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Metabolism-dependent cytotoxicity of citrinin and ochratoxin A alone and in combination as assessed adopting integrated discrete multiple organ co-culture (IdMOC)

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ABSTRACT

Citrinin (CTN) and ochratoxin A (OTA) can be present as co-contaminants in cereals, foods and feed commodities, and can affect human health. Metabolism-dependent toxicity of these two mycotoxins, separately as well as in combination, is not yet understood. To fill this gap we adopted integrated discrete multiple organ co-culture (IdMOC) technique, which obviates animal experiments from the perspectives of species difference as well as animal welfare concerns. IdMOC facilitates co-culture of a metabolically competent cell (HepG2) and a metabolically incompetent cell (3T3) that are physically separated but provides for extracellular product(s) from one cell to interact with the other. After ascertaining that HepG2 is metabolically competent and 3T3 is not, adopting luciferin-IPA metabolism assay, CTN and OTA were tested separately and in combination in the co-culture set-up, when both proved to be metabolism-dependent cytotoxic agents. Hepatocytes metabolize CTN into a diffusible product that is cytotoxic to 3T3 cells but the cytotoxicity of OTA appears to be limited to the hepatocytes, *i.e.*, local acting. As a combination at a concentration of 20% of IC_{50} of each, CTN forms a reactive metabolite that diffuses out of HepG2 to cause cytotoxicity to 3T3 cells synergistically with OTA parent molecule. The CYP isoenzymes involved in the metabolism OTA and CTN were identified adopting *in silico* methods which indicated that OTA and CTN can bind CYP proteins at specific sites.

1. Introduction

CTN and OTA are mycotoxins that can co-occur in fungus-contaminated grains, cereal products and feeds. The toxicity of CTN and OTA primarily depends on the frequency of exposure and the daily intake of contaminated foods and feed commodities. Mycotoxin toxicity particularly affects the organs concerned with excretion *viz.*, liver and kidney, in view of susceptibility to overt toxicity compared to other tissues. The nephrotoxic and hepatotoxic potentials of both CTN and OTA, separately as well as in combination, have been elucidated rather elaborately. Pathogenesis of human Balkan Endemic Nephropathy, which in turn is associated with urinary tract tumor in man, is connected with additive/synergistic interaction between OTA and CTN (Knecht et al., 2005; Follmann et al., 2007; Bouslimi et al., 2008a, 2008b; Golli-Bennour et al., 2010; Klaric et al., 2013; Kumar et al., 2014). Experimental evidences for hepatotoxicity of these two mycotoxins have also been provided (Ehrlich et al., 2002; Knasmuller et al., 2004; El Golli Bennour et al., 2009; Chopra et al., 2010; Anninou et al., 2014; Gayathri et al., 2015). Though the mechanism underlying hepatotoxicity of mycotoxins has been resolved in fair detail, a factor that complicates the prediction of hepatotoxicity is the metabolic fate of the mycotoxins. A given mycotoxin can potentially give rise to one or more metabolites during hepatic metabolism. Usually, most such metabolites are not toxic and are excreted. However, sometimes one or more metabolites can be toxic. For example, Aflatoxin B1 (AFB1) by itself is not hepatotoxic but on hepatic metabolism by CYP450 isoenzymes 1A2 and

Abbreviations: AFB1, aflatoxin B1; CTN, citrinin; CYP450, cytochrome P450; DMSO, dimethyl sulfoxide; IC_{50}, the concentration at which 50% of cells are dead; IdMOC, integrated discrete multiple organ co-culture; MTT, 3-4,5-dimethylthiazole-2-yl, 2,5-diphenyl tetrazolium bromide; MD, molecular dynamics; OTA, ochratoxin A; PBS, phosphate-buffered saline

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3A4 it results in AFB1–8, 9 epoxide, which is toxic to the hepatic cells themselves (Li et al., 2012). Thus, liver is the major organ concerned with biotransformation and detoxification of toxic chemicals including mycotoxins.

Interaction between the toxic metabolites and other macromolecules in the target cells can increase/decrease the cytotoxicity. Hence, during hepatic metabolism CTN, OTA and their combination may produce bioactive metabolite(s) that can affect liver itself and/or other organs/tissues which are sensitive to the bioactive metabolite(s). Though hepatic and renal metabolites of CTN and OTA have been reported, there are only a few studies concerned with metabolism-dependent cytotoxicity of CTN and OTA individually, and none in respect of combination of the two (Hansen et al., 1982; Stormer et al., 1981; Li et al., 2000; Simarro Doorten et al., 2004; Follmann et al., 2014). Therefore, understanding the metabolism-dependent toxicity of CTN and OTA individually as well as in combination would make it clear whether the parent CTN and OTA molecules induce cytotoxicity directly or the hepatic metabolite(s) of CTN and/or OTA induce cytotoxicity. Thus, we were interested in finding if CTN, OTA and their mixtures really require hepatic metabolism to induce toxicity in sensitive organs/tissues.

Metabolism of chemical entities and multi-organ toxicity are conventionally investigated in animal models. However, during the more recent times animal experiments have come to suffer criticism in view of species difference (Li, 2007, 2009b) and animal ethics issues (Li, 2004). Alternative methods in the context of 3Rs are strongly advocated (Russell and Burch, 1959). Hence, there is a paradigm shift in toxicology and pharmacology experiments where traditional animal-based toxicity testing methods are replaced by modern rationalized non-animal toxicity testing methods. *In vitro* systems are useful for comparative toxicity testing. Even then, the conventional *in vitro* techniques/methods are not adequate to study the metabolism-dependent cytotoxicity of a xenobiotic compound because monoculture of hepatocytes in isolation, without well-to-well interconnection, prohibits metabolites from reaching the sensitive organs/cells that are distally located (either hepatic or extrahepatic) (Li, 2008; Li et al., 2012; Gayathri et al., 2015). This issue could be resolved by co-culturing two or more cell types wherein the wells are not directly connected but connection can be provided for. Thus, it is possible to coculture hepatic cell with any other target cell, physically separated from each other. The xenobiotic that is included in the experimental system may get metabolized by the liver cells and the metabolite(s) thus produced can diffuse out to reach the distal target cells to induce toxicity.

Thus, in this study we used a next generation *in vitro* technique, IdMOC, to assess the comparative cytotoxicity of OTA and CTN alone as well as in combination on metabolically competent (HepG2) cells co-cultured with metabolically incompetent (3T3) cells. We chose HepG2 cells which, though sourced from a hepatocellular carcinoma, still mimic normal hepatocytes to a fairly good extent (see Gayathri et al., 2015). 3T3 fibroblasts lack drug metabolizing enzymes and have been used in earlier IdMOC studies as an extra-hepatic target cell (Li et al., 2012). In order to validate the IdMOC technique for metabolism-dependent toxicity studies Li et al. (2012) tested tamoxifen, aflatoxin B1 (AFB1) and cyclophosphamide, which study has been taken as a positive reference here. Our data suggest that the differential cytotoxicity assessment using metabolically competent HepG2 and metabolically incompetent 3T3 cells in the IdMOC represents an effective approach to find metabolism-dependent xenobiotic toxicity. Co-culture of cells in IdMOC system allows classification of mycotoxins as direct acting mycotoxins that produce adverse effect directly on sensitive cells without any metabolic activation; mycotoxins that require metabolic activation to exert toxicity only on cells in which they are metabolized; and mycotoxins that require metabolic activation, and the toxic metabolite(s) is/are stable enough to diffuse out of the cell/tissue/organ of metabolism and inflict toxicity on other/distal sensitive cells (Li et al., 2012).

Thus, the major objective of the study is to assess metabolism-dependent toxicity of CTN and OTA alone as well as in combination by co-culturing HepG2 cells (metabolically fairly competent hepatocytes, representing liver) and 3T3 cells (metabolically incompetent peripheral tissue fibroblasts) in the IdMOC system so as to provide for product(s) of hepatic metabolism to induce toxicity on the hepatocytes themselves as well as the distal fibroblasts (Li et al., 2012; Gayathri et al., 2015).

It was felt to be of interest to study the CYP450 enzyme(s) that is/are involved in the metabolism of CTN and OTA. Thus, we aimed at finding lead(s) for metabolic processing of the two mycotoxins through CYP450 isoenzymes. Since the conventional wet lab approach in this regard is cost-intensive and time-consuming, we resorted to *in silico* methods of molecular docking and molecular dynamics (Ayed-Boussema et al., 2012). Using these *in silico* methods the binding affinity and adaptability of the ligands to the binding pockets of important CYP proteins CYP1A1, CYP1A2, CYP2B6, CYP2C9 and CYP3A4 were analyzed by calculating the docking score, binding energy of protein complexes, hydrogen bond formations and RMSD analysis. This analysis threw light on the interaction pattern of OTA and CTN with CYP proteins that would lead to scrutinize the CYP isoenzymes in wet lab experiments in the future. Thus, a combination of advanced *in vitro* (IdMOC technology) and *in silico* approaches offered insights into metabolism-dependent toxicity of OTA and CTN individually and in combination.

2. Materials and methods

2.1. In vitro studies

2.1.1. Chemicals

CTN, OTA, and DMEM were obtained from Sigma Chemical Company (St. Louis, MO, USA). Phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from HiMedia Laboratories (Mumbai, India). Fetal bovine serum (FBS), trypsin-EDTA, penicillin and streptomycin were obtained from Invitrogen (USA). All other chemicals and reagents were of analytical grade.

2.1.2. Cell culture and IdMOC plate design

Human hepatocarcinoma cell HepG2, and mouse fibroblast cell 3T3, were obtained from National Center for Cell Science (NCCS), Pune, India. The cells were maintained in DMEM supplemented with 10% FBS, and 100 U/mL each penicillin and streptomycin, in a humidified atmosphere of 5% CO_2 and 95% air, in a CO_2 incubator (Thermo Scientific, USA). Both HepG2 and 3T3 cells were introduced into IdMOC-96 well plate which has 16 containing wells, each with 6 inner wells (Fig. 1). Five thousand cells, cells in a volume of 35 μL of DMEM, were plated per well. After an attachment period of 4 h, 750 μL of DMEM was added to each containing well to initiate the cytotoxicity study (Li et al., 2012; Gayathri et al., 2015).

2.1.3. P450-dependent metabolism

To demonstrate that HepG2 cells co-cultured with 3T3 cells in the IdMOC are competent in metabolism of xenobiotics, luciferin-IPA metabolism assay was carried out (Li, 2009a). Of the six wells in each containing well three were seeded with 3T3 cells and co-cultured with 0, 1, 2 and 3 wells of HepG2, and each was done in triplicates. After cell plating, an aliquot of 1000 μL of medium containing 5 μM of the CYP3A4 substrate luciferin-IPA was added to each containing well. After each designated incubation period, such as 1 h, 2 h, 3 h and 4 h, an aliquot of 50 μL of the medium was transferred to 96-well opaque white plate, followed by the addition of 50 μL of Luciferin Detection Reagent (Promega, Madison, WI). Luciferin formation was quantified in terms of luminescence using a multichannel plate reader (Perkin Elmer's VICTOR × 2). Based on a standard curve generated from luciferin standard (Promega; Madison, WI) the luminescent signals of luciferin were converted to pmol.