INTRODUCTION

Aflatoxins (AFs) refer to a group of highly toxic, mutagenic and carcinogenic compounds. They are the secondary metabolites of Aspergillus flavus and Aspergillus parasiticus, which are found worldwide to infect both living and dead plants and animals (Asao et al., 1963; Turner et al., 2005; Méndez-Albores et al., 2005). To present, a large number of AFs have been identified, among which, aflatoxin B₁ (AFB₁) is the most teratogenic, mutagenic and hepatocarcinogenic (Ma et al., 2014, 2015) and has been classified as a group 1 carcinogen by the International Agency for Research on Cancer (1993), Chang et al. (2013) and Corcuera et al. (2015). To remove the toxins, multiple physical, chemical and biological approaches have been proposed (Samarajeewa et al., 1990; Magnoli et al., 2008; Alberts et al., 2009; Wu et al., 2009; Saalia and Phillips, 2010; Diao et al., 2013). However, these methods suffer many disadvantages such as inconvenient operation, high equipment cost and no fulfilling the food safety requirements, especially regarding the safety of the degradation products and safeguarding the nutritional properties of treated foods and feeds, which greatly limited their practical applications (Liu et al., 2011; Luo et al., 2014).

Plasma is a partially ionized, low-pressure gas that contains ions, electrons, UV photons and reactive neutral species with sufficient energy to break covalent bonds and initiate various chemical reactions. In a previous work, we found that AFB₁ could be effectively degraded by Low-Temperature Radio Frequency Plasma (LTRFP). According to the structure-toxicity relationship of AFB₁, we proposed that the degradation products should have reduced toxicity, which required further experimental confirmation (Wang et al., 2015). Hence, the purpose of this study is to investigate the toxicity of the LTRFP degradation products of AFB₁ in Wistar rats and the HepG2 cell. We believe that this study could provide useful information for the practical application of LTRFP in the detoxification of AFB₁.

MATERIALS AND METHODS

Materials: Standard AFB₁ (2, 3, 6a, 9a-tetrahydro-4-methoxycyclopenta [c] furo [2, 3-h]
chromene-1, 11-dione; C_{17}H_{12}O_{6}; purity>99 \% was purchased from Sigma (St. Louis, MO, USA). The human hepatocellular carcinoma cell line (HepG2) was obtained from Shandong Analysis and Testing Center (Shandong, China). Acetonitrile of UPLC grade was obtained from Merck (Merck KGaA, Germany). Standard AFB\textsubscript{1} was dissolved in acetonitrile to make a stock solution of 100 mg/L. Dimethyl sulphoxide (DMSO) was purchased from Sigma (St Louis, MO, USA). Dulbecco’s Minimum Essential Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (10000 U/mL penicillin and 10000 U/mL streptomycin; Invitrogen), β-mercaptoethanol and methyl thiazolyl tetrazolium (MTT) were purchased from Gibco Life Technologies. Milli-Q deionized water with 18 MΩ/cm was used throughout the work and all other reagents were of analytical grade except otherwise specified.

Degradation of AFB\textsubscript{1} by LTRFP:

Degradation procedure: The stock solution of AFB\textsubscript{1} (100 mg/L) was transferred to a weighing bottle and dried by nitrogen purge. Then, the powder was exposed to the LTRFP as described in our previous work (Wang et al., 2015). Several minutes later, the powder was collected for degradation rate determination and toxicity evaluation.

Degradation rate determination: The degradation rate was determined by using an Agilent 1100 HPLC system (Agilent, Palo Alto, CA, USA) equipped with a 4.6×250 mm Agilent Eclipse XDB-C18 column. The degradation product was dissolved in acetonitrile and the elution was carried out isocratically using a mixture of distilled water, methanol and acetonitrile in 50:40:10 (v/v/v) as the mobile phase. The elution lasted 10 min at room temperature in a flow rate of 0.8 mL/min and the absorbance at 365 nm was monitored.

The degradation rate of AFB\textsubscript{1} was determined according to the variation of the peak area by using the following equation:

\[
\text{Degradation rate} = \frac{\text{Peak area of AFB}_1 \text{ before treatment} - \text{Peak area of AFB}_1 \text{ after treatment}}{\text{Peak area of AFB}_1 \text{ before treatment}} \times 100\%
\]

Toxicity evaluation in Wistar rats: Forty male and 40 female 3-week Wistar rats, weighing about 60-70 g, were obtained from the Better Biotechnology Co., Ltd (Nanjing, China). Upon arrival, all the animals were examined for health condition and acclimatized for 5 d on the standard diet prior to experiment. The rear conditions were temperature 25±1°C, 12-h light/dark cycles, humidity 50±5% and free access to standard commercial diet and drinking water throughout the acclimation and experimental periods. The rats were randomly grouped into 8 groups with 5 males and 5 females each group. All the procedures for animal experimentation were carried out in strict compliance with China’s guidelines for animal care.

According to preliminary experiments, the actual toxicity of the degradation product was evaluated using the dosages 17.50, 22.68, 29.40, 38.10, 49.38, 64.00, 82.96 and 107.52 mg/kg·bw. The degradation product was dissolved in Dimethyl Sulphoxide Solution (DMSO) to yield an 11 mg/mL solution and administered intragastrically to the rat on the first day of the experiment. The rats were fasted for 6 hours prior to toxin administration, but were allowed free access to standard diet and tap water 2-3 h after drug administration. The experiment lasted 14 days in total and the rats were observed for behavioral changes, signs of toxicity, or death during the experiment. The acute toxicity of the degradation product was calculated using the Karber method (Zhang et al., 2012; Ahmed, 2015).

Toxicity evaluation in HepG2 cells:

Cell culture: The HepG2 cells were maintained in DMEM containing 10% fetal bovine serum, 1% penicillin-streptomycin solution and 5 mM β-mercaptoethanol. The cells were grown in 63-cm² cell culture dish coated with type 1 collagen at 37°C under 5% CO\textsubscript{2} and 95% humidified environment. Prior to the experiment, the degradation product was dissolved in DMSO and applied to the HepG2 culture. The culture medium was refreshed every 3-4 d and subcultured a ratio of 1:4 once a week. After progressing to the logarithmic phase, the cells were exposed to difference concentrations of AFB\textsubscript{1} or its degradation products dissolved in DMSO for toxicity evaluation. The concentration of DMSO in the medium did not exceed 1% (v/v) throughout the experiment.

Measurement of cell viability: The cell viability was determined according to MTT assay. The cells were added at a density of 1.0×10^4 cells per well on a 96-well plate and allowed to attach for 4-6 h. After complete attachment to the wells, AFB\textsubscript{1} or its degradation products of different concentrations were transferred to the wells and incubated at 37°C for 24 h or 48 h. Then, 20 μL of 5 mg/mL MTT was added to each well and the contents were further incubated at 37°C in a 5% CO\textsubscript{2} humidified incubator. Four hours later, the medium was removed and replaced with 150 μL DMSO to dissolve the purple crystals. The plate was shaken for 15 min at 150 rpm and the absorbance of each well was measured on a microplate reader at 570 nm.

The cell viability was calculated according to the following equation:

\[
\text{Cell viability} = \frac{\text{MTT OD value of treated cells}}{\text{MTT OD value of untreated cells}} \times 100\%
\]
Table 1: Effect of LTRFP treatment on the content of AFB1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time (min)</th>
<th>Peak area (mAU*min)</th>
<th>Peak height (mAU)</th>
<th>Peak width (mAU)</th>
<th>Symmetry factor</th>
<th>Degradation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1 before treatment</td>
<td>8.72</td>
<td>6784.05</td>
<td>225.53</td>
<td>0.41</td>
<td>2.87</td>
<td>29.32%</td>
</tr>
<tr>
<td>AFB1 after treatment</td>
<td>8.80</td>
<td>4794.77</td>
<td>159.73</td>
<td>0.41</td>
<td>2.82</td>
<td></td>
</tr>
</tbody>
</table>

Peak area referred to the integral value of the peak height and retention time.

Fig. 1: Total ion chromatograms of AFB1 before (a) and after treatment by LTRFP (b)

**Optimization of degradation condition:** According to preliminary work, we found that the initial concentration of the AFB1 solution greatly affected the degradation efficiency. Hence, the effect of initial AFB1 concentration as well as the plasma generator power and the exposure duration the on the degradation rate was concerned in this study. The stock AFB1 solution was diluted to 0.2, 0.4, 0.5, 0.6, 0.8, 1.0 mg/L with acetonitrile and dried with nitrogen purge. Then, the powder was exposed to LTRFP under different input power (100, 200, 300, 400 W) and for different durations (2, 4, 6, 8, 10 min). The degradation rate was then determined.

**Statistical analysis:** All the measurements were performed in at least triplicate and all the values were expressed as the mean±SD. The comparisons among different groups were performed by analysis of variance using a Duncan test and SPSS 17.0 statistical software. The results were considered significant in p<0.05.

**RESULTS AND DISCUSSION**

**Degradation rate of AFB1:** The degradation rate after exposing 100 mg/L AFB1 to 300 W LTRFP for 8 min was given in Table 1. It could be seen that the degradation rate was only 29.32%, which was much lower than the values reported in our previous work (Wang et al., 2015) and could be possibly ascribed to the higher initial concentration of AFB1. The HPLC chromatograph of the degradation product was illustrated in Fig. 1. It could be seen that five products...
Table 2: LD$_{50}$ and 95% confidence limit of AFB$_1$ after degradation by LTRFP

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Logarithmic dose (x)</th>
<th>Mortality (D/T)</th>
<th>Mortality (%)</th>
<th>LD$_{50}$ values and 95% confidence limits (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.50</td>
<td>1.24</td>
<td>0/10</td>
<td>0</td>
<td>LD$_{50}$ = 29.41 mg/kg; 95% confidence limits: 25.15-34.40 mg/kg</td>
</tr>
<tr>
<td>2</td>
<td>22.68</td>
<td>1.36</td>
<td>4/10</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>29.40</td>
<td>1.47</td>
<td>7/10</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>38.10</td>
<td>1.59</td>
<td>8/10</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>49.38</td>
<td>1.70</td>
<td>8/10</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>64.00</td>
<td>1.81</td>
<td>9/10</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>82.96</td>
<td>1.92</td>
<td>9/10</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>107.52</td>
<td>2.03</td>
<td>10/10</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

D/T: dead/treated rats

Acute toxicity in rats: The 14-day acute toxicity of the degradation product of AFB$_1$ was presented in Table 2. It could be seen that the mortality increased progressively as the dose increased from 17.50 to 107.52 mg/kg. On day 14, the survival rate of the rats in the eight group were 100, 60, 30, 20, 20, 10, 10 and 0% respectively. During the experiment, we found that the rats showed irreversible signs of toxicity, including asthenia, piloerection, ataxia, anorexia, syncope, urine yellow, weight loss and finally death. Such symptoms have been reported in similar researches (Stanley et al., 1993).

Many works have showed that AFB$_1$ could reduce the activity of some pancreatic enzymes, inhibit the synthesis of enzymes and other hormones and hinder the absorption and metabolism of nutrients in the body, resulting in impaired digestion, loss of appetite, weight gain and growth and development of stagnation (Peters and Teel, 2003; Williams et al., 2004; Supriya et al., 2014). A similar variation, including decreased feed and water intake as well as declined body weight was also recorded in this study (data not shown). LD$_{50}$ is not only the main indicator of chemical toxicity, but also the most important quantitative index in acute toxicity evaluation. From Table 2, we could see that the LD$_{50}$ of the AFB$_1$ degradation product with degradation rate 29.32% was calculated to be 29.41 mg/kg·bw for rats. It has been reported that LD$_{50}$ of untreated AFB$_1$ ranged from 0.5 to 10 mg/kg·bw (Costanzo et al., 2015), which was much lower than that of treated AFB$_1$. Hence, LTRFP degradation could effectively reduce the toxicity of AFB$_1$ in Wistar rats.

Preliminary toxicity evaluation in HepG2 cell: To evaluate the toxicity of the degradation product in HepG2 cells, a preliminary research was carried out. The AFB$_1$ degradation product with degradation rate 29.32% was applied to the HepG2 cells and the cell viability was measured on 24 h and 48 h using the MTT assay. In Fig. 2, the survival rate of the HepG2 cells decreased with degradation product concentration increase, but was always higher than that of untreated AFB$_1$. For example, in toxin concentration 0.78 μmol/L, the survival rate of cells in the degradation product group was 98.41%, whereas that of the native AFB$_1$ group was only 94.46%. When the toxin concentration increased to 100 μmol/L, the viability of the degradation product group decreased significantly to 65.76%, but was still higher than that of the native AFB$_1$ group (p<0.05).

The effect LTRFP treatment on the 24-h and 48-h acute toxicity of AFB$_1$ in HepG2 cells was shown in Fig. 3. It could be seen that the survival rates of the HepG2 cell in the native AFB$_1$ group on 24 h and 48 h were significantly lower than that on 0 h (p<0.05), indicating that both the untreated AFB$_1$ and the degradation product possessed certain toxicity. Compared with the native AFB$_1$ group, the degradation product group displayed significantly higher cell viability.
Fig. 3: Effect of exposing 100 μmol/L AFB1 to 300 W LTRFP for 8 min on the 24-h and 48-h acute toxicity of AFB1 in HepG2 cells. Letters in lowercase indicated the comparisons between the values of the three groups in the same exposure duration and those in uppercase represented the comparisons between the values of the same group in different exposure durations.

Fig. 4: Effect of initial AFB1 concentration on its degradation by LTRFP viability (p<0.05), indicating that LTRFP degradation effectively reduced the toxicity of AFB1. This result was consistent with the toxicity evaluation results in rats.

**Effect of initial AFB1 concentration on AFB1 degradation by LTRFP:** As revealed in Table 1, exposing 100 μmol/L AFB1 to 300 W LTRFP for 8 min resulted in a degradation rate of only 29.32%. To explore the relationship between the degradation rate and toxicity of the degradation product, the effects of various parameters, including initial AFB1 concentration, plasma generator power and exposure duration, on the degradation rate were carried out in this study.

AFB1 solutions with initial concentration 0.2, 0.4, 0.6, 0.8 and 1.0 mg/L were exposed to 300 W plasma for 8 min and the degradation rates were shown in Fig. 4. It could be seen that the degradation rate declined along with the increase of initial AFB1 concentration. In initial AFB1 concentration 0.2 mg/L, the degradation rate of AFB1 reached up to 95.34%, which was significantly higher than that of other groups (p<0.05). This result disagreed with the photo degradation pattern of AFB1, in which the AFB1 degradation was not affected by its concentration (Liu et al., 2011). The difference could be related to the collision and reaction between particles. When the plasma generation power was constant, the number of active ions generated by the plasma was essentially the same and was sufficient for the degradation of only limited amount of AFB1. Hence, when the moiety of AFB1 increased, the degradation rate declined accordingly; whereas in photogradation, sufficient reactive ions were available and consequently the substrate moiety was no longer a limiting factor.

**Effect of plasma generator power on AFB1 degradation by LTRFP:** AFB1 in initial concentration 0.5 mg/L was exposed to 100, 200, 300, or 400 W LTRFP for 8 min and the resultant degradation rates were illustrated in Fig. 5. It could be seen that the degradation rate of AFB1 increased significantly along with plasma generator power rise (p<0.05). In generator power 100 W, the degradation rate was 64.35%; when the power was raised to 400 W, the degradation rate increased by 41.34% and reached up to 90.95%. The density of reactive ions in plasma was proportional to the generator power. When the quantity of AFB1 was constant, more reactive ions could lead to more complete substrate degradation.
Effect of radiation time on AFB₁ degradation by LTRFP: AFB₁ in initial concentration 0.5 mg/L was exposed to 300 W for different times and the degradation rates were demonstrated in Fig. 6. It could be seen that, similar to the effects of plasma generation power, the degradation rate increased significantly with exposure time elongation (p<0.05). When the exposure time was 2 min, the degradation rate of AFB₁ was as low as 32.56%; when the exposure time was elongated to 10 min, the degradation rate amounted up to 89.13%, indicating that the exposure time was a critical time that affected AFB₁ degradation.

Toxicity evaluation in HepG2 cells: The degradation product with degradation rate 95.34% was subjected to acute toxicity evaluation in HepG2 cells. The effects of degradation product concentration on the viability of HepG2 cells were shown in Fig. 7. It could be seen that the cell viability decreased with degradation product concentration increase and that of the degradation product group was always lower than the AFB₁ group. When the degradation production concentration increased from 0.78 μmol/L to 100 μmol/L, the cell viability declined slightly from 98.24% to 93.37% without significant difference (p>0.05), whereas that of
The 24-h and 48-h acute toxicity of the AFB₁ degradation product in initial concentration 100 μmol/L was shown in Fig. 8. We could see that exposure to the degradation product for 24 h and 48 h resulted in survival rate of 93.37 and 91.11%, respectively, which was slightly lower than the control, but was significantly higher than that of the AFB₁ group, whose cell viabilities were 63.72 and 33.12%, respectively (p<0.05). These results were consistent with the toxicity evaluation results in Wistar rats, that is, degradation by LTRFP reduced the toxicity of AFB₁.

CONCLUSION

The degradation efficiency of AFB₁ by LTRFP and the acute toxicity of the degradation products in Wistar rats and HepG2 cells were explored in this study. It was found that AFB₁ could be effectively degraded by LTRFP and the exposure of 100 mg/L AFB₁ to 300 W plasma for 8 min could result in a degradation rate of 29.32%. The degradation product was subjected to acute toxicity evaluation in Wistar rats and the estimated median lethal dosage (LD₅₀) was found to be 29.41 mg/kg·bw with 95% confidence limit 25.15-34.40 mg/kg·bw, which was much higher than that of untreated AFB₁. The initial AFB₁ concentration, plasma generator power and exposure duration greatly affected the degradation efficiency of LTRFP and the degradation rates in the selected experimental conditions ranged from 29.32 to 95.34%. The toxicity of the degradation products with the lowest and highest degradation rates were evaluated in HepG2 cells. LTRFP degradation significantly reduced the toxicity of AFB₁ in HepG2 cells and the 24-h and 48-h acute toxicity was inversely proportional to the degradation rate. It was concluded that LTRFP was potential alternative to the detoxification of AFB₁ contaminated products due to its high degradation efficacy and reduced toxicity of the degradation product.

ACKNOWLEDGMENT

This study was supported by the National Natural Science Foundation of China under Grant Nos. 31101391 and 31271963.

REFERENCES


