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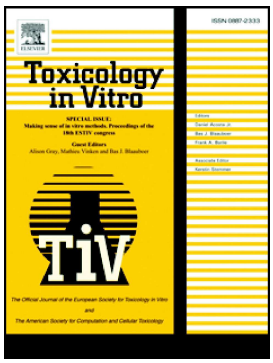

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Toxicological effects of regulated mycotoxins and persistent organochloride pesticides: In vitro cytotoxic assessment of single and defined mixtures on MA-10 murine Leydig cell line

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ABSTRACT

Epidemiological studies show that there is global decline in male fertility primarily as a result of poor sperm quality and this is attributed to exposure to endocrine disrupting chemicals (EDCs) in the environment, food and pharmaceutical products, including mycotoxins and pesticides. The Leydig cells in the male testes are responsible for producing androgens, hormones that play major roles in male development and reproductive function. Therefore, any toxin that affects the function and morphology of the Leydig cells may result in sub-fertility or infertility. The cytotoxic effects of single and binary mixtures of aflatoxin B_1 (AFB_1), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEN), alpha-zearalenol (α-ZOL), beta-zearalenol (β-ZOL), 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (p,p'-DDT) and 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (p,p'-DDE) on a model cell line, the MA-10 Leydig cells, were evaluated using 3-[4,5-dimethylthiazol-2-yl]-2,5-dipenyltetrazolium bromide (MTT) assay after 48 hours of exposure. With single toxin treatment at doses between 0.1 µM and 64 µM for 48 hours, DON was the most cytotoxic to MA-10 cells with a half maximal inhibitory concentration (IC_{50}) value of 12.3µM followed by α-ZOL (IC_{50}: 28 µM) and OTA (IC_{50}: 30 µM) while the IC_{50} of AFB_1, p,p'-DDT and p,p'-DDE were above the highest concentration tested (64 µM). Co-exposure with p,p'-DDT or p,p'-DDE enhanced the toxicity of DON, OTA and ZEN to MA-10 Leydig cells, particularly at higher concentrations. This highlights the possible adverse effects on male reproductive health following co-exposure to these toxins.

Key words: Mixture toxicity; Mycotoxins; Pesticides; Cytotoxicity; Leydig cells; MA-10 cell line; Reproductive toxicity

1. Introduction

Epidemiological studies show there is an increase in infertility worldwide, with the prevalence higher in developing countries than in developed countries (Mascarenhas et al., 2012). For instance, infertility prevalence is about 6% in the USA (Chandra et al., 2013), 10 – 15% in UK (Oakley et al., 2008) and 20 – 35% in many African countries (Larsen, 2000). It is estimated that in 20% of infertility cases, the pathology is solely from the man but both male and female factors also contribute 30% - 40% (Phillips and Tanphaichitr, 2008). Therefore, the male factor is at least partly responsible for infertility in about 50% of cases. The causes of male infertility are multi-factorial and these are still poorly understood. Several reports have shown that there is a general decline in male fertility mainly due to poor sperm quality (Carlsen et al, 1992; Swan et al, 1997, 2000). The global decline in male fertility and the occurrence of testicular dysgenesis syndrome can be attributed to exposure to endocrine disrupting chemicals (EDCs) in the environment, food and pharmaceutical products, including mycotoxins and pesticides (Ibe et al., 1994; Uriah et al., 2001; Skakkebæk et al., 2001; Phillips and Tanphaichitr, 2008; Martenies and Perry, 2013; Eze and Okonofua, 2015).

Exposure to various mycotoxins and pesticides is common and can be high in some populations, especially in developing countries. Aflatoxins (AFs), fumonisins (FUMs), ochratoxin A (OTA), deoxynivalenol (DON) and zearalenone (ZEN) are considered to be the major mycotoxins of public health concern due to their frequent occurrence in cereals and cereal-based products. Whilst there is evidence of health effects of these different mycotoxins (Rodrigues and Naehrer, 2012; Wild and Gong, 2010), there have been few studies of the role of mycotoxins on adverse

reproductive health in human populations. AFB_1 in serum (4.8 µM) and semen (5.3 µM) have been linked to abnormalities in sperm count, morphology and motility among infertile males in Nigeria (Ibe et al., 1994; Uriah et al., 2001). Studies have reported high levels of exposure to OTA (Steyn, 1993; Maaroufi et al., 1995) or DON (Sarkanj et al., 2013; Gong et al, 2015; Wells et al., 2016), but there is currently no data on the potential role of these mycotoxins in reproductive health.

ZEN is known to cause adverse reproductive effects in farm animals (Zinedine et al., 2007) and as ZEN and metabolites are known to bind to oestrogen receptors disrupting the binding of 17β-oestradiol (Kuipers et al., 1998), there has been some interest in possible effects on human reproductive health. Mean concentrations of ZEN (167.0±17.1 ng/mL; 0.525±0.054 µM) were detected in endometrial tissues of patients with endometrial hyperplasia (Tomaszewski et al., 1998) while concentration of ZEN (200 – 475 ng/mL; 0.628 - 1.492 µM) have been reported in individuals with breast and cervical cancer (Pillay et al., 2002). A concentration of ZEN at 18.9 - 103 ng/mL (0.059 - 0.324 µM) was associated with early thelarche/mastopathy in young girls (Szuets et al., 1997) while ZEN (0.7235 – 1.1439 ng/mL; 0.002 - 0.004 µM) and α-ZOL (0.1045 -0.1085 ng/mL; 0.00033 - 0.00034 µM) were linked to precocious puberty in young girls from North-West Tuscany, Italy (Massart et al., 2008, 2010). Similar concentrations of ZEN as found in human blood (150 ng/mL; 0.471 µM) caused reduction in sperm concentration and increase in sperm abnormalities in male mice (Zatecka et al., 2014) while ZEN concentration as low as 30 ng/mL (0.094 µM) caused sperm DNA instability in boars (Tsakmakidis et al., 2008).

In sub-Saharan Africa, in addition to mycotoxin exposure, exposure to organochloride pesticides is also common due to poor regulation of their use (Bouwman et al., 2011). The organochloride pesticide 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (p,p'-DDT) is a pesticide/insecticide previously used globally in the control of insects on agricultural crops as well as in mitigating insect vectors of diseases (ATSDR, 2002). Its metabolite, 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (p,p'-DDE) is found in both the biological systems and the environment, and has longer half-life compared to p,p'-DDT (ATSDR, 2002). In humans, p,p'-DDE levels of 232 ng/mL (0.729 µM) has been linked to reduction in testosterone levels and free androgen index (Martin et al., 2002). In agreement with the above study, Ayotte et al. (2001) reported that a mean serum p,p'-DDE of 600 ng/mL (1.887 µM) in Mexican men resulted to decrease in serum and bioavailable testosterone, decreased semen ejaculation volume and reduced sperm count. In subsequent studies, p,p'-DDT and p,p'-DDE had adverse effects on human sperm motility, morphology, count, and semen volume (De Jager et al., 2006; Aneck-Hahn et al., 2007), and reduced the production of reproductive hormones, especially testosterone (Giwercman et al., 2006; Asawasinsopon et al., 2006). Furthermore, an increased risk of birth defects, including reduced anogenital distance, cryptorchidism and urogenital malformation has also been associated with p,p'-DDT (Salazar-Garcia et al., 2004; Bornman et al., 2010) and p,p'-DDE (Longnecker et al., 2002).

As co-exposures of mycotoxins with p,p'-DDT and p,p'-DDE is common in many low and middle income countries, we have investigated the effects of such co-exposures in vitro, at doses consistent with concentrations that have been reported in human blood. The Leydig cells in the males' testes are responsible for producing androgens (testosterone and dihydrotestosterone) and

these hormones play major roles in male sexual differentiation (during embryogenesis), pubertal development, spermatogenesis and other reproductive function (Akingbemi, 2005). The MA-10 Leydig cell line was chosen for this study as it has been recommended as a useful model for assessing Leydig cell function and impacts of environmental toxins on the Leydig cells (Dankers et al., 2013; Roelofs et al., 2014, 2015). To the best of our knowledge, this is the first report of a detailed cytotoxicity assay of single and mixtures of mycotoxins and the pesticides p,p'-DDT and p,p'-DDE using MA-10 murine Leydig cell line as a model.

2. Materials and methods

2.1. Chemicals and cell line

Aflatoxin B_1 (AFB_1; purity ≥ 98%), deoxynivalenol (DON; purity ≥ 98%), zearalenone (ZEN; purity ≥ 98%), ochratoxin A (OTA; purity ≥ 98%), alpha-zearalenol (α-ZOL; purity ≥ 98%) and beta-zearalenol (β-ZOL; purity ≥ 98%), 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (p,p'-DDT; purity ≥ 98%), and 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (p,p'-DDE; purity ≥ 98%), absolute ethanol, dimethyl sulfoxide (99.99%) and charcoal-stripped foetal bovine serum were purchased from Sigma-Aldrich (Dorset, England). The MA-10 Leydig cell line (ATCC® CRL-3050™), Dulbecco's phosphate buffered saline (PBS) and 0.1% Gelatin solution (sterile) were purchased from LGC Standards (Middlesex, UK). The 3-[4,5-dimethylthiazol-2-yl]-2,5-dipenyltetrazolium bromide solution (MTT; 5mg/mL in PBS) and Solubilising reagent [10% sodium dodecyl sulfate (SDS) and 0.01 M hydrochloric acid (HCl)] were purchased from Sigma-Aldrich (Dorset, England). Dulbeco's Modified Eagle Medium/F-12 nutrient mixture (DMEM/F-12) without phenol red, DMEM/F-12 GlutaMax supplement with phenol red, HEPES (1M), TrypLE™ Express, Penicillin-Streptomycin and trypan blue were obtained from Invitrogen™

Life Technologies (Paisley, UK). All other chemicals are of analytical grade and used without further purification or treatment. AFB_1, DON, ZEN, α-ZOL, β-ZOL, OTA, p,p'-DDT and p,p'-DDE were dissolved in DMSO (99.99%) to make stock solutions and working solutions were prepared in assay media to give a DMSO concentration of 0.1% (v/v). The final concentration of DMSO (0.1% v/v single toxin treatment and 0.2% v/v in binary toxins treatment) equivalent to the highest DMSO concentration of working dilutions was tested and results were not significantly different from untreated media controls.

2.2. Cell culture

The MA-10 Leydig cell line (ATCC® CRL-3050™) was routinely cultured in DMEM/F-12 GlutaMax supplement (with phenol red) containing 15% charcoal-stripped foetal bovine serum, 2% HEPES, and 1% penicillin-streptomycin. Cells were maintained in a 75 cm^2 cell culture flask (Corning, Corning Incorporated, New York) at 37 °C in a humidified atmosphere (95%) with 5% CO_2. The flasks were pre-coated at room temperature with sterile 0.1% gelatin solution.

2.3. MTT cytotoxicity assay

The MTT assay have been used widely in in vitro toxicological studies to determine the cytotoxic effects of chemical exposure. The principle of MTT assay is that yellow tetrazollium salt can be converted to a photometrically measurable purple formazan derivative by mitochondrial enzyme succinate dehydrogenase of viable cells (Mosmann, 1983).

MA-10 cells at an 80% confluence were detached from flasks using TrypLE™ Express trypsin. The cells were counted for viability by trypan blue staining using an improved Neubauer counting chamber (Hawksley, Sussex, UK). Then cells were seeded into the sterile 0.1% gelatin-coated 96-well culture plates (Nunc, Roskilde, Denmark) at a density of 3 x 10^4 cells per well in 200 µL cultural media and allowed to attach for 24 hours before treatment with mycotoxins and pesticides. Final concentrations of mycotoxins and pesticides were achieved by adding DMEM/F12 media giving a final DMSO concentration of 0.1% (v/v). Media used to dilute mycotoxins and pesticides were free from phenol red and contained only 5% charcoal-stripped foetal bovine serum in order to avoid hormonal stimulation of cell proliferation which may be induced by phenol and general foetal bovine serum. Cells were treated with individual mycotoxins concentrations from 0.1 µM to 64 µM; AFB_1, DON, ZEN, α-ZOL, β-ZOL and OTA, and pesticides (0.1 - 64 µM); p,p'-DDT and p,p'-DDE and incubated at 37 °C in a humidified atmosphere (95%) with 5% CO_2. Binary mixtures (p,p'-DDT/p,p'-DDE, p,p'-DDT/AFB_1, p,p'-DDT/DON, p,p'-DDT/OTA, p,p'-DDT/ZEN, p,p'-DDT/α-ZOL, p,p'-DDT/β-ZOL, p,p'-DDE/AFB_1, p,p'-DDE/DON, p,p'-DDE/OTA, p,p'-DDE/ZEN, p,p'-DDE/α-ZOL, p,p'-DDE/β-ZOL, AFB_1/DON, AFB_1/OTA, AFB_1/ZEN, AFB_1/α-ZOL, AFB_1/β-ZOL, DON/OTA, DON/ZEN, DON/α-ZOL, DON/β-ZOL, OTA/ZEN, OTA/α-ZOL, OTA/β-ZOL, ZEN/α-ZOL, ZEN/β-ZOL, and α-ZOL/β-ZOL) with concentrations varying from 0.1 µM to 64 µM were also prepared and both the individual and binary mixtures were tested on the cell line for 48 hours. DMSO (0.1% and 0.2% v/v) was used as a vehicle controls for single toxin treatment and binary toxins treatment, respectively. The doses of mycotoxins and pesticides used were based on concentrations reported in human exposure studies as well as in previous in vitro studies.

After 48 hours, 10 µL of MTT solution was added into each well and plates covered with aluminum foil. The plates were incubated for 4 hours at 37 °C in a humidified atmosphere (95%) with 5% CO_2. Thereafter, 100 µL of solubilising reagent (10% SDS and 0.01 M HCL) was added to each well and incubated overnight at 37 °C in a humidified atmosphere (95%) with 5% CO_2. The plates were then read at 540 nm with a reference wavelength of 690 nm using an iEMS microplate reader (Thermo Scientific, Langenselbold, Germany). Viability of each sample was calculated as the percentage (%) absorbance when compared with the absorbance of the 0.1% DMSO (single treatment) or 0.2% DMSO (binary treatment) vehicle control. There was no significant difference between the viability of cells exposed to DMSO (0.1%; 0.2%) vehicle controls and those exposed to media.

2.4. Statistical analysis of data

Each toxin treatment was done in triplicates in three independent experiments. The reported values are mean viability (%) ± standard deviation (Mean±SD) with 0.1% DMSO (for single toxin treatment) or 0.2% DMSO (for binary combinations) as vehicle control. Data were analysed with GraphPad PRISM software version 6.0 (San Diego, CA). Differences between groups were analysed by one-way ANOVA followed by Dunnett's procedure for multiple comparisons. Significant effects are represented by $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). The IC_{50} for the different compounds were determined by fitting dose-response curves using GraphPad PRISM version 6.0.

2.5. Calculation of interactive effects of combined toxins

The effects and interactions of binary mixtures of mycotoxins, p,p'-DDT and p,p'-DDE were calculated using the model of independent joint action and Dose/Concentration-addition or response-addition as described by Clarke et al. (2014). The Combination index-Isobolgram (CI-IS) method as described by Chou (2006) could not be applied because some data sets exceeded 100% and negative values or values greater than 100% growth cannot be entered into CalcuSyn (Biosoft®, Cambridge, UK) or CompuSyn (ComboSyn Inc., USA) softwares, making the calculation of the CI not applicable.

2.5.1. Calculation of expected cell viability

The expected cell viability of binary mixtures was calculated through the addition of the mean viability (%) after exposure to one toxin with the mean viability (%) after exposure to second toxin. Toxins interactions were calculated as described by Clarke et al. (2014) as shown in the formulae below.

The model is as follows:

Mean viability of binary mixtures (expected in % of substance 1 + substance 2) = mean viability (substance 1 in %) + mean viability (substance 2 in %) - 100%.

2.5.2. Calculation of standard error of the mean of measured and expected values

The standard error of mean (SEM) was calculated using the model described by Weber et al. (2005) as shown below:

Expected SEM (substance 1 + substance 2) = $[(\text{SEM of substance 1})^2 + (\text{SEM of substance 2})^2]^{1/2}$

In order to evaluate the effects and interactions of the combined toxins below or above additivity, expected additive values were compared to actually measured values using an unpaired t-test (one tailed), with $p < 0.05$.

The results were interpreted as follows:

- **Additive effects:** measured cell viability values were not significantly above or below the expected values.
- **Synergistic effects:** measured cell viability values were significantly below expected values.
- **Antagonistic effects:** measured cell viability values were significantly above expected values.

3. Results

3.1. Effects of individual mycotoxins and pesticides on cell viability

With single toxin treatment at doses between 0.1 to 64 µM for 48 hours, DON was the most cytotoxic to MA-10 Leydig cells (IC_{50}: 12.3 µM), reducing the cell viability in dose dependent manner compared to 0.1% DMSO vehicle control (**Fig. 1D**). α-ZOL was the second most cytotoxic to MA-10 cells with an IC_{50} of 28.0 µM followed by OTA (IC_{50}: 30.0 µM), ZEN (IC_{50}: 50.0 µM) and β-ZOL (IC_{50}: 62.0 µM). ZEN, α-ZOL and β-ZOL significantly stimulated the proliferation of MA-10 cells ($P \leq 0.01$) at 16 µM (112.0%, 117.0% and 127.0%, respectively) with all the 3 toxins causing strong toxicity at 64 µM, decreasing the cell viability to 18.0%, 11.0% and 47.0%, respectively (**Figs. 1F – 1H**). p,p'-DDT showed slight toxicity between 0.1 and 32 µM, and significantly reduced cell viability to 56.0% at 64 µM ($P \leq 0.0001$). However,

p,p'-DDE and AFB_1 only showed slight toxicity to the MA-10 cells, even at the highest dose tested (64 µM) when compared to 0.1% DMSO control (**Figs. 1A & 1C**).

3.2. Effects of binary combinations of p,p'-DDT or p,p'-DDE with mycotoxins on MA-10 Leydig cell viability

Neither p,p'-DDT nor p,p'-DDE induced much cytotoxicity when the cells were treated with the compound individually, except that at 64 µM p,p'-DDT induced around 40% cell death (**Fig. 1A**). Cytotoxicity at this dose combination (64 µM) was greatly enhanced when cells were treated with p,p'-DDT and p,p'-DDE together (about 95% cell death, **Fig. 3**). For all other compounds tested, co-treatment with p,p'-DDT enhanced toxicity, whereas co-treatment with p,p'-DDE did not, except at high doses. For example, whereas little cytotoxicity was seen for p,p'-DDT or AFB_1 individually below 64 µM (**Figs. 1A, 1C**), a dose response was seen for p,p'-DDT + AFB_1, with significant cytotoxicity at 16 µM ($P \leq 0.0001$; **Fig. 2A**). In contrast, the cytotoxicity of the various mycotoxins was hardly altered by the addition of p,p'-DDE and in cases where it occurred, it was only at the highest doses tested (**data not shown**).

3.3. Effects of binary mycotoxin mixtures on MA-10 Leydig cell viability

OTA did not induce much cytotoxicity up to 16 µM when cells were treated with only this compound, but DON/OTA toxicity was increased at 8 µM - 32 µM compared to DON alone (**Fig. 4**A). OTA also had a synergistic effect on toxicity induced by ZEN, α-ZOL and β-ZOL (**Fig. 5A**). There was no increase in cytotoxicity seen for co-treatment with OTA and AFB_1. Co-treatment with none of the other mycotoxins had a noticeable impact on the cytotoxicity of ZEN,

α-ZOL or β-ZOL. Interestingly, AFB_1, which showed no toxicity up to 32 µM, reduced the toxicity due to DON when cells were treated with both mycotoxins at the same time (**Fig. 3A**). Such a reduction in toxicity was not seen when cells were treated with AFB_1 together with OTA.

3.4. Interactive effects of binary mycotoxin and pesticide mixtures on MA-10 Leydig cell viability

The measured and expected additive combinatory effects of the different binary mycotoxins and/or pesticide treatments at the examined doses can be seen in supplementary file 1 (**Supplementary figs. S1-S4**). It was observed that the combinatory effects of the mycotoxin (ZEN) and pesticide (p,p'-DDT) on MA-10 Leydig cells was synergistic in nature for all the doses tested, except at 64 µM when there was an exhibition of antagonistic effect (**Table 2**). In addition, combinations of DON/OTA, DON/ZEN, DON/α-ZOL, DON/β-ZOL, OTA/ZEN, OTA/α-ZOL, and OTA/β-ZOL were generally additive and synergistic at low concentrations, but antagonistic at 64 µM combinations. In contrast, combinations of p,p'-DDE with all the mycotoxins tested showed antagonistic effects at lower concentrations (1 – 8 µM) but were either additive or synergistic at concentrations ranging from 16 µM to 64 µM combinations (**Table 2**). In general, most of the mycotoxins and pesticides combinations showed antagonistic effects either at low or high doses.

4. Discussion

Mycotoxins co-exposure has been reported in food and feedstuff as well as in human exposure studies worldwide. In addition, simultaneous contamination of agricultural products by mycotoxins and pesticides has been reported (Musaiger et al., 2008; Romero-González et al., 2011). Therefore, this study investigated the individual and combined cytotoxic effect of selected mycotoxins (AFB_1, DON, ZEN, α-ZOL, β-ZOL and OTA) and pesticides (p,p'-DDT and p,p'-

DDE), and to evaluate their interactive effects on MA-10 Leydig cells. These mycotoxins and pesticides were selected because they are common contaminants in food, with reproductive toxicity being indicated in previous studies (Ibe et al., 1994; Yang et al., 1997a, 1997b; Uriah et al., 2000; De Jager et al., 2006; Giwercman et al., 2006; Asawasinsopon et al., 2006; Aneck-Hahn et al., 2007; Chakraborty and Verma, 2009; Schoevers et al., 2010; Frizzell et al., 2011; Zatecka et al., 2014).

4.1. Cytotoxicity of individual mycotoxins on MA-10 Leydig cells

In our experiments we found that DON was the most cytotoxic mycotoxin tested, followed by α-ZOL, OTA, ZEN and β-ZOL, with limited toxicity for AFB_1 at the doses tested. Cytotoxicity to MA-10 cells has previously been reported for both DON and ZEN, with lower IC_{50} values (0.25 µM and 34 µM) than reported here (Savard et al., 2016). The low IC_{50} reported by Savard et al. is possibly as a result of the very low number of cells used (1 X 10^4 cells/well) and the treatment of the cells in the same day without allowing 24 hours incubation for the cells to recover. DON has been shown to be cytotoxic to other cell types including renal proximal tubule epithelial cells, human lung fibroblast and Chinese hamster ovary K1 cells after 48 hours exposure (Konigs et al., 2007; Ruiz et al., 2011). Using WST-1 assay, Vejdovszky et al. (2016) showed that DON was highly cytotoxic to Caco2 cells (IC_{50}: 13.0 µM) while ZEN showed lower cytotoxic effect (IC_{50}: 49.5 µM). These IC_{50} values are similar to the ones reported in this study. Lei et al. (2013) reported that DON induced the highest significant cytotoxic effects on PK-15 cell line, followed by AFB_1 and then ZEN. The reduced cell viability caused by DON was speculated to be as a result of inhibition of DNA synthesis (Ranzenigo et al. 2008; Ndossi et al. 2012). However, we emphasise that the cytotoxicity observed for mycotoxins at concentrations ≥ 32

µM should be interpreted with caution as human beings are unlikely to be exposed to such concentrations in real life.

Although our assay was not set up to specifically measure cell proliferation, our results show that ZEN and its metabolites induced MA-10 cell proliferation at 16 µM. This result is similar to that from a panel of reporter gene assay (RGA) cell lines (MMV-Luc, TARM-Luc, TGRM-Luc and TM-Luc) which demonstrated that ZEN induced cell proliferation, but was in contrast to the effect observed in H295R cell line (Frizzell et al., 2011). We found that for ZEN, α-ZOL and β-ZOL, there was an increase in proliferation at 16 µM dose, but severe toxicity at either 32 µM (α-ZOL) or 64 µM (ZEN and β-ZOL). Savard et al. (2016) also reported that ZEN induced cell proliferation at 10 µM in MA-10 cells. Low concentrations of ZEN and its metabolites (α-ZOL and β-ZOL) are known to induce cell-cycle progression and proliferation in cells that have oestrogen receptors (ER), especially in MCF-7 cells due to their oestrogenic capacity (Dees et al., 1997). However, higher concentrations of ZEN and metabolites were reported to induce apoptosis leading to cell death (Ahamed et al., 2001). It has been reported that ERα/β mRNA and proteins are found in MA-10 Leydig cells (Lin et al., 2014; Milon et al., 2017). Similarly, another Mouse Leydig cell line, BLTK1 (a K1 clone of BLT-1 cells) is also known to express ER-α mRNA (Forgacs et al., 2012). The BLT-1 cells is suggested to have close characteristics with MA-10 cells (Kananen et al., 1996; Rahman and Huhtaniemi, 2004) and therefore, it is possible that the presence of ER in MA-10 cells may have caused the induction of cell proliferation after exposure to oestrogenic ZEN and its metabolites at 16 µM. The significant toxicity of ZEN and its metabolites at higher concentrations could also be attributed to their ability to induce apoptosis through the up-regulation of Bax expression, promotion of

cytochrome c release in cell cytosol, and activation of caspase-3 and caspase-9 in Leydig cells (Wang et al. 2014).

OTA cytotoxicity reported here is similar to that for OTA in other cell lines, including Caco-2 cells, mouse RAW264.7 macrophages and Madin-Darby Bovine Kidney (MDBK) cells (Clarke et al., 2014), Vero cells (Bouslimi et al., 2008; Golli-Bennour et al., 2010) and porcine PK15 cells (Klarić et al., 2008, 2012). The cytotoxic effect of OTA, particularly at high concentrations can be attributed to the fact that OTA has the ability to inhibit protein synthesis (Creppy et al., 1983), impair mitochondrial phosphorylation (Wei et al., 1985; Aleo et al., 1991); disrupt the nuclear factor-erythroid 2 p45-related factor 2 (Nrf2)-dependent pathway resulting to lipid peroxidation, proteolytic stress and oxidative DNA damage (Cavin et al., 2007, 2009; Limonciel and Jennings, 2014), induce cell apoptosis (Schwerdt et al., 1999; Schilter et al., 2005; Rached et al., 2006; Malir et al., 2016), and dysregulate mitosis by interfering with the activity of histone acetyltransferases (Adler et al., 2009; Czakai et al., 2011; Mally, 2012).

In this study, AFB_1 was not very cytotoxic to MA-10 cells, with only a slight increase in toxicity seen above 8 µM. Similar non-cytotoxic behaviour of AFB_1 on Caco-2 and RAW 264.7 cells have been described (Clarke et al., 2014). However, AFB_1 exerted higher toxicity in other studies using intestinal, kidney PK-15 cell line, immune, lung, human umblical vein epithelial, human lung fibroblasts, human bronchial epithelial and Caco-2 cell lines (Liao and Chen, 2005; Mckean et al., 2006a, 2006b; Braicu et al., 2010; Golli-Bennour et al., 2010; Lei et al. 2013; Halbin et al., 2013). The differences in the toxicological effect of AFB_1 in various cells could be attributed to the differences in the capacity of the cell to transform AFB_1 to its toxic metabolite such as

aflatoxin-8,9-epoxide. AFB_1 is usually converted to its toxic metabolites through the stimulation of aryl hydrocarbon receptor (AhR) in the liver or extra-hepatic tissues and subsequent activation of cytochrome P450 (CYP450) enzymes, including CYP1A1, CYP1A2, CYP1B1 and CYP3A4 (Mary et al., 2015). Studies indicate that the CYP1A1 and CYP1B1 mRNA and protein are present in MA-10 Leydig cells in relatively low levels (Mandal et al., 2001; Fan et al., 2010; Deb et al., 2011). This is corroborated by the failure of an AhR agonists such as 2,3,7,8-tetrachlorodibenzo-p-dioxin to induce CYP1B1 mRNA levels in MA-10 cells, but upregulated CYP1B1 mRNA expression in rat liver cells (Deb et al., 2010). Since CYP1A1 and CYP1B1 are regulated by AhR, it is possible that AhR stimulation by AFB_1 does not result in the activation of these CPY450 enzymes in MA-10 cells due to their low levels. Therefore, the low toxicity of AFB_1 observed in MA-10 cells could possibly be due to its non-conversion to toxic metabolites such as AFB1-8,9-epoxide, aflatoxin Q1, aflatoxicol and AFB1-dialdehyde.

4.2. Cytotoxic effects of individual pesticides

The pesticide p,p'-DDT can act through both oestrogen receptor (ER)-dependent and -independent mechanisms (Frigo et al., 2005; Strong et al. 2015). Exposure to p,p'-DDT has been associated with the disruption of reproductive organ morphology and function in domestic roosters (Blomqvist et al., 2006). It also has the ability to induce proliferation of ER-competent cells such as breast cancer cells (MCF-7) in vitro (Mrema et al., 2012). It should be mentioned that p,p'-DDT exposure disrupts ER signalling in the testes; consequently, it has been associated with the disruption of sperm quality (Ayotte et al., 2001; Martin et al., 2002). Its metabolite (p,p'-DDE) is a known antiandrogen and androgen receptor antagonist both in vivo and in vitro, and causes reduction in anogenital distance and increases nipple retention in male rat offspring

exposed in utero (Kelce et al., 1995). p,p'-DDE can also activate the ER and induce cell proliferation in ER-competent cells suggesting it is also a weak oestrogen (Kelce et al., 1995; Strong et al., 2015). Here, we found that p,p'-DDT was cytotoxic to MA-10 cells above 8 µM but limited cytotoxicity was seen with p,p'-DDE up to 64 µM.

4.3. Cytotoxic and interactive effects of binary mixtures of mycotoxins and pesticides

Few studies have reported the effect of exposure of combinations of mycotoxins to different cell lines and none has reported their cytotoxic and interactive effects on MA-10 Leydig cell line in combination with p,p'-DDT and p,p'-DDE. In this study, the cytotoxicity of the most common mycotoxins (AFB_1, DON, ZEN, α-ZOL, β-ZOL and OTA) and pesticides (p,p'-DDT and p,p'-DDE) on MA-10 Leydig cells were evaluated in their binary mixtures.

Although no longer used in most developed countries due to its known toxicity, p,p'-DDT is used officially for disease vector control in some tropical countries, and may be misused in agriculture in certain places. This raises the possibility of co-exposure of humans to p,p'-DDT and mycotoxins. In our experiments, the combination of p,p'-DDT with mycotoxins mostly increased the cytotoxicity of the mycotoxin compared to the mycotoxin alone. In contrast, the combination with p,p'-DDE has generally not increased mycotoxin cytotoxicity. In the presence of DON or OTA, the proliferative effects of ZEN, α-ZOL and β-ZOL were no longer evident, but AFB_1 did not prevent the proliferation induced by certain doses of ZEN or its metabolites. Interestingly, DON cytotoxicity was reduced in the presence of equivalent doses of AFB_1, showing that the results of interactions between mycotoxins can be complex.

AFB_1/OTA showed induction in cell proliferation at 0.1 - 8 µM but was cytotoxic at 16 - 64 µM combinations, although this was not very different to the cytotoxicity of OTA alone at these doses. Clarke et al. (2014) reported significant cytotoxic effect when MDBK cells were exposed to binary mixtures of AFB_1 and OTA after 48 hours of exposure but no cytotoxicity for RAW 264.7 and Caco-2 cells exposed to the same concentration. Additive cytotoxic effect was observed after exposure of AFB_1 + OTA to Green Monkey Vero cells (Golli-Bennour et al., 2010) and HepG2 cells (Corcuera et al., 2011). Our results found that the combination of AFB_1 and DON gave lower cytotoxicity than DON alone, which suggests that for some reason cells exposed to AFB_1 are less susceptible to DON induced cytotoxicity than cells not exposed to AFB_1, although it is difficult to speculate on the possible mechanism for such a result. Nevertheless, similar findings were seen for AFB_1 and ZEN or its metabolites, particularly at 0.1 µM – 16 µM. This contrasts with an additive effect of AFB_1 and DON on Cyprinus carpio primary hepatocytes (He et al., 2010) and a synergistic effect on PK-15 cells (Lei et al. 2013) that have been previously reported. The later study also reported synergy for AFB_1 + ZEN, which was not seen in the MA 10 cells in our study (Lei et al., 2013).

In conclusion, the results of this study indicate that exposure to DON and OTA in particular are toxic to MA-10 Leydig cells, and this toxicity is enhanced by co-exposure with either p,p'-DDT or p,p'-DDE. This raises the possibility that such exposures could contribute to adverse male reproductive health in exposed populations.

Abbreviations

$AFB_{1:}$ aflatoxin B_1; OTA: ochratoxin A; DON: deoxynivalenol; ZEN: zearalenone; α-ZOL: alpha-zearalenol; β-ZOL: beta-zearalenol; p,p'-DDT: 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane and p,p'-DDE: 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-dipenyltetrazolium bromide solution; DMSO: Dimethyl sulfoxide ; PBS: Dulbecco's phosphate buffered saline; CIN: Chronic interstitial nephropathy; FAO: Food and Agricultural Organisation; WHO: World Health Organisation; IC_{50}: half maximal inhibitory concentration; SDS: Sodium dodecyl sulphate; HCl: hydrochloric acid; AhR: aryl hydrocarbon receptor; ER: Oestrogen receptor; ER-α: oestrogen receptor alpha ERβ: oestrogen receptor beta; ANOVA: Analysis of variance

Conflict of interest statement

The authors declare that there are no conflict of interest.

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Table 1. Concentrations of mycotoxins and pesticides used in the study

Toxins	Concentrations (µM)	Concentrations (ng/mL)
p,p'-DDT	0.1	35.449
	1	354.49
	8	2,835.92
	16	5,671.84
	32	11,343.68
	64	22,687.36
p,p'-DDE	0.1	31.803
	1	318.03
	8	2,544.24
	16	5,088.48
	32	10,176.96
	64	20,353.92
DON	0.1	29.632
	1	296.32
	8	2,370.56
	16	4,741.12
	32	9,842.24
	64	18,964.48
OTA	0.1	40.381
	1	403.81
	8	3,230.48
	16	6,460.96
	32	12,921.92
	64	25,843.84
AFB_1	0.1	31.227
	1	312.27
	8	2,498.16
	16	4,996.36
	32	9,992.64
	64	19,985.28
ZEN	0.1	31.836
	1	318.36
	8	2,546.88
	16	5,093.76
	32	10,187.52
	64	20,375.04
α-/β-ZOL	0.1	32.038
	1	320.38
	8	2,563.04
	16	5,126.08
	32	10,252.16
	64	20,504.32

Abbreviations. $AFB_{1:}$ aflatoxin B_1; OTA: ochratoxin A; DON: deoxynivalenol; ZEN: zearalenone; α-ZOL: alpha-zearalenol; β-ZOL: beta-zearalenol; DDT (p,p'-DDT): 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane and DDE (p,p'-DDE): 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene

Table 2. Interactive effects of binary mixtures of mycotoxins and pesticides

Toxin combinations	Dose combinations (μM) and interaction effects of binary mixtures					
	0.1: 0.1	**1 : 1**	**8 : 8**	**16 : 16**	**32 : 32**	**64 : 64**
DDT + DDE	Antagonistic	Antagonistic	Antagonistic	Antagonistic	Antagonistic	Synergistic
DDT + AFB1	Antagonistic	Additive	Antagonistic	Additive	Additive	Antagonistic
DDT + DON	Additive	Additive	Additive	Additive	Synergistic	Antagonistic
DDT + OTA	Additive	Additive	Synergistic	Synergistic	Synergistic	Antagonistic
DDT + ZEN	Synergistic	Synergistic	Synergistic	Synergistic	Synergistic	Antagonistic
DDT + α-ZOL	Additive	Additive	Additive	Additive	Synergistic	Antagonistic
DDT + β-ZOL	Antagonistic	Additive	Additive	Synergistic	Synergistic	Antagonistic
DDE + AFB1	Antagonistic	Antagonistic	Antagonistic	Additive	Additive	Synergistic
DDE + DON	Antagonistic	Antagonistic	Additive	Additive	Additive	Synergistic
DDE + OTA	Antagonistic	Antagonistic	Additive	Additive	Synergistic	Synergistic
DDE + ZEN	Antagonistic	Antagonistic	Antagonistic	Additive	Synergistic	Synergistic
DDE + α-ZOL	Antagonistic	Antagonistic	Antagonistic	Additive	Additive	Additive
DDT + β-ZOL	Antagonistic	Antagonistic	Antagonistic	Additive	Additive	Additive
AFB1 + DON	Antagonistic	Antagonistic	Antagonistic	Antagonistic	Antagonistic	Antagonistic
AFB1 + OTA	Antagonistic	Antagonistic	Antagonistic	Additive	Additive	Antagonistic
AFB1 + ZEN	Antagonistic	Antagonistic	Antagonistic	Additive	Antagonistic	Antagonistic
AFB1 + α-ZOL	Antagonistic	Additive	Antagonistic	Antagonistic	Antagonistic	Antagonistic
AFB1 + β-ZOL	Antagonistic	Antagonistic	Antagonistic	Antagonistic	Antagonistic	Antagonistic

DON + OTA	Additive	Antagonistic	Synergistic	Synergistic	Antagonistic	Antagonistic
DON + ZEN	Additive	Additive	Synergistic	Synergistic	Antagonistic	Antagonistic
DON + α-ZOL	Additive	Additive	Additive	Additive	Antagonistic	Antagonistic
DON + β -ZOL	Additive	Additive	Synergistic	Synergistic	Synergistic	Antagonistic
OTA + ZEN	Additive	Additive	Synergistic	Synergistic	Synergistic	Antagonistic
OTA + α-ZOL	Synergistic	Additive	Synergistic	Synergistic	Antagonistic	Antagonistic
OTA + β -ZOL	Additive	Additive	Synergistic	Synergistic	Synergistic	Antagonistic
ZEN + α-ZOL	Antagonistic	Antagonistic	Antagonistic	Synergistic	Synergistic	Antagonistic
ZEN + β -ZOL	Antagonistic	Antagonistic	Antagonistic	Synergistic	Synergistic	Antagonistic
α-ZOL+ β -ZOL	Antagonistic	Additive	Additive	Synergistic	Synergistic	Antagonistic

Keys. Additive: measured cell viability values were not significantly above or below the expected values; **Synergistic:** measured cell viability values were significantly below expected values; **Antagonistic:** measured cell viability values were significantly above expected values. AFB_1: aflatoxin B_1; OTA: ochratoxin A; DON: deoxynivalenol; ZEN: zearalenone; α-ZOL: alpha-zearalenol; β-ZOL: beta-zearalenol; p,p'-DDT: 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane and p,p'-DDE: 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene

Highlights

- We examined the effects of single and binary mixtures of mycotoxins and pesticides.
- Deoxynivalenol was the most cytotoxic to MA-10 Leydig cells (IC_{50}: 12.3µM).
- Zearalenone and its metabolites at a dose of 16 µM stimulated cell proliferation.
- Aflatoxin B_1 reduced the cytotoxic effects of deoxynivalenol.
- The toxicity of deoxynivalenol, ochratoxin A and zearalenone is enhanced by pesticides**.**

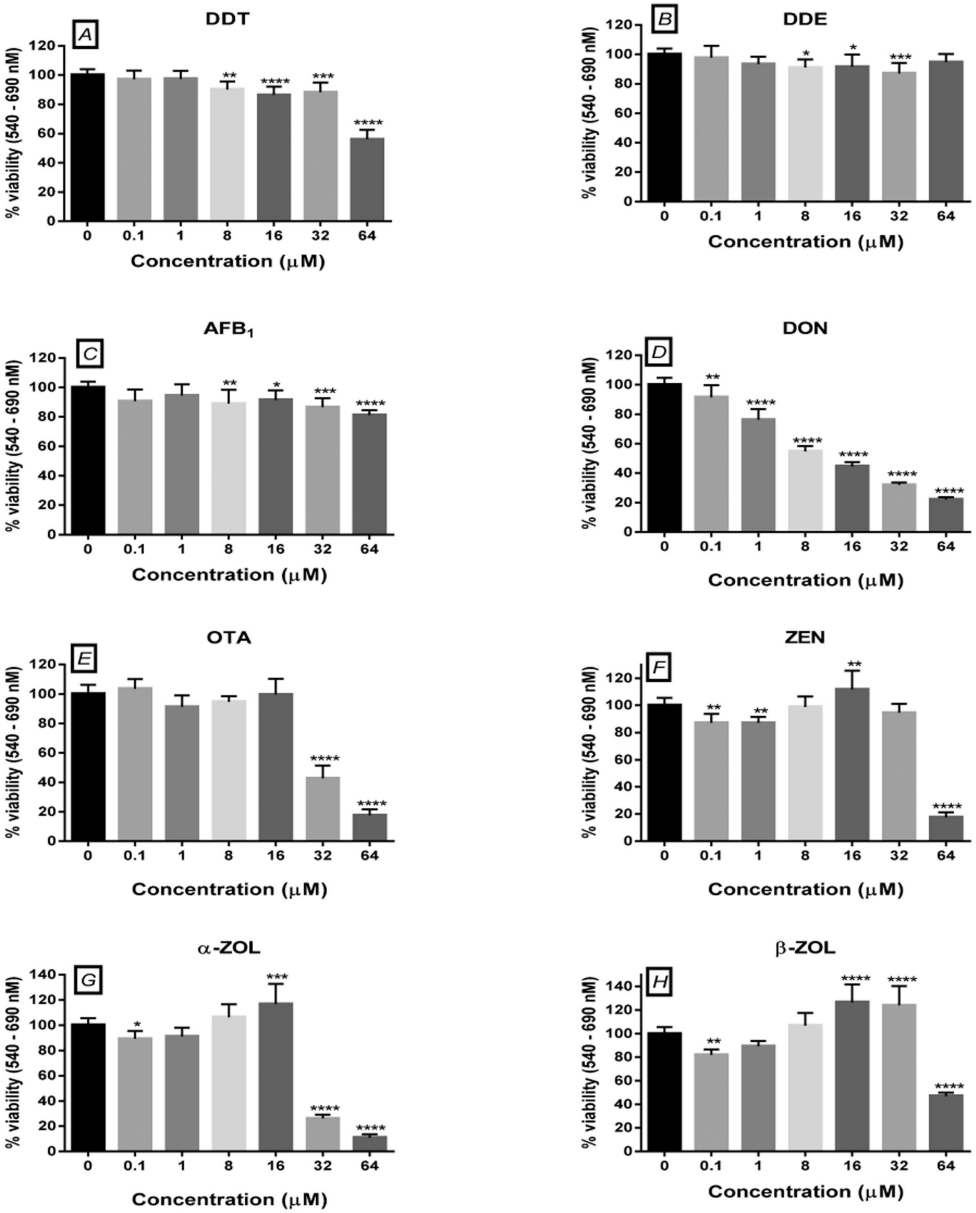
A
DDT
% viability (540 - 690 nM)
Concentration (μM)
B
DDE
C
AFB1
D
DON
E
OTA
F
ZEN
G
α-ZOL
H
β-ZOL
0
0.1
1
8
16
32
64

Figure 1

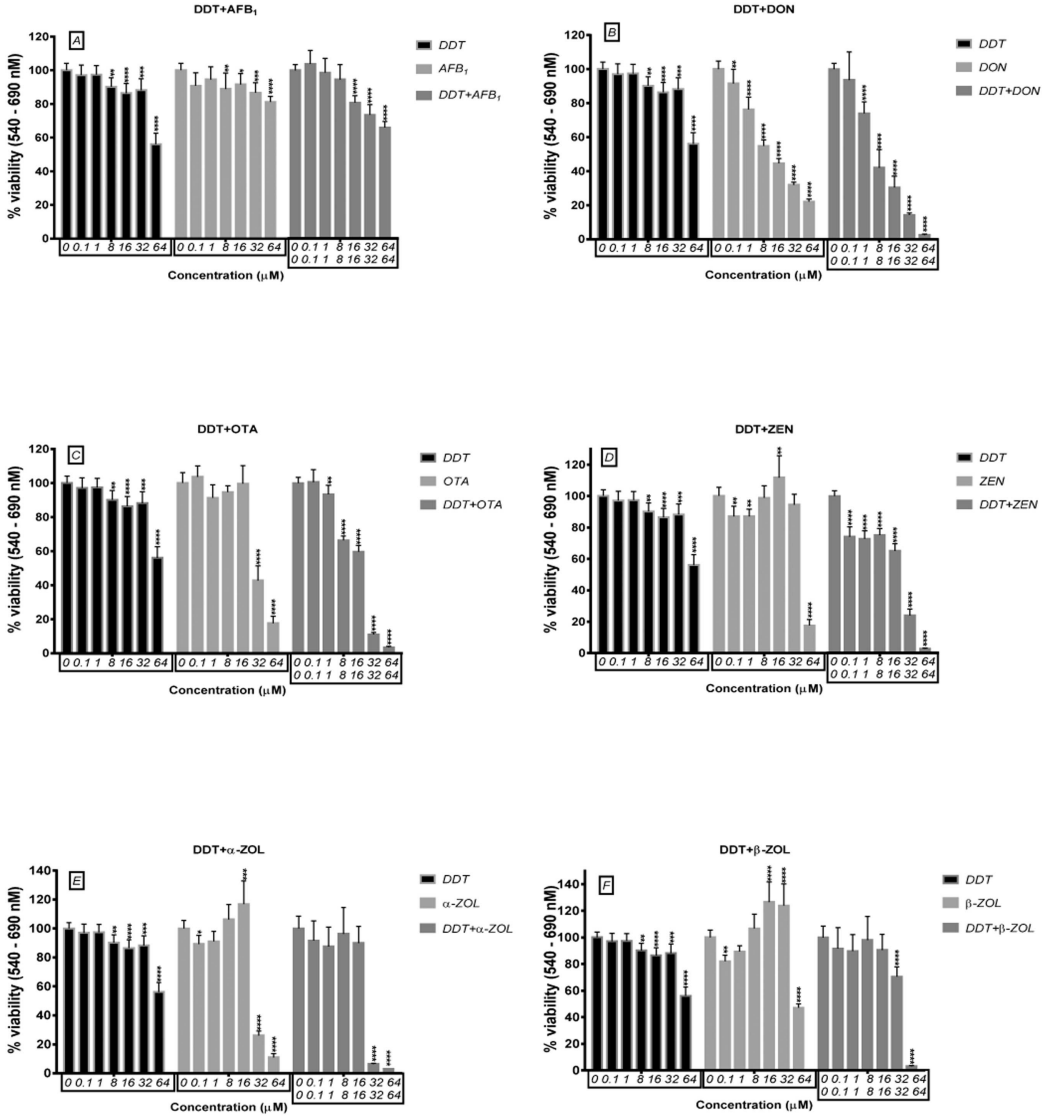
DDT+AFB1
A
DDT
AFB1
DDT+AFB1
% viability (540 - 690 nM)
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
Concentration (μM)
DDT+DON
B
DDT
DON
DDT+DON
% viability (540 - 690 nM)
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
Concentration (μM)
DDT+OTA
C
DDT
OTA
DDT+OTA
% viability (540 - 690 nM)
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
Concentration (μM)
DDT+ZEN
D
DDT
ZEN
DDT+ZEN
% viability (540 - 690 nM)
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
Concentration (μM)
DDT+α-ZOL
E
DDT
α-ZOL
DDT+α-ZOL
% viability (540 - 690 nM)
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
Concentration (μM)
DDT+β-ZOL
F
DDT
β-ZOL
DDT+β-ZOL
% viability (540 - 690 nM)
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
0 0.1 1 8 16 32 64
Concentration (μM)

Figure 2

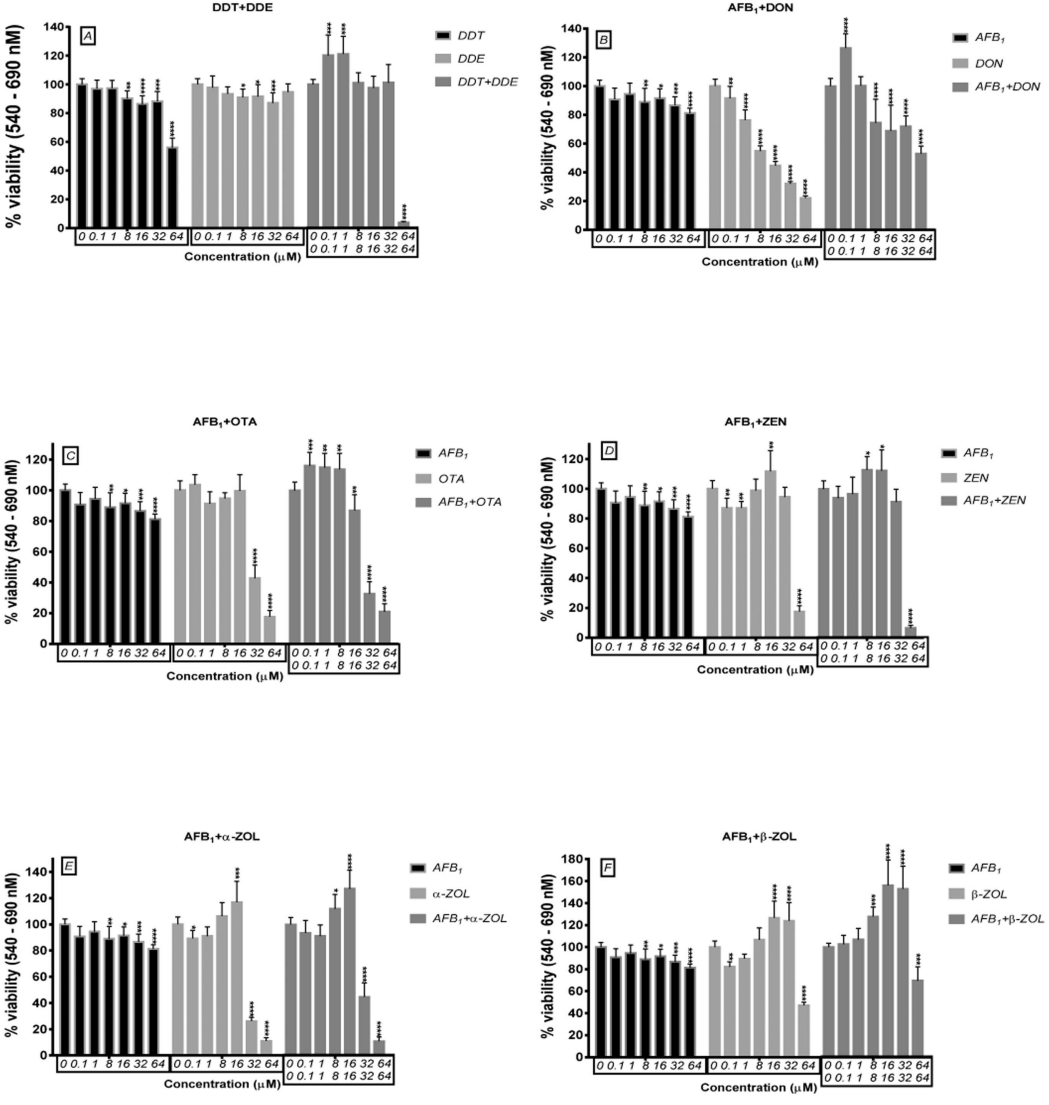
DDT+DDE
A
DDT
DDE
DDT+DDE
% viability (540 - 690 nM)
Concentration (μM)
AFB1+DON
B
AFB1
DON
AFB1+DON
AFB1+OTA
C
AFB1
OTA
AFB1+OTA
AFB1+ZEN
D
AFB1
ZEN
AFB1+ZEN
AFB1+α-ZOL
E
AFB1
α-ZOL
AFB1+α-ZOL
AFB1+β-ZOL
F
AFB1
β-ZOL
AFB1+β-ZOL

Figure 3

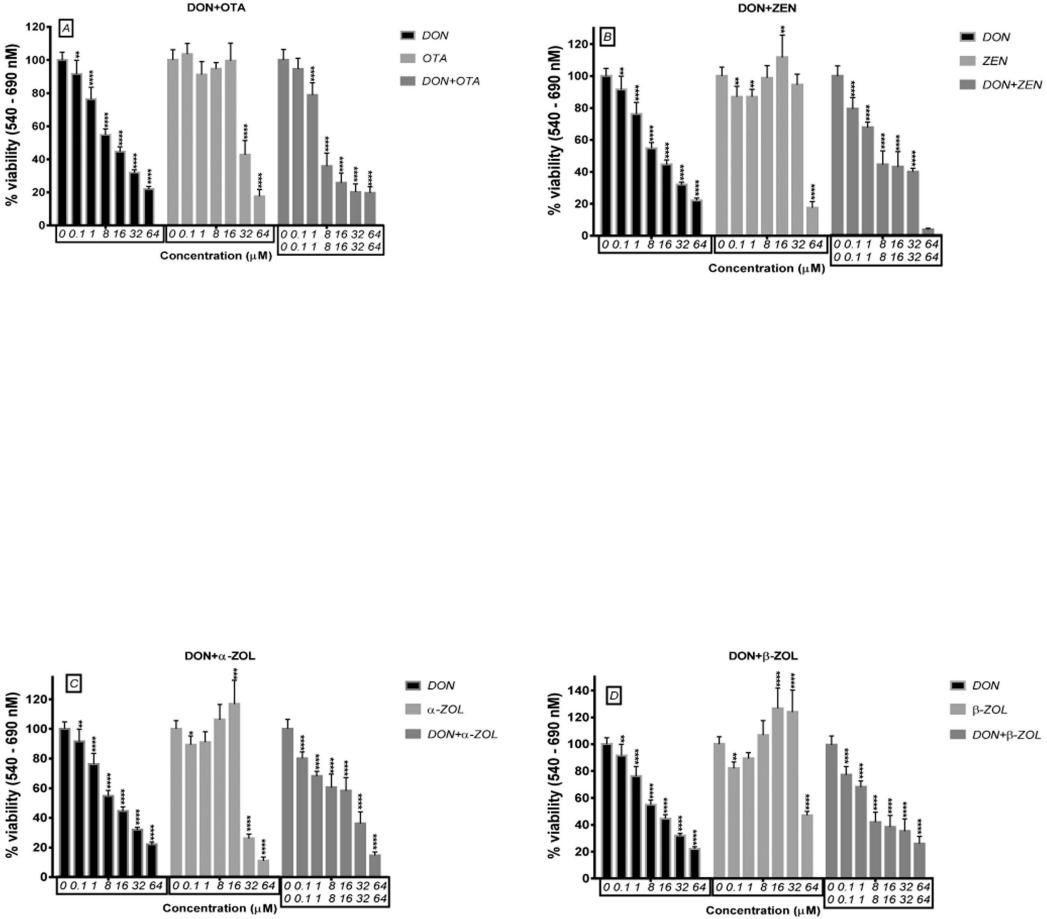
DON+OTA
A
% viability (540 - 690 nM)
Concentration (μM)
DON
OTA
DON+OTA
DON+ZEN
B
ZEN
DON+ZEN
DON+α-ZOL
C
α-ZOL
DON+α-ZOL
DON+β-ZOL
D
β-ZOL
DON+β-ZOL

Figure 4

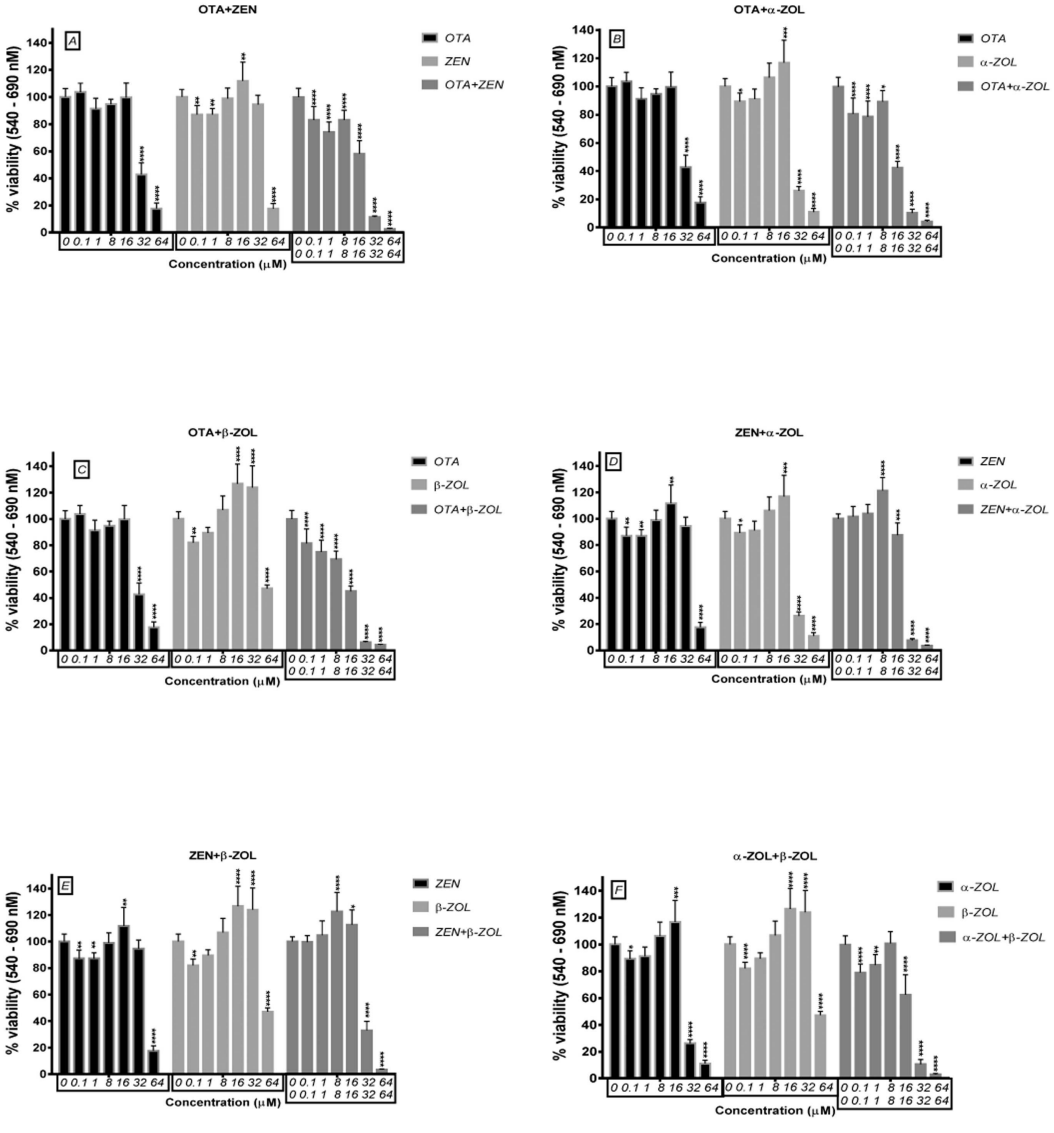
OTA+ZEN
A
OTA
ZEN
OTA+ZEN
OTA+α-ZOL
B
OTA
α-ZOL
OTA+α-ZOL
OTA+β-ZOL
C
OTA
β-ZOL
OTA+β-ZOL
ZEN+α-ZOL
D
ZEN
α-ZOL
ZEN+α-ZOL
ZEN+β-ZOL
E
ZEN
β-ZOL
ZEN+β-ZOL
α-ZOL+β-ZOL
F
α-ZOL
β-ZOL
α-ZOL+β-ZOL
% viability (540 - 690 nM)
Concentration (μM)
0 0.1 1 8 16 32 64

Figure 5