RESEARCH COMMUNICATION

Mutagenicity and Mutagens of the Red Chili Pepper as Gallbladder Cancer Risk Factor in Chilean Women

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Abstract

High consumption of red chili pepper has been shown to be a risk factor for gallbladder cancer (GBC) in Chilean women with gallstones, and included mutagens may be important in this context. We aimed to investigate the mutagenicity and mutagens in Chilean red chili pepper in the Ames test using *Salmonella typhimurium* strains TA98, TA1537, TA100, and TA1535 with and without metabolic activation (S9 mix). Pure capsaicin was tested for mutagenicity using strain TA98. The presence of aflatoxins was evaluated by two-dimensional thin layer chromatography, and then the concentrations of aflatoxins B1, B2, G1, and G2 were measured by an HPLC system. In strain TA98, the mean numbers of revertant colonies with and without the S9 mix were 2.5- and 2.2-fold higher than those of each negative control, respectively. However, pure capsaicin did not show mutagenic activity in strain TA98. Aflatoxin contamination of red chili pepper was confirmed, and the concentrations of aflatoxins B1 and G1 were 4.4 ng/g and 0.5 ng/g, respectively. Our findings suggest that low-level but protracted exposure to aflatoxins may be associated with the development of GBC in Chilean women who carry gallstones.

Keywords: Ames test - *Salmonella typhimurium* TA98 - aflatoxin contamination - red chili pepper - gallbladder cancer

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Introduction

There is a prominent worldwide geographical and racial variability in the incidence of gallbladder cancer (GBC), which correlates with the prevalence of cholelithiasis (Wistuba and Gazdar, 2004). Furthermore, female gender is one of the risk factors for GBC, and the incidence rate is 2-6 times higher in women than men, especially in countries and regions with the highest rates of GBC (Wistuba and Gazdar, 2004). Therefore, the urgent task is to clarify the etiology of GBC in women in the high incidence areas.

Recent studies have shown that the incidence rate for GBC is higher in Chile than in other countries (Kaushik, 2001; Misra et al., 2003; Andiaet al., 2008). In a previous study, we explored the risk factors for GBC in Chilean men and women using a hospital-based case-control study (Serra et al., 2002). In that study, only high consumption of red chili pepper was identified as a significant risk factor for GBC in Chilean women who carry gallstones (GS), among 60 questions that included type of cooking oil used, medical history, family history, daily activity, and so on. However, the pathogenic mechanism by which GBC occurs via red chili pepper consumption in the presence of GS remains uncertain.

Chili pepper originated in Latin America, where it has traditionally been grown as a tropical vegetable, but now it is grown in many places around the world. Chili pepper is commonly consumed as a spice because of its strong pungent taste. However, striking physiological effects caused by capsaicin (8-methyl-N-vanillyl-6-nonenamide), which is a pungent ingredient of chili pepper, have been shown in previous studies as follows: excitement by irritation of sensory nerves (Buck and Burks, 1986), bodily heat production (Osaka et al., 2000), effectiveness as a spice for persons on special diets or those needing to limit their salt intake (Osada el al., 1997; Reinbach el at., 2009; Snitker et al., 2009), antioxidant action (Howard et al., 2000), antibacterial action (Careaga et al., 2003), maintenance of body strength (Kim et al., 1997), immune depressive effect (Singh et al., 1996), inhibition of tumor cell growth (Morre et al., 1995), analgesic action (Craft and Porreca, 1992). In addition to these reports, there have been several reports on the mutagenicity or carcinogenicity of red chili pepper or capsaicin (Buchanan et al., 1981; Toth et al., 1984; Nagabhushan and Bhide, 1985; Lawson and Gannett, 1989; Miller et al., 1993; Azizan and Blevins, 1995; Chanda et al., 2004; Proudlock et al., 2004), although the findings are inconsistent. Some researchers have reported that red chili pepper and capsaicin show mutagenic activity by the Ames test (Toth et al., 1984; Nagabhushan and Bhide, 1985; Lawson and Gannett,

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1989; Miller et al., 1993; Azizan and Blevins, 1995), while others have reported the opposite outcome, namely non-mutagenicity or non-carcinogenicity (Buchanan et al., 1981; Chanda et al., 2004; Proudlock et al., 2004).

Although conflicting results regarding the mutagenicity or carcinogenicity of chili pepper or capsaicin have been reported, the presence of some mutagens or carcinogens in red chili pepper may be associated with the increased risk of GBC in Chilean women. Furthermore, Chilean red chili pepper may be contaminated with aflatoxins because conditions favorable for natural aflatoxin contamination of foods occur at latitudes between 40°N and 40°S of the equator (Williams et al., 2004).

We hypothesized that GBC in Chilean women is in some cases caused by the high consumption of red chili pepper including such mutagens or carcinogens. To date, no study has examined the mutagenicity of red chili pepper or the presence of mutagens in red chili pepper consumed by Chilean women. We therefore examined the mutagenicity of Chilean red chili pepper and pure capsaicin, and aflatoxins levels in Chilean red chili pepper.

Materials and Methods

Materials

Dry crushed red chili pepper was purchased at a central market in Santiago (Mercado Central), Chile.

Extraction of mutagens from chili pepper

Red chili pepper was powdered using an ultra centrifugal mill, model ZM 200 (RETSCH Co., Ltd., Tokyo, Japan). Extraction of mutagens in red chili pepper was performed using the following process reported by Villasenor et al., (1994): 20 g of powdered red chili pepper was mixed with 200 ml of 95% ethanol on a stirrer for 60 min. The red chili pepper-ethanol mixture was filtered and then concentrated under reduced pressure at 40ºC using a rotary evaporator (Eyken, Tokyo, Japan). The residue after the evaporation to dryness was re-extracted with 200 ml of water and hexane (1:6, v/v). The aqueous layer was further extracted with 50 ml of chloroform. The chloroform layer was concentrated under reduced pressure at 40ºC using the rotary evaporator. The residue was dissolved in 1 ml of dimethyl sulfoxide (DMSO). This dissolved solution was diluted twice with DMSO, and we then investigated the mutagenicity of red chili pepper at concentrations of 2, 1, 0.5, 0.25, 0.125, and 0.0625g red chili pepper equivalents/plate.

Mutagenicity assay

The mutagenicity assay was performed using the *Salmonella typhimurium*/microsome assay (Ames test) described by Ames et al., (1975). *Salmonella typhimurium* strains TA98, TA1537, TA100, and TA1535 were used to evaluate the mutagenicity of red chili pepper. The *Salmonella* strains were obtained from Dr. Takehiko Nohmi, Division of Genetics and Mutagenesis, National Institute of Health Sciences. The strains stored in nutrient broth containing 8% DMSO at -80°C were suspended in nutrient broth and incubated with shaking (180 rpm) at 37°C for 9 hours until the absorption at 620 nm reach a bacterial cell population density of 1-2 x 10^8/ml.

For metabolic activation, we used an S9 mixture (S9 mix) that included S9 (Oriental Yeast Co., Ltd., Tokyo, Japan) and co-factors (Oriental Yeast Co., Ltd.). The contents of the S9 mix per ml were as follows: 0.1 ml of phenobarbital and 5,6-benzoflavone-induced male Sprague-Dawley rat-liver S9, 8 μmols of $MgCl_2$, 33 μmols of KCl, 5 μmols of glucose-6-phosphate, 4 μmols of NADPH, 4 μmols of NADH, and 100 μmols of sodium phosphate, pH 7.4. Positive controls (Oriental Yeast Co., Ltd.) with or without the S9 mix were as follows: 2-aminoanthracene (2-AA) for the 4 strains with the S9 mix, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) for strains TA98 and TA100, 9-aminoacridine (9AA) for strain TA1537, and sodium azide (NaN_3) for strain TA1535 without the S9 mix.

The procedures for the Ames test were as follows: the mixture with 500 μl of the S9 mix or 0.1 mole sodium phosphate (pH 7.4), 100 μl of each dilution of the residue dissolved with DMSO , and 100 μl of the tester strain was incubated at 37°C with shaking (120 rpm) for 20 min. We then added 2 ml of molten top agar to the incubation mixture. After mild mixing, the tube contents were poured onto a plate of minimal glucose agar medium (CLIMEDIA AM-N, Oriental Yeast Co., Ltd.). The plates were incubated for 48 hours at 37°C, and bacterial colonies that appeared on the plates were counted. The assays were performed with triplicate plates.

Mutagenicity was evaluated by mutagenic ratio (MR). Namely, mutagenicity was judged to be positive when the revertants in red chili pepper or pure capsaicin increased more than 2-fold compared with the level in the negative control (Claxton et al., 1987).

Analysis of capsaicin in the chloroform layer and mutagenicity assay of pure capsaicin

The residue after evaporation of the chloroform layer was dissolved with pure methanol in place of DMSO. The concentration of capsaicin was measured using a high-performance liquid chromatography (HPLC) system. In addition, mutagenicity of pure capsaicin (Wako Pure Chemicals, Ltd., Osaka, Japan) was evaluated using strain TA98 with or without the S9 mix.

Qualitative and quantitative analyses of aflatoxins

To confirm whether Chilean red chili pepper contains aflatoxins, we followed the AOAC CB method (Cunniff, 1995) and used a two-dimensional thin-layer chromatography (TLC). The procedure was as follows: a total of 50 g of powdered red chili pepper was extracted with 400 ml of acetonitrile:water (9:1, v/v). The mixture of red chili pepper and extraction liquid was blended by a high-speed blender for 5 min and filtered. A portion of the filtrate was subjected to TLC using a glass plate coated with silica gel (silica gel 60, Merck KGaA, Darmstadt, Germany). The spots were observed by a two-dimensional TLC method using chloroform:acetonitrile (9:1, v/v) and toluene:ethyl acetate:formic acid (6:3:1, v/v/v) as developing solvents, and evaluated using a 365-nm UV lamp.

Because we detected aflatoxin in red chili pepper,

we measured the precise levels of aflatoxins based on the Official Method of Japan for the determination of aflatoxins (Shoku-An No. 0728004) (Ministry of Health, Labour & Welfare, 2008). The procedure of quantitative analysis was as follows: a 5-ml aliquot of the filtrate was transferred to a MultiSep #228 cartridge multifunctional column (Romer Labs, Inc., USA) and passed at a flow rate of 1 ml/min. Then, a 0.5-ml portion of the first 1-ml elution was evaporated under reduced pressure at 40ºC using the rotary evaporator. A 0.1-ml aliquot of trifluoroacetic acid (TFA) was added to the residue, which was then stored at room temperature for 15 min in darkness. A 0.4-ml portion of acetonitrile:water (1:9, v/v) was added to the TFA mixture solution, and then 20 μl of the solution and aflatoxins standard solutions were subjected to HPLC analysis.

The concentrations of aflatoxins B1, B2, G1, and G2 in red chili pepper were determined by HPLC analysis using a Hitachi D-2000 Elite system (Hitachi High-Technologies Corporation, Tokyo, Japan). The operating conditions were as follows: column: Atlantis T3 C18 (5-μm particle size, 250 mm x 3.0 mm, Waters Corporation, Milford, MA, USA); column temperature: 40ºC; mobile phase: acetonitrile:methanol:water (1:3:6, v/v/v); flow rate: 0.4 ml/min; detection wavelength: excitation wavelength 365 nm/emission wavelength 450 nm; injection volume: 20 μl. Concentrations of aflatoxins were calculated by comparing with the aflatoxins standards. The recovery rates for 5 ng/g of aflatoxins B1, B2, G1, and G2 were 92%, 97%, 100%, and 118%, respectively. The detection limit of the assay was 0.5 ng/g. The detected aflatoxins were identified by liquid chromatography-mass spectrometry (Quattro micro API, Waters Corporation, Milford, MA, USA).

Results

Table 1 shows the mutagenicity of Chilean red chili pepper with the S9 mix. Values in the negative control (DMSO) and the positive controls were acceptable approximations as compared with the reference values reported by Oriental Yeast Co., Ltd. In strain TA98, the numbers of revertant colonies at concentrations of 1 and 2 g/plate increased 2.5-fold compared with that of the negative control. In strain TA1537, the MR was less than 1.3 at any concentration we evaluated. In strains TA100 and TA1535, the MR was 1.9 at concentrations of 1 g/plate and 0.5 g/plate. Bacterial growth inhibition in strains TA1537 and TA1535 was found at concentrations of 1 and 2 g/plate and 2 g/plate, respectively, because the MR was less than 1.0.

Table 2 shows the mutagenicity of Chilean red chili pepper without the S9 mix. In strain TA98, the MR was 2.2 at concentrations of 0.25 and 0.5 g/plate. In other strains, the MR was less than 1.0 at any concentration we evaluated. Bacterial growth inhibition was found in strains TA1537, TA100, and TA1535. The revertant colonies in strain TA1537 were especially notably decreased with the increase of concentration.

Figure 1 shows the mutagenicity of pure capsaicin, using strain TA98 with or without the S9 mix. Since red

Table 1. Mutagenicity of Chilean Red Chili Pepper with the S9 Mix

Chemicals	Concentration	TA98		TA1537		TA100		TA1535	
	(g/plate) [a)]	Mean ± SD	MR	Mean ± SD	MR	Mean ± SD	MR	Mean ± SD	MR
DMSO (100 μl)		30 ± 2		23 ± 4		136 ± 7		11 ± 4	
Red Chili Pepper	0.06	33 ± 3	1.1	26 ± 1	1.1	151 ± 8	1.1	14 ± 3	1.3
	0.13	30 ± 6	1.0	31 ± 1	1.3	151 ± 6	1.1	11 ± 4	1.1
	0.25	33 ± 2	1.1	31 ± 1	1.3	158 ± 9	1.2	15 ± 3	1.4
	0.5	41 ± 6	1.4	30 ± 8	1.3	183 ± 11	1.3	20 ± 3	1.9
	1	76 ±12	2.5	22 ± 8	0.9	261 ± 7	1.9	19 ± 8	1.8
	2	75 ± 7	2.5	17 ± 3	0.7	166 ± 7	1.2	6 ± 2	0.6
Positive control (100 μl)		304 ± 38		142 ± 3		598 ± 27		217 ± 20	

[a)] Values are expressed in terms of the powdered red chili pepper extracted; DMSO: dimethyl sulfoxide; Revertant colonies are represented as the mean ± standard deviation (SD) of triplicate generations; MR: mutagenic ratio. The MR was calculated by the following equation: mean number of revertant colonies/mean number of spontaneous revertant colonies (DMSO); Positive controls used 0.5 μg/plate of 2-aminoanthracene (2-AA) for strain TA98, 2 μg/plate of 2-AA for strain TA1537, 1 μg/plate of 2-AA for strain TA100, and 2 μg/plate of 2-AA for strain TA1535

Table 2. Mutagenicity of Chilean Red Chili Pepper without the S9 Mix

Chemicals	Concentration	TA98		TA1537		TA100		TA1535	
	(g/plate) [a)]	Mean ± SD	MR	Mean ± SD	MR	Mean ± SD	MR	Mean ± SD	MR
DMSO (100 μl)		18 ± 1		24 ± 4		155 ± 14		8 ± 1	
Red Chili Pepper	0.06	18 ± 2	1.0	20 ± 3	0.8	154 ± 11	1.0	13 ± 4	1.7
	0.13	24 ± 3	1.4	15 ± 3	0.6	152 ± 6	1.0	11 ± 2	1.4
	0.25	38 ± 3	2.2	10 ± 2	0.4	164 ± 12	1.1	8 ± 4	1.0
	0.5	39 ± 6	2.2	8 ± 1	0.3	103 ± 15	0.7	10 ± 6	1.3
	1	23 ± 4	1.3	6 ± 1	0.2	87 ± 5	0.6	9 ± 3	1.2
	2	17 ± 3	1.0	6 ± 3	0.2	84 ± 5	0.5	6 ± 3	0.8
Positive control (100 μl)		221 ± 12		280 ± 19		529 ± 29		585 ± 7	

[a)] Values are expressed in terms of the powdered red chili pepper extracted; DMSO: dimethyl sulfoxide; Revertant colonies are represented as the mean ± standard deviation (SD) of triplicate generations; MR: mutagenic ratio. The MR was calculated by the following equation: mean number of revertant colonies/mean number of spontaneous revertant colonies (DMSO); Positive controls used 0.1 μg/plate of 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) for strain TA98, 80 μg/plate of 9-aminoacridine for strain TA1537, 0.01 μg/plate of AF-2 for strain TA100, and 0.5 μg/plate of sodium azide for strain TA1535

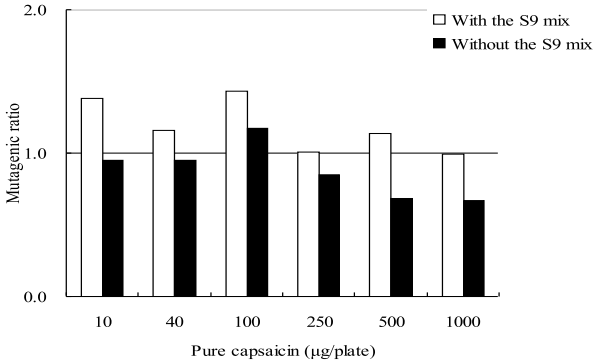

Figure 1. Mutagenicity of Pure Capsaicin, Using *Salmonella Typhimurium* Strain TA98 With or Without the S9 Mix. Negative (solvent/vehicle) control: number of spontaneous colonies in 100 μl of dimethyl sulfoxide (DMSO); The mutagenic ratio was calculated using the following equation: mean number of revertant colonies/ mean number of spontaneous revertant colonies (DMSO)

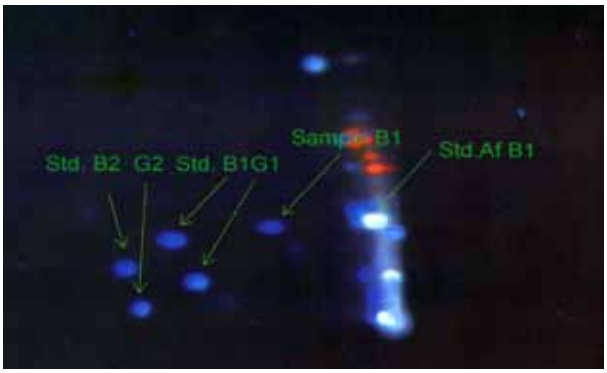

Figure 2. Spot of Aflatoxin B1 in the Chili Pepper Imaged by Two-dimensional Thin Layer Chromatography. The fluorescent spot in the red chili pepper extract corresponds to aflatoxins B1, B2, G1, and G2 standards; Std. Af B1 or Std. B1, B2, G1, G2 represent the spots of aflatoxin B1 or aflatoxins B1, B2, G1, G2 standards, respectively; Aflatoxin B1 was detected, but the concentrations of aflatoxins B2, G1, and G2 were below the detection limit of the method of analysis

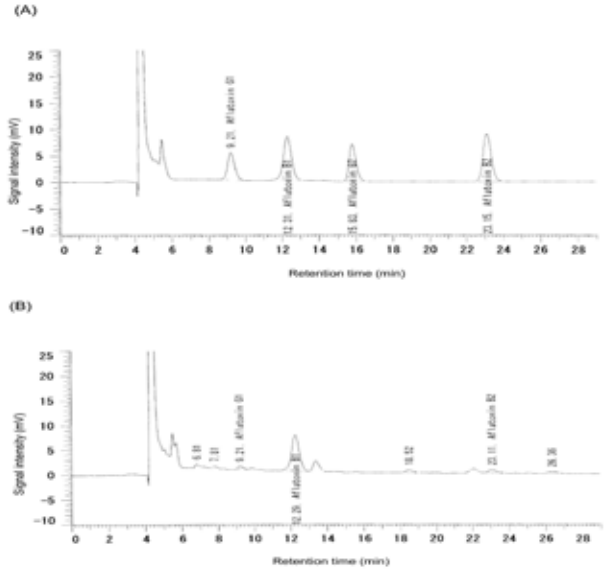

Figure 3. HPLC Chromatograms of Aflatoxin Standards of 5 ng/g (A) and Red Chili Pepper (B). Aflatoxins B1 and G1 were detected at levels of 4.4 ng/g and 0.5 ng/g, respectively

chili pepper showed mutagenic activity in strain TA98 with or without the S9 mix, mutagenicity of pure capsaicin was estimated under the same analytical conditions. The MRs were between 1.0 and 1.4 with the S9 mix, and between 0.7 and 1.2 without the S9 mix. Pure capsaicin did not show mutagenic activity at any concentration in strain TA98, even though 90 μg of capsaicin per gram of red chili pepper was detected from the residue-methanol solution (data not shown).

Figure 2 shows the spot of aflatoxin B1 in the chloroform layer by two-dimensional TLC analysis. We tested for the most prevalent aflatoxins B1, B2, G1, and G2, but only aflatoxin B1 was detected. Since aflatoxin B1 was detected in the residue-methanol solution, quantitative analysis by the HPLC system was performed to determine details of the aflatoxin contamination.

Figure 3 shows the chromatograms of the HPLC analysis corresponding to each 5 ng/g of aflatoxin standard solution (A) and red chili pepper (B). Aflatoxins B1 and G1 were detected at concentrations of 4.4 ng/g and 0.5 ng/g, respectively. The concentrations of aflatoxins B2 and G2 were less than the detection limit of our analysis method. Our results revealed that red chili pepper is contaminated with low concentrations of aflatoxins B1 and G1.

Discussion

In addition to previous studies that have examined the associations between chili pepper consumption and gastric cancer (Lopez-Carrillo et al., 1994), gastric cancer and liver cancer (Archer and Jones, 2002), or esophageal cancer (Ghadirian et al., 1992), the authors of at least two studies conducted in Chile and India have reported the association of red chili pepper consumption and an increased risk of GBC (Pandey and Shukla, 2002; Serra et al., 2002). Our previous epidemiological study demonstrated that a high level of consumption of red chili pepper is a risk factor for GBC in Chilean women who carry GS (Serra et al., 2002). Red chili pepper is a widely consumed spice among the Chilean population. Our previous study reported that 15.4% of Chilean female GBC patients consume red chili pepper every day (Baez et al., 2010). However, the mechanism of the development of GBC by red chili pepper consumption is not yet known. The presence of certain mutagens or carcinogens in red chili pepper may be related to the increased risk of GBC in Chilean women. Therefore, under the assumption of the multi-factorial causation hypothesis, we initiated this study to demonstrate whether red chili pepper shows mutagenic activity or contains mutagens.

As we expected, red chili pepper was mutagenic by the Ames test, although mutagenic activity levels were low. The authors of several previous studies have examined mutagenicity of chili pepper or capsaicin, but their findings have been inconsistent. Some studies have demonstrated that chili pepper or capsaicin is mutagenic (Toth et al., 1984; Nagabhushan and Bhide, 1985; Lawson and Gannett, 1989; Miller et al., 1993; Azizan and Blevins, 1995), but some have shown them to be nonmutagenic (Buchanan et al., 1981; Chanda et al., 2004; Proudlock

et al., 2004). Toth et al., (1984) reported that both chili pepper and capsaicin at concentrations of 250, 500, and 750 μg/plate were mutagenic in strain TA98 with S9 mix. The authors of two other studies have shown that mutagenicity of capsaicin is positive at a concentration of 40 μg/plate in strain TA100 (Azizan and Blevins, 1995) and in strains TA98, TA100, and TA1535 (Nagabhushan and Bhide, 1985) with S9 mix. Since red chili pepper showed mutagenic activity and contained capsaicin in the residue-methanol solution, we conducted a comparative study of the mutagenicity of red chili pepper and pure capsaicin. While our results demonstrated mutagenicity of red chili pepper in strain TA98, pure capsaicin did not show significant mutagenic activity under the same analytical conditions.

Aflatoxins are carcinogenic, mutagenic, teratogenic, and immunosuppressive to humans. Aflatoxin B1 shows the strongest toxicity among this group of mycotoxins. In fact, aflatoxin B1 was shown to be mutagenic in strains TA98 and TA100 (Ames et al., 1975) and in strain TA 100 (Sawada et al., 1993) with S9 mix. Aflatoxins are produced by various *Aspergillus* section *Flavi* present in the soil of tropical areas. Red chili pepper is produced in the central part of Chile, where *Aspergillus* section *Flavi* can grow. Based on the evidence of previous studies, we examined aflatoxins as the mutagens of red chili pepper. However, our results did not show mutagenicity of red chili pepper in strain TA100, although the MR was 1.9 and was very close to 2.0. The difference from the previous findings may have been caused by the amount of aflatoxins detected in red chili pepper (4.4 ng/g), or by an anti-mutagenic activity. On the other hand, the capsaicin content of some chili varieties ranges up to 0.53% (Toth and Gannett, 1992), and these varieties are reported to have anti-mutagenic activity (Buchanan et al., 1981; Chanda et al., 2004; Proudlock et al., 2004). In the present study, growth inhibition was found in strains TA1537, TA100, and TA1535 at concentrations of 1 and 2 g/plate with or without the S9 mix. These results show that red chili pepper contains anti-mutagenic agents that have a bacterial growth inhibitory effect, very probably capsaicin (Careaga et al., 2003), chlorophyllin (Ong et al., 1986), and others. Therefore, our results by the Ames test reflect an antagonistic interaction between the mutagenic and anti-mutagenic activities of red chili pepper. This is another reason why our results did not show mutagenic activity in strain TA100.

Aflatoxin B1 is the major risk factor for liver cancer (Newberne and Bulter, 1969), and it is changed into a carcinogen after metabolism by cytochrome P450 enzymes. Because a part of the metabolite is excreted in gallbladder bile, the aflatoxin metabolite may be related to the development of GBC, especially in patients with GS. Although our results show that aflatoxin contamination of red chili pepper and the development of GBC in Chilean women may be associated in a cause-and-effect manner, at this time we can only speculate as to the correctness of our assessment of this relationship, because of the low levels of aflatoxins contamination we observed. However, evidence regarding the association between aflatoxin B1 and the development of GBC has been published (Sieber et al., 1979; Olsen et al., 1988). A previous study demonstrated that the numbers of revertant colonies at the concentration of 0.1 μg of aflatoxin B1 are 1940/plate and 2280/plate in strains TA98 and TA100, respectively (McCann et al., 1975). In the present study, the concentration of aflatoxin B1 detected from red chili pepper was 4.4 ng/g, and was almost one-twentieth of the concentration used by MacCann et al., (1975) (0.1μg). Our results in strains TA98 (76/plate) and TA100 (261/plate) were roughly equivalent to one-twentieth of the values obtained by MacCann et al. In addition, chili pepper is produced and consumed by people who live in countries or regions with a high incidence of GBC, such as Bolivia, Peru, India, and State of New Mexico. Since these are all located between 40°N and 40°S of the equator, red chili peppers and other agricultural produce from this area may be contaminated with aflatoxins. Interesting test results showing that chili peppers marketed in Portugal and Hungary are contaminated with aflatoxin B1 have been reported (Martins et al., 2001; Fazekas et al., 2005). Hungary was one of the countries with the highest mortality rates of GBC (4-5/100,000 women) in central Europe during 2000 and 2004 (La Vecchia et al., 2010). Therefore, additional studies on aflatoxin contamination of red chili pepper produced in other high GBC incidence regions will help us to understand whether aflatoxin contamination of red chili pepper is a causative factor for the development of GBC.

Since the recent mortality rate of GBC in Chilean women is almost the same as that for the last several decades (Andia et al., 2008), it remains a matter of urgency to determine whether aflatoxin-contaminated red chili pepper consumption is the cause of the development of GBC. Aflatoxins are metabolized and changed to carcinogens in the human liver, and the metabolite is excreted into the bile. Our findings suggest that low-level but protracted exposure to aflatoxins in Chilean women with GS may be associated with the development of GBC. While our findings provide evidence for a critical role of aflatoxins B1 and G1 in the mutagenicity of red chili pepper, the relationship of this role to GBC risk is uncertain, and further studies, such as testing for aflatoxin B1-associated adducts in GBC patients or experimental animals, are needed.

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