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Association Between Aflatoxin M_1 Excreted in Human Urine Samples with the Consumption of Milk and Dairy Products

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Abstract This study aimed to find the association between urinary aflatoxin M_1 level and milk and dairy products consumption. Of 160 morning urine samples collected, aflatoxin M_1 was detected in 61.3 % samples (n = 98) [mean ± SD = 0.0234 ± 0.0177 ng/mL; range = 0–0.0747 ng/mL]. Of these positive samples, 67.3 % (n = 66) had levels above the limit of detection. Respondents with intake of milk and dairy products above median (67.79 g/day) had significantly high level of AFM_1 compared to those with low intake. A significant and positive association ($\varphi = 0.286$) was found between milk and dairy products consumption and urinary aflatoxin M_1 level.

Keywords Aflatoxin · Aflatoxin M_1 · Milk and dairy products consumption · Malaysia

Aflatoxin is one of the mycotoxins and human exposure to this contaminant has become a global issue as it is a potent carcinogen and linked to the development of liver cancer (IARC 1993). Aflatoxin is a toxic compound produced naturally by *Aspergillus* species of fungi mainly *Aspergillus flavus*, *Aspergillus paraciticus* and *Aspergillus nomius* (Reddy et al. 2011). Aflatoxin M_1 (AFM_1), which is a hydroxylated metabolite of AFB_1 produced from animal and human metabolism after the ingestion of AFB_1, can be found in urine, milk and blood (de Romero et al. 2010). Since aflatoxin is associated with the etiology of hepatocellular carcinoma (HCC), Sun et al. (1999) indicated that the risk of HCC is increased by 3.3-folds in those with detectable urinary AFM_1 (above 3.6 ng/L) and infected chronically with hepatitis B virus (HBV).

The main route of aflatoxin exposure among human is through the diets (de Romero et al. 2010) and milk products are among the top foods that are commonly found to be contaminated with aflatoxin. A recent study by Sadia et al. (2012) for instance found that AFM_1 is detected in milk and sweets in Pakistan. In fact, 32.3 % and 78.3 % of milk and sweets respectively had levels above the European Union permissible level (Sadia et al. 2012). Although there is no data on the contamination level of aflatoxin reported in milk and dairy products in Malaysia, these food commodities are among the top five daily consumed foods in Malaysia (Norimah et al. 2008). Additionally, the latest national food consumption statistic indicated high prevalence of condensed milk intake among Malaysians (MOH 2006). Since AFM_1 can be found in milk, the humans are at risk to aflatoxin exposure as they are used in manufacture of many products such as cheese, powdered milk and other dairy products. Given that as the epitome for our research, the purpose of this study was to screen the level of AFM_1 detected in human urine samples. Besides, this study also aimed to find the association between milk and dairy products consumption and the level of AFM_1 detected in human urine samples.

Materials and Methods

This research had received ethical approval from the Medical Research Ethics Committee of Universiti Putra Malaysia. There were 160 non-academic and support staffs from a faculty in Universiti Putra Malaysia who participated

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in this research. Prior to samples collection, written informed consent was obtained. An aliquot of 15 mL morning urine sample was collected and frozen at −80°C.

The respondents' milk and dairy products consumption was assessed through a semi-quantitative food frequency questionnaire (FFQ). The items in the FFQ were foods that are normally consumed and used by Malaysian such as fresh milk and condensed milk. The conversion of food frequency list to the amount of food intake (g/day) was calculated as described by Norimah et al. (2008) with the references used from the Atlas of Food Exchanges and Portion Sizes (Suzana et al. 2002) and Nutrient Composition of Malaysian Foods (Tee et al. 1997).

The level of AFM_1 was determined using the Helica Aflatoxin M_1 ELISA kit (Helica Biosystem, Inc., Santa Ana, CA, USA). In order to remove debris and precipitate, urine samples were centrifuged at 3,000*g* for 5 min (Kubota Centrifuge Model 2810, Tokyo, Japan) and supernatant was then used for the determination of AFM_1 accordingly as specified in the manual kit. An automated microplate washer (Drop ELISA Washer, RADIM, Italy) was used during the washing step. The absorbance was measured at 450 nm using microplate reader (SIRIO S Microplate Reader, RADIM, Italy). As validation, urine samples were spiked with 0.15 ng/mL AFM_1 standard. The fortified samples were covered from light and kept overnight at 2–8°C. The level of AFM_1 was measured as mentioned above. The limit of detection (LOD) was 0.011 ng/mL and the recovery ranged from 73 % to 88 % and was within the range stated on the provided manual. For the value below LOD, half of the LOD value was assigned. The urinary AFM_1 concentration was expressed as pg/g Cr (creatinine) in order to correct for variations in urine dilution among individual samples. The creatinine level was measured using Roche Cobas C311 Chemistry Analyzer (Roche Diognastics Limited, West Sussex, England).

The milk and dairy products consumption was categorized into low and high categories based on the respondents' median intake. Mann–Whitney *U* test was used to see if there was significant difference between milk and dairy products consumption and urinary AFM_1 level. Then, the correlation between these two variables was assessed based on Pearson Chi-square association.

Results and Discussion

There were 74 male and 86 female respondents, with the average age of 35.2 ± 10.15 years (Range = 23–57 years) who participated in this study. Of 160 morning urine samples collected, 61.3 % (n = 98) of the samples were found to have AFM_1 (mean ± SD = 0.0234 ± 0.0177 ng/mL; range = 0–0.0747 ng/mL). Moreover, 67.3 % of the positive samples (n = 66) had AFM_1 level above LOD. Besides that, all the respondents either with detected urinary AFM_1 or not had normal kidney function based on the creatinine level.

The AFM_1 values in urine observed in this study were low than those previously reported in China (Qian et al. 1994; Zhu et al. 1987), Ghana (Jolly et al. 2006) and Sierra Liones (Jonsyn-Ellis 2000). For instance, Qian et al. (1994) reported that the AFM_1 level detected in the urine varied from 0.17 to 5.2 ng/mL, which was 7–70 times higher than ours. However, the level found in this study was higher compared to the urinary AFM_1 level detected by de Romero et al. (2010) in Brazil (Range = 1.8–39.9 pg/mL) and Desalegn et al. (2011) in Sri Lanka (Mean = 0.012 ng/mL). These evidences show that aflatoxin exposure is ubiquitous. Due to that, it can be postulated that respondents from this study were moderately exposed to aflatoxin.

On the other hand, we found that AFM_1 was detected mostly among the younger respondents (below 40 years) rather than the older ones (Fig. 1). Nevertheless, there was no significant correlation between these two variables. It is indeed corroborated with the finding by Sun et al. (1999) as AFM_1 levels detected in male with hepatitis B are not correlated with age. Furthermore, Sun et al. (1999) indicated that the highest level of AFM_1 was found in men, age ranged of 30–45 years.

A positive association was found between AFM_1 detected in human urine samples and dietary AFB_1 intake (Zhu et al. 1987). There was between 1.23 % and 2.18 % of dietary AFB_1 found to be present as AFM_1 in human urine samples (Zhu et al. 1987). Therefore, based on the equation of $y = 0.007 + 0.0214x$ [where y represents the amount of AFM_1 in urine samples (ng/mL) while x represent dietary AFB_1 intake (μg/day)] by Sabran et al. (2012) and average body weight of Malaysian adult of 62.65 kg (Azmi et al. 2009), the estimated dietary AFB_1 exposure computed from the mean (0.0234 ng/mL) was 0.0122 μg/day kg body weight. The value was slightly low compared to the one indicated by Sabran et al. (2012) and the range stated by Liu and Wu (2010) in Malaysia. The period of collecting the samples might be one of the reasons for such observation. Jonsyn-Ellis (2000) for instance found a significant contribution of seasonal variation with the level of aflatoxin detected in human biological samples. In fact in Malaysia, humans are probably exposed to aflatoxin especially during the festive season such as during *Eidul Fitr* and holidays (Reddy et al. 2011), where certain foods that signify the celebrations are abundant and frequently consumed.

Our finding showed that respondents with milk and dairy products consumption above median (67.79 g/day) had significantly high level of AFM_1 compared to its counterpart (Table 1). Additionally, there was also statistically significant and positive association between milk

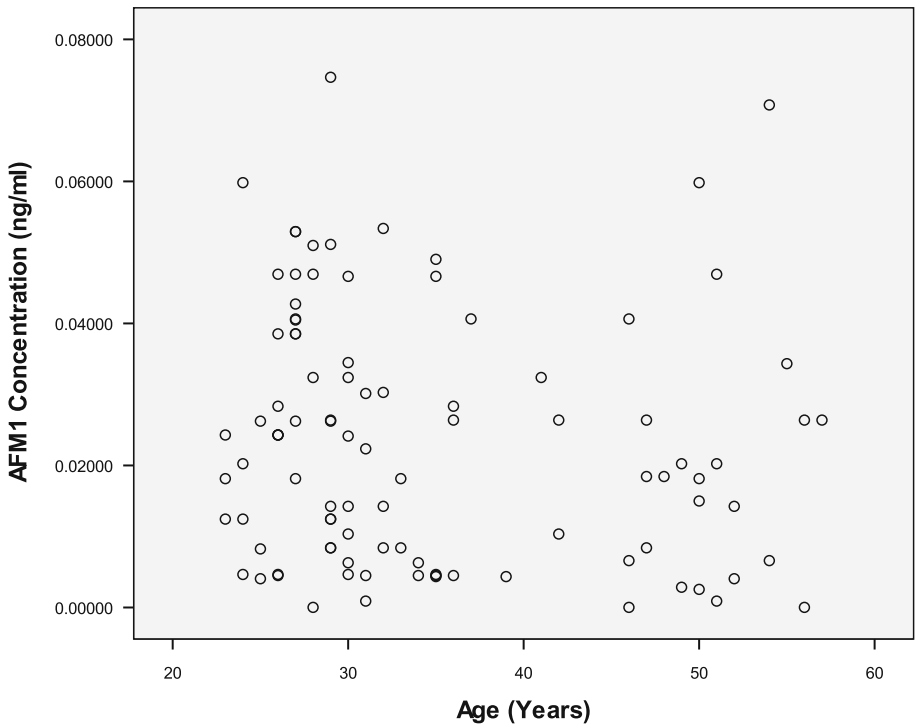

Fig. 1 Distribution of urinary AFM_1 according to respondents' age

Table 1 The AFM_1 level according to the consumption of milk and dairy products (n = 98)

Milk and dairy products consumption[a]	AFM_1 level, pg/g Cr (mean ± SD)	*p* value[b]
Low	2.00 ± 2.38	0.027
High	2.67 ± 2.27	

[a] Classification is based on median intake of 67.79 g/day

[b] Value obtained from Mann–Whitney *U* test

and dairy products consumption with the level of AFM_1 detected in urine samples (Table 2).The finding is in contrast to a study that revealed lack of association between urinary AFM_1 and food consumption (de Romero et al. 2010). In fact, milk and dairy products are one of the commodities that have high probability to be contaminated by aflatoxin and its contamination levels have been documented (Beltran et al. 2011; Heshmati and Milani 2010; Kim et al. 2000). Even though the data is not available yet in Malaysia and considering that high incidence of AFM_1 in milks and dairy products is a serious public health problem, a maximum permissible level of 5 μg/kg has been adopted in the Malaysia Food Regulations 1985 as one of the enforcements to control the contamination of aflatoxin (FoSIM 2012).

Although aflatoxin is a controllable risk factor in foods, we believe that some of the contaminated food might "escape" and persist in the food chain, where humans can be directly or indirectly exposed to this contaminant. Milk for example can be contaminated by aflatoxin when the animals such as cow, goat, and sheep are fed with AFB_1-contaminated feed. As this happens, the ingested AFB_1 is hydrolyzed into AFM_1, where it can be excreted in milk. Without realizing the incidence, the milk is used in manufacturing dairy products such as cheese, powdered milk and other milk-based products. Albeit many steps are taken during the food processing such as boiling and frying or even pasteurization, Kabak (2009) indicated that aflatoxin can still be found in the food commodities. One of the reasons is aflatoxin is heat-resistant and has high decomposition temperature, ranging from 237 to 306°C (Kabak 2009). For instance, following pasteurization at 62°C for 30 min, Purchase et al. (1972) only observed 32 % of AFM_1 reduction in the milk. Surprisingly, as the cycle goes on, humans at the end of food chain are vulnerable to be exposed to this toxic compound through these contaminated products. Indeed, newborn baby and children are at greater risk as the milk and dairy products are the main source of diets during their period of growth.

There were some limitations of the present study that should be noted for conducting a better research in the future. Firstly, we are only looking at the association of milk and dairy products consumption with the urinary AFM_1 level. It is noteworthy that further research on other "suspected" food commodities such as nuts, cereals and spices can provide expressive contribution toward exposure to aflatoxin through the diets. Secondly, the level of aflatoxin in the milk and dairy product was not investigated. Due to that, it is suggestible to investigate the level of aflatoxin in this food commodity; hence the data can

Table 2 Correlation between milk and dairy products consumption and urinary AFM_1 level (pg/g Cr)

Milk and dairy products	Median intake (g/day)	Pearson Chi-square[a]	Correlation[b]
Fresh/UHT milk	16.67	1.366	0.118
Condensed milk	5.71	0.384	−0.063
Evaporated milk	0.72	5.900*	0.245
Powdered milk	0.13	0.582	−0.077
Cheese	0.18	0.326	0.058
Others[c]	6.00	0.582	0.077
Overall	67.79	8.030**	0.286

* Significant at $p < 0.05$; ** Significant at $p < 0.01$

[a] Computed only for a 2×2 based on median intake and urinary AFM_1 level (1.53 pg/g Cr)

[b] Correlation is based on Phi value of association

[c] Includes yoghurt, dadih and other dairy products

provide a better and clear picture regarding human dietary exposure to aflatoxin. Thirdly, by investigating other aflatoxin biomarkers such as aflatoxin albumin adduct in serum can permit us to make a better assessment and validation on the extent of human exposure to aflatoxin in Malaysia.

In essence, the consumption of milk and dairy products cannot be said as the only source of occurrence of AFM_1 in the urine. However, it is believed that these findings provide an interesting insight regarding the occurrence of aflatoxin biomarker in human biological sample in Malaysia and its association with food consumption. Even though the levels detected are not that high and AFM_1 is less mutagenic and carcinogenic as compared to AFB_1, long term exposure to this contaminant could pose detrimental health effects. The incidence of aflatoxicosis case involving humans that happened around two decades ago (Lye et al. 1995) should be taken as a lesson for us to be more cautious about fungal and aflatoxin contamination. By creating the awareness about the harmful effect of aflatoxin among the public, it is possible to prevent the contamination. Furthermore, an early intervention should be adopted in order to prevent the "flow" of aflatoxin in the food chain.

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Conflict of interest All the authors have declared that they do not have any potential conflicts of interest.

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