Moctezuma-Zárate et al., J Food Nutr Disor 2015, 4:3
http://dx.doi.org/10.4172/2324-9323.1000174

Journal of Food & Nutritional Disorders

Research Article

A SCITECHNOL JOURNAL

Role of pH in the Mutagenicity of Aflatoxin B1 in Maize Tortillas during *In Vitro* Human Digestion Model

María de Guadalupe Moctezuma-Zárate[1], Magda Carvajal-Moreno[1*], Javier J. Espinosa-Aguirre[2], María Eugenia Gonsebatt-Bonaparte[2], Francisco Rojo-Callejas[3], Pável Castillo-Urueta[1] Israel Pérez-López[4] and Silvia Ruiz-Velasco[5]

Abstract

Introduction: Aflatoxins (AFs) are potent mutagens, carcinogens and teratogens for humans; thus their presence in food is of great concern. A maize tortilla survey in Mexico revealed that 17% are contaminated with AFs. The initial process to prepare tortillas includes boiling maize grains with lime and to assumpt that AFs in maize tortillas are destroyed, protecting consumers from their mutagenic effects.

Objective: The purpose of this study was to investigate the mutagenicity of aflatoxin B1 (AFB1) in tortillas during digestion.

Methods: Two *in vitro* models of human digestion were used: one with maize naturally contaminated by AFB1, and another with AFB1 standard spiked to them. The pH at each step was measured, and a neutral pH range (5.8 to 7.5) reactivated the AFB1 mutagenicity. AFB1 was quantified by HPLC, and its mutagenicity was determined by Ames test. Human digestion treatments were saliva (pH 7.0), artificial gastric juice with pepsin (pH 1.2), pancreatic fluid with pancreatin (pH 7.5) and combinations of saliva with gastric juice (pH 1.8), gastric and pancreatic fluids (pH 2.4), and saliva with gastric and pancreatic fluids (pH 5.8).

Results: The alkalinity of the lime treatment (pH 12.0) and the acidity of gastric fluid (pH 1.2) inhibited AFB1 mutagenicity. The neutral pH of saliva increased mutagenicity, and of pancreatic fluid returned the mutagenicity to untreated levels. The mixture of saliva with gastric and pancreatic fluids (pH 5.8) also rendered the AFB1 mutagenic.

Conclusion: The pH during human digestion plays an important role in the mutagenicity of AFB1.

Keywords: Human digestion; Aflatoxins; Mutagenicity; Carcinogenesis; pH

Introduction

Aflatoxins (AFs) are inevitable food mutagens, carcinogens and teratogens, it is almost impossible to avoid the ingestion of them, so the research about the changes in the mutagenicity of AFs during the digestion process is of upmost importance. On the other hand, cancer is the group of diseases that causes the highest mortality in humans from just born to old people and all the studies that tend to control these diseases are welcome for human survival.

Aflatoxins (AFs) are bisdihydro-furancoumarins produced from some strains of maize-contaminating molds *Aspergillus flavus* [1], *A. parasiticus* [2], *A. nomius* [3], *A. pseudotamarii* [4] and *A. bombycis* [5]. The properties of AFs, including their biosynthesis, production conditions and toxic effects, are well-characterized [6]. They are teratogens [7] that cause chromosomal aberrations and DNA breakage [8], and act as biological carcinogens [9] in animal and human organs such as the liver, colon, pancreas, rectum [10], lung [11] and cervix [12]. Aflatoxin B1 (AFB1) requires bioactivation to achieve mutagenicity [13].

Surveys have demonstrated the presence of AFs in maize tortillas, the staple food of Mexico [14]. Maize grains are cooked with lime, and it is thought that this treatment eliminates AFs. Therefore, this has been proposed as a method of detoxification [15]. In fact, maize tortillas appear to contain smaller amounts of AFB1 than expected for their maize content, possibly because the AFB1 is lost from the maize pericarp and released into residual water during boiling and washing, and because the alkaline conditions present in tortillas open the lactone ring of AFs and inhibit their fluorescence and detection [16,17]. Additionally, lime treatment is not effective for detoxification, as acidification in the stomach reactivates AFs [18].

During digestion the mutagenicity of AFB1 in maize tortillas changes in relation to exposure to saliva or gastric and pancreatic fluids, either alone or in combination. Saliva contains the enzyme ptyalin, an amylase that maintains a pH of 6.5 to 7.0 in the mouth [19]. Saliva can transport hepatitis B virus passing also AFs, that can be potential causative agents for hepatitis and liver cancer [20]. There are contradictory reports regarding the role of saliva; it has been suggested to be mutagenic and teratogenic [21], to inactivate the mutagenicity of AFB1 [22] and provide protective antibacterial activities [23] through antioxidant enzymes [24]. Gastric fluid contains pepsin, a protease that degrades food proteins into peptides [25]. Chloride creates the acidic environment (pH 1.5 to 3.0) necessary for pepsin activity, and the lowest pH recorded for this secreted acid is 0.8 [26]. Pancreatic fluid contains the enzymes trypsin and chymotrypsin, which digest proteins into peptides and amino acids in the duodenum at a pH of 7.5 to 8.5 at 37°C [27]. The effect of AFB1 mutagenicity on human digestion has not been clarified; our aim in this study was to determine the changes in AFB1 mutagenicity due to pH using an *in vitro* model of human digestion.

Materials and Methods

In vitro model of human digestion. Ingredients, enzymes and the pH naturally present in the digestive tract were used. Two maize sources were used to make tortillas, one was naturally contaminated with AFB1 (Figure 1A), and the other was uncontaminated ground maize but spiked with AFB1 standard (Sigma-Aldrich, Mexico) at dough stage (32 µg AFB1 + 390 mL MeOH/H_2O, 80:20 v/v) (Figure 1B), homogenizing the mixture with care. Tortillas from both sources

***Corresponding author:** Dr. Magda Carvajal-Moreno, Laboratorio de Micotoxinas, Departamento de Botánica, Instituto de Biología, Universidad Nacional Autónoma de México (UNAM). 04510 México,Tel: (5255) 5622 9138; Fax: (5255) 5550 1760; E-mail: magdac@ib.unam.mx

Received: December 23, 2014 **Accepted:** May 18, 2015 **Published:** May 22, 2015

were ground and subjected to a simplified *in vitro* model of human digestion following the protocols of physicians from the Institute of Pediatrics Health Ministry of Mexico:

a) Control tortilla with AFB1.

b) To resemble the model of human digestion in the mouth, 250 mL of natural saliva with ptyalin was collected from two persons fasted for 12 hr, adjusted to pH 7.0, added to the mash that were 3 replications of 75 g of ground tortilla (Figure 1), and the mixture was incubated for 2 min. Complete artificial saliva with α-amylase is not recommended in *in vitro* toxicology research [28], so we used the natural saliva. Natural saliva is watery substance located in the mouth, secreted by salivary glands and composed 99.5% by water, and 0.5% consists from electrolytes, mucus, glypoproteins, enzymes and antibacterial compounds such as secretory IgA and lysozymes [26].

c) For a model of digestion in the stomach, the mash was used plus 250 mL of simulated gastric juice with 0.032 % pepsin solution and 2.0 g of NaCl (Merck) and 3.2 g of pepsin (Sigma-Aldrich, Mexico) in 7 mL of HCl at 37.4% (J.T. Baker). The volume was increased to one L with distilled water (H_2Od), and adjusted to pH 1.2. This mixture was incubated for 2 hr in the dark to resemble the digestive process in the stomach.

d) To simulate digestion in the intestines, tortilla with AFB1 plus pancreatic fluid (250 mL) was prepared by diluting 6.8 g of KH_2O4 (Merck) in 250 mL of H_2Od. Then, 190 mL of 0.2 N NaOH (Merck), 400 mL of H_2Od and 10.0 g of pancreatin (Sigma-Aldrich, Mexico) were added and mixed, and the solution was adjusted to pH 7.5 ± 0.2 with NaOH and incubated for 5 hr in darkness (Figure 1).

These same digestive fluids were tested in several combinations:

e) To simulate sequential digestion in the mouth and stomach, the mash was mixed with 83 mL of saliva (pH 7.0) incubated for 2 min, then supplemented with 83 mL gastric juice (pH 1.2) for 2 hr in the dark at a final pH of 1.8.

f) To simulate the passage of mash from the stomach to the intestine, a tortilla with AFB1 was mixed with 83 mL of gastric juice (pH 1.2) incubated for 2 hr, then blended with 83 mL of pancreatic fluid (pH 7.5), and incubated for 5 hr at a final pH of 2.4.

g) To simulate complete digestion of the maize tortillas from mouth to gut, the spiked tortilla was incubated for 2 min with 83 mL of saliva (pH 7.0), mixed with 83 mL of gastric juice (pH 1.2) and incubated for 2 hr, then mixed with 83 mL of pancreatic fluid (pH 7.5) for 5 hr in the dark at room temperature and a final pH of 5.8.

The method for AFB1 extraction has been described previously [29]. Quantification of AFB1 was carried out by high-performance liquid chromatography (HPLC) [30], and its mutagenicity was determined by the Ames test [31-34].

Maize naturally contaminated with AFB1. Maize from the warehouse `Las Yescas´ in Valle Hermoso, State of Tamaulipas, Mexico, was sieved and used to make tortillas. Of the maize used, 98% contained ≥ 20 µg/kg AFB1, with an average of 66 µg/kg [35]. *In vitro* models of human digestion were applied. AFB1 was quantified by HPLC, and the mutagenic effect of one of ten tortillas made with naturally AFB1-contaminated maize was tested in triplicate by the Ames test (Figure 1A).

Maize dough spiked with AFB1. As described in Figure 1B, because it is not normally present during digestion, methanol (MeOH) was evaporated under an extraction chamber for 24 hr. Tortillas were then prepared, dried, ground and digested by the *in vitro* human digestion models (Figure 1B). To counteract the pH effect two different extraction assays and column washes were performed, with either H_2Od or PBS. Distilled water maintains the natural pH of the digestive fluid solutions used, which ensures that the *in vitro* digestion model approximates human digestion. In the second assay, ground dry tortilla was blended with 50 mL MeOH/ PBS at 0.25 M (80:20 v/v) to compare the role of the pH in the human digestion model. PBS is not normally present and neutral pH can affect the mutagenicity of AFB1. The two assays were independently filtered and evaporated to 8 mL. Later, the AFB1-spiked tortilla, or mash from both extraction assays were subjected to the *in vitro* models of human digestion.

Chemical extraction

Two extraction assays were done by blending dry ground tortilla (25 g) + 1.25 g NaCl in two independent diluting solvents: a) 50 mL MeOH/ H_2O (80:20 v/v) and b) 50 mL MeOH/PBS at 0.25 M (80:20 v/v) (Figure 1).

Both digested mixtures were later filtrated and monitored for the presence of AFB1 by applying each treatment to separate immunoaffinity columns (IAC) previously equilibrated with 20 mL of 0.25 M PBS at pH 7.4. The IACs were washed with one of two different solvents: a) H_2Od, or b) 10 mL of 5% Tween 20-PBS at and later with 10 mL PBS (pH 7.4).

AFB1 was eluted with 1.5 mL of HPLC MeOH and 1.5 mL of H_2Od, and the resulting 3 mL of eluate was then dried at 40°C in a Lab-Line Ambi HI-LO Chamber (Novatech, USA). Dry eluate was resuspended in 600 µL of MeOH and divided equally into two vials. One vial was used for HPLC chemical analysis. From this sample, 100 µL were derivatized as previously described [36], and triplicates of 30 µL were quantified by HPLC analysis [30].

The second vial was dried at 40°C, then resuspended in 100 µL dimethyl sulfoxide (DMSO) for to the Ames test with *Salmonella typhimurium* strain TA-98 (Figure 1B).

HPLC analysis was validated and the conditions were a Series 400 pump, LC-10 Fluorescence detector (Exc362 nm, Emis450 nm), LCI-100 Laboratory computing integrator, Lambda 3A UV/Vis spectrophotometer (Perkin Elmer) and HS-10 C18 Phenomenex column 0258-0172. The mobile phase was H_2Od /ACN/ MeOH (65:15:20 v/v/v) with a flow rate of one mL/min for 20 min. AFB1 standard (Sigma-Aldrich, Mexico) was included, and all samples were analyzed in triplicate.

Ames test for mutagenicity: The Ames test was used to determine the effect of pH on the mutagenicity of AFB1 by microsomal S9 activation. *Salmonella typhimurium* TA98 [hisD 3052, gal, (chl, uvrB, bio) rfa, pKM101 (MucA/B Amp)] was donated by Bruce Ames to the Experimental Oncology laboratory at the National Institute of Pediatrics, Ministry of Health, Mexico.

Spontaneous reversion test: Top agar (2 mL) mixed with 100 µL of wild type (Wt) or *S. typhimurium* TA98 bacteria in minimum glucose medium (MGM) was incubated for 48 hr. Reverted colonies (rc) were counted with an electronic colony counter (New Brunswick Scientific). Reference values for spontaneous reversion of the control strain TA98 without S9 were 20 to 50 rc [34,37]. Three colonies that

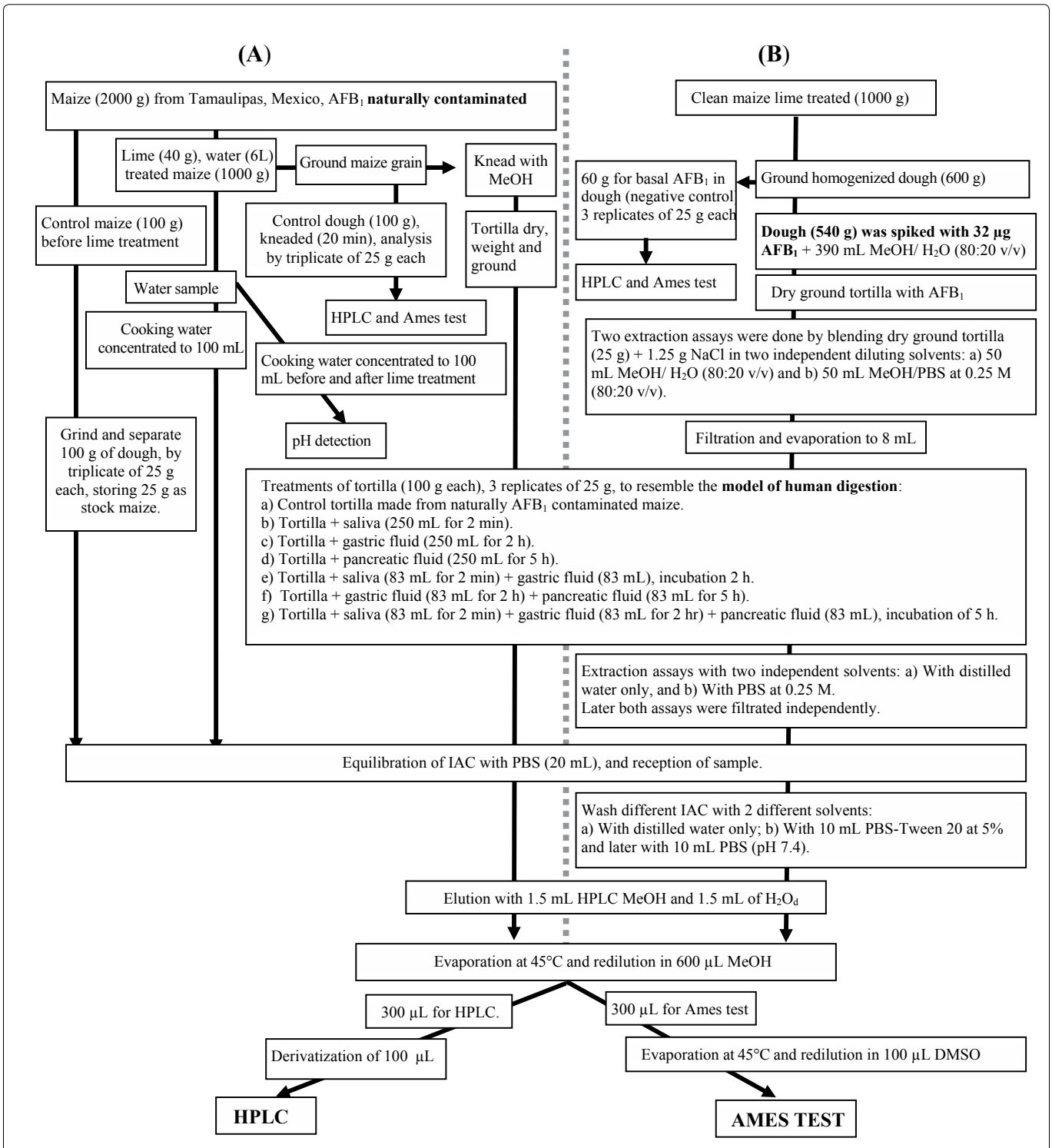

Figure 1: Model of human digestion of tortillas *in vitro* processed from: A) Maize naturally contaminated by AFB1. B) Tortilla prepared from 1000 g of clean maize dough spiked with 32 µg of AFB1 and treated in a model of human digestion. IAC = immunoaffinity column; DMSO = Dimethylsulfoxide; PBS = Phosphate buffered saline; MeOH = Methanol.

showed the genetic markers were chosen as stocks kept on plates at 4 °C and in cryovials at -80°C.

Cryovial stock: TA98 (200 µL) from an overnight culture was gently homogenized in sterile cryovials with 1800 µL DMSO (Nalgen NY EUA) at 4°C for one hr, frozen at -20°C and stored at -80°C prior to use. To start an overnight culture, a frozen sample from a cryovial stock was inoculated in Oxoid 2 nutritive medium [38].

Master stock plate *S. typhimurium* TA98: Bacteria from an

overnight culture were plated on agar enriched with ampicillin and incubated at 37°C for 48 hr. Following incubation, the plates were allowed to cool, flipped upside down, wrapped in aluminum foil and then frozen.

Verification tests of genetic markers of *S. typhimurium* TA98 were performed in comparison with those of the Wt strain [37]. The tests performed were: spontaneous reversion, dependence on histidine, dependence on biotin, dependence on histidine/biotin; sensitivity to crystal violet; presence of the rfa mutation marker; sensitivity to UV light (deletion of the gene uvr-B-bio) and verification of the presence of the plasmid pKM101 [31, 39-41].

Verification of *S. typhimurium* TA98 with mutagenicity controls: TA98 bacteria (100 µL) from surface agar was mixed with 100 µL AFB1, diluted in 500 µL DMSO and S9 microsomal extract, vortex homogenized and plated in triplicate on MGM. Plates were incubated inverted for 48 hr at 37°C, and rc were counted. *S. typhimurium* TA98 was verified with negative controls for mutagenicity that included surface agar (1.9 mL) with TA98 night culture (100 µL), and positive controls for mutagenicity with and without metabolic activation: surface agar (1.3 and 1.8 mL) + 2-aminofluoren, benzo (α) pyrene (100 L) + TA98 night culture (100 L) + S9 (100 and 500 µL). The direct treatments without metabolic activation were surface agar (1.8 mL) + 100 µL of AFB1 (10, 100 and 500 µg/mL) + TA98 (100 µL) and direct treatments with metabolic activation of surface agar (1.3 mL) + 100 µL of AFB1 (10, 100 and 500 µg/mL) + TA98 (100 µL) + S9 (500 µL).

Controls for *in vitro* human digestion model: Controls were AFB1 standard (150 µL) incubated in 500 µL of the *in vitro* human digestion model solutions described above; lime (2% $Ca(OH)_2$, pH 12.0, incubated for 40 min and boiled for 15 min), and regulating solution (potassium acetate/acetic acid, pH 5.0, incubated for 5 hr). All samples were dried and processed six times in triplicate for HPLC analysis, and three replicates were dissolved in 100 µL DMSO for the Ames test.

AFB1 standards with enzyme-free digestive solutions: AFB1 was tested as follows: AFB1 in H_2Od; AFB1 in pepsin-free gastric solution, pH 1.2, for 2 hr; AFB1 in pancreatin-free pancreatic fluid, pH 7.5, for 5 hr; and AFB1 with the pepsin–free gastric solution for 2 hr followed by pancreatin-free pancreatic fluid for 5 hr at pH 2.4, solutions were then dried. A second assay was performed in which H_2Od was replaced with PBS, and samples were dried before treatment with the digestive solutions to maintain the pH of digestion.

Testing of digested tortilla samples by Ames test: The digested samples were dried, resuspended in 100 µL DMSO and analyzed in triplicate to determine the level of mutagenicity. AFB1 (1.6 to 150 ng/mL) in DMSO was used as a positive control with S9 induced by Aroclor 1254 and NADP. Negative controls, applied alone, were: H_2Od, PBS, S-9 microsomal rat concentrate, saliva, gastric fluid, pancreatic fluid, saliva + gastric fluid, saliva + pancreatic fluid, saliva + gastric + pancreatic fluids, AFB1 + NaCl + HCl, AFB1 + KH_2PO4 + NaOH and AFB1 + NaCl + KH_2PO4 + NaOH.

Statistical analysis

Standard deviations (SD) and percentage of relative standard deviation (% RSD) were calculated in revertant colonies from maize tortillas naturally contaminated with AFB1 (µg/kg), in the presence and absence of digestive fluids, controls without tortillas and from tortillas made from dough spiked with AFB1.

A two way analysis of variance was performed using the different treatments (and controls) as one factor and the blended of the tortillas, as the second one, plus the interaction. After fitting the model test differences of means with Bonferroni corrections were used in all the pairs of treatment by blended.

Results

Validation of HPLC method for AFB1 quantification (12). The limits of detection (LOD) and quantification (LOQ) were LOD=0.5 µg/kg and LOQ=1.4 µg/kg, the coefficient of variation repeatability percentage was 1.8%, the equation of regression of the calibration curve (lineality) was y=4.367x – 2.874 with R2=0.999.

The recovery percentage of AFB1 to determine the exactitude of the method was done with three spiked amounts of AFB1 (9.9, 19.9 and 29.7 µg/kg), the recovered AFB1 corresponding to each amount were 8.6, 17.2 and 28.4 µg/kg, the corresponding recovered percentages were 86.4, 86.5 and 95.4 %, the standard deviations were ± 1.5, ± 0.8 and ± 1.2, the percentages of the coefficients of variation were 1.8, 0.9 and 1.2 % and the confidence intervals were 82.6 to 90.3, 84.6 to 88.4 and 92.5 to 98.3.

Spontaneous reversion of *S. typhimurium* TA98

The strain TA98 (INP B), with an average of spontaneous reversion of 24.33 rc was chosen because it provided better results in the verification tests of genetic markers than the other strains. Because a three-fold increase in rc over controls constituted a positive Ames test result, 73 rc was the lowest limit of detected mutations that could be considered positive.

Naturally AFB1-contaminated maize in the model of human digestion

Most of the AFs were eliminated in the residual limed water, and although 11 µg/kg of AFB1 were found in the dough by HPLC, no mutagenic effect was observed, Figure 2. A cytotoxic effect was present in only some replicates, shown by cell deformations or ruptures; thus it is possible that proteins or lipids from the maize inhibited AFB1 mutagenicity, because the CYPs are involved in converting AFB1 to electrophiles, e.g. diol-epoxide, responsible for forming DNA adducts and causing mutagenesis, although lipid oxidation cannot be considered in the stomach separately. The average concentration of

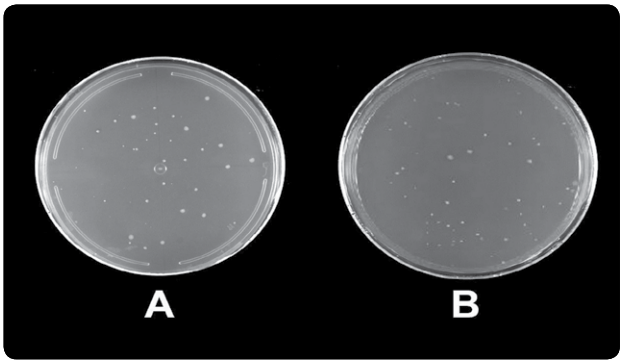

Figure 2: Lack of mutagenic effect of tortillas (pH 11.6) made from AFB1 naturally contaminated maize by Ames test. Extract from one tortilla digested with pancreatic fluid at 7.5 pH by HPLC (A= 12.8 ng AFB1 and 43 revertant colonies) or ten tortillas (B= 128 ng AFB1 and 49 revertant colonies) without mutagenic effect

AFB1 in tortillas made with naturally contaminated maize is given in Table 1. No AFB1 was detected in ground, limed maize because the lactone ring of the AF structure opened as a result of alkaline hydrolysis, which in turn caused a loss of fluorescence [17]. When this ground limed maize was kneaded into dough, 1.2 ng/g of AFB1 appeared.

The amount of AFB1 (5.3 ng/g) detected in cooked tortillas increased in the presence of saliva (11.6 ng/g) or pancreatic fluid (12.8 ng/g). Similar amounts of AFB1 (5.3 ng/g) were found with tortillas alone at pH 11.6 and with gastric fluid at pH 1.2 (5.7 ng/g); therefore, both alkaline and acidic pH inhibit AFB1. Saliva (pH 7.0) activated and increased AFB1 to twice the amount found in tortilla alone (11.6 ng/g). Tortillas made with naturally AFB1-contaminated maize lacked mutagenicity in one and the addition of 10 tortillas extracts, and only their digestion with pancreatic fluid (pH 7.5) recovered AFB1 (12.8 µg/kg) (Table 1).

Mutagenic effect of controls, without tortillas. In the AFB1 experiment without tortillas (Table 2), we observed no mutagenicity in the controls with AFB1 standard alone or enzymatic solutions at an alkaline or acidic pH without AFB1.

Treatments in a neutral pH with AFB1 standard in the presence of saliva (pH 7.0), pancreatic fluid (pH 7.5) and saliva + gastric juice + pancreatic fluid (pH 5.8) caused a mutagenic effect, (Table 3). Therefore, pH can be considered a key factor along with the enzyme itself in producing mutagenicity. We found a mutagenic effect of AFB1 with saliva (254 rc, pH 7.0) and with pancreatic fluid (115 rc, pH 7.5). The gastric juice (25 rc, pH 1.2) inhibited AFB1 mutagenicity and acidified saliva (pH 1.8) and pancreatic fluid (pH 2.4).

Mutagenic potential of tortillas made from AFB1-spiked dough, in the model of human digestion. Positive results only occurred with tortillas made from dough spiked with AFB1, shown in Table 3.

The dilution liquid used, either H_2Od or PBS, influenced both pH and mutagenicity. Saliva with a neutral pH of 7.0 with H_2Od gave a more AFB1-based mutagenic response (254 rc) than saliva with PBS buffer (pH 6.9, 112 rc) (Table 3), a result consistent with a previous study [17]. However, saliva did not protect, detoxify or attenuate the mutagenicity of AFB1 as previously reported [22]. Complete artificial saliva (CAS) with α-amylase is not recommended because artificial saliva alters expression of proinflammatory cytokines in human dermal fibroblasts, so it is important to carefully evaluate the "vehicle effects" of CAS and its components in *in vitro* toxicology research [28]. The mutagenicity of AFB1 was inhibited when saliva was mixed with gastric fluid in H_2Od (pH 1.8, 20 rc). When the pH increased to 6.1, due to the use of PBS, the activity of AFB1 recovered (63 ng/g), and the mutagenic effect was restored (99 rc). These results again demonstrate that the role of pH on the mutagenicity of AFB1 is important.

The greatest recovery of, and increase in, AFB1 mutagenicity was with saliva (pH 7.0), followed by pancreatic fluid at pH 7.5, which resembles the pH present in the duodenum, (Figure 3). Similar results were observed for AFB1 with the combination of saliva, gastric and pancreatic fluids at pH 5.8, whereas each of these digestive fluids alone did not render the maize mutagenic. The mutagenicity of AFB1 did not recover at pH of 1.2, as found in the gastric fluid dissolved in H_2Od, but it was restored by pH 6.0 when the gastric juice was dissolved in PBS.

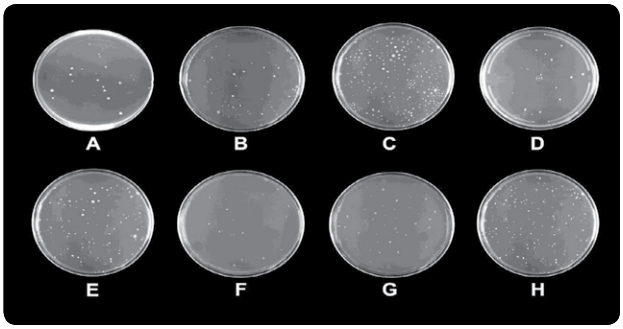

Figure 3: Effect of pH, from the *in vitro* human digestion model, over the AFB1 mutagenicity in tortilla made from AFB1 spiked dough, shown by revertant-mutated colonies (rc) of *Salmonella typhimurium*: A) Tortilla without AFB1 as negative control pH 11.6 with 25 rc; B) Tortilla with lime and AFB1 pH 11.6 with 53 rc; C) Tortilla with saliva pH 7 with 254 rc; D) Tortilla with gastric fluid pH 1.2 with 25 rc; E) With pancreatic fluid pH 7.5 with 115 rc; F) With saliva and gastric fluid pH 1.8 and 20 rc; G) With gastric and pancreatic fluids pH 2.4 and 51 rc; H)With saliva, gastric and pancreatic fluids pH 5.8 with 184 rc.

Table 1: Revertant colonies from maize tortillas naturally contaminated with AFB_1 (µg/kg), in the presence and absence of digestive fluids.

Samples of maize and tortillas naturally contaminated with AFB_1	pH	Average of AFB_1 amount (µg/kg) by HPLC	Revertant colonies (number)	SD	% RSD
Maize before lime treatment	12	12.8	52	1	1.92
Maize in water with lime	12	18.7	21	1	4.76
Residual water after lime treatment	12	11.0	31	1.5	4.98
Ground maize with lime treatment	11.6	0	21	2	9.52
Ground maize with lime treatment kneaded with methanol/water 60:40 v/v	11.6	1.2	35	1	2.86
Tortilla	11.6	5.3	32	1.53	4.82
Tortilla + saliva (mouth)	7.0	11.6	32	0.58	1.82
Tortilla + gastric fluid (stomach)	1.2	5.7	31	1.53	4.98
Tortilla + pancreatic fluid (duodenum)	7.5	12.8 [a]	43	0.58	1.35
10 Tortillas + pancreatic fluid (duodenum)	7.5	128 [b]	49	0.56	1.40
Tortilla + saliva + gastric + pancreatic fluids	5.8	2.9	40	0.58	1.46

SD = Standard deviation; % RSD = Percentage of relative standard deviation; [a] = Average AFB_1 amount (µg/kg), as determined by HPLC in one tortilla; [b] = Average of AFB_1 amount (µg/kg), as determined by HPLC, in ten tortillas made from maize naturally contaminated with AFB_1 without mutagenic effect.

Table 2: Effect of controls, enzymatic solutions and treatment with AFB_1 standard, without tortillas, in the human digestion model.

Controls/Treatments	pH	Average AFB_1 amount (ng/mL) by HPLC	Revertant colonies (number)	SD	% RSD
Controls with AFB_1					
With Na Cl + HCl	1.2	40	21	2	9.52
With KH_2PO_4 + NaOH	7.5	30	52	1	1.92
With (NaCl + HCl)+ (KH_2PO_4 + NaOH)	2.4	103	16	2	12.5
With K acetate/acetic acid	3.5	123	41	2	4.88
With Ca $(OH)_2$	12.0	12	33	1	3.03
With Ca $(OH)_2$ + heat	12.0	11	33	0.58	1.73
Enzymatic solutions without AFB_1					
Saliva	7.0	0	33	1.53	4.68
Gastric fluid	1.2	0	14	1	7.14
Pancreatic fluid	7.5	0	29	1	3.45
Saliva + gastric fluid	1.8	0	13	1	7.69
Gastric + pancreatic fluids	2.4	0	24	1	4.17
Saliva + gastric + pancreatic fluids	5.8	0	38	1	2.63
Treatments with AFB_1 (150 ng/mL) standard in human digestion model					
With saliva	7.0	116	**156***	2.83	1.81
With gastric fluid	1.2	66	38	2	5.26
With pancreatic fluid	7.5	89	**74***	1.41	1.91
With saliva + gastric fluid	1.8	54	38	0.58	1.51
With gastric + pancreatic fluids	2.4	102	40	2	5
With saliva +gastric + pancreatic fluids	5.8	116	**84***	1.41	1.68

*= Positive result in the Ames test; SD = Standard deviation; % RSD = Percentage of relative standard deviation

Table 3: Mutagenic changes in AFB_1 owing to pH in tortillas treated in the human digestion model, made from dough spiked with AFB_1.

Human digestion model control treatments with AFB_1 contaminated tortilla.	Mutagenic effect of AFB_1 (ng/g) in relation to the pH									
	Tortillas blended with methanol/ distilled water (60:40 v/v)					Tortillas blended with methanol/PBS [a] (60:40 v/v)				
	pH	AFB_1 [a]	rc [b]	SD	% RSD	pH	AFB_1 [a]	rc [b]	SD	% RSD
Without AFB_1	11.6	0	25 [d]	2	8	9.5	0	31	1	3.23
Made from AFB_1 spiked dough	11.6	37	53 [c]	1	1.89	9.4	35	68 [f]	0.58	0.85
+ Saliva (mouth)	7.0	147	**254***	2.83	1.11	6.9	75	**112*[e]**	1.41	1.26
+ Gastric juice (stomach)	1.2	36	25 [d]	1.41	5.66	6.0	68	**121***	1.41	1.17
+ Pancreatic fluid (duodenum)	7.5	67	**115***	2.83	2.46	7.0	44	66 [f]	1.41	2.14
+ Saliva + gastric juice (stomach)	1.8	35	20	1.41	7.07	6.1	63	**99***	0.71	0.72
Gastric + pancreatic fluids (pylori, duodenum)	2.4	70	51 [c]	1.41	2.77	6.4	19	66 [f]	2.83	4.29
Saliva + gastric + pancreatic fluids (pylori, duodenum, colon)	5.8	83	**104***	2.83	2.72	6.5	30	**110*[e]**	1.41	1.29

PBS = Phosphate buffer saline; ng/g = nanograms per gram; * = mutagenic effect; a = by HPLC; b= average of triplicate counts; rc = revertant colonies; SD = Standard deviation; % RSD = Percentage of relative standard deviation. c with c, d with d, e with e, and f with f = Treatments that were statistically the same

The effect of pH in human digestion with water, which is the normal solvent, increased mutagenicity. A neutral pH (6-7) favored the mutagenicity of AFB1 while acidic or basic conditions inhibited it (Figure 4). The exception was with the pancreatic fluid with H_2Od (pH 7.5), which produced a positive mutagenic result (115 rc), and with PBS buffer where although a pH 7.0 was obtained, only 66 rc were produced. These results demonstrate that pancreatic fluid itself, and not only pH, plays a role in mutagenicity.

When PBS was used as the dilution solvent, the resulting pH of 6.0 was in the neutral range and the mutagenicity of AFB1 was restored to 121 rc in the gastric juice step. PBS diminished the effect of saliva on mutagenicity, from 254 rc to 112 rc. However, saliva dissolved in both H_2Od and PBS was mutagenic (Table 3). A pH dose-response curve is displayed in Figure 4.

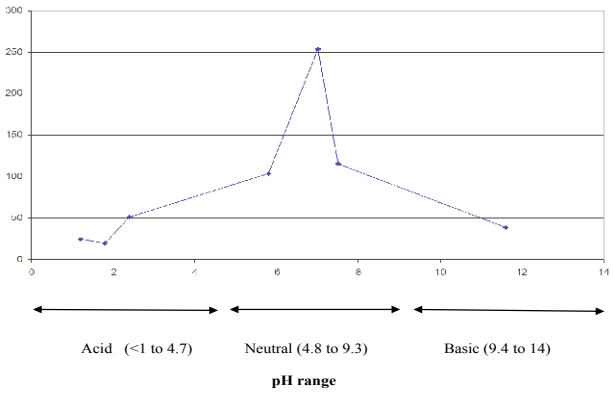

Figure 4: Dose response curve of the relationship between the pH in the human digestion model and the mutagenicity of AFB1 (revertant colonies of *Salmonella typhimurium*) in the Ames test.

Statistical analysis

Table 3 included the statistical analysis of the count of the

reverted colonies, there was no influence of the solution fluid shown when tortillas were blended with methanol or distilled water that were not statistical significant different from the control made from AFB1 spiked dough (c), and treatments with gastric + pancreatic fluids (pylori, duodenum) (c), as well as for the tortillas digested with the gastric fluid (stomach) (d) and the control (without AFB1) (d). Whereas the case of tortillas blended with methanol/PBS there were not significant differences between control tortillas made from AFB1 spiked dough (f), with pancreatic fluid (duodenum) (f) and with gastric + pancreatic fluids (pylori, duodenum) (f). All the others differences of means were statistically different ($p < 0.05$).

Discussion

Although the absorption of the AF mainly occurs in the intestine, AFB1 mutagenicity of the chewed maize tortilla bolus, can change in the different organs where the contaminated food passes or distributes and the accumulation of the AFB1–FAPY adducts in the same place can initiate a cancer [10,12]. Cytochrome P450 CYP1A2 is responsible for the metabolism of various xenobiotics, and is related to the mutagenicity of AFB1 [42]. Cytochrome P450 CYP3A4 is involved in the oxidation of wide range of substrates, and cytochrome P450 CYP2B1 converts AFB1 into mutagenic metabolites [43]. These enzymes convert AFB1 into a genotoxic epoxide that forms N7-guanine DNA adducts that were studied with the Ames test [44]. Low hepatic glutathione S-transferase (GST) and increased hepatic AFB1-DNA adduction correlate with hepatocyte proliferation and contribute to increased tumorigenicity of AFB1 in newborn and partially hepatectomized mice [45].

The human digestion model may provide means to assess physical and chemical changes of the food and AFB1 when they pass through the digestive tract. However, metabolic activation or mutation occur only when the toxin is absorbed into the body and reached to the site of action. About the susceptibility to AFB1 adduct formation, there are different organ responses. Liver and kidney might be the probable target organs for AFB1 with the highest formation and persistence of DNA adducts [46]. There is a extrahepatic bioactivation of AFB1 in fetal, infant and adult rats in the mucosas of the nasopharynx, some glands in the nose, and of the glandular stomach trachea, bronchioles, colon and caecum, but not in the small intestine, oesophagus or Harderian gland [47]. The nasal olfactory mucosa had a much higher capacity than the liver to form AFB1-metabolites which bound to DNA and protein [47]. Several of the extrahepatic tissues able to bioactivate AFB1 have been reported to be targets for the carcinogenicity of the substance. The extrahepatic carcinogenicity of AFB1 is correlated to a local bioactivation in the sensitive tissues [47]. Liver is by far the AF target organ and the induction of hepatocellular carcinoma [48]. AFB1-DNA binding was observed in both liver cell and was 3 to 5 fold higher in parenchymal cells than in non-parenchymal cells [48]. In contrast no tumours arising from the sinusoidal cell population have been reported after exposure to AFB1. AF activate mainly in the liver, but also in other organs such as lungs [49,50], small intestine [51], nasal mucosa [47], etc. Donnelly et al. [50] investigated AFB1 bioactivation, the role of enzymes, detoxification and pulmonary susceptibility to AFB1 in human lung tissue obtained from patients undergoing clinically indicated lobectomy. Appropriate pharmacokinetic model (including the rate of absorption and metabolism) to assess the mutagenic potential of the AFB1 absorbed at each organ, discussing the role of pH in the mutagenicity of the toxin in each part of the digestive system is a necessary research to clarify AFB1 role in the carcinogenesis. The space in the digestive system in the *in vitro* model is considered outside, not inside of the body and there might be some changes in the toxicological standpoint. Although stomach and intestine were tested separately in the model a sequential digestion would be plausible, therefore the mixture of enzymes and pH of the different steps (saliva with pepsin pHs, gastric and pancreatic fluids pH's and all of them) shows a closer approach to real human digestion. Microsomal cytochrome P450 has a minor role in the bio activation of AFB1 in human lung. AFB1 is a pulmonary carcinogen in experimental animals, and epidemiological studies have shown an association between AFB1 exposure and lung cancer in humans [50].

Intracellular AFB1 adducts are formed in the small intestine, and this reflects, at least in part, the catalytic activity of cytochrome P450 (CYP) 3A enzymes. The small intestine may not play a significant role in the metabolism of AFB1 [51]. Because these AFB1 adducts should ultimately pass in stool, enterocyte CYP3A may represent a regulatable barrier to dietary AF [52].

Short-term tracheal explant cultures from the rabbit were used to study the metabolism of AFB1 and to determine the cell types that are susceptible to damage by AFB1 and their relative contents of monooxygenase enzymes [53]. The conclusions of these studies were: a) rabbit tracheal explants are able to metabolize AFB1; (b) the nonciliated secretory cell population in this tissue is the target cell for cytotoxicity of this carcinogen; and (c) as is the case in the more distal airways, the nonciliated epithelial cells appear to have a high content of components of the pulmonary cytochrome P450 monooxygenase system, which may be an important factor in the susceptibility of these cells and this region of the airways to suspected airborne carcinogens [53].

AFB1-DNA adducts in human placenta (58%) and (9%) of adducts in cord blood, readily available specimens that respond to maternal environmental insult and are being used to investigate metabolism, bioactivation, and transplacental transfer of procarcinogens [54]. Thus, monitoring adduct levels in human specimens may provide information not only on carcinogen exposure but also on the relationship among infection with hepatitis B/C virus, dietary exposure to AFB1, and liver cancer.

Dietary and caloric restriction has been recommended to reduce cancer risk [55-57]. Sixty percent dietary restriction reduced AFB1 metabolizing enzyme activity and decreased the AFB1-DNA adduct formation in young rats treated with AFB1. A protective effect due to caloric restriction reduced the metabolic activation of AFB1, this fact brought a decrease of AFB1-DNA binding by more than 50% [57]. Thus, the contributions of caloric restriction are the lower initial AFB1-DNA binding and less DNA damage, presumably by the less apurinic sites formed during the depurination process of AFB1-DNA adducts. Species and tissue specificities exist regarding the metabolic activation of AFB1 [57]. The effects of food restriction on the metabolic activation of AFB1 in rats and mice, which are AFB1-sensitive and -resistant rodent species, respectively has been reported [55]. In a comparison of food restriction and *ad libitum* food consumption treatments there was a reduction of metabolic activation of AFB1 in both rats and mice, causing formation of hepatic AFB1-DNA adducts to be 43% and 31% lower, respectively.

Human gastric juice is an excellent medium for the oxidation of lipids. Cholesterol sulfate exhibits gastroprotective activity [16,58,59]; in the Ames test of the present study, its low pH inhibited

the mutagenicity of AFB1, producing a negative result with 25 rc. However, when gastric juice was dissolved in PBS, the pH increased to 6.0, and the mutagenicity was restored (121 rc).

The present study demostrated that AFs from tortillas, although not detectible by fluorescence, are still present in gastric and pancreatic fluids and their chemical structures and toxicity can be restored. The action of HCl (pH 1.2) alone on maize following lime processing of tortillas did not yield greater levels of AF. Our results demonstrated that there was a 60-76% recovery of AFB1 with digestive solutions in the pH range of 5.8-7.5, data found in agreement with Price and Jorgensen [17] but not with an earlier study [60].

Traditional lime treatment does not appear to be an adequate process for AF detoxification. AFB1 can produce a mutagenic effect in both the mouth and duodenum, as assays with and without tortillas demonstrated (Tables 3 and 4). Lime treatment blocked the mutagenic effect but did not prevent risk of AFB1 ingestion. A neutral pH, but not an acidic pH, restored the mutagenic effect, as was previously reported [60]. Incubation with pancreatin or pepsin did not affect the amount of AF associated with casein [61]. Pancreatin causes a reduction in the digestibility of maize and an increase in AF content [62].

The acidity of gastric fluid was suitable for forming nitrosamines, a potent carcinogen for many animal species [63]. However, acidic pH inhibited the mutagenic effect of AFB1. It seems that stomach cancer is more closely associated with nitrosamines, and AFs with colorectal cancer [10], but the etiological role of AFB1 in stomach cancer remains unclear because there are multiple changes in pH during digestion. Further studies on AFB1-DNA adducts in stomach cancer DNA are needed to clarify this issue. Pepsin has no effect in mutagenicity, requiring acid pH to act properly. In contrast, chloride lowers the pH and inhibits the genotoxic properties of AFB1, in agreement with previous findings [64]. The ingestion of maize may cause several mutations, but the human body has protective mechanisms to limit them. In susceptible individuals eating tortillas as a staple food, the risk for cancer increases.

Many phytochemical compounds from vegetables and fruits can block mutagenic and carcinogenic processes [65-67]. Natural substances in maize tend to protect humans against AFB1 mutagenicity and anti-mutagenic activity of extracts from natural maize have been reported [68]. The linoleic acid (9,12 acid–octadecanoic) found in maize and tortillas, as well as a the compound that could contain linoleic acid in its structure, have been reported as anti-mutagenic and antioxidant compounds in AF-contaminated maize [69]. These compounds might protect humans exposed to high amounts of AFs in their diet by blocking the mutagenicity of AFs. Tortillas can contain these compounds and therefore continue acting as anti-mutagens when AFs are extracted. Other reports recommend the control of toxigenic *Aspergillus flavus* and aflatoxin accummulation in peanuts using a native atoxigenic *A. flavus* strain based on competitive exclusion of the toxigenic strains [70].

Conclusion

The pH of each digestion step plays an important role in inducing or inhibiting the mutagenic effect of AFB1. Tortillas prepared from AFB1-spiked dough had a mutagenic effect in the Ames test, but this effect was not observed in tortillas prepared with maize naturally contaminated by AFs. The alkaline pH (11.6 to 12.0) of the lime treatment of tortillas inhibited the mutagenic effect of AFB1, whereas the neutral pH of both saliva (7.0) and pancreatic fluid (7.5) reactivated AFB1 mutagenicity. The acidic pH 1.2 of gastric juice inhibited AFB1 activity, but using PBS to increase the pH to 6.0 restored the mutagenicity of AFB1. This research shows also that the pancreatic fluid has a role in activating the mutagenicity of AFB1, not only the pH.

Acknowledgments

The authors thank the Instituto de Biología, Universidad Nacional Autónoma de México, which supported this work. We also thank the Universidad Autónoma de San Luis Potosí for the PhD scholarship Supera from Promep given to María de Guadalupe Moctezuma. Thanks also to IBUNAM´s personnel: Noemí Chávez from the Secretaría Técnica, Carmen Loyola, Joel Villavicencio, Jorge López, Alfredo Wong, Diana Martínez and Julio César Montero provided valuable assistance with imaging, computer analysis and design. Additionally, we thank Georgina Ortega Leite and Gerardo Arévalo for library information.

References

1. Chang P-K, Horn BW, Dorner JW (2005) Sequence breakpoints in the aflatoxins biosynthesis gene cluster and flanking regions in nonaflatoxigenic *Aspergillus flavus* isolates. Fungal Genet Biol 42: 914-923.
2. ICMSF International Commission on Microbiological Specifications for Foods (1996) Toxigenic Fungi: *Aspergillus*. Academic Press. London UK.
3. Kurtzman CP, Horn BW, Hesseltine CW (1987) *Aspergillus nomius*, a new aflatoxin-producing species related to *Aspergillus flavus* and *Aspergillus tamarii*. S Antonie Van Leeuwenhoek 53: 147-158.
4. Ito Y, Peterson SW, Wicklow DT, Goto T (2001) *Aspergillus pseudotamarii*, a new aflatoxin producing species in *Aspergillus* section Flavi. Mycol Res 105: 233-239.
5. Peterson SW, Ito Y, Horn BW, Goto T (2001) *Aspergillus bombycis* a new aflatoxigenic species and genetic variation in its sibling species, *A.nomius*. Mycologia 93: 689-703.
6. OPS, Organización Panamericana de la Salud (1983) Criterios de Salud Ambiental II. pp. 2-83. Oficina Regional Sanitaria. Organización Mundial de la Salud (WHO). [Panamerican Health Organization. (1983). Environmental Health Criteria II. Region Sanitary Office. World Health Organization (WHO)].
7. Llewellyn GC, Stephenson GA, Hofman JW (1977) Aflatoxin B1 induced toxicity and teratogenicity in japanese Medaka eggs (*Oryzias latipes*). S Toxicon 15: 582-587.
8. Wong JJ, Hsieh DP (1976) Mutagenicity of aflatoxins related to their metabolism and carcinogenic potential. S Proc Natl Acad Sci U S A 73: 2241-2244.
9. Wogan GN (1992) Aflatoxins as risk factors for hepatocellular carcinoma in humans. S Cancer Res 52: 2114s-2118s.
10. Harrison JC, Carvajal M, Garner RC (1993) Does aflatoxin exposure in the United Kingdom constitute a cancer risk? S Environ Health Perspect 99: 99-105.
11. Massey TE, Smith GB, Tam AS (2000) Mechanisms of aflatoxin B1 lung tumorigenesis. S Exp Lung Res 26: 673-683.
12. Carvajal M, Berumen J, Guardado-Estrada M (2012) The presence of aflatoxin AFB1-FAPY adduct and human papilloma virus in cervical smears from cancer patients in Mexico. S Food Addit Contam Part A Chem Anal Control Expo Risk Assess 29: 258-268.
13. Massey TE, Stewart RK, Daniels JM, Liu L (1995) Biochemical and molecular aspects of mammalian susceptibility to aflatoxin B1 carcinogenicity. S Proc Soc Exp Biol Med 208: 213-227.
14. Castillo-Urueta P, Carvajal M, Méndez I, Meza F, Gálvez A (2011) Survey of aflatoxins in maize tortillas from Mexico City. S Food Addit Contam Part B Surveill 4: 42-51.
15. Park DL, Jemmali M, La Farge-Frayssinet C, Yvon M (1981) Decontamination of aflatoxin-contaminated peanut meal using monomethyl amine: Ca(OH)2. In: Memorial Symposium on Mycotoxins. 72th Anual Meeting of AOAC. New Orleans, USA. J Am Oil Chem Soc 58:995A-1002A.
16. Machorro VL, Valdivia LA (1984) Cambios cuantitativos en las aflatoxinas

durante el proceso de nixtamalización y elaboración de la tortilla. Tecnol Alim 19: 10-14.

17. Price RL, Jorgensen KV (1985) Effects of processing on aflatoxin levels and on mutagenic potential of tortillas made from naturally contaminated corn. J Food Sci 50: 347-349.
18. Parker WA, Melnick D (1966) Absence of aflatoxin from refined vegetable oils. S J Am Oil Chem Soc 43: 635-638.
19. Venturi 1, Venturi M (2009) Iodine in evolution of salivary glands and in oral health. S Nutr Health 20: 119-134.
20. Tsega E (1977) Current views on liver diseases in Ethiopia. S Ethiop Med J 15: 75-82.
21. Yim SH1, Hee SS (1995) Genotoxicity of nicotine and cotinine in the bacterial luminescence test. S Mutat Res 335: 275-283.
22. Nishioka H, Nishi K, Kyokane K (1981) Human saliva inactivates mutagenicity of carcinogens. S Mutat Res 85: 323-333.
23. Karincaoglu 1, Batcioglu K, Erdem T, Esrefoglu M, Genc M (2005) The levels of plasma and salivary antioxidants in the patient with recurrent aphthous stomatitis. S J Oral Pathol Med 34: 7-12.
24. Suschek CV, Schewe T, Sies H, Kröncke KD (2006) Nitrite, a naturally occurring precursor of nitric oxide that acts like a 'prodrug'. S Biol Chem 387: 499-506.
25. Iwamori M, Suzuki H, Ito N, Iwamori Y, Hanaoka K (2005) Lipid compositions of human gastric fluid and epithelium: the role of sulfated lipids in gastric cytoprotection. S J Clin Gastroenterol 39: 129-133.
26. Guyton AC, Hall JE (2006) Textbook of Medical Physiology. (11th edtn), Philadelphia: Elsevier Saunders, The Netherlands. ISBN 0-7216-0240-1.
27. Rodriguez J, Gupta N, Smith RD, Pevzner PA (2008) Does trypsin cut before proline? S J Proteome Res 7: 300-305.
28. Malpass GE, Arimilli S, Prasad GL, Howlett AC (2013) Complete artificial saliva alters expression of proinflammatory cytokines in human dermal fibroblasts. Toxicol Sci 134: 18-25.
29. Shepherd MJ, Mortimer DN, Gilbert J (1987) A review of approaches to the rapid analysis of aflatoxins in foods. JAPA 25: 129-142.
30. AOAC Association of Official Analytical Chemists (2005) Official methods of analysis. (17th edtn), Horwitz W, ed. Food Composition, Additives; Natural Contaminants. Subchap 1: Mycotoxins. Subchapter 2: Aflatoxins. II. Chap 49. Washington (DC) USA.
31. Ames BN, Gurney EG, Miller JA, Bartsch H (1972) Carcinogens as frameshift mutagens: metabolites and derivatives of 2-acetylaminofluorene and other aromatic amine carcinogens. S Proc Natl Acad Sci U S A 69: 3128-3132.
32. Ames BN, McCann J, Yamasaki E (1975) Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutat Res 31: 347-363.
33. Bloching M1, Stephan D, Agha-Mir-Salim P, Berghaus A, Lautenschläger C, et al. (2001) [Ames test as biomarker]. S HNO 49: 440-446.
34. Mortelmans K, Zeiger E (2000) The Ames Salmonella/microsome mutagenicity assay. S Mutat Res 455: 29-60.
35. Juan-López M, Carvajal M, Ituarte B (1995) Supervising programme of aflatoxins in Mexican corn. S Food Addit Contam 12: 297-312.
36. Akiyama H, Goda Y, Tanaka T, Toyoda M (2001) Determination of aflatoxins B1, B2, G1 and G2 in spices using a multifunctional column clean-up. S J Chromatogr A 932: 153-157.
37. McCann J, Choi E, Yamasaki E, Ames BN (1975) Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. S Proc Natl Acad Sci U S A 72: 5135-5139.
38. Koenig GL (2003) Viability of and plasmid retention in frozen recombinant *Escherichia coli* over time: a ten-year prospective study. S Appl Environ Microbiol 69: 6605-6609.
39. Ames BN, Lee F, Durston WE (1973 a) An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc Natl Acad Sci USA 70:782-786.
40. Ames BN, Durston WE, Yamasaki E, Lee F (1973 b) Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. Proc Natl Acad Sci USA 70: 2281-2285.
41. Langer PJ, Shanabruch WG, Walker GC (1981) Functional organization of plasmid pKM101. S J Bacteriol 145: 1310-1316.
42. Peterson S, Lampe JW, Bammler TK, Gross-Steinmeyer K, Eaton DL (2006). Apiaceous vegetable constituents inhibit human cytochrome P-450 1A2 (hCYP1A2) activity and hCYP1A2-mediated mutagenicity of aflatoxin B1. Food Chem Toxicol 44: 1474-1484.
43. Horn RC, Vargas VM (2003) Antimutagenic activity of extracts of natural substances in the *Salmonella*/microsome assay. S Mutagenesis 18: 113-118.
44. Guengerich FP (2005) Principles of covalent binding of reactive metabolites and examples of activation of bis-electrophiles by conjugation. S Arch Biochem Biophys 433: 369-378.
45. Shupe T, Sell S (2004) Low hepatic glutathione S-transferase and increased hepatic DNA adduction contribute to increased tumorigenicity of aflatoxin B1 in newborn and partially hepatectomized mice. S Toxicol Lett 148: 1-9.
46. Choi MJ, Lee JW, Lee BM (1996) Comparative assessment of DNA adduct formation, *Salmonella* mutagenicity, and chromosome aberration assays as short-term tests for DNA damage. S J Toxicol Environ Health 49: 271-284.
47. Larsson P, Tjälve H (1995) Extrahepatic bioactivation of aflatoxin B1 in fetal, infant and adult rats. S Chem Biol Interact 94: 1-19.
48. Jennings GS, Oesch F, Steinberg P (1992) *In vivo* formation of aflatoxin B1-DNA adducts in parenchymal and non-parenchymal cells of rat liver. S Carcinogenesis 13: 831-835.
49. Chelcheleh M, Allameh A (1995) In vivo biotransformation of aflatoxin B1 and its interaction with cellular macromolecules in neonatal rats. S Mech Ageing Dev 78: 189-196.
50. Donnelly PJ, Stewart RK, Ali SL, Conlan AA, Reid KR, et al. (1996) Biotransformation of aflatoxin B1 in human lung. S Carcinogenesis 17: 2487-2494.
51. Salbe AD, Bjeldanes LF (1989) Effect of diet and route of administration on the DNA binding of aflatoxin B1 in the rat. S Carcinogenesis 10: 629-634.
52. Kolars JC, Benedict P, Schmiedlin-Ren P, Watkins PB (1994) Aflatoxin B1-adduct formation in rat and human small bowel enterocytes. S Gastroenterology 106: 433-439.
53. Coulombe RA Jr, Wilson DW, Hsieh DP, Plopper CG, Serabjit-Singh CJ (1986) Metabolism of aflatoxin B1 in the upper airways of the rabbit: role of the nonciliated tracheal epithelial cell. S Cancer Res 46: 4091-4096.
54. Hsieh LL, Hsieh TT (1993) Detection of aflatoxin B1-DNA adducts in human placenta and cord blood. S Cancer Res 53: 1278-1280.
55. Chou MW, Chen W (1997) Food restriction reduces aflatoxin B1 (AFB1)-DNA adduct formation, AFB1-glutathione conjugation, and DNA damage in AFB1-treated male F344 rats and B6C3F1 mice. S J Nutr 127: 210-217.
56. Chou MW, Shaddock JG, Kong J, Hart RW, Casciano DA (1995) Effect of dietary restriction on partial hepatectomy-induced liver regeneration of aged F344 rats. S Cancer Lett 91: 191-197.
57. Gao P, Chou MW (1992) Effect of caloric restriction on hepatic nuclear DNA damage in male Fischer 344 rats treated with aflatoxin B1. S Toxicol Lett 61: 233-242.
58. Maron DM, Ames BN (1983) Revised methods for the *Salmonella* mutagenicity test. S Mutat Res 113: 173-215.
59. Stoloff L, Trager W (1965) Recommended decontamination procedures for aflatoxin. Notes. J Assoc Off Anal Chem 48: 681-682.
60. Méndez-Albores JA, Arámbula-Villa G, Loaraca-Piña MG, González-Hernández J, Castaño-Tostado E, et al. (2004) Aflatoxins' fate during the nixtamalization of contaminated maize by two tortilla-making processes. J Stored Prod Res 40: 87-94.
61. Blanc B, Lauber E, Sieber R (1983) Binding of aflatoxin to milk proteins. (Fixation de l´aflatoxine sur les proteins du lait). Microbiol Aliment Nutr 1: 163-177.
62. Panigrahi S, Bestwick LA, Davis RH, Wood CD (1996) The nutritive value of stackburned yellow maize for livestock: tests *in vitro* and in broiler chicks. S Br J Nutr 76: 97-108.

63. Zeisel SH, daCosta KA, LaMont JT (1988) Mono-, di- and trimethylamine in human gastric fluid: potential substrates for nitrosodimethylamine formation. S Carcinogenesis 9: 179-181.

64. Tilch C, Elias PS (1984) Investigation of the mutagenicity of ethylphenylglycidate. S Mutat Res 138: 1-8.

65. Catterall F, Copeland E, Clifford MN, Ioannides C (2003) Effects of black tea theafulvins on aflatoxin B(1) mutagenesis in the Ames test. S Mutagenesis 18: 145-150.

66. Gomes-Carneiro MR, Dias DM, Paumgartten FJ (2006) Study on the mutagenicity and antimutagenicity of beta-ionone in the *Salmonella*/microsome assay. S Food Chem Toxicol 44: 522-527.

67. Namiki M (1994) Antimutagen and anticarcinogen research in Japan. Food Phytochemicals for Cancer Prevention I. Fruits and Vegetables, ACS Symposium Series 546, edited by Huang MT, Osawa T, Ho CT and Rosen RT Washington, DC.

68. Burgos-Hernández A, López-García R, Njapau H, Park DL (2001) Anti-mutagenic compounds from corn. S Food Addit Contam 18: 797-809.

69. Burgos-Hernández A, López-García R, Njapau H, Park DL (2001) Partial chemical/structural elucidation of anti-mutagenic compounds from corn. S Toxicology 166: 161-170.

70. Chulze SN, Palazzini JM, Torres AM, Barros G, Ponsone ML, et al. (2015) Biological control as a strategy to reduce the impact of mycotoxins in peanuts, grapes and cereals in Argentina. S Food Addit Contam Part A Chem Anal Control Expo Risk Assess 32: 471-479.

Author Affiliation

Top

[1]*Laboratorio de Micotoxinas, Departamento de Botánica, Instituto de Biología, Universidad Nacional Autónoma de México (UNAM). 04510 México.*
(Mycotoxins Laboratory, Botany Department, Institute of Biology, National Autonomous University of Mexico (UNAM). 04510, México)
[2]*Laboratorio de Mutagénesis, Instituto de Investigaciones Biomédicas, UNAM.04510 México.*
(Mutagenesis Laboratory, Biomedical Research Institute, UNAM, 04510 Mexico)
[3]*Departamento de Química Analítica, Facultad de Química, UNAM. 04510 México.*
(Department of Analytical Chemistry, Faculty of Chemistry, UNAM. 04510, Mexico)
[4]*Instituto Nacional de Pediatría, Secretaría de Salud. México.*
(National Institute of Pediatrics, Health Ministry, Mexico)
[5]*Departamento de Probabilidad y Estadística, Instituto de Investigaciones en Matemáticas Aplicadas y en Sistemas, 04510 UNAM.*
(Probability and Statistics Department, Applied Mathematics and Systems Research Institute, UNAM. 04510 Mexico)