Carcinogenic and Mutagenic Activities of Milk from Cows Fed Bracken Fern (*Pteridium aquilinum*)

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**ABSTRACT**

Carcinogenicity, toxicity, and mutagenicity of the milk of bracken fern (BF)-fed cows were determined. Four Brown Swiss cows were fed a BF-containing diet, and two cows were fed a normal hay-grain diet. The milk obtained was fed to rats as either fresh or freeze-dried powdered milk mixed with grain diet: 9 of 34 fed whole milk and 11 of 56 fed powdered milk diet developed small intestine, kidney, or urinary bladder carcinomas within 117 weeks, while 0 of 70 rats fed either whole or powdered milk from cows receiving a normal diet and 0 of 20 rats fed a basic grain diet displayed neoplasia of these organs.

Six organic solvent extract fractions of milk were prepared from BF- or normal diet-fed cows. Diethyl ether and ethyl acetate fractions displayed the highest murine acute toxicity. The murine bladder carcinogenicity of prepared fractions was assessed by the pellet implantation technique. Diethyl ether fractions from milk of BF-fed cows induced bladder carcinoma in 10 of 24 mice compared with the corresponding fraction of normal diet-fed cows (3 of 19) or mice exposed to only the cholesterol vehicle (5 of 30, 6 of 40) (p < 0.01).

Four organic solvent extract fractions of milk obtained from BF- or normal diet-fed cows were prepared and tested for mutagenic activity in *Salmonella typhimurium* TA 98 and TA 100. The chloroform:methanol fraction from BF-fed cows demonstrated mutagenic activity for TA 100 but not for TA 98.

**INTRODUCTION**

BF is a food delicacy and salad green in the United States, Canada, and Japan (10, 12, 15). In the Miye, Nara, and Wakayama prefectures of Japan, human BF consumption was correlated with an increased incidence of esophageal carcinoma (T. Hirayama, personal communication). BF is a forage contaminant responsible for a high rate of spontaneous urinary bladder cancer in cattle and water buffalo (11) and intestinal and urinary bladder cancer in sheep (3, 6). Deliberate feeding of BF to cows, rats, mice, guinea pigs, and Japanese quail produced a wide variety of urinary bladder and intestinal tumors (11, 18).

The carcinogen may also be present in the milk of cows pastured in the BF-infested areas of the world, e.g., Turkey, Yugoslavia, and Bulgaria. These cows subsequently excrete toxic metabolites in their milk (4) and carcinogenic substances in their urine (5, 9). In this study the toxicity, carcinogenicity, and mutagenicity of milk obtained from BF-fed cows are demonstrated.

**MATERIALS AND METHODS**

**Preparation of Feed.** BF collection and processing has been described (11, 13). Six Brown Swiss cows (body weight, 300 to 350 kg) served as milk sources. Four were fed the BF-containing diet and hay. Each cow ingested 1 g BF per kg body weight per day, and the total biennial BF intake/cow was 218 to 252 kg. Two cows received a normal grain diet and hay. Milk obtained (13 to 15 liters/cow/day) was stored at 4°C and then fed to rats either fresh in their water bottles in place of water (approximately 65 to 75 ml milk per rat per day) or as freeze-dried powdered milk (90 g powdered milk obtained from 1 liter whole milk) mixed in the grain diet (12) at 1:1 (w/w). The latter was kept in a cold room to prevent rancidity of milk before use.

Clinical and cystoscopic examinations of 4 BF-fed and 2 control cows were performed as described (11).

**Carcinogenicity Test.** Male and female rats of the Norwegian strain (12) (Refik Saydam Institute of Hygien, Ankara, Turkey), weighing 100 to 105 and 90 to 95 g, respectively, were used. They were housed in screen-bottomed metal cages with no more than 4 rats/cage and were fed their diet and water or whole milk *ad libitum*.

Rats were divided into 5 groups. Group 1 consisted of 24 males and 10 females; Group 2, 35 males and 21 females; Group 3, 15 males and 20 females; Group 4, 20 males and 15 females; and Group 5 (negative control), 10 males and 10 females. Groups 1 and 3 were fed normal diet (12) and were given whole fresh milk from BF-fed cows and from cows that received normal diet, respectively. Groups 2 and 4 were fed basic diet containing powdered milk prepared from the milk of BF-fed cows and basic diet containing powdered milk of cows on a normal diet, respectively. Approximately 7.5 g powdered milk were consumed by each rat each day. Group 5 received a basic grain diet (12). This regimen of feeding was continued until the animals died or were killed, at which time necropsy was performed. Representative histological sections of various organs were made and studied (12).

**Toxicity Test.** Powdered milk (450 g) dissolved in distilled water was continuously extracted with various organic solvents as indicated in Chart 1. Dried pentane (I), methanol (II), benzene (III), diethyl ether (IV), ethyl acetate (V), or residue (VI) fractions were dissolved in high-grade DMso.
Mutagenicity Test for the Milk Fractions. Powdered milk prepared from the milk of BF-fed cows or from cows on normal diet was dissolved in water and extracted 3 times with pentane. The combined pentane extracts were evaporated under reduced pressure at low temperature (36°C); all evaporation were carried out under these conditions. The remaining residue was extracted 5 times with methanol, and the combined methanolic extracts were evaporated. The concentrated methanol fraction was extracted 5 times with diethyl ether and evaporated. Prior to use, diethyl ether was treated with a 40% solution of FeSO₄ and Na₂S₂O₅ to remove peroxides; the ether phase was separated from the water phase and then shaken with 40% NaOH solution, collected, and washed with distilled water.

The following substances were then consecutively well mixed with 25 ml of the concentrated diethyl ether fraction: 10 ml H₂O, 3 ml NH₄OH, 25 ml ethanol, 50 ml diethyl ether, and 50 ml petroleum ether. The mixture was centrifuged for 5 min, and the ether phase was siphoned. The remaining water phase was reextracted 3 more times with half the amounts of ethanol, diethyl ether, and petroleum ether. The combined ethanol, diethyl ether, and petroleum fractions were washed with water and centrifuged to aid with phase separation. The organic phase was collected and evaporated, and the dried organic fraction was purified by being dissolved in 100 ml chloroform, mixed with 50 g silica gel, and filtered batchwise through a No. 1 sintered glass filter. The filter was then washed 3 times with 250 ml chloroform. The combined chloroform filtrate was dried under reduced pressure at low temperature. Then the filter was washed with chloroform: methanol (1:1) 3 times with 250 ml of the solvent mixture. The combined chloroform:methanol filtrates were dried under reduced pressure at low temperature (C. Y. Wang, A. M. Pamukcu, and G. T. Bryan, unpublished data).

The dried pentane, methanol, chloroform, and chloroform:methanol fractions were dissolved in a spectrophotometric grade of DMSO (Aldrich Chemical Co.) and tested for mutagenicity. Spot tests with S. typhimurium strains TA 98 and TA 100 were performed (1, 7, 17). Solutions (50 µl) containing the different milk fractions were put on the center of the plates, and the plates were inverted and incubated at 37°C for 2 days. The control plates were treated with 50 µl DMSO without the milk fractions. The colonies of his+ revertants on the plate were counted. Fractions inducing more than twice the number of revertants than control were considered mutagenic. The diameter of the clear zone produced by antibacterial activity was also measured. 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (7, 17, 18) was used as a positive control.

Silica gel thin-layer chromatography of these fractions of milk was performed in solvent systems of benzene:acetic acid:water (2:2:1), n-butanol:acetic acid:water (12:5:3), or chloroform:methanol (49:1). The chromatograms were air-dried and cut into 1- x 1.5-cm strips. The strips were tested for mutagenicity on an agar plate containing TA 100 or TA 98. The chromatograms were studied under visible or UV light.

RESULTS

Effects of BF on Cows. Clinical and hematological...
changes induced in 4 cows by BF feeding were similar to those described previously (11, 13). Cystoscopic examination revealed that all 4 experimental cows developed papillary tumors of the urinary bladder during a period of 1.5 (Cows 1 and 3) and 2 years (Cows 2 and 4). There were no clinical, hematomatological, or vesical changes in 2 control cows receiving a normal diet.

**Carcinogenicity of Whole or Processed Milk.** All rats consumed approximately equal quantities of milk (65 to 75 ml/rat/day) and the diet containing powdered milk (15 to 20 g/rat/day) from cows fed either BF or a normal diet during the same period of time. During the first 10 months of the experiment, the average weights of the rats in Groups 1 to 5 were comparable. After this time the animals of Groups 1 and 2 gained weight at a comparable rate but grew more slowly than did rats in Groups 3, 4, and 5.

The rat number, tumor distribution, and mean survival time are presented in Table 1. Only rats in Groups 1 (8 of 34) and 2 (11 of 56) fed whole or powdered milk from BF-fed cows displayed intestinal, renal pelvic, or vesical tumors alone or in combination at 47 to 141 and 31 to 117 weeks of feeding. The male:female ratio of rats bearing 1 or more of these tumor types that characteristically appear in rats fed BF directly (12, 13) was 6:3 and 8:3 for Groups 1 and 2, respectively.

Intestinal tumors in Groups 1 and 2 rats were grossly visualized, mostly in the ileum, multiple, and 5 to 15 mm in diameter. They were histologically similar to those described previously (12, 13). The most advanced lesions present in each of the afflicted animals were: Group 1, 1 adenocarcinoma, 1 adenocarcinoma and fibrosarcoma, and 1 fibroadenoma; and Group 2, 3 adenocarcinoma, 1 fibroadenoma, 1 hemangiosarcoma, and 1 fibrohemangiosarcoma. Adenocarcinomas penetrated through all layers of the small intestine including the serosa. Lymphatic metastases were detected in 2 rats of Group 2.

The distribution of bladder tumors in Group 1 rats was: 4 alone, 2 combined with intestinal tumors, and 1 combined with renal pelvic transitional cell carcinoma. In Group 2 rats bladder tumors were: 3 alone, 2 combined with intestinal tumors, and 1 combined with intestinal and renal pelvic tumors. Bladder tumors, visualized after bladder distension with fixative, were either papillary or sessile. Group 1 rats had 3 papillomas and 4 transitional cell carcinomas that penetrated into the muscle wall. Group 2 rats had 5 papillary transitional cell carcinomas and 1 squamous cell carcinoma, each of these penetrating through the bladder wall and into subserosal tissues. Histological features of these neoplasms were described (12). No nodal or distant metastases from these tumors were detected. Renal pelvis transitional cell carcinoma was present in 2 rats of Group 1 and 4 rats of Group 2.

Incidental tumors present were: mammary adenofibroma, 3 in Group 1, 2 in Group 2, 2 in Group 3, and 1 in Group 4; and ear duct tumor, 1 in Group 5.

Nine Group 1 and 11 Group 2 tumor-bearing rats died from either pulmonary infections (10 rats) or intestinal obstruction (6 rats) and malnutrition (4 rats). Rats in Groups 3 to 5 died periodically from pneumonia. The remaining rats from all groups were killed at 150 weeks.

**Toxicity of Milk Fractions.** The results obtained from murine toxicity tests of pentane (I), methanol (II), benzene (III), diethyl ether (IV), ethyl acetate (V), and residue (VI) fractions of powdered milk obtained from cows fed BF or normal diet are shown in Table 2. The mice died within 6 hr to 5 days after the injection. The mice given injections confined themselves to a corner of the cage and remained there with closed eyes. Their fur became rough. They revealed lesions of acute toxicity, vascular engorgement, petechial hemorrhages of serous membranes, and fatty degeneration of livers. Diethyl ether and ethyl acetate fractions of milk from cows fed BF had high toxicity in mice. No toxicity was observed with milk fractions of cows on normal diet.

**Carcinogenicity of Milk Fractions.** Milk fractions obtained from BF-fed cows and control cows were implanted into mouse bladders.

The number of animals subjected to the surgical implantation of pellets, the total number of animals surviving 52 weeks after surgery, and the incidence of bladder carcinomas are presented in Table 2. The percentage of mice that lived 52 weeks was about 50%. About 30% of the animals died within 30 days after surgery from impaction of the pellets into the urethra or from the toxicity of diethyl ether and ethyl acetate fractions of milk from BF-fed cows. Since the bladders were inspected after the mice had survived 52 weeks, no data were collected to determine the earliest time or the average time at which carcinomas were noted. The incidence of bladder carcinomas was significantly greater in the mice in Group IV, which received implants of diethyl ether fractions of milk from BF-fed cows, than it was in the other implanted as well as in the control groups (Table 2). The diethyl ether fraction of milk from BF-fed cows was the most toxic and carcinogenic (p < 0.01). The histological characteristics of the mouse bladder tumors were similar to those reported previously (2, 9, 10, 16). Carcinomas were mostly of transitional cell type, although squamous cell carcinomas were occasionally seen.

**Mutagenicity of Milk Fractions.** Four milk fractions, including pentane, methanol, chloroform, and chloroform:methanol (1:1), were tested for mutagenic activity in S. typhimurium TA 98 and TA 100. The results of these tests showed that only the chloroform:methanol fraction of milk from BF-fed cows demonstrated mutagenic activity for TA 100, but it did not for TA 98. The mutagen was invisible under visible or UV light on the chromatogram. The other fractions of milk from BF-fed cows and cows on normal diet were not mutagenic.

![Table 1](cancerres.aacrjournals.org)
shikimic acid, fumaric acid, succinic acid and tiliroside, and rutin, catecholamines and pteroquilin, pterolactam, zones of the world. It produces tumors at several sites in a variety of animals. The chemicals astragalin, isoquercitrin identified from BF. The condensed tannin isolated from BF and tannin (16), and pterosin and indanone (14) have been shown to be mutagenic metabolite(s) not present in the milk of normal cows. Feeding the milk of BF-fed cows to the rats showed the highest toxicity in mice (Table 2). Evans et al. (4) also demonstrated the toxicity of milk from BF-fed cows. On BF feeding, the ether-soluble fractions of cow milk and rat urine were identical.

Recent studies in Japan suggest that human BF consumption creates an increased risk of esophageal carcinoma (15). However, the role of milk obtained from cows freely exposed to BF in predisposing human populations to a higher risk of gastrointestinal tract or urinary bladder cancer remains to be determined.

**DISCUSSION**

BF is a highly carcinogenic plant that grows in temperate zones of the world. It produces tumors at several sites in a variety of animals. The chemicals astragalin, isoquercitrin and rutin, catecholamines and pteroquin, pterolactam, shikimic acid, fumaric acid, succinic acid and tiliroside, and tannin (16), and pterosin and indanone (14) have been identified from BF. The condensed tannin isolated from BF induced sarcomas in rats by s.c. injections (C. Y. Wang, A. M. Pamukcu, and G. T. Bryan, unpublished data). We clearly demonstrate that milk of cows ingesting BF contains carcinogenic, toxic, and mutagenic metabolite(s) not present in the milk of normal cows. Feeding the milk of BF-fed cows to the rats induced intestinal and urothelial carcinomas (Table 1). Diethyl ether and ethyl acetate fractions of such milk showed the highest toxicity in mice (Table 2). Evans et al. (4) also demonstrated the toxicity of milk from BF-fed cows. On BF feeding, the ether-soluble fractions of cow milk and rat urine were found to be mutagenic for S. typhimurium TA 100, and thin-layer chromatography suggested that mutagens in the cow milk and rat urine were identical.

**REFERENCES**

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