Reduction of Mutagenic Potentials in Milk: Effects of Ammonia Treatment on Aflatoxin-Contaminated Cottonseed

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ABSTRACT

Milk obtained from cows fed rations containing aflatoxin-contaminated cottonseed, ammonia-treated aflatoxin-contaminated cottonseed, and uncontaminated cottonseed were tested for mutagenic potential using the Salmonella/mammalian microsome mutagenicity assay using Salmonella typhimurium strains TA98 and TA100. Standard assay protocol was used with S-9 liver homogenate added. Samples including whole milk, nonfat dry milk powder, and reconstituted whole milk were applied directly to the plates in triplicate. As a control, samples of whole milk, reconstituted whole milk, and nonfat dry milk powder from cows fed uncontaminated feed were spiked with aflatoxin and tested for mutagenic activity. High levels of mutagenic activity were observed in all samples from cows exposed to aflatoxin-contaminated cottonseed and the aflatoxin-spiked milks. This high activity was not evident in whole milk and whole milk component samples from cows exposed to aflatoxin-contaminated cottonseed or nonaflatoxin-containing cottonseed. A low level of mutagenic potential was evident in whole milk from the ammonia treated group using TA100 tester strain.

The purpose of this study was to determine the mutagenic potentials of the milks obtained from animals consuming ammonia-treated aflatoxin-contaminated cottonseed and compare these results to milks from cows fed aflatoxin-contaminated cottonseed, nonaflatoxin contaminated feed, and spiked milk.

MATERIALS AND METHODS

Ammoniation procedure

Whole cottonseed containing 1200 μg/kg total aflatoxins was ammoniated using the closed vessel, high temperature/high pressure procedure (8). The conditions of the procedure were: a) moisture content of the cottonseed, adjusted to 14%; b) ammonia concentration, 4% w/w-ammonia/cottonseed mixture; c) pressure, 40 psi; d) temperature, 100°C; and e) time, 30 min. Aflatoxin analysis was done, in duplicate, on both ammoniated and nonammoniated cottonseed using AOAC Methods (1).

Animal diets

Lactating dairy cows (Holstein-Friesian) from the University of Arizona Dairy Farm were fed one of the following diets: #1 Whole cottonseed, 1200 μg aflatoxins/kg, at 25% of ration, (300 μg/kg in final rations); #2 Same as #1 - except the whole cottonseed was treated with ammonia (less than 10 μg total aflatoxins/kg after ammonia treatment).

The cows were first fed the ammonia-treated cottonseed diet for 10 d and then switched to the aflatoxin-contaminated diet for 10 d. The cows were milked twice a day, with the milks for each milking being pooled, and labeled for day and milking. Milks collected at each milking were tested daily for aflatoxin content. To insure complete removal of any residues from the first feeding period, the milk from the interim period (approximately 3 d) was discarded.

Milk samples

Milk and milk product samples in this study were obtained as follows:
(a) Milk from cows fed Diet 1 (3.2 μg AFM₁/L); (b) Milk from cows fed Diet 2 (AFM₁, non detectable); (c) Milk obtained from commercial outlets produced under normal operations for which feed rations contained cottonseed at 13.5% of the diet at levels <20 g total aflatoxins/kg cottonseed (personal communication), (AFM₁, nondetectable); (d) Nonfat dry milk powder was
prepared from the three milks described above. Powder from milk from cows fed aflatoxin-contaminated cottonseed contained 32 μg AFM₁/kg. The solids-not-fat skim milk concentrate test portion was prepared at 30% concentration with distilled water; (e) Cream prepared from the three milks described above containing 46% milk fat; (f) Reconstituted whole milk prepared from nonfat dry milk powder and cream for each treatment described above; and (g) Aflatoxin B₁ spiked samples - 50 μg aflatoxin B₁ added to nonaflatoxin containing whole milk, reconstituted milk, and nonfat dry milk powder concentrate. After initial testing, the milks were defatted and spray dried (Niro Atomizer Spray Dryer with a flow rate of 7 L/h). The inlet and outlet temperatures were 180°C and 90°C, respectively. Following the drying procedure, samples of dried milk were analyzed for aflatoxin content and stored at -18°C until analysis.

**Aflatoxin analysis**

All milk samples were analyzed, in duplicate, for aflatoxin M₁ and B₁ using the AOAC Methods (26.095-26.100, M₁ and 26.031, B₁). Aflatoxin B₁ identity was confirmed by the TFA procedure (26.083).

**Salmonella/mammalian microsome mutagenicity assay**

Salmonella typhimurium tester strains TA98 and TA100 were used in the presence of Aroclor 1254-induced Sprague-Dawley rat liver homogenate (S9) according to the standard assay (6). One hundred μl aliquots of each sample were added to the top agar containing the S-9 mix and bacteria then plated. This was done in triplicate for each sample. Nonfat dry milk powder was tested by extracting 15 g with 125 ml methylene chloride, evaporating to dryness under N₂, and dissolving in 750 μl DMSO of which 100 μl was added to each plate.

**RESULTS AND DISCUSSION**

The number of revertants observed for S. typhimurium tester strains TA98 and TA100 for each of the samples tested is presented in Fig. 1 and 2. Mutagenic potentials for aflatoxins B₁ and M₁ were confirmed under these test conditions (Fig. 3). Mutagenic potentials of milks tested in this study show that the ammonia treatment of aflatoxin-contaminated cottonseed either eliminated or significantly reduced the mutagenic potentials of the milk when tested with the Salmonella/microsome mutagenicity assay using tester strains TA98 and TA100. This correlates with results of earlier research which showed that ammoniation of aflatoxin-contaminated cottonseed lowers aflatoxin M₁ levels found in milk (8,9).

Results obtained using tester strain TA 98 showed that naturally-occurring, aflatoxin-contaminated milk had a high mutagenic potential (Fig. 1). Mutagenic potentials of milks from cows fed the ammonia-treated cottonseed and milks obtained from commercial outlets were equivalent. Milks prepared from nonfat dry milk powder (reconstituted) and milk fat showed no increased mutagenicity.

Results obtained with Salmonella tester strain TA100 showed the same high mutagenic potential with the naturally-contaminated milk (Fig. 2). The mutagenic potential of the milk from the cows fed the ammonia-treated cottonseed was significantly decreased; however, a positive mutagenic potential was observed. The mutagenic potential in the milk from the cows fed the ammoniated cottonseed observed with the strain could be due to the increased sensitivity of the tester strain (TA100), the type of mutagen formed, or other substances in the milk. The types of mutagenic events observed for tester strains TA98 and TA100 are frame-shift and base-pair substitution, respectively. Addition of aflatoxin B₁ (50 μg/L) to whole milk, dry milk, and reconstituted milk showed a mutagenic potential similar to that observed naturally in the dry-milk powder, reconstituted milk, and milk fat but not near the level observed for the naturally-contaminated whole milk. This suggests that although the ammonia treatment either eliminated or significantly reduces this potential, aflatoxin may not be the only source of mutagenicity observed. What is not known is whether the mutagenicity observed is associated with the residual aflatoxin or other metabolites, or the components of the rations. Equivalent mutagenic po-
tentials were observed for milks containing 3.2 g aflatoxin M\textsubscript{1} /L (whole milk), 32 μg aflatoxin M\textsubscript{1} /kg (dry milk powder), and 3.2 μg/L (reconstituted milk). The cream was not tested for aflatoxin. These results also suggest other mutagenic compounds are in the milks from cows fed aflatoxin-contaminated cottonseed.

One possible explanation for this observation is that a portion of the compounds causing the mutagenicity was affected by the spray-drying process. The extremely high mutagenic potential observed in the whole milk samples has not been analyzed to determine whether the compounds are water soluble or heat sensitive.

**Figure 3. Dose responses of tester strains TA98 and TA100 to aflatoxins B\textsubscript{1} and M\textsubscript{1}. Average spontaneous revertants per plate were 24 and 144 for TA98 and TA100, respectively.**

By comparing the results obtained in this study to the work done with aflatoxin B\textsubscript{1} and aflatoxin-contaminated cottonseed, the mutagenic potential with this assay as cows fed naturally-contaminated cottonseed. The ammination treatment levels of aflatoxin B\textsubscript{1} in the feed (1200 vs 10 μg total aflatoxins/kg cottonseed) as well as the mutagenic potential.

**REFERENCES**


