Folic Acid Supplementation Reduces the Mutagenicity and Genotoxicity Caused by Benzo(a)pyrene

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Summary Folate is a vital vitamin for the human being and its deficiency can lead to a variety of clinical abnormalities ranging from neural tube defects to cancers. Benzo(a)pyrene (BaP), a strong mutagen and carcinogen, is considered one of the common contaminants in food. The aim of this study was to investigate the positive effect of folate on cancer prevention at a fundamental level. In the present study, we investigated the impact of folic acid on BaP-induced mutagenicity and genotoxicity by means of in vitro and in vivo experiments. The reformulated Ames test was applied to study the antimutagenicity of folic acid against BaP. The protective effect of folic acid on cytotoxicity caused by BaP in human liver cell line L02 was evaluated by MTT assay. In addition, the effect of folic acid on the BaP-induced genotoxicity in vivo was assessed by mouse bone marrow micronucleus assay. The results indicated that folic acid significantly inhibited the reverse mutation of Salmonella typhimurium strains TA98 and TA100, and protected the viability of human liver cells against BaP \( (p<0.01) \). The micronucleus test showed that all doses of folic acid had a remarkable protective effect for the female mice \( (p<0.01) \). In conclusion, folic acid was found to reduce the mutagenicity and genotoxicity induced by BaP.

Key Words folic acid, Ames test, MTT assay, micronucleus test, benzo(a)pyrene

Benzo(a)pyrene (BaP), a prototypical polycyclic aromatic hydrocarbon (PHA), is a strong mutagen and carcinogen as revealed in numerous studies (1, 2). Its metabolites are capable of forming DNA adducts and thus causing DNA damage (3). It is this property that is generally believed to account for the mutagenic and genotoxic activity of BaP. BaP can be easily formed in foods during baking, frying and smoking processes in which combustion products come into direct contact with the food substance (4). Additionally, BaP is a crucial contaminant in air pollution, which has attracted more and more attention for the public health in developing countries (5). Therefore, it is necessary and urgent to explore prevention strategies for BaP.

Folate, a water-soluble B vitamin, including endogenous food folate and its synthetic form, folic acid (FA), is a necessary micronutrient for normal human growth and development (6, 7). Early in 1965, Hibbard et al. (8) reported the association between apparent folate deficiency and neural tube birth defect (NTD). Research spanning decades suggests that the exogenous or periconceptional supplementation of FA can reduce a woman’s risk of having an infant with NTD (9). In addition, dietary intake and blood level of folate appear to be negatively correlated to the risk of tumorigenesis in several organs, such as the breast, colon, and lung (10–12). Recently, a number of studies have reported that high folate supplementation might cause epigenetic changes that may promote carcinogenesis (13–17). However, little was done about the antimutagenic effects of folate supplementation.

In the present study, the inhibiting effects of mutagenesis induced by BaP were investigated with different doses of folic acid supplementation. The antimutagenic and antigenotoxicity effects of folic acid supplementation on benzo(a)pyrene were assessed by the Ames Test, MTT assay and the micronucleus test.

MATERIALS AND METHODS

Ethics statement. This study was carried out in strict accordance with the Regulations for the Care and Use of Laboratory Animals. The protocol was approved by the Ethics Committee of Wuhan Polytechnic University, China.

Materials. Folic acid, benzo(a)pyrene, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fenamino-sulf, 2-aminofluorene, 1-glutamine sodium azide, β-nicotinamide adenine dinucleotide phosphate disodium salt, D-glucose 6-phosphate disodium (G6P) salt hydrate, and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich. Polychlorinated biphenyl and Giemsa stain were acquired from Sigma Chemical Co. Fetal bovine serum was from Gibco Chemical Co. Other reagents and solvents used during the experiment were obtained from various commercial suppliers and were of the highest purity available. Salmonella typhimurium strains TA98 and TA100, human liver cell line L02 and lab mice were procured from Tongji Medical College, Huazhong University of Science and...
Preparation of different concentrations of folic acid. Folic acid (5.5 mg) was weighed accurately in a sterile environment and dissolved in distilled water before setting the volume to 250 mL. The concentration of folic acid solution was 50 μM until dissolved completely and then diluted to four different concentrations (50, 25, 12.5, 6.25 μM) with distilled water.

Tested bacterial strains. Salmonella typhimurium strains TA98 and TA100 were maintained as described by Maron and Ames (18). The genotypes of the test strains were checked routinely for their histidine requirement, deep rough (rfa) character, ultraviolet sensitivity (uvrB mutation), and presence of the R factor. They were stored at −80°C.

Activation mixture. The exogenous metabolic activation system (S9 mixture) was used as the activation mixture. The S9 microsome fraction was prepared from mouse liver treated with polychlorinated biphenyls (PCBs). The liver was minced and homogenized in 0.1 M KCl (3 mg/g liver wet). The homogenates were centrifuged at 9,000 × g for 10 min at 0–4°C. The supernatants were stored at −80°C for use.

Ames test. A modified plate incorporation procedure (19) was used to determine the effects of folate supplementation on the mutagenesis induced by BaP. Briefly, 0.5 mL of the S9 mixture for BaP (10 μg/plate) was distributed in sterilized capped tubes in an ice bath, then 0.1 mL of mixture (BaP and/or folate) and 0.1 mL of bacterial culture (2–5)×10^8 cells/mL were added. After gentle vortexing and preincubation at 37°C for 30 min, 2 mL of top agar supplemented with 0.05 M L-histidine and d-biotine was added to each tube and vortexed for 3 s. The entire resulting mixture was overlaid on the minimal agar plate. The plates were incubated at 37°C for 48 h, and the revertant bacterial colonies on each plate were counted. Three control groups were included: blank control (spontaneous mutation), negative control (DMSO) and positive control (BaP). There were four dishes for each concentration of folic acid.

The inhibition rate of mutagenicity (%) was calculated by the following formula:

Percent inhibition (%) = ( revertant number of BaP positive control plates − revertant number of test plates)/ revertant number of BaP positive control plates) × 100.

Cell culture. Human liver cell line L02 was cultured in an incubator with RPMI 1640 culture medium plus 10% fetal bovine serum, supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin, in a humidified incubator at 37°C and an atmosphere enriched with 5% CO₂. The culture medium was renewed every 2 to 3 d.

MTT assay. Logarithmic phase human liver cells of L02 were treated essentially according to the method described previously (19). Cells were seeded into 96 well plates and incubated overnight. Different concentrations of folate (5, 50, 500 nM) and BaP (10 μM) were added to the final solution. After 48 h incubation, cells were washed once before adding 50 μL fetal bovine serum–free medium containing 5 mg/mL MTT. After 4 h of incubation at 37°C, the medium was discarded, and the formazan blue formed in the cells was replaced by adding 150 μL DMSO. A negative control without the tested compound was prepared in the same manner. Optical density (OD) was measured at 490 nm with the microplate reader. The cytotoxic effect (%) was calculated according to the following equation:

Cell viability (%) = OD of treated cells/OD of negative control × 100.

Mouse bone marrow micronucleus assay. Healthy male and female mice weighing 25–30 g, aged 7–12 wk were selected and randomized into 5 groups with 5 males and 5 females. Folic acid was administered by gavage together with BaP. In the experiment, animals were treated with folic acid in doses ranging from 0.05 to 5 mg/kg. The negative control group was treated with distilled water, while the positive control group was treated with BaP at the concentration of 40 mg/kg body weight. Mouse bone marrow micronucleus assay was carried out two times for 30 h and at an interval of 24 h. Animals were killed by cervical dislocation at 6 h after the second dosing. Femurs were removed and bone marrow was collected in glass slices containing fetal bovine serum (FBS). The smear was blended and air dried. Glass slices were put into methanol solution for 15 min and stained with Giemsa stain for 15 min. The stain was removed and dried naturally (20).

After the smears dried, 5,000 polychromatic erythrocytes (PCEs) for every animal were counted using oil microscopy. The normochromatric erythrocytes (NCEs) were also counted, and the ratio PCE/NCE was calculated by counting a total of 200 erythrocytes. Micronucleus frequency was expressed as the total number of micronucleated polychromatophilic erythrocytes (MNPCeS) per 1,000 cells.

Statistical analysis. All experiments were done in triplicate, and data are presented as means ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance followed by the least significant difference test. In all tests, the criterion for significance was set at p<0.05 and p<0.01.

RESULTS

Mutagenic activity of folic acid

In a series of experiments preceding the antimutagenicity studies, the mutagenicity of folate was investigated. The result showed that folate, at concentrations ranging from 6.25 to 50 μM, was non-toxic to Salmonella typhimurium TA98 and TA100 strains and exhibited no mutagenicity to the strains.

Ames test

A dose of 10 μg/plate of BaP was chosen for the antimutagenicity study because this dose was not toxic and induced 52.66±7.57 revertants in Salmonella typhimurium TA98 and 309.66±17.0 revertants in Salmonella typhimurium TA100 strains.

The addition of folic acid at concentrations of 6.25, 12.50, 25, and 50 μM reduced the mutagenicity by BaP and a weak dose-response effect was observed (Figs. 1
and 2). FA supplement of all concentrations showed a very significant \((p<0.01)\) antimutagenic effect against BaP in the presence of the TA100 assay system, whereas high-dose FA (25, 50 \(\mu\)M) treatment reduced the mutagenicity significantly \((p<0.05)\) in the TA98 assay system. In addition, at the concentrations of 25 and 50 \(\mu\)M folic acid, the relative revertants induced by BaP in TA98 and TA100 strains decreased by 32.66\%, 38.61\%, 31.65\%, and 33.05\%, respectively. The inhibitory effects of different concentrations of FA on the mutagenesis induced by BaP using the plate incorporation are illustrated in Table 1.

**MTT assay**

Human liver cells of L02 were treated with BaP alone and in combination with 5, 50, and 500 nm folic acid. Cell viability was evaluated by the MTT assay. The positive control group was exposed to 10 \(\mu\)M of BaP. The results showed that the cytotoxic effect of folate supplementation increased slightly from 5 to 500 nm (Fig. 3). The viability of human liver cells of L02 treated with three concentrations of folic acid increased significantly \((p<0.01)\) compared with the BaP group. In addition, at the high dose of folic acid (500 nm), the cell viability reached 72.73\%, as illustrated in Table 2.

**Bone marrow micronucleus assay**

The effects of folic acid in different doses on the bone marrow micronucleus formation ratio induced by BaP are illustrated in Table 3. The results showed that folic acid was able to inhibit the genotoxicity induced by BaP for mouse marrow cells. It could be observed that the protective effect of all doses of folic acid on female mice was very significant \((p<0.01)\). The effects of folic acid on male mice exposed to medium and high doses was significant \((p<0.01)\). It indicated that the protective effect varied by mouse gender.

**DISCUSSION**

Present knowledge about nutrition has indicated that supplementation of folic acid in the diet for women before pregnancy will effectively reduce the risk of NTDs...
For mutagenesis and carcinogenesis, scholars have conducted a lot of research to prove that there existed a certain degree of correlation with folate deficiency. In short, it will be of benefit to intervene in the early stage of mutagenesis for the prevention of cancer.

In order to explore the inhibitory effect of folic acid on mutagenicity induced by BaP, an antimutagenicity assay was conducted. The results revealed that high doses (25 and 50 μM) of folic acid were able to significantly inhibit mutagenicity induced by BaP in Salmonella typhimurium strains TA98 and TA100. This indicated that folic acid played an important role of antagonism to the BaP-induced mutagenicity. BaP acted as the indirect mutagen in the experiment and was activated by microsome oxidase into benzopyrene dihydrodiol epoxide (BPDE) that resulted in DNA damage. While folic acid, the necessary micro vitamin for DNA metabolic activity, was involved in the repair of DNA and was beneficial for the synthesis of DNA at certain doses, which might explain the antimutagenicity of folic acid.

The results of the MTT assay revealed that 10 μM BaP severely inhibited the viability of human liver cell line L02 against BaP. Negative control (NC); positive control (PC); folic acid (FA). Positive control was treated with BaP (10 μg/plate). *p<0.05, **p<0.01 statistically significant difference from positive control group (ANOVA LSD test).
line L02. Adding different doses of folic acid significantly increased the cell activity (63.64%, 68.18% and 72.73%). This indicated that folic acid restored the vitality of L02 cells against BaP. The reason responsible for the above results may be that folic acid is required for the synthesis of nucleic acid and it also plays an important role in cell growth and division (26). In addition, folic acid is an essential cofactor for the biosynthesis of S-adenosylmethionine (SAM), which is the principal methyl donor in DNA methylation (27).

Bone marrow micronucleus assay has been widely used as a screening procedure to evaluate the structural integrity of chromosome and detect potential chromosome damaging agents (28). As the main nucleus of an erythroblast is extruded, it develops into a polychromatic erythrocyte (PCE); if the PCE has the micronucleus in the cell body, it is called a micronucleated polychromatic erythrocyte (MNPCE). In the present study, the micronucleus assay was applied to assess the antigenotoxicity of the food fortifier folic acid. A significant decrease was observed in the micronucleus frequency for groups of folic acid intervention compared with the positive control group. It was noted that higher protective effect was observed in females than in males. Fender-related differences in the micronucleus frequency may account for the differential response to BaP exposure in males and females, which is consistent with the report of Fenech et al. (29). In summary, the results obtained indicated that folic acid can serve as a protectant against a comparable extent of chromosomal instability induced by BaP in culture mice. This is in agreement with the research conducted by Donya et al., showing that folic acid significantly inhibited the percentage of chromosomal aberrations and DNA fragmentation (30).

In conclusion, our present study demonstrates that folic acid possess antimutagenicity and antigenotoxicity. Folic acid plays an important role in inhibiting mutagenicity induced by BaP both in vivo and in vitro. The study of vitamins with chemopreventive effects and a better understanding of health-related interactions could lead to a better use of dietary intervention in the prevention of cancers.

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REFERENCES
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### Table 3. Effects of folic acid on the bone marrow micronucleus formation ratio induced by BaP.

<table>
<thead>
<tr>
<th>Parts</th>
<th>Dose (mg/kg bw)</th>
<th>Animals</th>
<th>PCE/NCE</th>
<th>MNPCE(‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>NC</td>
<td>—</td>
<td>5</td>
<td>5</td>
<td>0.7</td>
</tr>
<tr>
<td>PC</td>
<td>—</td>
<td>5</td>
<td>5</td>
<td>0.9</td>
</tr>
<tr>
<td>FA</td>
<td>0.05</td>
<td>5</td>
<td>5</td>
<td>1.2</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td>5</td>
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<tr>
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<td>5</td>
<td>5</td>
<td>1.4</td>
<td>1.1</td>
</tr>
</tbody>
</table>

*Negative control (NC); positive control (PC); folic acid (FA); polychromatic erythrocyte (PCE); normochromatic erythrocyte (NCE); micronucleated polychromatic erythrocyte (MNPCE). Positive control was treated with BaP (40 mg/kg body weight). In all cases, 5,000 PCEs per animals were analyzed. **p<0.01 statistically significant difference from positive control group (ANOVA LSD test).
Folic Acid, Mutagenicity, Genotoxicity, Benzo(a)pyrene


