Detection of apoptosis in citrinin, aflatoxin and their combined effects in broiler chicken

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ABSTRACT


Immunomodulatory effects of citrinin (CTN) 5 ppm and aflatoxin (AF) 0.5 ppm in 3 week old broiler chicken were studied by their toxin induced apoptosis and necrosis in the spleen and thymus at 6, 12, 24 and 48h by flow cytometry and light and electron microscopic studies. In the spleen, by flow cytometry technique, peak induction of apoptosis was seen at 48h in the CTN group and 24h in the AF and CTN+AF groups. Peak induction of necrosis was noted at 48h post treatment in the spleen of CTN+AF when compared to the other groups. In thymus, the entire mycotoxin fed groups showed peak induction of apoptosis at 48h post treatment. The per cent necrosis of thymus increased significantly (P<0.05) in all the mycotoxin treated groups when compared to the control at 48h. Light and electron microscopic studies revealed apoptotic cells characterized by chromatin margination along the nuclear envelope shrinkage of cells with a clear halo formation around them and without any inflammatory reactions in the CTN, AF and CTN + AF birds. Lymphocytolysis in the spleen and thymus was also found in all mycotoxin fed groups. The changes in the CTN+AF mycotoxicoses were comparable to the individual toxicities. Combined toxicity effect was less than additive.

Keywords: Citrinin, aflatoxin, broiler chicken, apoptosis, immunity, ultrastructure and histopathology

INTRODUCTION

The hepato-nephrotoxic mycotoxins Citrinin (CTN) and aflatoxin (AF) are secondary metabolites of fungi Penicillium citrinum and Aspergillus parasiticus respectively. Co-occurrence of these two mycotoxins affects the growth and productivity of broiler chicken. The natural occurrence of CTN and AF in the feed ranged from 40 and 4800 ppb\(^1\) and 1 ppb to 12 ppm\(^2,3\) and their co-occurrence in the feed was 9.3% and in combination with AF, the CTN concentration ranged from 40-800 ppb\(^1\). Aflatoxin affects both cellular and humoral immunity of chicken, while CTN causes lymphopaenia. Immunomodulatory effects of these mycotoxins need more study since the toxins can cause immunosuppression and predispose to infectious diseases. Apoptosis is one of the proposed mechanisms. With this background and considering the recent reports on the increased frequency of co-occurrence of CTN and AF in feed, the present study was conducted with an objective of finding the apoptotic effects of CTN (5 ppm) and AF (0.5 ppm) in the spleen and thymus of three weeks old broiler chicken.

MATERIALS AND METHODS

Citrinin was produced on maize\(^4\) and rice\(^5\) and the AF was produced on rice\(^6\). Thirty-two newly hatched broiler chicks were fed on control diet up to three weeks of age. Subsequently the birds were randomly distributed to four groups of 8 chicks each and fed with control, AF (0.5 ppm), CTN (5 ppm) and AF (0.5 ppm) + CTN (5 ppm) mixed diets. Two birds from each group were sacrificed at 6, 12, 24 and 48h intervals. The spleen and thymus were removed aseptically in cold phosphate buffered saline.

Flow cytometric detection of apoptosis was performed using annexin V apoptosis detection kit–SC-4252 AK (M/s. Santa Cruz Biotechnology Inc., Germany). Lymphocyte suspensions were prepared from spleen and thymus\(^7\) and the cell concentration was adjusted to 1x10\(^5\) cells/mL in RPMI-1640 medium (Himedia, India) supplemented with 5% foetal calf serum (Sigma, USA). A 100 µL aliquot of cells (1x10\(^5\) cells) was transferred to 5 mL polystyrene round tubes (Becton Dickinson, USA) containing 900 µL cold phosphate buffer saline.

Briefly, the cell suspensions were centrifuged at 1500 rpm for 5 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in 500µL of 1 x assay buffer. The cell pellet was gently vortexed. The cells were then stained by adding 0.5µg of annexin V fluoroisothiocyanate (FITC) and 500 µg of propidium iodide. The samples were gently vortexed and incubated for 15 min at room temperature in the dark.

The annexin V FITC and propidium iodide fluorescence of individual cells was measured using cell quest software in Fluorescence Activated Cell Sorting (FACS) caliber flow cytometer (Becton Dickinson, USA).
The forward and side scatter profiles were adjusted to ensure that the lymphocytic population was clearly displayed. Fluorescence was measured on FL₁ and FL₂ channels. A total of 10,000 events in the gated population of lymphocytes were analysed for each sample. All measurements were done under the same settings in the instrument. At analysis, the dot plot of forward scatter versus side scatter was displayed and the lymphocyte population was identified by its typical location and selection by gating. A quadrant statistics was then obtained for the gated region to identify the apoptotic cell population.

For semi-thin sections of 500-700 nanometer (0.5 - 0.7µm) thickness, the spleen and thymus samples were prefixed in 3 per cent glutaraldehyde and stored at 4°C as per standard method and were stained by toluidine blue⁶. The ultrathin sections of the spleen and thymus were stained with uranyl acetate and lead citrate and examined under Philips (Teknai-10) computer augmented transmission electron microscope operated at 60-kilowatt ampere (KVA). The data generated from experimental trial were statistically analysed using SPSS version 9.0 software for windows.

**RESULTS**

Mean ± SE of the per cent apoptosis and necrosis detected by FACS in the spleen and thymus of broiler chicks fed with control, CTN, AF and CTN+AF diets are shown in Tables 1-4 respectively.

### Table 1. Detection of Apoptosis in spleen of broiler chicken fed with control, CTN, AF and CTN+AF feeds [Mean ± SE per cent]

<table>
<thead>
<tr>
<th>Groups</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.09 ± 0.92</td>
<td>1.21 ± 0.86</td>
<td>1.03 ± 0.92</td>
<td>0.38 ± 0.27</td>
</tr>
<tr>
<td>CTN(5 ppm)</td>
<td>2.25 ± 1.29</td>
<td>4.07 ± 2.39</td>
<td>4.37 ± 1.15</td>
<td>6.56 ± 5.14</td>
</tr>
<tr>
<td>AF (0.5 ppm)</td>
<td>3.07 ± 3.03</td>
<td>3.61 ± 1.68</td>
<td>2.88 ± 1.34</td>
<td>1.50 ± 0.20</td>
</tr>
<tr>
<td>CTN+ AF</td>
<td>2.46 ± 0.33</td>
<td>2.49 ± 0.29</td>
<td>7.74 ± 1.73</td>
<td>7.71 ± 5.59</td>
</tr>
</tbody>
</table>

Means with different superscripts within a column differ significantly (P<0.05)

### Table 2. Detection of Necrosis in spleen of broiler chicken fed with control, CTN, AF and CTN+AF feeds [Mean ± SE per cent]

<table>
<thead>
<tr>
<th>Groups</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.29 ± 0.09</td>
<td>0.16 ± 0.07</td>
<td>0.43 ± 0.04</td>
<td>0.37 ± 0.05</td>
</tr>
<tr>
<td>CTN(5 ppm)</td>
<td>0.51 ± 0.04</td>
<td>0.75 ± 0.04</td>
<td>0.54 ± 0.02</td>
<td>1.19 ± 0.77</td>
</tr>
<tr>
<td>AF (0.5 ppm)</td>
<td>0.72 ± 0.13</td>
<td>0.91 ± 0.03</td>
<td>0.54 ± 0.04</td>
<td>0.54 ± 0.01</td>
</tr>
<tr>
<td>CTN+ AF</td>
<td>1.03 ± 0.18</td>
<td>0.59 ± 0.19</td>
<td>0.75 ± 0.04</td>
<td>1.88 ± 0.69</td>
</tr>
</tbody>
</table>

Means with different superscripts within a column differ significantly (P<0.05)

### Table 3. Detection of Apoptosis in thymus of broiler chicken fed with control, CTN, AF and CTN+AF feeds [Mean ± SE per cent]

<table>
<thead>
<tr>
<th>Groups</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.02 ± 0.01</td>
<td>0.25 ± 0.22</td>
<td>0.11 ± 0.57</td>
<td>0.26 ± 0.23</td>
</tr>
<tr>
<td>CTN(5 ppm)</td>
<td>0.49 ± 0.21</td>
<td>1.57 ± 0.21</td>
<td>1.74 ± 1.12</td>
<td>29.49 ± 10.34</td>
</tr>
<tr>
<td>AF (0.5 ppm)</td>
<td>0.35 ± 0.13</td>
<td>1.19 ± 0.42</td>
<td>1.09 ± 0.07</td>
<td>33.36 ± 17.33</td>
</tr>
<tr>
<td>CTN+ AF</td>
<td>0.54 ± 0.35</td>
<td>0.66 ± 0.38</td>
<td>2.53 ± 0.02</td>
<td>44.47 ± 3.78</td>
</tr>
</tbody>
</table>

Means with different superscripts within a column differ significantly (P<0.05)

### Table 4. Detection of Necrosis in thymus of broiler chicken fed with control, CTN, AF and CTN+AF feeds [Mean ± SE per cent]

<table>
<thead>
<tr>
<th>Groups</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.05 ± 0.01</td>
<td>0.15 ± 0.05</td>
<td>0.16 ± 0.04</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>CTN(5 ppm)</td>
<td>0.24 ± 0.02</td>
<td>0.32 ± 0.09</td>
<td>0.49 ± 0.01</td>
<td>1.68 ± 0.11</td>
</tr>
<tr>
<td>AF (0.5 ppm)</td>
<td>0.20 ± 0.05</td>
<td>0.34 ± 0.07</td>
<td>0.80 ± 0.08</td>
<td>2.40 ± 0.21</td>
</tr>
<tr>
<td>CTN+ AF</td>
<td>0.17 ± 0.02</td>
<td>0.80 ± 0.08</td>
<td>0.46 ± 0.02</td>
<td>12.0 ± 0.13</td>
</tr>
</tbody>
</table>

Means with different superscripts within a column differ significantly (P<0.05)
Flow cytometry showing apoptosis and necrosis percentage in spleen of Control, CTN, AF and CTN+AF fed birds; Peak induction of apoptosis was seen at 48h in CTN group. Peak induction of necrosis was seen at 48h post treatment in the spleen of CTN+AF. Lower right quarter: Apoptosis and Upper right quarter: Necrosis (Annexin V+ PI−). **Fig. 1.** Flow cytometry showing apoptosis and necrosis percentage in spleen of Control, CTN, AF and CTN+AF fed birds; Peak induction of apoptosis was seen at 48h in CTN group. Peak induction of necrosis was seen at 48h post treatment in the spleen of CTN+AF. Lower right quarter: Apoptosis and Upper right quarter: Necrosis (Annexin V+ PI−).

Significant (P<0.05) differences were observed between the control and mycotoxin treated groups at 24 and 48h post-treatment. Comparison of means revealed that there was no significant difference among the control, CTN and AF groups for 24h and between the control and AF, CTN and AF and CTN and CTN+AF for 48h (Fig. 1). The CTN+AF differed significantly (P<0.05) from the control and AF groups at 24 and 48 h post-treatment. Peak induction of apoptosis was observed at 48h in CTN group and 24h in CTN+AF groups.

Flow cytometry for spleen necrosis

Significant (P<0.05) differences were observed between the control and CTN+AF groups at 48h post-treatment. No significant difference was observed among the control, CTN and AF groups at 48h post-treatment. Significant (P<0.05) induction of necrosis was observed at 48h post-treatment in the CTN+AF group when compared to control and AF (Fig. 1).

Flow cytometry for thymus apoptosis

Significant (P<0.05) differences between the control and mycotoxin treated groups were observed at 24 and 48 h post-treatment. The CTN+AF significantly (P<0.05) differed from the CTN and AF group at 24h post-treatment and from the CTN group at 48h post-treatment. No significant differences were observed among the control, CTN and AF groups at 24h and CTN and AF and CTN and CTN+AF groups at 48h post-treatment.
Fig. 3. Section of spleen showing apoptosis of splenic cells with margination of chromatin and halo formation (arrow) at 48h in the 3 weeks old chick fed with CTN. Toluidine blue x1000; Fig. 4. Splenic cells showing apoptosis with margination of chromatin and halo formation (arrow) at 48h in the 3 weeks old chick fed with CTN+AF. Toluidine blue x1000; Fig. 5. Section of thymus showing apoptosis at 48h in the 3 weeks old chick fed with CTN+AF. Toluidine blue x400; Fig. 6. Thymus cells showing apoptosis (arrow) at 48h in the 3 weeks old chick fed with CTN+AF. Toluidine blue x1000; Fig. 7. Section of thymus showing lymphocytolysis at 48h in the 3 weeks old chick fed with AF. Toluidine blue x400; Fig. 8. Section of thymus showing necrosis (arrow) at 48h in the 3 weeks old chick fed with CTN+AF. Toluidine blue x400; Fig. 9. Transmission Electron Micrograph (TEM) of spleen showing apoptosis (arrow) and karyorrhexis (arrow head) at 48h in the 3 weeks old chick fed with CTN+AF. Uranyl acetate-Lead Citrate stain x4200; Fig. 10. TEM of thymus showing apoptosis (arrow) at 48h in the 3 weeks old chick fed with CTN+AF. Uranyl acetate-Lead Citrate stain x7000.

All mycotoxin fed groups showed peak induction of apoptosis at 48h post-treatment (Fig. 2) when compared to the control.

**Flow cytometry for thymus necrosis**

Significant (P<0.05) difference between the control and mycotoxin treated groups were observed at 24 and 48 h post-treatment. Significant (P<0.05) difference was observed only between the control and AF groups at 24 h post-treatment. The mycotoxin fed groups significantly (P<0.05) differed from the control for per cent necrosis in thymus with peak induction at 48 h post-treatment (Fig. 2). The per cent necrosis of thymus increased significantly (P<0.05) in all the mycotoxin treated groups when compared to the control at 48 h in the ascending order of CTN, AF and CTN+AF.

**Light and electron microscopy**

Findings of splenic tissue sections showed presence of apoptotic cells characterized by chromatin margination along the nuclear envelope, shrinkage of cells with a clear halo formation and without any inflammatory reactions, the characteristics of apoptosis, in the spleen of CTN (Fig.3), AF and CTN+AF (Fig.4) and in the thymus of CTN (Fig.5), AF and CTN+AF (Fig.6) fed birds. Lymphocytolysis was also observed in the spleen and thymus of CTN, AF (Fig.7) and CTN+AF (Fig.8) fed birds. Electron microscopical examination also revealed apoptotic bodies in the splenocytes and thymocytes in all mycotoxin fed birds (Fig.9,10).

**DISCUSSION**

The CTN, AF and CTN+AF induced apoptosis and necrosis in splenocytes and peak induction of apoptosis was observed after 24h post treatment and necrosis at 48h. In the AF group, the peak induction of necrosis was observed at 24h which was comparable with earlier
reports. AF, CTN and CTN+AF induced peak apoptosis and necrosis in thymocytes at 48h post treatment. However, peak induction of apoptosis and necrosis in thymocytes at 24h post treatment was reported in layer chicks fed 0.5 ppm AF$^{9,10}$. Though, the CTN was reported to induce apoptosis in time-dependent manner in the kidneys of rabbits at a dose of 15 ppm by earlier workers$^{11}$, in this study, the induction of apoptosis by CTN (5 ppm) in broiler chicken was observed even at a three times lesser dose level. Similar to this study with citrinin toxicity, Kamalavenkatesh et al.$^{12}$ found a significant induction of apoptosis in the thymus and spleen of broiler chicken at 24h post-treatment but with other mycotoxins viz. 1 ppm T-2 toxin and 10 ppm cyclopiazonic acid respectively. The morphological evidence of apoptosis observed in this study agreed with Yu et al.$^{13}$ in CTN toxicity studies in human promyelocytic leukaemia cells. The present study showed that CTN+AF effected comparatively more severe induction of apoptosis and necrosis in the spleen and thymus than individual toxicity. These findings also implied that the apoptosis induced at low level exposure to AF+CTN in chicks could compromise the immunity of birds.

Flowcytometric quantification of the apoptosis in splenocytes and thymocytes was also supported by histopathological and ultrastructural changes of apoptosis and necrosis. These observations also agreed with the findings of earlier workers$^{9,10}$ who reported apoptosis in thymus of layer chicks 24h post treatment with 0.5ppm AF. The apoptotic and necrotic lesions noticed in the semithin and ultrathin sections of spleen concurred with the findings of previous workers with T-2 and CPA toxicoses$^{12}$. No such studies were found for CTN and CTN+AF apoptosis in broiler chicken. However, Kumar et al.$^{11}$ reported renal ultrastructural lesions of apoptosis such as nuclear fragmentation and cytoplasmic blebbing in rabbits fed with 15 ppm CTN and 0.75 ppm ochratoxin A.

In conclusion, the present study showed that CTN+AF affected apoptosis and necrosis in the splenocytes and thymocytes and thereby these mycotoxins can play a vital role in decreasing the immune response of the broiler chicken. The combined toxicity effect was found to be less than additive.

REFERENCES