# Effects of Sterigmatocystin, Deoxynivalenol and Aflatoxin $G_1$ on Apoptosis of Human Peripheral Blood Lymphocytes *in vitro*[1]

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**Objective** To explore the effects of Sterigmatocystin (ST), Deoxynivalenol (DON) and Aflatoxin $G_1$ ($AFG_1$) on apoptosis of human peripheral blood lymphocytes (HPBLs) *in vitro* and thus to further elucidate the putative roles of these three mycotoxins on human immunosystem. **Methods** The effects of ST, DON and $AFG_1$ on apoptosis of HPBLs were studied with cell culture, flow cytometric (FCM) DNA analysis and DNA agarose gel electrophoresis. **Results** DNA agarose gel electrophoresis results showed the characteristic "ladder" pattern of apoptosis in HPBLs treated with ST, DON and $AFG_1$. Flow cytometric DNA analysis revealed that typical subdiploid peaks of apoptosis in DNA histogram could be seen in all groups treated with the three mycotoxins. Significant time-effect and dose-effect relationships were found between the apoptosis rates and treatment time as well as concentrations of the three mycotoxins. **Conclusion** ST, DON and $AFG_1$ can induce apoptosis of HPBLs *in vitro* and may have some negative effects on human immunosystem.

**Key Words:** Sterigmatocystin; Deoxynivalenol; Aflatoxin $G_1$; Apoptosis; Human; Lymphocytes

## INTRODUCTION

Many studies have showed that foodstuffs of the residents in high incidence areas of esophageal, gastric and liver cancers in China were quite frequently contaminated with fungi and mycotoxins[1-4]. Sterigmatocystin (ST), Deoxynivalenol (DON) and Aflatoxin $G_1$ ($AFG_1$) are the most frequently seen contaminating mycotoxins[1,4,5]. To explore their possible effects on human immunosystem, their effects on apoptosis of human peripheral lymphocytes (HPBLs) *in vitro* were studied with cell culture, flow cytometric (FCM) DNA analysis and DNA gel electrophoresis.

## MATERIALS AND METHODS

### *HPBL Culture*

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HPBLs were obtained by Ficoll-Hypaque gradient centrifugation from sodium citrate antiagglutinated fresh venous blood of healthy donors. The seperated lymphocytes were then washed 3 times with centrifuge in PBS, and initially cultured in 1 640 medium (GIBCO,USA) supplemented with 15% new-born calf serum (NBCS),insulin (100 μg/mL), penicillin (100U/mL) and phytohemagglutinin (PHA, 25 μg/mL) at 37℃ in a $CO_2$ incubator (Forma Scientific Co.) containing 5% $CO_2$.

*Mycotoxin Treatment*

Fourty-eight hours after initial culture, the medium was replaced by new medium without PHA, and then the flasks were randomly divided into different groups. The following experiments were conducted.

*Effect of Different Mycotoxin Treatment Times on Apoptosis of HPBL in vitro*. The cells in experimental groups were respectively treated with ST (2,000ng/mL), DON (1,000ng/mL) and $AFG_1$ (1,000ng/mL. The three mycotoxins were purchased from Sigma Co.), while those in control groups were treated with equal amount of solvent PBS. Cells were harvested at 2, 4, 6, 12, 24, 36, 48 and 72 h respectively after mycotoxin treatment for detection of apoptosis with FCM and DNA gel electrophoresis. Duplicate samples were collected for each group at each time point.

*Effect of Different Mycotoxin Concentrations on Apoptosis of HPBL in vitro*. The cells in experimental groups were treated with different mycotoxins respectively. The concentration ranges for each mycotoxin were determined according to the authors' previous works[4-9] and the final concentrations for the mycotoxins were as follows:

ST: 3,000ng/mL, 2,000ng/mL, 1,000ng/mL, 250ng/mL and 125ng/mL.

DON: 2,000ng/mL, 1,000ng/mL, 500ng/mL, 200ng/mL, 100ng/mL and 50ng/mL.

$AFG_1$: 2,000ng/mL, 1,000ng/mL, 500ng/mL, 200ng/mL, 100ng/mL, 50ng/mL, 12.5ng/mL and 3.12ng/mL.

The cells in control groups were treated with equal amount of solvent PBS correspondently. The lymphocytes were respectively harvested at 6 and 24 h after the mycotoxin treatment with two flasks for each concentration.

All the harvested cells were washed three times in PBS, fixed in 70% ethanol and stored at 4℃ for FCM analysis.

*FCM DNA Analysis*

Flow cytometric cellular apoptosis rates were assessed by flow cytometric method as Described[10] with improvements. Briefly, the ethanol fixed lymphocytes were first washed with PBS and then treated with pepsin for dissociation of cells. The lymphocytes were then stained with 1% ethidium bromide solution (containing 1% Triton-X 100 and 20mg/mL RNase). Nuclear DNA contents were measured with a FACS 420 cytometer (Becton-Dickinson Co. USA) and all the data were processed in Consort 30 with single DNA histogram statistics software (Becton-Dickinson Co., USA). Apoptosis was identifed by the appearance of subdiploidy peak in the DNA histogram and the apoptosis rates were calculated with the software.

*DNA Agarose Gel Electrophoresis*

Cellular DNA extraction and gel electrophoresis were performed according to Smith *et al*[11]. The HPBLs in mycotoxin treated groups (ST 2 000ng/mL; DON 1 000ng/mL and $AFG_1$ 1 000ng/mL) and corresponding control groups were harvested respectively at 6 and 24 h after mycotoxin treatment, and washed twice with PBS. The cells were then suspended

in 400 μL lysis buffer containing 1% NP40, 20mmol/L EDTA and 50mmol/L Tris • HCl (pH7.5), vortexed for 10 s and centrifuged for 5 min at 8 000 rpm to separate DNA fragments (supernatants) from intact chromatin (pellets). The pellet was treated with lysis buffer containing 0.1mg/mL proteinase K,1% SDS,10mmol/L EDTA and 10mmol/L Tris • HCl, and 10mmol/L NaCl (pH 7.5) for 3h at 56℃. DNA in the pellet was extracted two times with phenol-chloroform. The supernatant was treated with 0.1mg/mL proteinase K and 1% SDS for 1h at 37℃ and subsequently treated with 0.2mg/mL RNase A for 1 h at 37℃. DNA from both the pellet and supernatant was precipitated with ethanol and 0.3mol/L NaAc at -20℃ overnight. The DNA precipitate was centrifuged at 12,000rpm for 10 min, washed in 80% ethanol, centrifuged again to get rid of supernant and dried. The DNA was dissolved in 20 μl of Tris- EDTA buffer (10mmol/L Tris-HCl(pH8.0) and 1mmol/L EDTA). DNA was electrophoresed at 5V/cm on a 1.5% agarose gel containing 0.375 μg/mL ethidium bromide in 1×Tris-EDTA buffer for 2h and detected by UV transillumination and photographed. All of the above experiments were repeated twice.

*Statistical Analysis*

POMS statistic analysis software was used for the statistical analysis. Differences between groups were analyzed with one-way ANOVA, while linear regression was used for the analysis of dose-effect and time-effect relationship.

## RESULTS

*FCM Analysis*

FCM DAN analysis showed that typical subploid peaks of apoptosis in DNA histogram of HPBLs could be found in all the groups, and they were significantly higher in mycotoxin treated cells than those in the control groups.

The effects of ST (2 000ng/mL), DON (1 000) ng/mL and $AFG_1$ (2 000ng/mL) on apoptosis of HPBLs *in vitro* at different treating times were shown in Fig 1. The results revealed that all the apoptosis rates of HPBLs in three mycotoxin treated groups were significantly higher than those in their corresponding control groups. The apoptosis rates of HPBLs *in vitro* increased as the treatment time of mycotoxins prolonged. The apoptosis rates were positively correlated with the time of mycotoxin treatment. Significant time-effect relationships could be found between apoptotic rates and mycotoxin treatment time. Differences in the treatment time ranges existed for different mycotoxin (DON: 2~72 h, $r$=0.8782, $P$<0.05; $AFG_1$: 2 ~24 h, $r$=0.9218, $P$<0.05; ST 2~ 48 h, $r$=0. 9629, $P$<0.0001).

The effects of ST, DON and $AFG_1$ on apoptosis of lymphocytes at different concentrations were shown in Fig 2. The results showed that 6 and 24 h after the mycotoxin treatment, apoptosis rates of HPBLs *in vitro* in ST, DON and $AFG_1$ treated groups at different concentrations were higher than those in the control groups. The apoptosis rates of HPBLs increased as the concentration of mycotoxin increased. Significant dose-effect relationships could be found at both 6 and 24 h between mycotoxin concentration and apoptosis rate within certain concentration ranges (ST: 125~1 000ng/mL, 6 h, $r$=0.7788, $P$<0.01; 24 h, $r$=0.9827, $P$<0.01; $AFG_1$: 3.12 -2,000ng/mL, 6 h, $r$=0.7584, $P$<0.05, 24 h, $r$=0.7280, $P$<0.05; DON: 50-1,000 ng/mL, 6 h, $r$=0.8261, $P$<0.05; 24 h, $r$=0.8976, $P$<0.05).

*DNA Agarose Gel Electrophoresis*

In DNA agarose gel electrophoresis, characteristic "ladder" patterns of apoptosis were found in HPBLs treated with ST (2 000ng/mL), DON (1 000ng/mL) and $AFG_1$(1 000ng/mL) (Fig. 3).

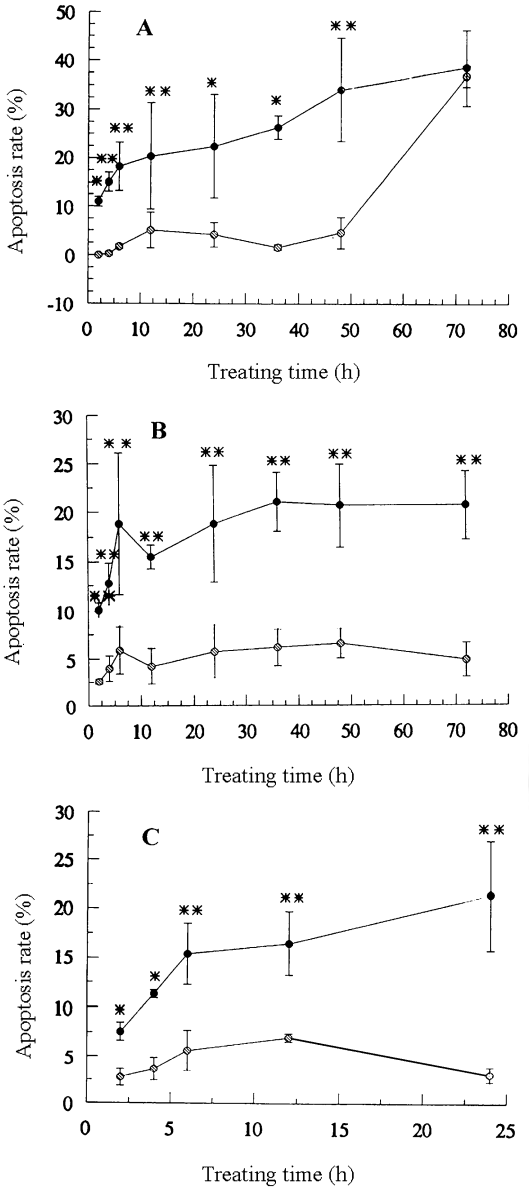


FIG. 1. The effects of ST, DON and $AFG_1$ on apoptosis of lymphocytes at different treating times. The apoptosis rates of lymphocytes in three mycotoxin treated groups (— —) are significantly higher than those in the corresponding control groups (—o—). The apoptosis rates are positively correlated with the time of mycotoxin treatment.
A: Sterigmatocystin (2 000ng/mL), B: Deoxynivalenol (1 000ng/mL), C: Aflatoxin $G_1$, (2000ng/mL), * $P<0.05$: ** $P<0.01$.

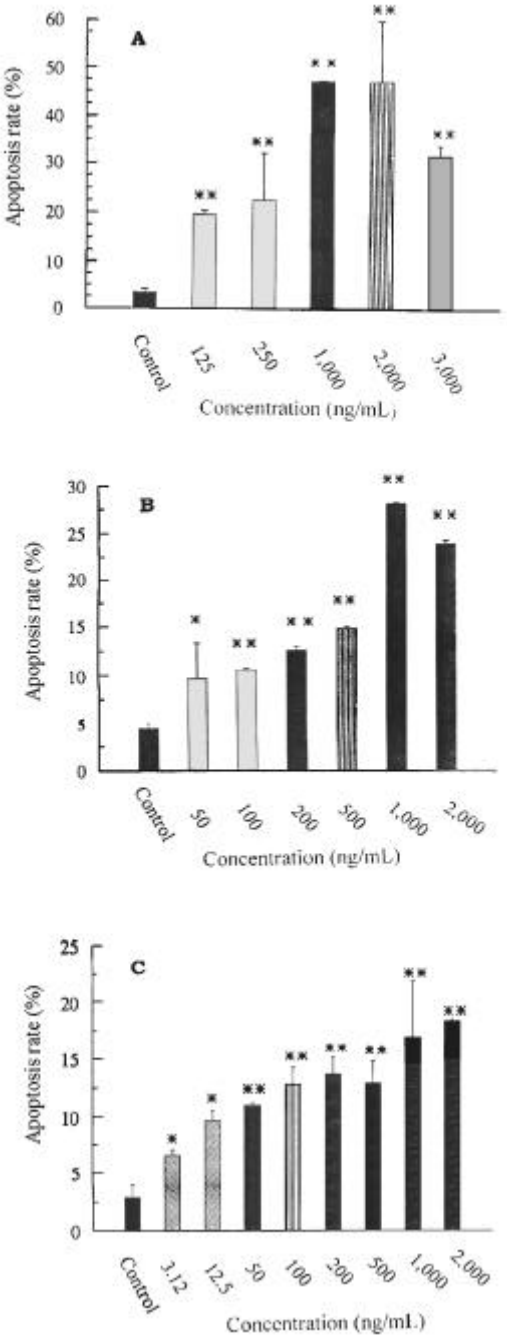


FIG. 2. The effects of ST, DON and $AFG_1$ on apoptosis of lymphocytes at different concentrations at 6th hour are shown. The significant dose-effect relationship could be found between mycotoxin concentration and apoptosis rate within certain concentration range.
A: Sterigmatocystin, B: Deoxynivalenol, C: Aflatoxin $G_1$, * $P<0.05$ ** $P<0.01$.

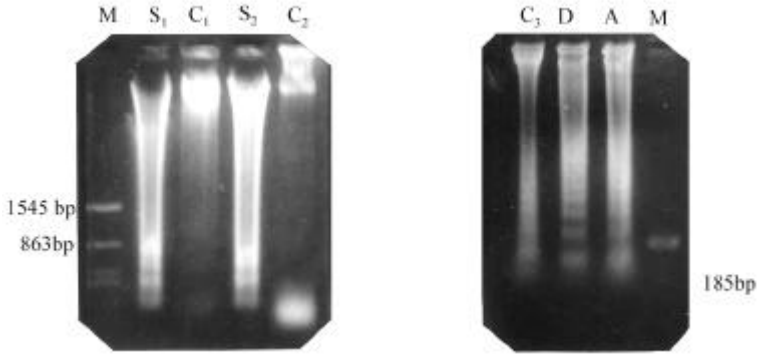


FIG. 3. DNA agarose gel electrophoresis shows the ladder pattern in mycotoxins treated HPBLs. M: Marker, $S_1$: ST treated group (2 000ng/mL, 6h), $C_1$: Control group (6h), $S_2$: ST treated group (2 000ng/mL, 24 h), $C_2$: Control group (24 h), A: $AFG_1$ treated group (1 000ng/mL, 24 h), D: DON treated group (1 000ng/mL, 24 h), $C_3$: Control group (24 h).

## DISCUSSION

Fungi and mycotoxin contamination of food and grains in the high incidence areas of esophageal, gastric and lung cancers in China has been considered as the possible etiological factors of cancer. The past several decades have witnessed many progresses on the detection of contaminating mycotoxins and studies of the putative roles of different mycotoxins such as aflatoxin $B_1$, T-2, ST etc, in the carcinogenesis of stomach, esophagus, liver and lung. Previous works had shown that both the fungi contaminated food and the culture of isolated predominant contaminating fungi in the foodstuffs from high risk areas of cancer could induce precancerous lesions and carcinomas in esophagus, forestomach, glandular stomach as well as lungs in different experimental animals[2,12,13]. Among the contaminating mycotoxins, ST, DON and $AFG_1$ were the most frequently detected ones. Their contamination rates and contents in the grains were quite high[3-5]. There have been several studies on the carcinogenic effects of ST on human tissues[6-9]. Recently, immunotoxicologic effects of some mycotoxins have caused the concern of biomedical workers. It was found that some trichothecene could affect secretion of cytokines, inhibit synthesis of protein and secretion of IgG in domestic animals and induce apoptosis of immune cells in experimental mice[14-20]. Yet, up to now, the effects of mycotoxins on human immunity have not been fully investigated.

Apoptosis is characterized morphologically by increased cytoplasmic granularity, cell shrinkage and nuclear condensation. The most prominent feature of apoptosis is the activation of an endogenous endonuclease which cleaves chromatin into nucleosome-sized fragments of proximately 200bp[21-23]. The DNA fragments could be detected by agarose gel electrophoresis, which is a basic and very reliable assay for the determination of apoptosis[11]. A "ladder-like" banding pattern indicates the presence of DNA fragmentation. Flow cytometric DNA analysis is another commonly used method in the determination of apoptotic cells. Appearance of the characteristic subdiploid DNA peak before $G_0/G_1$ peak in the DNA histogram is a generally accepted marker of cell apoptosis[10]. FCM DNA analysis and DNA agarose gel electrophoresis for apoptosis detection as described by Smith and Nicoletti were adopted in this study to explore the effects of ST, DON and $AFG_1$ on apoptosis of human peripheral blood lymphocytes *in vitro*. FCM DNA analysis showed that the apoptosis rates of HPBLs treated with ST, DON and $AFG_1$ were significantly higher than

those of the controls. Significant time-effect and dose-effect relationships were found between the apoptosis rates and mycotoxin treatment time as well as mycotoxin concentration at certain ranges. DNA agrose gel electrophoresis revealed that characteristic "ladder" banding pattern could be found in the HPBLs treated with ST, DON and $AFG_1$. The results in this study showed that ST, DON and $AFG_1$ in the high incidence areas of malignancies in China could induce apoptosis in human peripheral blood lymphocytes *in vitro*, and thus, exposures to these three mycotoxins in food and grain may have certain effects on human immunosystem in a negative way.

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