

Immunotoxicity of *Penicillium* Mycotoxins on Viability and Proliferation of Bovine Macrophage Cell Line (BOMACs)

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Abstract: *Penicillium* mycotoxins are natural contaminants found in grains, crops, fruits, and fermented products, especially during post harvest as well as storage periods. Contamination by individual and combinations of these toxins is likely to compromise food quality and safety. In this study, the potential immunotoxicity of citrinin (CIT), ochratoxin A (OTA), patulin (PAT), mycophenolic acid (MPA) and penicillic acid (PA) was evaluated using a bovine macrophage cell line (BOMACs) by assessing their potential cytotoxicity and then their effects on cell proliferation. The BOMACs were exposed to a range of mycotoxin concentrations, and then to different mycotoxin combinations for 48 hrs. Some cytotoxicity was evident at concentrations greater than 2.4 μ M for PAT, and 160 μ M for PA, however, at the IC50 (concentration that inhibits 50% cell proliferation), no cytotoxicity was observed for either of these mycotoxins. The mycotoxin IC50s from most potent to least potent were 0.56 μ M (PAT), 12.88 μ M (OTA), 29.85 μ M (PA), and 91.20 μ M (CIT). Concentrations of MPA greater than 80 μ M did not inhibit cell proliferation enough to calculate an IC50. Significant higher inhibition of cell proliferation was observed from the combinations of CIT+OTA, OTA+PAT, and OTA+PA compared to the effects of individual mycotoxins suggesting additive and in some cases synergistic activity between these paired mycotoxins.

Keywords: Bovine, Macrophages, *Penicillium* mycotoxins.

INTRODUCTION

Mycotoxins are metabolites of filamentous fungi that naturally contaminate a wide variety of crops, and therefore cereals and livestock feeds, worldwide [1,2]. The Food and Agriculture Organization of the United Nations (FAO) estimated that approximately 25% of the food and food stuffs in the world are compromised by mycotoxin contamination [3]. Economic losses from mycotoxin contamination occur due to reduced crop yield and value, and reduced livestock productivity and animal health from the consumption of contaminated feed [4]. Environmental conditions associated with high humidity and temperature favor fungal contamination of feed stuffs, and this can significantly affect the safety of both human and animal feed [5]. The effects of individual mycotoxins, such as carcinogenicity, teratogenicity, genotoxicity, nephrotoxicity as well as immunotoxicity have been studied, but their combinational effects have not been assessed [6-8]. One of the difficulties associated with characterizing the toxicity of mycotoxins is that contaminated feedstuffs may contain various combinations of mycotoxins produced by different fungal species [9]. For example,

Penicillium expansum produces citrinin (CIT), ochratoxin A (OTA), and patulin (PAT), while *Aspergillus ochraceus* produces OTA and PA [10-12]. Additionally, *Fusarium* mycotoxins are typically more abundant in corn silage immediately post-harvest, whereas *Penicillium* mycotoxin contamination increases during storage (Haladi, personal communication).

Among many fungal species, the *Penicillium*, *Aspergillus*, and *Fusarium* fungi are the most predominant species found in livestock feed such as corn silage [13-16]. Many studies have characterized the impact of oral exposure to *Fusarium* and *Aspergillus* mycotoxins on livestock species and examples of genotoxicity and immunotoxicity have been reported [15,17]. However, little is known about the impact of exposure to *Penicillium* mycotoxins, despite their potential to modulate immune function [6,18,19]. It is assumed that mycotoxins in general are detoxified by rumen microflora [20], since commensal microbes within ruminants have capacity to neutralize mycotoxins produced from *Fusarium* or *Aspergillus* mold before they enter the systemic circulation via the hepatic portal vein [21]. Many *Penicillium* mycotoxins on the other hand, are known to have antimicrobial properties, which could disrupt the normal function of these microbes, including their detoxification of *Penicillium* mycotoxins [22, 23]. Therefore, *Penicillium* mycotoxin exposure may be especially relevant to the health of

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ruminant species, especially since they are frequently found in contaminated silage.

In this study, we evaluated the cytotoxicity and combined effect of exposure to CIT, OTA, PAT, mycophenolic acid (MPA), and penicillic acid (PA) on the proliferation of a bovine macrophage cell line (BOMACs). We hypothesized that *Penicillium* mycotoxins will alter the proliferation of BOMACs in a dose-dependent manner, and that certain combinations of these mycotoxins will additively and/or synergistically inhibit the cell proliferation.

METHODS

Cell Preparation and Mycotoxin Exposure

The BOMACs (provided by J.R. Stabel and T.J. Stabel, 1995) were cultured in RPMI 1640 supplemented with 2.0 mM L-glutamine, 10% heat inactivated fetal bovine serum (FBS), 100 unit/ml of penicillin, 100 µg/ml of streptomycin, 0.25 µg/ml of amphotericin B, and 25 mM HEPES buffer (Invitrogen, ON, Canada) [24]. After reaching confluence, the BOMACs were dislodged with 0.05% trypsin-EDTA (Invitrogen, ON, Canada), seeded into 96-well flat bottom plates (10,000 cells per well), and incubated at 37°C with 5% CO₂ for 1 hr. The BOMACs were then exposed to a range of concentrations of CIT, OTA, PAT, MPA, and PA (Sigma, ON, Canada, Table 1) dissolved in DMSO for 47 hrs at 37°C with 5% CO₂. The ranges of exposure concentration were selected based on cited IC50s determined for bovine and porcine lymphocytes [6,19], and several preliminary trials using BOMACs that were used to optimize the toxic range of concentrations for each mycotoxin.

Table 1. Mycotoxin Concentrations Used for BOMAC Exposure Study

Mycotoxins	Concentrations (µM)
CIT	2.5, 5, 10, 20, 30, 40, 60, 80, 160, 320
OTA	0.30, 0.60, 1.2, 2.4, 4.8, 7.0, 9.6, 14, 19.2, 38.4
PAT	0.0038, 0.0075, 0.015, 0.038, 0.075, 0.15, 0.2, 0.3, 0.4, 0.5, 0.6, 1.2, 2.4, 4.8
MPA	0.05, 0.10, 0.2, 0.4, 0.6, 0.8, 1.6, 3.2, 32, 160, 320
PA	2.5, 4, 5, 10, 15, 20, 40, 80, 160, 320

Assessing Cytotoxicity of CIT, OTA, PAT, MPA, and PA to BOMACs

After 47 hrs of mycotoxin exposure, another batch of BOMACs was seeded into 96-well plates (50,000, 37,500, 25,000, 12,500, 0 cells per well) and incubated for 1 hr at 37°C with 5% CO₂- these cells were used as viable standards for the cytotoxicity assay. A parallel standard for dead cells was also prepared by incubating BOMACs in 70% methanol at room temperature for 1 hr- these cells were stained with 0.1% trypan blue and viewed under a light microscope to confirm cell death. The standard for dead cells was prepared by seeding the dead cells into the 96-well plates already

containing the standard for live cells such that the live cell proportion was equivalent to 100, 75, 50, 25, and 0%. At 48 hrs post mycotoxin exposure, all plates including the standards were washed with PBS, and cytotoxicity was assessed using a commercially available cytotoxicity kit (Invitrogen, ON, Canada). The number of live cells was estimated using a 1420 Victor2 Multilabel Counter (Beckman Coulter, Inc. California) by measuring the fluorescence of calcein AM (excitation 494/emission 517 nm), once it was hydrolyzed by intracellular esterases, and the number of dead cells was estimated by measuring the fluorescence of ethidium homodimer-1 (excitation 528/emission 617 nm) bound to nucleic acids. When possible, the LC50 (concentration that kills 50% of cells) was calculated for each mycotoxin using the Graphpad Prism software (La Jolla, California, U.S.A.).

Assessing the Inhibitory Effect of CIT, OTA, PAT, MPA, and PA on BOMAC Proliferation

The BOMACs were exposed to the mycotoxins as described above, and after 47 hrs of exposure, a standard for the proliferation assay was prepared by seeding another batch of BOMACs (50,000, 25,000, 12,500, 6,250, 3,125, 1,563, 782, 0 per well) into the 96-well plates for 1 hr of incubation at 37°C with 5% CO₂. The media was then removed from all plates by blotting, and the plates were frozen for 24 hrs at -80°C prior to the proliferation assay. The number of cells was estimated using a commercially available proliferation kit (Invitrogen, ON, Canada) that uses CyQUANT® GR dye (excitation 494/emission 517 nm) to label nucleic acids. The fluorescence intensity was measured with a 1420 Victor2 Multilabel Counter. The IC50 (concentration that inhibits 50% cell proliferation) for each mycotoxin was calculated using the Graphpad Prism software.

Assessing the Combined Effects of *Penicillium* Mycotoxin on BOMAC Proliferation

The IC25 for each mycotoxin was calculated using the Graphpad Prism software and is shown in the toxicity curves in Fig. (1). The BOMACs were then subjected to exposure by various combinations of CIT, OTA, PAT, MPA, and PA at their respective IC25s and to the individual mycotoxins (Table 2); cell proliferation was then assessed as described above.

Table 2. The LC50, IC50, and IC25 Various *Penicillium* Mycotoxins (µM)

	CIT	OTA	PAT	MPA	PA
LC50	N/A	N/A	4.46	N/A	175.79
IC50	91.20	12.88	0.56	N/A	29.85
IC25	52.72	8.91	0.32	0.50	13.90
R-Square*	0.945	0.981	0.841	0.871	0.987

LC50s estimated from Fig. (1A); IC50s and IC25s estimated from Fig. (1B).

*R-square: the fitness of curve with the data points from Fig. (1B)

Data Analysis

All data are presented as percent cytotoxicity and percent cell proliferation, based on the combined results of three

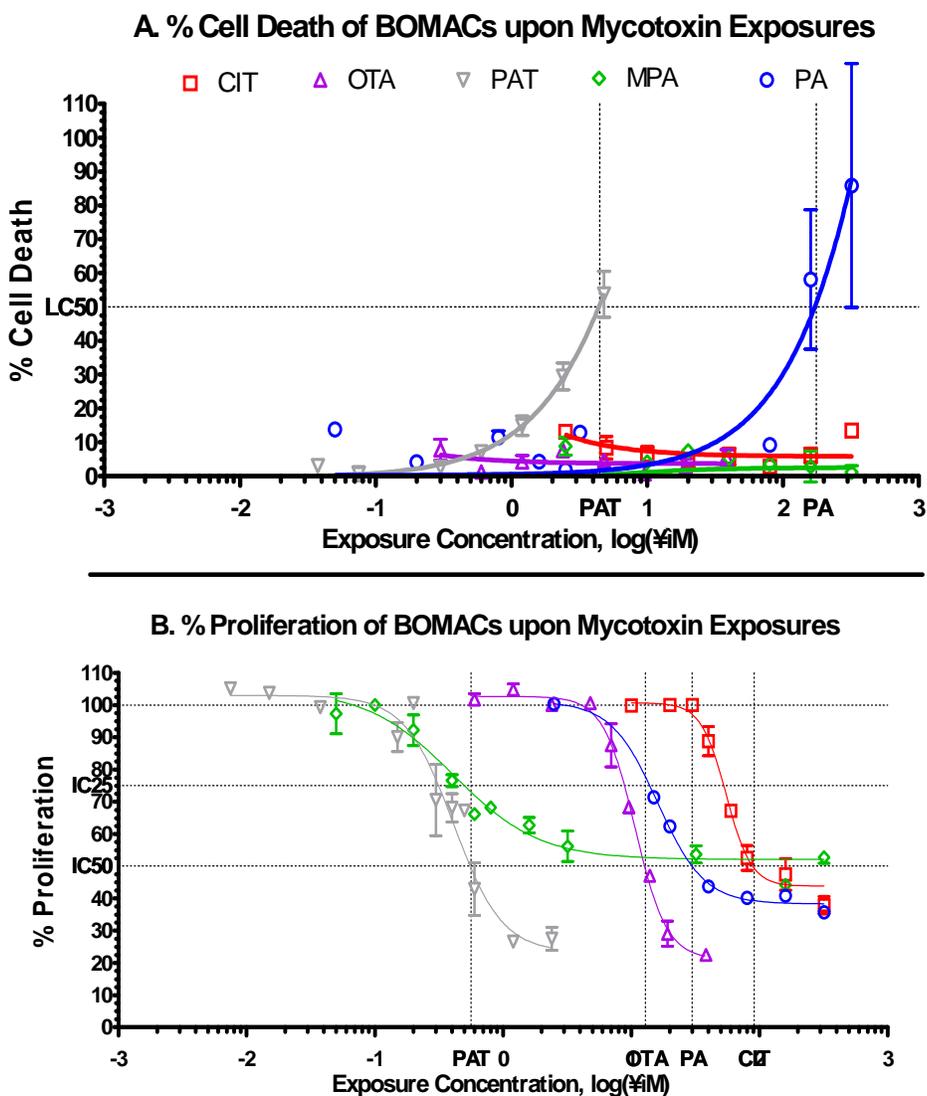


Fig. (1). A. Cytotoxicity and B. Proliferation of BOMACs following 48 hrs of Mycotoxin Exposure. CIT(□), OTA(□), PAT(▽), and MPA(◇), PA(○). LC50s (the concentraion that kills 50% of cells) are indicated in A; IC25s (The concentrations that inhibits 25% cell proliferation) and IC50s (The concentrations that inhibits 50% cell proliferation) are indicated in B with dotted lines.

independent experiments. For the dose-response data, an ANOVA followed by Dunnett’s test was used to test for statistical significance between the solvent controls and treatments (b), and a T-test was used to make comparisons between the control and solvent control (a). For the combined mycotoxins, an ANOVA followed by Tukey’s test for comparisons was used to compare treatment groups (c) (Fig. 2). The concentration data were log transformed for the analysis, and significance was determined at a p-value ≤ 0.05 using the Graphpad Prism software.

RESULTS AND DISCUSSIONS

Cytotoxicity and Inhibition of the Cell Proliferation due to Individual Mycotoxin Exposure

Cytotoxicity was evident at concentrations greater than 2.4 µM for PAT, and 160 µM for PA; the LC50s for PAT and PA were estimated to be 4.46 µM and 175.79 µM, respectively (Table 2). The cytotoxicity of PAT and PA may

be due to their genotoxic effect on BOMACs. In support of this, Liu *et al.*, (2003) concluded PAT as a potent clastogen with the ability to cause oxidative damage to DNA in hamster ovary cells and human peripheral blood lymphocytes, while CIT did not exhibit any significant evidence of such genotoxicity [8]. In the present study, CIT, OTA, and MPA did not induce any cytotoxicity within the concentration ranges tested (Fig. 1A). It is possible that higher concentrations of these mycotoxins would have induced sufficient cytotoxicity to estimate their respective LC50s, however, the concentration of DMSO at these higher concentrations would have also inhibit the proliferation of BOMACs, biasing the results of the proliferation assay (Data not shown).

In the context of cell proliferation, the mycotoxin IC50s calculated in this study occurred at concentrations where no overt cytotoxicity occurred, and their order of potency from highest to lowest was: 0.56 µM (PAT) > 12.88 µM (OTA) > 29.85 µM (PA) > 91.20 µM (CIT) (Table 2). When these IC50s were compared to IC50s determined from other stu-

dies, it appears that BOMACs are less sensitive to CIT, OTA, and PA, but are more sensitive than porcine and bovine lymphocytes to PAT (Table 3). The exposure concentrations of some of the mycotoxins investigated and IC50s determined in this study, such as OTA, were higher than circulating concentrations reported from *in vivo* studies [25, 26]. However, there is the potential for their bioaccumulation in certain target tissues, such as kidney, intestine, and cutaneous fat [27, 28], as well as their synergistic and additive interaction among other mycotoxins within these tissues [29].

Table 3. Comparison of Mycotoxin IC50s for Cell Proliferation of Porcine and Bovine Lymphocytes (μM)

	Porcine Lymphocyte [6]	Bovine Lymphocyte [19]	BOMACs
CIT	38.00	44.27	91.20
OTA	1.30	4.41	12.88
PAT	1.20	3.63	0.56
PA	18.00	N/A	29.85

Based on the observations of this study, MPA was found to have little effect on BOMACs. Although MPA concentra-

tions of 32 μM and 320 μM did inhibit cell proliferation, the response was not significantly different between these concentrations, and was not substantial enough to estimate an IC50 (Fig. 1B). The inhibitory mechanism of MPA on cell proliferation has been well studied. Mycophenolic acid and its commercial analogue, mycophenolate mofetil (MM), block the *de-novo* purine biosynthesis pathway, which is required for lymphocyte proliferation, by reducing cellular NF- κB levels [30]. While the proliferation of B- and T-lymphocytes relies heavily on the *de novo* pathway, BOMACs may equally utilize both *de-novo* and salvage pathways for their proliferation [31]. Furthermore, a more gradual reduction in cell proliferation was observed at the lower MPA concentrations when compared to the other mycotoxins (Fig. 1B). These distinct characteristics may suggest why MPA and MM have been successfully used for treatment of allogeneic transplantation as well as other immune-mediated diseases [32, 33].

Combined Effect of Penicillium Mycotoxins on the Proliferation of BOMACs

When the ten different combinations of mycotoxins were evaluated in the present study, three combinations significantly inhibited the proliferation of BOMACs when compared to the individual mycotoxins. These combinations included; CIT+OTA, OTA+PAT, and OTA+PA, (Fig. 2). Combinations of CIT, OTA, and PA were of interest because

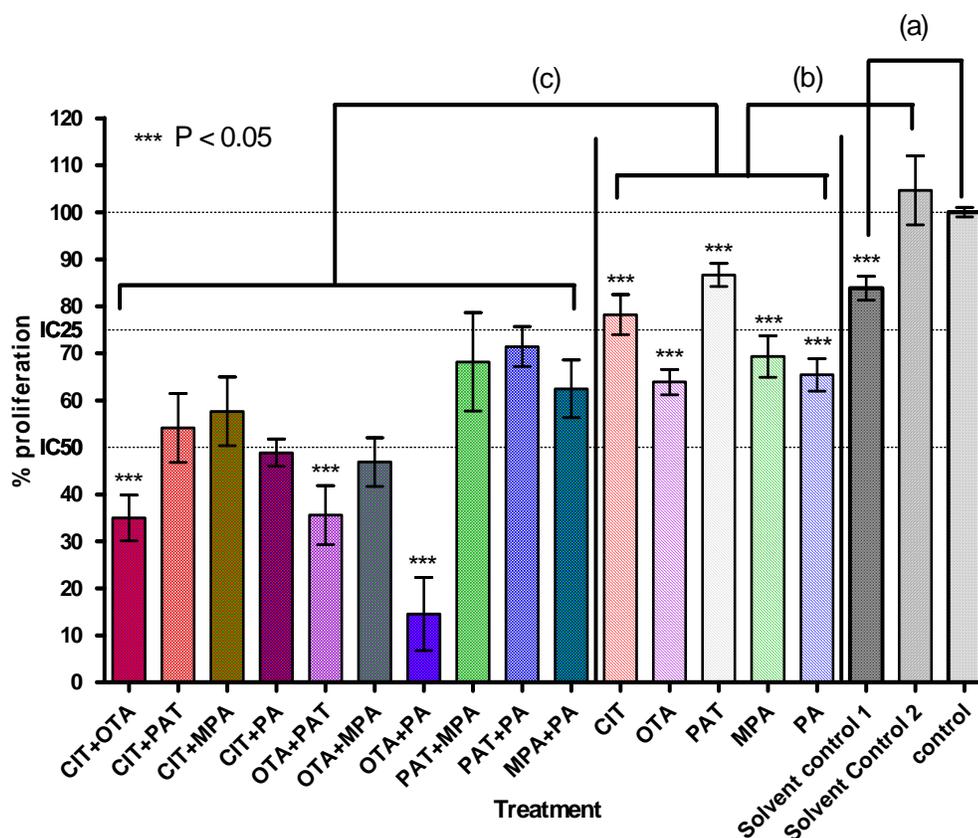


Fig. (2). The combined Effects of Mycotoxins on the Proliferation of BOMACs. Solvent control 1 is only for the combinations of CIT+OTA. Solvent control 2 is for all other combinations and individual mycotoxins. (a) control vs. solvent controls; (b) the solvent controls vs. individual treatments; (c) combined vs. individual treatments.

these mycotoxins can be produced by *Penicillium viridicatum*, whereas *Aspergillus ochraceus* produces OTA and PA [10, 34]. *Penicillium Expansum*, which is commonly found in rotten apples, is also capable of producing CIT, PAT, and OTA [11, 12]. The combination of CIT+OTA was previously demonstrated to increase chicken embryonic toxicity compared to CIT or OTA, alone [35]. Additionally, Sansing *et al.*, (1976) reported that CIT+OTA and OTA+PA inhibited orotic acid incorporation into liver and kidney tissues, while this inhibitory effect was not observed from any of the individual mycotoxins [10]. Furthermore, the synergistic and additive interaction between CIT and OTA as well as OTA and PA on nephrotoxicity, hepatotoxicity and teratogenicity has been well addressed in other studies [36, 37]. In the present study, the proliferation of BOMACs was shown to be inhibited in an additive manner with the combinations of CIT+OTA and OTA+PAT, while the synergistic activity was observed from the combination of OTA+PA (Fig. 2).

CONCLUSIONS

The inhibitory effect of the following *Penicillium* mycotoxins, CIT, OTA, PAT, MPA, and PA, as well as their combinations, on the cytotoxicity and proliferation of BOMACs was evaluated in this study. All mycotoxins except MPA inhibited cell proliferation by at least 50% with the evidence of cytotoxicity from PAT and PA for the concentration ranges used in this study. Most of these toxic concentrations determined in the study are higher than circulating concentrations from *in vivo* studies, yet the continuous consumption of feed contaminated with these toxins may lead to their accumulation in host tissues potentially increasing their bioavailability to the host immune cells. Of greater concern, however, was the additive or synergistic effects of various combinations of mycotoxins including; CIT+OTA, OTA+PAT, and OTA+PA. Since these mycotoxins can be naturally found in these combinations, their effects on the immune system warrant further investigation.

CONFLICT OF INTEREST

None declared.

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ABBREVIATION

CIT	=	Citrinin
OTA	=	Ochratoxin A
PAT	=	Patulin
MPA	=	Mycophenolic acid
PA	=	Penicillic acid
MM	=	Mycophenolate mofetil

BOMACs	=	Bovine macrophage cell line
IC50	=	Concentration that inhibits 50% of cell proliferation
IC25	=	Concentration that inhibit 25% of cell proliferation
LC50	=	Concentration that kills 50% of cells

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