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Assessing interactions of binary mixtures of *Penicillium* mycotoxins (PMs) by using a bovine macrophage cell line (BoMacs)

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ABSTRACT

Penicillium mycotoxins (PMs) are toxic contaminants commonly found as mixtures in animal feed. Therefore, it is important to investigate potential joint toxicity of PM mixtures. In the present study, we assessed the joint effect of binary combinations of the following PMs: citrinin (CIT), ochratoxin A (OTA), patulin (PAT), mycophenolic acid (MPA) and penicillic acid (PA) using independent action (IA) and concentration addition (CA) concepts. Previously published toxicity data (i.e. IC25; PM concentration that inhibited bovine macrophage (BoMacs) proliferation by 25%) were initially analyzed, and both concepts agreed that OTA + PA demonstrated synergism ($p < 0.05$), while PAT + PA showed antagonism ($p < 0.05$). When a follow-up dilution study was carried out using binary combinations of PMs at three different dilution levels (i.e. IC25, 0.5 * IC25, 0.25 * IC25), only the mixture of CIT + OTA at 0.5 * IC25 was determined to have synergism by both IA and CA concepts with Model Deviation Ratios (MDRs; the ratio of predicted versus observed effect concentrations) of 1.4 and 1.7, respectively. The joint effect of OTA + MPA, OTA + PA and CIT + PAT complied with the IA concept, while CIT + PA, PAT + MPA and PAT + PA were better predicted with the CA over the IA concept. The present study suggests to test both IA and CA concepts using multiple doses when assessing risk of mycotoxin mixtures if the mode of action is unknown. In addition, the study showed that the tested PMs could be predicted by IA or CA within an approximate two-fold certainty, raising the possibility for a joint risk assessment of mycotoxins in food and feed.

1. Introduction

Penicillium mycotoxins (PMs) have been shown to cause a wide range of toxic effects in animals. Nephrotoxicity and hepatotoxicity are the most common clinical signs reported in animals fed PM-contaminated feed (Braunberg et al., 1992; Dickens and Jones, 1965; Sansing et al., 1976). Some studies have also reported immunomodulatory effect of PMs (Al-Anati and Petzinger, 2006; Ferrante et al., 2008; Herzog-Soares and Freire, 2004; Oh et al., 2015). For example, our previous studies determined that PMs can differentially affect various macrophage biological activities, such as proliferation, viability, reactive oxygen production (ROS) production, phagocytosis as well as the gene expression of epigenetic enzymes and cytokines (Oh et al., 2012, 2013). The macrophage is one of the key regulators of immune system (Kelsall, 2008; Wynn et al., 2013), and therefore, changes in the function of macrophages could potentially predispose animals to secondary diseases (Wynn et al., 2013).

Until now, most toxicity studies involving mycotoxins have focused on toxicity of individual mycotoxins. However, contaminated animal feeds are in reality usually contaminated with various combinations of mycotoxins. Mansfield et al. (2008) for example, reported that >50% of maize silage collected from 30 different Pennsylvania dairies between 2001 and 2002 contained more than one mycotoxin. Therefore, the potential risk of exposure to mycotoxins may be underestimated when only assessing single mycotoxin presence and toxicity, and not their joint effect.

In the context of PMs, various interactions have been reported. Specifically, citrinin (CIT) and penicillic acid (PA) kidney and liver toxicity have been shown to increase in mice as well as embryotoxicity in chicks

Abbreviation: 95% CI, 95% confidence limit; BoMacs, bovine macrophage cell line; CA, concentration addition; CIT, citrinin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IA, independent action; IC25, concentration that inhibit 25% cell proliferation; IC50, concentration that inhibit 50% cell proliferation; MDR, Model Deviation Ratios (MDRs), the ratio of predicted versus observed effect concentrations of IC50s extrapolated from toxicity curves; MPA, mycophenolic acid; OTA, ochratoxin A; P_A or P_B, observed proportion proliferaiton of a individual PM; PA, penicillic acid; PAT, patulin; P_{PM}, predicted proportion proliferation of a PM mixture; PM, *Penicillium* mycotoxin; P_{OM}, observed percent proliferation of a PM mixture; RPMI 1640, Roswell Park Memorial Institute 1640 media.

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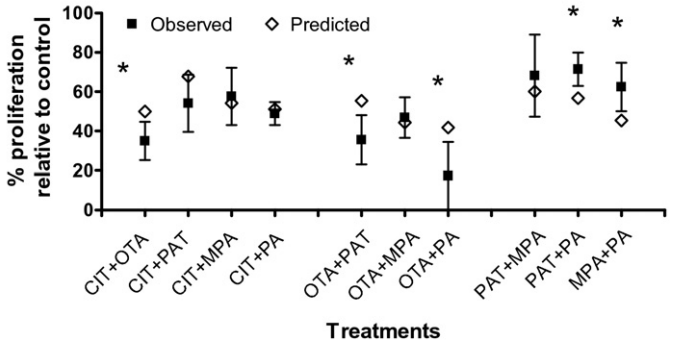

Fig. 1. The comparison between P_{OM} (observed) and P_{PM} (predicted) of PM mixtures at their respective IC25s by using the IA approach from the previous proliferation data (Oh et al., 2012). Clear squares indicate the mean of 'predicted' proliferation from the PM mixtures. Black squares represent the mean of 'observed' proliferation, and the error-bars are 95% CI.

when these animals were co-exposed with ochratoxin A (OTA) (Sansing et al., 1976; Veselá et al., 1983). There is no other previously reported case of mycotoxin interaction on immune parameters. However, using bovine macrophages (BoMacs), we previously found potential synergistic interactions of binary PM mixtures at their respective IC25s, the concentration of PM that inhibited BoMac proliferation by 25% (Oh et al., 2012). BoMacs that were used in previous studies and this study originated from bovine macrophages isolated from the peritoneal region along the intestinal gut lining (Stabel and Stabel, 1995). Peritoneal macrophages may come into contact with mycotoxins even before detoxification occurs within the liver and kidney, which makes these cells a biologically relevant cell population to consider the effect of mycotoxins in addition to the liver and kidney. In this study, proliferation was used since BoMac proliferation was the only endpoint to be commonly affected by the following PMs, CIT, OTA, patulin (PAT), mycophenolic acid (MPA) and PA. The previous proliferation study, however, was lacking in both experimental and statistical design to more precisely quantify interactions between PMs. Specifically, conclusions were solely based on single concentration data and therefore, did not take into account binary interactions at different PM concentrations (Meadows et al., 2002; Oh et al., 2012).

There are several different ways of determining interaction of compounds in a mixture. For all methods, mixture toxicity data is compared to a predicted mixture effect that assumes no interactions between chemicals (Berenbaum, 1981; Cedergreen et al., 2008; Stork et al., 2007). Two basic toxicity concepts for estimating the combined effects of mixtures include independent action (IA) and concentration addition (CA) (Cedergreen et al., 2008; Kjaerstad et al., 2010; Kortenkamp and Altenburger, 1998). The IA concept assumes that compounds in a mixture have a completely independent mode of action that affects a common endpoint, while the CA concept assumes that compounds have a similar mode of action (Cedergreen et al., 2008); both concepts assume that the chemicals do not interact.

The application of IA or CA concepts determines the predicted additive effect from individual exposure data (Cedergreen et al., 2008). The comparison between the predicted additive effect and the observed joint effect provides a statistical means of distinguishing interactions of synergism and antagonism among PM mixtures. Previously, we assessed the observed effect of binary PM mixtures at their respective IC25s at their sub-lethal levels (Oh et al., 2012). The previous study described the interaction of the PM mixtures at a single concentration, but it did not investigate interactions of mixtures at other concentrations. Therefore, in the present study, the mixture toxicity concepts were applied to re-analyze the proliferation data from the previous Oh et al. (2012) study, and were then used to re-examine potential interactions of binary mixtures of PMs at three different dilution levels (IC25, 0.5 * IC25, and 0.25 * IC25) to test the interactions. Since the mode of action of these PMs on the proliferation of BoMacs is currently unknown, both IA and CA concepts were applied to predict joint effects of PM mixtures from the Oh et al. (2012) study, and were then compared to the results from the present dilution study.

2. Methods

2.1. Data analysis and plotting of the previous PM toxicity data

The previous proliferation data (Oh et al., 2012) were analyzed using IA and CA concepts (Figs. 1 and 2). The mixture data was plotted together with predicted effects based on concentration-response data of the individual chemicals.

Based on the assumptions of the IA concept, the effect of the binary PM mixture was predicted from the observed effect of the individual PM exposure data using the following equation (Jørgensen, 2013): $P_{PM} = P_A * P_B$, where P_{PM} is the predicted proliferation of the administered mixture relative to the control treatment, and P_A and P_B are the relative proliferation of the individual PMs. The observed and predicted relative proliferation from the mixture (P_{OM}) and P_{PM} were plotted in Fig. 1. If the predicted cell proliferation fell within the 95% confidence limits (CI) of the observed cell proliferation, then the assumption of additivity was accepted, and PMs within the mixture were said to have an additive effect. In contrast, when the mixture prediction was outside of the 95% CI of P_{OM}, then significant interaction between two PMs occurred ($p < 0.05$) as either synergistic (P_{PM} being located above 95% CI of P_{OM}), or antagonistic (P_{PM} being located below 95% CI of P_{OM}).

For the CA concept, whole dose-response mixture predictions were constructed from the cell proliferation data of individual PM (Oh et al., 2012). The effect concentrations (ECs) for each proliferation response (e.g. the concentrations that inhibited BoMacs proliferation by 99%… 1%) were calculated from the curves giving the best fit, including biphasic curves using SigmaPlot 13 (Systat, CA, USA). To calculate a mixture CA curve from several curves with different slopes, the predicted curve is calculated from several EC concentrations. The mixture prediction for each effect concentration were then calculated by $1/(\frac{pA}{EC\times A} + \frac{pB}{EC\times B})$, where A and B represent different PMs with p being the proportion of the individual PM in the mixture ($\Sigma(pA + pB) = 1$) (Ohlson et al., 2010). The predicted data points were connected with a curve and plotted together with P_{OM} including 95% CI (Figs. 1 and 2). Significant deviations between observed and predicted data were evaluated as above, with predictions falling outside the 95% CI being considered as either synergistic (P_{PM} being located above 95% CI of P_{OM}) or antagonistic (P_{PM} being located below 95% CI of P_{OM}).

2.2. Assessing the interactions of PMs at a range of concentrations

2.2.1. Cell preparation. The BoMacs, provided by Stabel and Stabel (1995), were cultured in Roswell Park Memorial Institute (RPMI) in 1640 medium, 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. All cell culture products were purchased from Invitrogen, Canada. Cells were incubated at 37 °C with 5% CO_2 in a 75 cm^2 flask, and were grown to above 80% confluence prior to use for the study.

Fig. 2. (A–J). The comparison between theoretical curve of P_{PM} (predicted) and P_{OM} (observed) of PM mixtures at their respective IC25s by using CA approach from the previous proliferation data (Oh et al., 2012). Clear diamond with dotted lines indicate the mean of observed mean of 'predicted' proliferation from PM mixtures. Black squares represent the observed proliferation from IC25 mixtures of PMs, and the error-bars are 95% CI.