RESEARCH ARTICLE

Lack of an Apparent Association between Mycotoxin Concentrations in Red Chili Peppers and Incidence of Gallbladder Cancer in India : an Ecological Study

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Abstract

Our recent studies conducted in South America have shown that mycotoxin contamination of red chili peppers (RCPs) may be associated with an increased risk of gallbladder cancer (GBC). Whether this relationship exists in India, a country with a high incidence of GBC and high consumption of RCPs, is unclear. We therefore measured concentrations of aflatoxins (AFs) and ochratoxin A (OTA) in RCPs from areas of low, medium, and high incidence of GBC in India, and compared these concentrations with GBC incidence in each area. Twenty-one RCP samples were collected from nine cities (eight from a low-incidence area, five from a medium-incidence area, and eight from a high-incidence area). Concentrations of AFs and OTA were measured using high-performance liquid chromatography. No significant differences in mean concentrations of AFs and OTA were found in the three areas. AFB1 levels in the low-incidence area (10.81 μg/kg) and high-incidence area (12.00 μg/kg) were more than 2.2 and 2.4 times higher compared with the maximum permitted level of AFB1 in spices (5.0 μg/kg) set by the Commission of the European Communities, or that (4.4 μg/kg) obtained in our previous study in Chile. Our results show that the mean concentrations of mycotoxins in RCPs are similar among the three areas in India with different incidences of GBC. Further studies with human subjects are needed to evaluate any association between AFB1 and GBC.

Keywords: Mycotoxin contamination - Indian red chili peppers - aflatoxin B1 - ochratoxin A - HPLC

Asian Pac J Cancer Prev, **17 (7)**, 3499-3503

Introduction

There is a marked worldwide geographic and racial variation in gallbladder cancer (GBC) incidence (Wistuba and Gazdar, 2004). High incidence of GBC is observed in specific countries (e.g., Chile, Bolivia, Peru) or in certain areas (e.g., South Chile, northern India). This characteristic suggests that GBC incidence is associated with geographic-specific environmental factors and environmental factor-related genetic factors.

Our previous study suggested that high consumption of red chili peppers (RCPs) is a significant risk factor for GBC in Chilean women with gallstones (Serra et al., 2002). However, the pathogenic mechanism by which GBC develops via RCP consumption in the presence of gallstones is not known. We conducted studies to reveal the pathogenic mechanism of GBC in subjects from Chile. We hypothesized that GBC in Chileans would be developed by high consumption of mycotoxin-contaminated RCPs. We measured concentrations of aflatoxin (AF)B1, AFB2, AFG1, and AFG2, or ochratoxin A (OTA) in Chilean RCPs. Contrary to our original expectation, Chilean RCPs had low levels of AFs and high levels of OTA (Tsuchiya et al., 2011; Ikoma et al., 2015). In addition, the mean OTA concentration in RCPs from Chile, Bolivia, and Peru was in the order Chile > Bolivia > Peru, which is in accordance with GBC incidence (Ikoma et al., 2015). These evidences suggest an association between high consumption of OTA-contaminated RCPs and GBC development in Chile, Bolivia, and Peru. Recently, Nogueira et al. (2015) demonstrated an association between AFs and GBC based on our previous findings (Foerster et al., 2016).

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India has a high incidence of GBC, especially in the north Indian women (Kapoor and McMichael, 2003; Indian Council of Medical Research, 2014). Indian people consume RCPs as an essential spice almost every day. According to the Food and Agriculture Organization Corporate Statistical Database, Indians consume dried chilies and peppers at a frequency of 2.50 g/capita/day (Honkawa Data Tribune, 2007). A guideline for GBC in India suggests that AF contamination of daily food is a risk factor for GBC (Indian Council of Medical Research, 2014). However, studies on the concentrations of mycotoxins (including AFs and OTA) in RCPs consumed widely by Indians are lacking. Therefore, we conducted a study to ascertain if mycotoxin concentrations in RCPs are associated with GBC in Indians.

Materials and Methods

Incidence of GBC in India

Based on the incidence rate of GBC per 100,000 subjects in each city of India (Kapoor and McMichael, 2003; Murthy et al., 2011; Sharma et al., 2014), we divided the incidence rate in females into three groups: low-incidence area, ≤4.0; medium-incidence area, 4.1-8.0; high-incidence area, ≥8.1. Grouping for cities that did not disclose the incidence rate was determined by reference to data published in adjacent cities.

Materials

Twenty-one powdered RCPs were purchased at central markets in nine cities (eight from Delhi, Lucknow, and Saharsa of the high-incidence area; five from Bhopal, Jaipar, and Orisa of the medium-incidence area; eight from Mumbai, Chennai, and Bangalore of the low-incidence area). All RCPs were sent to Japan, and concentrations of mycotoxins in samples measured in the way prescribed hereafter at the Mycotoxin Research Association (Yokohama, Japan).

Extraction of AFs and OTA from RCPs

AFs were extracted from RCPs using a method described previously in reference to Director Notice (Shoku-An number 0728004) (Ministry of Health, Labour and Welfare, 2008). OTA was extracted with methanol/water and 1% $NaHCO_3$ solution (70:30, v/v) and eluted with methanol (Sugita-Konishi et al., 2006).

Measurement of AF concentration

A high-performance liquid chromatography (HPLC) system (D-2000; Hitachi High Technology, Tokyo, Japan) was used to measure concentrations of AFB1, AFB2, AFG1, and AFG2 in RCPs by a method described previously by our research team (Asai et al., 2012). We used the following operating conditions: Atlantis T3 C18 column (5-μm particle size, 250 mm × 3.0 mm i.d.; Waters, Milford, MA, USA); column temperature, 40°C; mobile phase, acetonitrile: methanol:water (1:3:6, v/v/v); flow rate, 0.4 mL/min; excitation wavelength of 365 nm and emission wavelength of 450 nm for detection; injection volume, 20 μL.

Measurement of OTA concentration

The OTA concentration in RCPs was measured using the HPLC system as described previously (Sugita-Konishi et al., 2006). HPLC conditions for OTA detection were: Cadenza CD-C18 column (3 μm particle size, 250 mm × 4.6 mm, i.d.; Imtakt, USA); column temperature, 40°C; mobile phase, acetonitrile:water:acetic acid (55:43:2, v/v/v); flow rate, 1.0 mL/min; excitation wavelength of 333 nm and emission wavelength of 460 nm for detection; injection volume, 100 μL.

Recovery rates and limit of detection

Recovery rates for each 2.5 μg/kg of AFB1, AFB2, AFG1, and AFG2 were 85%, 86%, 90%, and 90%, respectively. The limit of detection of the each AF assay was 0.1 μg/kg. The recovery rate for 10 μg/kg of OTA was 87%. The limit of detection of the OTA assay was 0.1 μg/kg.

Statistical analyses

A one-way analysis of variance (ANOVA) was used to examine significant associations between mycotoxin concentrations in RCPs and GBC incidence. Statistical analyses were done using STATA SE14 (Stata, College Station, TX, USA). $P < 0.05$ (two-tailed) was considered significant.

Results

We measured mycotoxin concentrations in 21 RCPs collected from low-, medium-, and high-incidence areas, and then compared these concentrations with the incidence

Table 1. Concentration of Aflatoxin B1 in Red Chili Peppers Collected from Low-, Medium-, and High-Incidence Areas of Gallbladder Cancer in India

	Low-incidence area of GBC	Medium-incidence area of GBC	High-incidence area of GBC
Sample-1	4.7	1.1	25.2
Sample-2	0.6	0.6	10.5
Sample-3	19.7	5.1	3.5
Sample-4	20.7	4.9	3.6
Sample-5	4.4	0.7	0.7
Sample-6	26.9		33.2
Sample-7	9.4		16.4
Sample-8	< 0.1		2.9
No. of samples	8	5	8
Mean (SD), μg/kg	10.81 (10.25)	2.48 (2.31)	12.00 (11.94)
>5 μg/kg, No. (%)	4 (50.0)	1 (20.0)	4 (50.0)

GBC: gallbladder cancer, Mean value including the data below the detection limit (<0.1 μg/kg) was calculated using 0.1 as the value of the sample.

Table 2. Concentration of Total Aflatoxins in Red Chili Peppers Collected from Low-, Medium-, and High-Incidence Areas of Gallbladder Cancer in India

	Low-incidence area of GBC	Medium-incidence area of GBC	High-incidence area of GBC
Sample-1	5.2	1.4	26.7
Sample-2	0.9	0.9	11.8
Sample-3	21.0	5.5	3.9
Sample-4	22.0	5.5	4.7
Sample-5	4.8	1.0	1.0
Sample-6	28.5		35.7
Sample-7	10.0		17.4
Sample-8	0.4		3.3
No. of samples	8	5	8
Mean (SD), μg/kg	11.60 (10.77)	2.86 (2.42)	13.06 (12.61)
>10 μg/kg, No. (%)	4 (50.0)	0 (0)	4 (50.0)

GBC: gallbladder cancer

Table 3. Concentration of Ochratoxin A in Red Chili Peppers Collected from Low-, Medium-, and High-Incidence Areas of Gallbladder Cancer in India

	Low-incidence area of GBC	Medium-incidence area of GBC	High-incidence area of GBC
Sample-1	14.9	1.8	49.2
Sample-2	1.6	0.5	8.5
Sample-3	62.6	611.2	1.7
Sample-4	44.8	40.7	54.4
Sample-5	22.2	16.6	18.7
Sample-6	27.5		3.6
Sample-7	58.3		19.1
Sample-8	< 0.1		8.1
No. of samples	8	5	8
Mean (SD), μg/kg	29.00 (24.11)	134.2 (267.2)	20.41 (20.41)
>15 μg/kg, No. (%)	5 (62.5)	3 (60.0)	4 (50.0)

GBC: gallbladder cancer; Mean value including the data below the detection limit (<0.1 μg/kg) was calculated using 0.1 as the value of the sample.

in each area in India.

Table 1 shows AFB1 concentrations in RCPs collected from low-, medium-, and high-incidence areas in India. No significant difference in the mean concentration of AFB1 was found among the three areas, though the highest mean concentration of AFB1 was in the high-incidence area (12.00 μg/kg), followed by low- (10.25 μg/kg), and medium- (2.31 μg/kg) incidence areas. Values from low- and high-incidence areas were 2.5 and 2.7 times higher than that in Chile (4.4 μg/kg) (Tsuchiya et al., 2011). Samples that exceeded the maximum permitted level of AFB1 in spices (5.0 μg/kg) set by the Commission of the European Communities, Commission Regulation (EC) (the Commission of the European Communities, 2006) were detected in four RCPs (4/8, 50%) from the low-incidence area, in one RCP (1/5, 20%) from the medium-incidence area, and in four RCPS (4/8, 50%) from the high-incidence area.

Table 2 shows the concentrations of total AFs in RCPs collected from low-, medium-, and high-incidence areas in India. As in the case with AFB1, the highest mean concentration of total AF was in the high-incidence area (13.06 μg/kg), followed by the low-incidence area (11.60 μg/kg), and medium-incidence area (2.86 μg/kg). However, no significant differences were found among the three areas. Samples that exceeded the maximum permitted level of total AFs in spices (10 μg/kg) set by the EC were detected in four RCPs (4/8, 50%) from low- and high-incidence areas, but none in the medium-incidence area.

Table 3 shows OTA concentrations in RCPs collected from low-, medium-, and high-incidence areas in India. The highest mean OTA concentration was found in the medium-incidence area (134.2 μg/kg), and the lowest level was found in the high-incidence area (20.41 μg/kg). However, there were no significant differences in mean OTA concentrations among the three areas. Samples that exceeded the specific maximum limit for RCPs (15 μg/kg) set by the EC were detected in five RCPs from the low-incidence area (5/8, 62.5%), in three RCPs from the medium-incidence area (3/5, 60.0%) and in four RCPs from the high-incidence area (4/8, 50.0%).

These results suggested no significant association of mycotoxin concentrations of RCPs with GBC incidence in India.

Discussion

This ecological study demonstrated no significant associations between mycotoxin concentrations of RCPs and GBC incidence in India. However, mean levels of AFB1 in RCPs from low- and high-incidence areas were 2.5 and 2.7 times higher than that in Chilean RCP (4.4 μg/kg).

India has a high incidence of GBC, and the most common areas for GBC are in northern and northeastern India (Kapoor and McMichael, 2003; Indian Council of Medical research, 2014). Studies have shown the risk factors for GBC: demography; gallbladder abnormalities; patient exposure; infections (Kanthan et al., 2015). However, environmental risk factors for GBC in Indians are not well known. According to a guideline provided by

the Indian Council of Medical Research (Indian Council of Medical research, 2014), obesity, diabetes mellitus, addiction to smoking and alcohol, dietary contamination (e.g. AF exposure) are risk factors for GBC. Some researchers have examined the association between dietary habits and GBC development using case-control studies (Pandey and Shukla, 2002; Pandey, 2003; Rai et al., 2004; Kumar et al., 2006; Jain et al., 2013; Panda et al., 2013; Gupta et al., 2016), but no study has examined the association between consumption of RCPs contaminated with mycotoxins and GBC risk.

A high consumption of RCPs has been shown to be a significant risk factor for GBC in Chilean women with gallstones (Serra et al., 2002). However, the pathogenic mechanism by which GBC develops via RCP consumption in the presence of gallstones is not well defined. We hypothesized that GBC in Chileans would develop because of high consumption of RCPs contaminated with mutagens or carcinogens. Our first target of mutagens/carcinogens was mycotoxins such as AFs and OTA in RCPs. Our research team has shown that: (i) Chilean RCPs are mutagenic according to the Ames test, and that AFB1 and AFG1 are detected in RCPs at 4.4 and 0.5 μg/kg, respectively (Tsuchiya et al., 2011); (ii) AFs are detected in RCPs from Bolivia and Peru, which are countries with a high incidence of GBC (Asai et al., 2012); (iii) contamination by OTA in RCPs from Chile, Bolivia, and Peru is higher than that for AFs. GBC incidence in these countries is in accordance with the mean concentration of OTA in each country (Ikoma et al., 2015).

Based on our findings, a recent study conducted in Chile demonstrated an association between AFs and GBC (Nogueira et al., 2015). They measured levels of aflatoxin-albumin adducts in plasma collected from patients with GBC, patients with gallstones, and healthy subjects. They found that the level in patients with GBC was significantly higher than that in patients with gallstones or healthy subjects. Indians consume RCPs with extraordinary frequency, so high consumption of RCPs contaminated with high levels of AFs suggests the possibility of GBC development. However, the effect of AFs in RCPs on development of GBC in Indians has not been evaluated, so we measured mycotoxin concentrations in RCPs collected from areas with a different incidence of GBC, and compared the concentrations with the incidence in each area. However, contrary to initial expectations, our results showed no significant association between mycotoxin concentrations of RCPs and GBC incidence. The difference in results between Chile and India may be explained by four main reasons. In Chile, the association between AF level and GBC was evaluated directly through a study of patients and healthy volunteers, but our results from India were evaluated indirectly using an ecological study. Second, although RCP samples were collected from cities in different incidence areas, they may have been grown and produced in another region. Third, we used RCPs as samples, but other foods contaminated with mycotoxins may also be associated with GBC development. In our previous study, mycotoxins were not detected from fresh RCPs collected from Bolivia and Peru (Ikoma et al., 2015), so we should measure the concentrations of mycotoxins in dried foods, such as dry ginger and black pepper (Jaswal et al., 2015). Fourth, recent studies have suggested that gene-environment interactions are important for understanding cancer development (Brennan, 2002) because cancer risk due to environmental factors is modified by mutations of environmental factor-related genes. This hypothesis suggests that GBC could be caused among individuals with some specific genetic mutations. In humans, AFB1 is metabolized and changed to carcinogens in the liver, and AFB1-DNA adducts excreted into bile (Ruth et al., 1999; Guarisco et al., 2008). Thus, additional studies focusing on the metabolism of AF genes are needed to evaluate the association between AF and GBC incidence in Indians.

Our study had limitations. First, the sample size was small, so the results had reduced statistical power to detect significant associations between mycotoxin concentrations and GBC incidence. Otherwise, our results may have not reflected precisely the impact of mycotoxins on GBC incidence in Indians. A further study using larger sample sizes is required to clarify the association between mycotoxins and GBC incidence. Second, this was an ecological study, so we could not estimate the daily intake of mycotoxins from foods in Indians. In our next study, we will examine the daily intake and mycotoxin concentrations of RCPs consumed by the people living in three incidence areas.

This was the first ecological study to examine the association between mycotoxin contamination of RCPs and GBC incidence. Thus, direct evaluation of the association between AFs and GBC (such as the one conducted in Chile) are needed.

Acknowledgements

We are indebted to Dr. Ajit Mishra, Dr. Deepti V, and Dr. Swayam Prakash of the Sanjay Gandhi Post Graduate Institute of Medical Sciences (Lucknow), Dr. Paari Vijay Raghvan of the MIOT International Hospital (Chennai), Dr. Ajay Sharma of the SMS Medical College (Jaipur), Dr. Senthilnathan P of the GEM Hospital and Research Centre (Coimbatore), Dr. Joy Abraham of the CIMS Hospital (Ahmedabad), Dr. Shailesh Shrikhnade of the Shrikhande Clinic (Mumbai), Dr. Subhodh Varshney of the Bhopal Memorial Hospital & Research Centre (Bhopal) for their assistance in RCP collection. This study was supported in part by a Grant-in-Aid for Scientific Research (C) (2016, #16K09080) from the Japanese Ministry of Education, Science, Sports and Culture.

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