is replaced by a hydroxyl group; about 0.3 to 6.2% of AFB₁ ingested through animal feed has been reported to transfer in the form of AFM₁ into milk (6, 37). AFM₁ can also appear in urine, blood, and organ tissue of cows fed AFB₁-contaminated feed (24, 32). Hussein and Brasel reported that daily AFB₁ intake of ≥70 μg by cows will lead to AFM₁ levels in milk exceeding the regulatory limit (0.05 μg/liter) set by the European Union (EU) (21).

AFM₁ has 2 to 10% of the carcinogenic potency of AFB₁ and has almost the same liver toxicity as AFB₁ (17, 19, 33). The International Agency for Research on Cancer has classified it as a human carcinogen (group 1) (40). The main organ targeted by AFM₁ is the liver, where it induces hepatitis and hepatocirrhosis, but it is also toxic to other organs, like the kidney and thymus (31). Rat fetuses exposed to AFM₁ through placenta or breast milk have been found to be immunodeficient and susceptible to infection and particularly at risk for jaundice and myelodysplasia (38). Studies have reported that young animals are more susceptible to AFM₁ than adults (14, 19).

Common pasteurization methods (e.g., ultrahigh temperature or high-temperature–short-time processing) cannot effectively destroy AFM₁ in milk (28), so controlling AFB₁ contamination in feed being consumed by lactating cows may be the most appropriate preventive measure. To protect people (particularly children) from consuming contaminated dairy products, many countries have regulated the AFB₁ levels in feeds and AFM₁ in milk. The U.S. Food and Drug Administration has established an action level of 0.50 μg of AFM₁ per liter in whole, low-fat, and skim milk, while the EU has set a maximum allowed level of 0.05 μg/liter for raw milk, heat-treated milk, and milk used in the manufacture of milk-based products (13, 25, 36). In Taiwan, the maximum allowed levels in fresh milk and milk powder are 0.5 μg/liter and 5 μg/kg, respectively; AFM₁ in dairy baby food cannot be detected by the standard analytical methods (10, 11).

Taiwan is located in southeast Asia and has a typical subtropical climate suitable for mold growth. There are very few reports on AFM₁ levels in milk in the area of southeast Asia. This study investigated the occurrence of AFM₁ in milk and infant formula in Taiwan and assessed the potential risk of hepatocellular cancer (HCC) caused by ingesting AFM₁. This study is one of few surveys of AFM₁ levels in milk over an entire year and represents one of the first to assess HCC risk caused by AFM₁.

MATERIALS AND METHODS

Reagents. 4-Methylmorpholine was purchased from Aldrich (St. Louis, MO). Acetonitrile and methanol were high-pressure liquid chromatography (HPLC) grade (J. T. Baker, Phillipsburg,
Sample sources. We measured AFM$_1$ levels of 144 milk samples that were collected from supermarkets and convenience stores in Taipei City during the first and third weeks of each month during the entire year of 2005. All samples were refrigerated at 4°C and were analyzed before the expiration dates. These samples were from the three major brands (arbitrarily designated A, B, and C) representing 77.3% of the milk market in Taiwan in the year 2002 (8). We also collected two samples each of eight brands of infant formulae, both domestic products and products imported from the United States, Japan, Europe, and New Zealand in March and August 2005.

Preparation of samples. Levels of AFM$_1$ were determined using a published method (7). Briefly, 20 g of milk powder dissolved in 200 ml of deionized water at 40°C or 200 ml of fluid milk was divided into four 50-ml polyethylene tubes and centrifuged at 3,000 rpm (1,410 × g) for 25 min. Following the AOAC standard procedure (1), the floating lipids and condensed solids were discarded. The milk was then heated to 70°C and passed through activated 50-mm Speedisk C$_18$ (J. T. Baker) at 100 ml/min on a Diskmate II rotary extraction station (J. T. Baker). After washing with 10 ml of distilled and deionized water (Milli-Q water), the disks were moved to a vacuum manifold (J. T. Baker) and dried for 5 min (20-in. mercury vacuum) before being eluted with two portions of 5 ml of acetonitrile.

The disk eluate was concentrated to 0.5 to 1.0 ml at 45°C with a SpeedVac (Thermo Savant SPD 1010, Holbrook, NY). Milli-Q water was then added to bring the volume to 15 ml. The solution was passed through an AFM$_1$ immunoaffinity column (IAC) (capacity, 150 ng of AFM$_1$; Vicam, Watertown, MA), which was rinsed and followed by an additional 10 ml of Milli-Q water. After water residue was forced out of the column with a syringe, the IAC was eluted twice with 2 ml of acetonitrile-methanol (3:2 [vol/vol]), and the eluate was concentrated to 300 μl using the SpeedVac. It was necessary to use the solid-phase extraction of Speedisk plus IAC cleanup to eliminate the matrix effect, whereby the components in the sample influence the quantitation of the analyte; milk samples that were only extracted by IAC encountered up to 60% ion suppression on AFM$_1$ (details will be reported in a subsequent article).

Instrumental analysis. Fifty microliters of the concentrate was injected onto a Waters 600s HPLC pump attached to a TSQ 7000 triple-quadrupole mass spectrometer (Finnigan MAT, San Jose, CA). Separation was performed at 40°C on a PRP-1 polystyrene-divinylbenzene column (50 by 2.1 mm with a particle size of 3 μm; Hamilton, Reno, NV) with a guard column (25 by 2.3 mm; particle size, 12 to 20 μm) of the same material. The mobile phase consisted of 10 mM 4-methylmorpholine aqueous solution (pH 9.7) and acetonitrile at a flow rate of 0.2 ml/min. Initially, the organic portion was 20%, which linearly changed to 100% within 6 min and was left for 2 min. The retention time of AFM$_1$ was 5.1 min. The system was reequilibrated for 8 min before the next injection. AFM$_1$ was detected using negative electrospray ionization under selected reaction monitoring mode. The precursor ion was m/z 327.0 ([M – H$^-$])$^-$; the quantitation and confirmation ions were m/z 311.9 and 295.1 (327.0 > 311.9 and 327.0 > 295.1), respectively. The collision energy was 26 eV, and the dwelling time was 0.5 s. The sheath gas and the auxiliary gas were both nitrogen, at 70 and 20 psi, respectively.

An external calibration curve was built at each analysis by using seven standard solutions of AFM$_1$ (0.4, 2, 10, 40, 100, 200, and 400 ng/ml) in acetonitrile-water (1:4 [vol/vol]) ($r^2 > 0.995$). The limit of detection and limit of quantitation (LOD and LOQ) were defined as signal-to-noise ratio at 3 and 10, respectively. Data were acquired and analyzed using Finnigan Xcalibur Home Page v 1.1 and Microsoft Excel 2002. AFM$_1$ levels in different brands, lipid content, and seasons were tested with Student’s t test and one-way analysis of variance. The Scheffe post hoc test was used to compare the AFM$_1$ levels among seasons and brands.

Quality assurance and quality control. Glassware was soaked in 2 M sulfuric acid for 1 day before use to remove possible AFM$_1$ adsorption sites. Used glassware that had been in contact with AFM$_1$ standards or samples was soaked in aqueous NaOCl to destroy AFM$_1$ residue before cleaning. To deactivate the surface, we silylated the glass tubes and vials with 7% (vol/vol) dimethyldichlorosilane in toluene and rinsed them twice with toluene and methanol in sequence. With each batch of sample analysis, a Milli-Q water sample was analyzed and no experimental contamination was observed; two blank milk or milk powder samples (from the same bottle as the one used for spiking) were run to check existing residue levels. Two spiked samples at the level of 25 ng/liter using the same milk source were processed with other samples to calculate the recovery of the measurement; residue concentrations in milk blanks (if detected) were deducted in the calculation.

RESULTS

AFM$_1$ was detected in all of the 144 milk samples, and the average LOD and LOQ were 1.39 and 4.63 ng/liter, respectively (Table 1). There were no significant differences in detection limits between brands and between lipid contents of milk. Thirty-six samples (25.0%) contained less than 0.01 μg of AFM$_1$ per liter, and 107 samples (74.3%) contained between 0.01 and 0.05 μg of AFM$_1$ per liter.
TABLE 1. Levels of AFM1 in milk and infant formula

<table>
<thead>
<tr>
<th>Product</th>
<th>No. of samples</th>
<th>Positive samples (%)</th>
<th>&lt;0.01</th>
<th>0.01–0.05</th>
<th>0.05–0.25</th>
<th>&gt;0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>144</td>
<td>100</td>
<td>36</td>
<td>107</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Infant formulae</td>
<td>16</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a* The average detection limits of milk and infant formulae were 1.39 ng/liter and 11.9 ng/kg, respectively. ND, none detected.

Only one sample (0.055 µg/liter) slightly exceeded the EU action level, and none of these samples were above the action level set for Taiwan (0.5 µg/liter). The arithmetic and geometric means of the concentrations were 17.8 and 14.5 ng/liter, respectively. The AFM1 recovery rates from milk samples were 72.3 ± 18.9%. AFM1 was not detected in any infant formula sample (Table 1). The average LOD and LOQ were 11.9 and 39.7 ng/kg, respectively, and the average recovery rate was 88.5%.

AFM1 levels in low-fat milk (18.9 ± 11.4 ng/liter, n = 72) were statistically greater than those in whole-fat milk (16.7 ± 10.5 ng/liter, n = 72) (*P* < 0.05, paired *t* test). The mean level of AFM1 in brand C milk (12.3 ± 7.7 ng/liter) was significantly lower than that of either A (18.0 ± 11.0 ng/liter) or B (23.2 ± 11.3 ng/liter) (*P* < 0.05), although all average concentrations were lower than the maximum level set by the EU.

Regarding seasonal variations, AFM1 levels in milk in the autumn and winter were higher than those in the spring and summer (*P* < 0.01). We divided the 12 months into spring (March to May), summer (June to August), autumn (September to November), and winter (December to February); the AFM1 levels (mean ± standard deviation) for these seasons were 12.4 ± 9.0, 14.3 ± 10.4, 20.6 ± 11.1, and 23.9 ± 9.6 ng/liter, respectively (Table 2). The AFM1 levels in the spring were statistically lower than those in the autumn (P = 0.011) and the winter (P = 0.01). The levels in the summer were only significantly different from those in the winter (P = 0.011). There was no statistical difference in AFM1 levels between autumn and winter (Table 2).

The AFM1 exposure was different between males and females regarding age groups. The mean intake of AFM1 in females ranged from 3.25 to 4.95 ng/day; the lowest and highest groups were 45 to 64 and 19 to 44 years old, respectively. The average intake of AFM1 in males ranged from 3.58 to 5.67 ng/day; the lowest and highest groups were 19 to 44 and 6 to 9 years old, respectively. The average liver cancer risks of males and females with HBsAg− ranged from 1.65 × 10−8 to 5.5 × 10−8 and from 1.79 × 10−8 to 5.83 × 10−8, respectively, and were highest in 6- to 9-year-old girls, with the maximum-exposure individual (95% upper limit) at 1.60 × 10−7, because of their lower body weights. The average liver cancer risks of males and females with HBsAg− ranged from 5.49 × 10−10 to 1.83 × 10−9 and from 5.97 × 10−10 to 1.94 × 10−9, respectively, among different age groups. Consequently, even for those persons with HBsAg−, their HCC risks because of AFM1 ingestion were much lower than 1 × 10−8; for those people with HBsAg−, the HCC risks were lower than 1 × 10−8.

**DISCUSSION**

The high detection rate (100%) in our study can be ascribed to the improved sensitivity of our analytical method rather than to a serious contamination of AFM1 in milk in Taiwan. Several recent studies with low detection limits have reported high percentages of positive samples, but the levels fell within lower ranges (Table 3). For example, Lin et al. (25) detected AFM1 in 91% of the milk samples collected in 2002 in Taiwan in the range of 2 to 83 ng/liter; Nakajima et al. (26) reported the occurrence of AFM1 in milk in Japan to be 99.5%, but at low levels (1 to 20 ng/liter), a finding similar to that of Taiwan, where AFM1 is ubiquitous in milk but is in low concentrations. While the detection rate of AFM1 in milk in Turkey has been reported to be 58%, 81% of the positive samples exceeded the regulated level in Europe (0.05 µg/liter) (35) (Table 3).

We found some evidence possibly relating AFM1 levels and lipid content. AFM1 has been found in some milk derivatives like yogurt and cheese with a three- to fivefold increase over that in the milk, associated to the protein fraction (27). About 80% of the AFM1 is partitioned in the nonfat portion of milk, and about 30% of AFM1 in the portion is adsorbed to the nonlipid solids, particularly to the casein (16). The removal of lipids from milk may increase the relative portion of casein; because AFM1 has a low affinity to lipids (4), the relative concentration of AFM1 might increase in low-fat milk, as was observed in our study. Carvajal et al. (5), however, suggest that there is no significant correlation between milk lipid content and AFM1 levels, although there is a slightly higher probability of being contaminated by AFM1 with higher fat content. Because Carvajal et al. (6) considered only the samples contaminated above accepted levels (≥0.05 and ≥0.5 µg/liter), the difference between their findings and ours may result from dissimilar statistical analysis and distribution range of AFM1 levels.

**TABLE 2. Levels of AFM1 in different seasons**

<table>
<thead>
<tr>
<th>Season</th>
<th>Mean concn ± SD (ng/liter)</th>
<th>Significantly different from</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spring</td>
<td>12.4 ± 9.0</td>
<td>Autumn and winter</td>
</tr>
<tr>
<td>Summer</td>
<td>14.3 ± 10.4</td>
<td>Winter</td>
</tr>
<tr>
<td>Autumn</td>
<td>20.6 ± 11.1</td>
<td>Spring</td>
</tr>
<tr>
<td>Winter</td>
<td>23.9 ± 9.6</td>
<td>Spring and summer</td>
</tr>
</tbody>
</table>

*a* Comparison by Scheffe’s post hoc test.
Our finding on seasonal variations in AFM₁ levels was consistent with other studies, particularly for summer (2, 22, 23). In summer, lactating cows may ingest less AFB₁ when they are grazing than in autumn and winter; seasons when mixed feed is used (22, 23); temperature and moisture content could significantly affect the growth and toxification of Aspergillus spp. in compound feed for cattle. In contrast, Carvajal et al. (6) observed no significant difference in contaminating frequency (≥0.05 µg/liter) between the seasons.

One previous study indicated that the process of making milk powder (e.g., the freeze-dried and spray-dried milk) might reduce AFM₁ levels (30). Their finding may explain why we could not detect AFM₁ in either domestic or imported infant formulas. Another explanation may be that the stricter regulations for AFM₁ in dairy infant products in Europe (0.025 µg/liter) have taken effect in reducing AFM₁ levels in infant formulas.

According to the 2005 statistical data from the Department of Health, Taiwan (12), liver cancer was the second leading cause of death in all cancers (19.1%) and led to 7,108 deaths in Taiwan. Assuming that people in Taiwan are exposed to the existing regulatory level of AFM₁ (0.5 µg/liter), this will result in only 1.35 and 1.59 additional cases of HCC in men and women, respectively, therefore, lowering the limit to the EU regulatory level (0.05 µg/liter) will not achieve measurable reduction in liver cancer risk. A more suitable approach would be the vaccination against hepatitis B virus; after the universal vaccination of newborns and children, the incidence of liver cancer in Taiwan has begun to decrease (18).

This study found that the AFM₁ levels in milk in Taiwan apparently did not exceed EU regulatory limit (0.05 µg/liter), which demonstrated that the contamination of this natural toxin can be well controlled even in a subtropical country through good food practices and the enforcement of regulations. Although this survey did not detect AFM₁ in any sample of infant formula, we still need to pay attention to AFM₁ in baby food to protect the health of this vulnerable group of milk consumers. Children younger than 6 years old usually drink a relatively high amount of milk, but their consumption rates of milk are not available, unfortunately. Milk intake data for this age group would help us establish a better profile of cancer risk caused by AFM₁. Finally, the liver cancer risk in Taiwan caused by ingestion of AFM₁ in milk is quite low and acceptable based on the results of this first entire-year survey in Taiwan. Therefore, the consumption of milk, a nutritious foodstuff, should not be discouraged.

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REFERENCES


**TABLE 3.** Comparison of AFM₁ in milk in different countries in recent years

<table>
<thead>
<tr>
<th>Study</th>
<th>Country</th>
<th>Determination method</th>
<th>No. of samples</th>
<th>Limit of detection (ng/liter)</th>
<th>Detection rate (%)</th>
<th>Mean (range) (ng/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bognanno et al. (3)</td>
<td>Italy</td>
<td>HPLC-fluorescence and MS</td>
<td>240</td>
<td>1</td>
<td>81.3</td>
<td>15.4 (1.1–108)</td>
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<tr>
<td>Ghiasian et al. (15)</td>
<td>Iran</td>
<td>ELISA</td>
<td>186</td>
<td>5</td>
<td>64.0</td>
<td>43.3 (&lt;10–410)</td>
</tr>
<tr>
<td>Hussain and Anwar (20)</td>
<td>Pakistan</td>
<td>Fluorometer</td>
<td>168</td>
<td>NA</td>
<td>100</td>
<td>371 (&lt;10–700)</td>
</tr>
<tr>
<td>Nakajima et al. (26)</td>
<td>Japan</td>
<td>HPLC-fluorescence</td>
<td>208</td>
<td>1</td>
<td>99.5</td>
<td>9 (&lt;1–29)</td>
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<td>Lin et al. (25)</td>
<td>Taiwan</td>
<td>HPLC-fluorescence</td>
<td>44</td>
<td>2</td>
<td>90.9</td>
<td>NA (2–83)</td>
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<tr>
<td>Rastogi et al. (29)</td>
<td>India</td>
<td>ELISA</td>
<td>12</td>
<td>NA</td>
<td>33.3</td>
<td>86 (28–164)</td>
</tr>
<tr>
<td>Unusan (35)</td>
<td>Turkey</td>
<td>ELISA</td>
<td>129</td>
<td>10</td>
<td>58.1</td>
<td>108 (ND–544)</td>
</tr>
<tr>
<td>Zinedine et al. (41)</td>
<td>Morocco</td>
<td>HPLC-fluorescence</td>
<td>54</td>
<td>1</td>
<td>88.8</td>
<td>18.6 (1–117)</td>
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<tr>
<td>This study</td>
<td>Taiwan</td>
<td>HPLC/MS/MS</td>
<td>144</td>
<td>1</td>
<td>100</td>
<td>17.8 (1.1–55)</td>
</tr>
</tbody>
</table>

a MS, mass spectrometry; ELISA, enzyme-linked immunosorbent assay; NA, not available; ND, not detected.
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