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In vitro effects of single and binary mixtures of regulated mycotoxins and persistent organochloride pesticides on steroid hormone production in MA-10 Leydig cell line

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Abstract

Epidemiological studies have shown strong deterioration in male reproductive health globally due to compromised testosterone production leading to altered spermatogenesis and poor sperm quality. However, the effects and mechanisms through which mycotoxins and persistent organochloride pesticides contribute to poor reproductive health in males remain unclear. The effects of single and binary combinations of ochratoxin A, deoxynivalenol, zearalenone, alpha-zearalenol, beta-zearalenol and 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane on testicular steroidogenesis were evaluated using the MA-10 Leydig cell line after 48 h of exposure. Zearalenone exposure, especially at 16 μM, had a stimulatory effect on progesterone secretion (4.7 ± 0.48 ng/mL compared to 0.60 ± 0.07 ng/mL in control), but inhibited testosterone production after 48 h compared to the solvent control. Ochratoxin A treatment significantly increased both progesterone and testosterone levels. Combination of alpha-zearalenol with beta-zearalenol showed a synergistic stimulation of progesterone hormone level at 1 and 8 μM. The results presented here show that the MA-10 Leydig cell line is a useful model for assessing the effects of xenoestrogens on testicular steroidogenesis. In addition, the inhibitory effects of zearalenone, alpha-zearalenol and beta-zearalenol on testosterone production was enhanced by co-exposure with 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane, further compounding the threat posed by these mycotoxins to male reproductive health.

Key words: mycotoxins; endocrine activity; MA-10 Leydig cells; mixture toxicity; steroidogenesis; reproductive toxicity

1. Introduction

Epidemiological studies have shown a strong deterioration in male reproductive health globally (Kumar et al., 2015; Sengupta et al., 2017). In a recent and robust systematic review and meta-regression analysis, Levine et al. (2017) reported a significant reduction (50 - 60%) in both sperm concentration and total sperm count among men from North America, Europe, Australia and New Zealand from publications between 1973 and 2011. The decline in male reproductive health characterised by poor sperm quality and increased incidences of cryptorchidism, hypospadias and testicular germ cell tumours have been attributed to human exposure to endocrine disrupting chemicals (EDCs) in the environment, food and pharmaceutical products, including pesticides and mycotoxins (Kumar et al., 2011; Skakkebaek et al., 2016; Eze et al., 2016; 2018a).

Steroid hormones are critically essential for the proper development and function of the reproductive organs in both humans and animals. The production of steroid hormones (steroidogenesis) involves the conversion of cholesterol to progestagens by CYP11A1, CYP17A1 and 3β-HSD enzymes, and further metabolism to androgens and oestrogens (Ndossi et al., 2012). Alteration in steroidogenesis has been linked to poor reproductive function and developmental defects (Yeung et al., 2011). Here, we have evaluated the effects of single and co-exposures of a series of mycotoxins (OTA, DON, ZEN, α-ZOL and β-ZOL) and the pesticide p,p'-DDT on testicular steroidogenesis using MA-10 murine Leydig cell line as a model.

Mycotoxins are secondary toxic metabolites produced by some fungi, especially Aspergillus and Fusarium species during their growth on various agricultural commodities and are known to contaminate about 25% of agricultural products worldwide (Rai et al., 2012). Human exposures to multiple mycotoxins have been reported in several population studies (Warth et al. 2013; Shephard et al., 2013; Gong et al., 2015; Shirima et al., 2015). Previous studies have

demonstrated that Fusarium mycotoxins such as deoxynivalenol (DON), zearalenone (ZEN), zearalenol (α-ZOL), and β-zearalenol (β-ZOL) cause reproductive disorders in animals (Cortinovis et al., 2013; Eze et al., 2018a). For instance, ZEN and its derivatives α-ZOL and β-ZOL are known to negatively affect testosterone production, reduce testicular germ cells, cause poor sperm quality, alter the testicular morphology, and impair fertility in exposed mice (Yang et al., 2007; Long et al., 2017) and rats (Kim et al., 2003). Similarly, animal exposure to DON resulted in testicular germ cell degeneration, decrease in absolute cauda epididymal sperm numbers, reduction in caudal epididymal weights, and decline in serum testosterone levels (Sprando et al., 1999, 2005). However, there is little or no data on the potential role of these mycotoxins in human reproductive health.

Pesticides of different chemical categories, including the organochlorides, organophosphates, carbamates and pyrethroids are also widely studied for their endocrine disrupting activity (Wielogorska et al., 2015). The persistent organochloride pesticides (POPs) are of significant importance due to their persistent nature and long half-life which allows them to accumulate in the environment and biological systems in wildlife and human beings (ATSDR, 2002). Among the POPs, dichlorodiphenyltrichloroethane (DDT) and its isomers and metabolites have been of major public health concern due to their adverse effects on reproductive health (ATSDR, 2002). Although banned in many countries, the organochloride pesticide 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (p,p'-DDT) and its major metabolite 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (p,p'-DDE) are still found in the food chain and environment, and human exposure leads to their accumulation in adipose tissues (Mrema et al., 2012). Human exposure to p,p'-DDT and p,p'-DDE have been linked to various health effects such as infertility, poor semen quality, reduced testosterone level, cancer, spontaneous abortion and other reproductive health disorders (Ayotte et al., 2001; ATSDR, 2002; Giwercman et al., 2006; Aneck-Hahn et al., 2007).

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 due to its ability to produce progesterone (P4) and testosterone, and express mRNA for steroidogenic enzymes, including steroid acute regulatory protein (StAR), 17α-hydroxylase/17,20-lyase type 1 (Cyp17a1), cytochrome P450 cholesterol side-chain cleavage enzyme (Cyp11a1), 3β-hydroxysteroid dehydrogenase type 1 (3β-hsd1) and 17β-hydroxysteroid dehydrogenase type 3 (17β-hsd3) (Clewell et al., 2010; Dankers et al., 2013; Roelofs et al., 2015). To the best of our knowledge, this is the first report of a comprehensive study of the effects of single and mixtures of mycotoxins and the pesticide p,p′-DDT on Leydig cell steroidogenesis.

2. Materials and methods

2.1. Chemicals and cell line

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%), absolute ethanol, dimethyl sulfoxide (DMSO; purity: 99.99%), 8-Bromoadenosine 3′,5′-cyclic monophosphate (purity ≥ 98%), 0.1% sterile gelatin solution, Dulbecco's phosphate buffered saline (PBS) and charcoal-stripped foetal bovine serum were purchased from Sigma-Aldrich (Dorset, England). The MA-10 Leydig cell line (ATCC® CRL-3050™) was purchased from LGC Standards (Middlesex, UK). The 3-[4,5-dimethylthiazol-2-yl]-2,5-dipenyltetrazolium bromide solution (MTT; 5 mg/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). Dulbecco's Modified Eagle Medium/F-12 nutrient mixture (DMEM/F-12)

without phenol red, DMEM/F-12 GlutaMax supplement with phenol red, HEPES (1 M), TrypLE™ Express, Penicillin-Streptomycin and trypan blue were obtained from Invitrogen™ Life Technologies (Paisley, UK). DON, ZEN, α-ZOL, β-ZOL, OTA, and p,p′-DDT were dissolved in DMSO 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 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. For experiments, MA-10 cells were cultured in DMEM/F-12 without phenol red containing 15% charcoal-stripped foetal bovine serum, 2% HEPES, and 1% penicillin-streptomycin and maintained for at least 24 hours at 37 °C in a humidified atmosphere (95%) with 5% CO_2 in a 75 cm^2 cell culture flask pre-coated with sterile 0.1% gelatin solution to further starve the cells of hormone.

2.3. Cell viability/cytotoxicity assessment

The cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-dipenyltetrazolium bromide solution (MTT) assay as previously described in Eze et al. (2018b). Briefly, 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 3×10^4 cells per well in 200 µL cultural media and allowed to attach for 24 h before treatment with mycotoxins and pesticides. After 48 h, 10 µL of MTT solution was added into each well and plates covered with aluminum foil. The plates were incubated for 4 h 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. Only treatments that did not reduce cell viability data to below 80% were used for the hormone assay, except for DON (8 and 16 µM) where cell viability of below 80% were found after treatment.

2.4. Treatment of cells for hormone assay

Before the commencement of the experiment, MA-10 cells were grown in T75 flasks with DMEM/F-12 media without phenol red and containing 15% charcoal-stripped foetal bovine serum, 2% HEPES, and 1% penicillin-streptomycin for at least 24 hours to starve the cells of exogenous hormones. All experiments were performed in 24-well cell culture plates (Corning, Corning Incorporated, New York) pre-coated with 0.1% sterile gelatin solution. A 1 mL of cell suspension in DMEM/F-12 without phenol red (containing 15% charcoal-stripped foetal

bovine serum, 2% HEPES, and 1% penicillin-streptomycin) at a concentration of 2×10^5 cells/mL was added to each well and the cells were allowed to attach for 24 h at 37 °C in a humidified atmosphere (95%) with 5% CO_2. After cell attachment, the media were changed and the experiment was commenced. Cells were exposed to individual mycotoxins concentrations from 0.1 μM to 16 μM; DON, ZEN, α-ZOL, β-ZOL and OTA, and p,p′-DDT pesticides (0.1–16 μM) diluted in DMEM/F-12 without phenol red (containing only 5% charcoal-stripped foetal bovine serum, 2% HEPES, and 1% penicillin-streptomycin) for 48 h in the same 24-well cell culture plates in duplicate. Binary mixtures (p,p′-DDT/ZEN, p,p′-DDT/α-ZOL, p,p′-DDT/β-ZOL, OTA/ZEN, OTA/α-ZOL, ZEN/α-ZOL, ZEN/β-ZOL and α-ZOL/β-ZOL) with concentrations varying from 0.1 μM to 8 μM were also prepared and both the individual and binary mixtures were tested on the cell line for 48 h. DMSO at 0.1% and 0.2 % were used as vehicle controls while 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP: 50 μM) was used as positive control. The positive control 8-Br-cAMP activates adenylyl cyclase enzyme and subsequent stimulation of cAMP production which indirectly upregulates steroidogenesis in Leydig cells (Dankers et al., 2013). After 48 h, the cell media supernatants were collected from each well and centrifuged for 5 minutes at 300 RPM to remove cell debris that may be present in the media. These were then transferred into plastic 2 mL vials and stored at -20 °C until ready for hormone analysis. Two independent exposures were carried out for each treatment.

2.5. Hormone quantification

Frozen media supernatants were thawed at room temperature prior to hormone analysis. Enzyme-linked immunosorbent assay (ELISA) kits for testosterone (Catalogue No. 582701, Cayman Chemical Company, Ann Arbor, MI; cross-reactivity: 100% for testosterone, 27. 4% for 5α-dihydrotestosterone, 18.9% for 5β-dihydrotestosterone (DHT), and 3.7% for

androstenedione) and progesterone (P4) (Catalogue No. EIA-1561, DRG Diagnostics, Germany; cross reactivity: P4 - 100%, 17α-hydroxylprogesterone - 0.30%, pregnenolone - 0.35%) were used according to manufacturers' instructions and standards were prepared in cell culture medium used in toxin treatment. All kits consisted of 96-well pre-coated antibody plates for which samples compete for binding with conjugated hormone. Following incubation, the plates were washed and measured at 405 nm for testosterone or 450 nm for P4 using microtitre plate reader (Thermo fisher, UK). Standards (3.9 - 500 pg/mL for testosterone or 0 - 40 ng/mL for P4) were used to generate a standard curve for quantification. Hormone levels were quantified in triplicate for each of the two independent exposures and results were calculated using 4 parameters Logistics curve fit. The inter-assay % coefficient of variation were less than 15% whereas the % coefficient of variation were less than 10%.

2.6 Statistical analysis of data

The hormone concentrations were determined for media supernatants collected from each toxin treatment done in duplicates in two independent experiments. Hormone levels were quantified in triplicates for each of the two independent exposures. The reported values are mean testosterone (pg/mL) ± standard deviation (Mean ± SD) and mean P4 (ng/mL) ± standard deviation (Mean ± SD) of two independent hormone assay with 3 technical replicates each. Difference in the hormone concentration (two independent experiments with 3 technical replicates each) of cells exposed to single toxins with the control (0.1% DMSO for single toxin treatment or 0.2% DMSO for binary combinations) were analysed using one-way ANOVA followed by Dunnett's procedure to correct for multiple comparisons. Significant effects are represented by $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

To determine the interactive effects of binary mixtures, the hormone concentrations were expressed as fold-induction compared to controls. The predictions for the combined treatments were made by assuming additive effects (Groten et al., 2001). In this model, it was assumed that FC_1 and FC_2 are the fold changes for exposure to single treatments 1 and 2, respectively (Ahmed et al., 2019). The additive model then predicts the fold change in their combined treatment to be $FC = FC_1 + FC_2 - 1$. Predicted expected values lower or greater than the measured values are regarded as antagonistic or synergistic, respectively. To test if the expected values were significantly different from the measured values, multiple t-test was performed and correction for multiple comparison was done using Holm-Šídák test method. $p \leq 0.05$ was accepted as significant interactive effects (**Supplementary file**).

3. Results

3.1 Effects of single mycotoxins and pesticide on progesterone (P4) secretion

The basal P4 level in the residual cell culture medium of MA-10 cells treated with DMSO (0.1%) for 48 h was 0.60 ± 0.07 ng/mL. After treatment with the positive control (8-Br-cAMP: 50 µM) for 48 h, the concentration of P4 secreted by the MA-10 cells was increased to 135.50 ± 44.34 ng/mL (680-fold). Generally, the maximum concentration of P4 occurred at 16 µM for p,p'-DDT, ZEN, β-ZOL, DON and OTA whereas peak P4 level was observed at 0.1 µM in α-ZOL with the lowest P4 concentration at 16 µM (**Fig. 1**). Among the tested compounds, the highest dose of ZEN employed (16 µM) induced the maximum P4 level in MA-10 cells followed by OTA at 16 µM (**Fig. 1B & 1F**). P4 production following ZEN exposure for 48 h was significantly increased in all the doses tested compared to the solvent control ($P \leq 0.0001$)

with the highest P4 secretion occurring at 16 μM (4.7 ± 0.48 ng/mL). At 8 and 16 μM of OTA, the level of P4 in MA-10 cells was significantly increased ($P \leq 0.0001$) with the highest concentration occurring at 16 μM of OTA (3.7 ± 0.6 ng/mL). The lowest concentrations of p,p'-DDT (0.1 - 8 μM) had no effect on P4 release in MA-10 cells, but p,p'-DDT (16 μM) significantly ($p \leq 0.001$) induced the level dose-dependently (**Fig. 1A**).

3.2 Effects single mycotoxins and pesticide on testosterone production

The basal testosterone level in the residual cell culture medium of MA-10 cells treated with DMSO (0.1%) was 251.6 ± 20.7 pg/mL after 48 h exposure. Exposure of MA-10 cells with the (8Br-cAMP: 50 μM) positive control induced testosterone level by 21-fold (5233.9 ± 594.63 pg/mL) after 48 h. Following OTA treatment at 0.1, 1 and 8 μM, the level of testosterone increased by approximately 2-fold compared to the vehicle control whereas no significant effect was observed at 16 μM (**Fig. 2F**). Generally, ZEN, α-ZOL, β-ZOL and DON at 8 and 16 μM strongly inhibited testosterone production in exposed cells ($p \leq 0.01$; **Fig. 2B – 2E**). However, there was a slight reduction of testosterone level after p,p'-DDT (8 and 16 μM) treatment, but this was not significantly different from the vehicle control.

3.3 Effects of binary mixtures of mycotoxins and pesticide on Leydig cell steroidogenesis

The concentrations of binary combinations of mycotoxins and pesticides used in these experiments were based on the cytotoxicity data reported previously (Eze et al., 2018b) with combinations that showed at least 80% cell viability at 0.1, 1 and 8 μM chosen to assess the effect of binary combinations of mycotoxins and/or pesticides on MA-10 Leydig cell steroidogenesis. When p,p'-DDT was combined with ZEN, α-ZOL or β-ZOL at equimolar concentrations (0.1 - 8 μM), there was a 1.5 - 3.0 fold increase in P4 secretion, but these effects were not different from the effects observed when cells were treated with p,p'-DDT, ZEN, α-

ZOL or β-ZOL, alone (**Fig. 3A – 3C**). Although combination of OTA with ZEN or α-ZOL had stimulatory effect on P4 production at all the doses tested ($p \leq 0.0001$), these were not significantly different from the effects on P4 release when MA-10 cells were exposed to each of the toxins alone (**Fig. 3D and 3E**). Combined treatment of ZEN and α-ZOL strongly inhibited P4 release at 0.1 and 1 μM ($p \leq 0.0001$) and this was significantly reduced compared to the effects each of the toxins exhibited alone. Interestingly, combination of α -ZOL with β-ZOL showed a synergistic stimulation of P4 hormone level and this was significantly higher than the effects mediated by each toxin in single treatments (**Fig. 3H**).

The concentration of testosterone was significantly reduced ($p \leq 0.0001$) when p,p'-DDT was combined with ZEN, α-ZOL or β-ZOL at equimolar concentrations (0.1 - 8 μM) compared to vehicle control, and these decline were also significantly different from the level of testosterone generated after exposure to each of p,p'-DDT, ZEN, α-ZOL or β-ZOL, alone (**Fig. 4A – 4C**). The stimulatory effect of OTA (0.1 - 8 μM) on testosterone secretion was inhibited by the addition of ZEN or α-ZOL (**Fig. 4D & 4E**). The inhibitory effects observed on testosterone release when ZEN and β-ZOL were exposed to MA-10 cells at 1 μM alone was not seen in co-treatment of ZEN with β-ZOL indicating antagonistic effects (**Fig. 4G**). In addition, the co-treatment of α -ZOL and β-ZOL at 1 μM antagonised the significant inhibitory effects on testosterone production seen when MA-10 cells were exposed to α -ZOL at 1 μM (**Fig. 4H**).

4. Discussion

4.1 MA-10 Leydig cell line produces progesterone and testosterone in both unstimulated and stimulated condition

In this study, we have shown that the MA-10 Leydig cell line can produce substantial amounts of P4 and testosterone both in basal and stimulated condition using our cell culture and treatment modifications. Levels of basal P4 and testosterone were similar to previous studies which reported that 8-Br-cAMP (100 μM) treated MA-10 Leydig cells had increased testosterone concentration in the medium up to 3200 ± 40 pg/ml, and expressed mRNA for steroidogenic enzymes, including StAR, Cyp17a1, Cyp11a1, 3β-hsd1 and 17β-hsd3 (Dankers et al., 2013; Roelofs et al., 2014, 2015).

4.2 Effects of single exposures on progesterone and testosterone production in MA-10 Leydig cells

Here, OTA significantly increased P4 level in MA-10 cells in a dose-dependent manner after 48 h exposure, suggesting that OTA is a potential endocrine disruptor as it interferes with testicular steroidogenesis. This finding is in accordance with a previous study in which 100 ng/mL of OTA significantly increased P4 production and upregulated 3β-hydroxysteroid dehydrogenase type 1 (3β-HSD1) mRNA and protein levels in JEG-3 placental cell line (Woo et al., 2013). In line with previous studies (Fenske and Fink-Gremmels, 1990, Frizzel et al, 2013), OTA (0.1 - 8 μM) did not significantly increase testosterone levels compared to the vehicle control.

In our study, ZEN or its metabolites α-ZOL and β-ZOL increased the ability of MA-10 cells to produce P4 in unstimulated condition. Frizzell et al. (2011) also showed that similar concentrations of ZEN, α-ZOL and β-ZOL (0.1 - 10 μM) significantly induced P4 levels in unstimulated H295R cells after 48 h treatment. We found that ZEN, α-ZOL and β-ZOL significantly inhibited testosterone production in MA-10 Leydig cells in dose-dependent fashion compared to vehicle control. The decreased production of testosterone, and the increased induction of P4 secretion in the MA-10 Leydig cell line exposed to ZEN and its

metabolites could be either as a result of inhibition of Cyp17a1 enzyme involved in the conversion of P4 to testosterone, the upregulation of the 5α-reductase type 1 (srd5α1) enzyme involved in converting testosterone to DHT or due to increased activity of aromatase (cyp19) enzyme resulting to the conversion of more testosterone to oestradiol. Previous studies reported that ZEN and its metabolites inhibited testicular testosterone secretion, impaired spermatogenesis, decreased sperm quality, impaired sperm DNA integrity, induced germ cell degeneration, infertility, and caused perturbation of the genes for enzymes involved in steroidogenesis and ATP-binding cassette efflux (ABC) transporters in vivo and in vitro (Cortinovis et al., 2013; Kim et al., 2003; Cheraghi et al., 2015; Zheng et al., 2016; Bielas et al., 2017; Pang et al., 2017). In males, testosterone level is critical in spermatogenesis, sperm maturation, and sexual function in adults and essential for the masculinisation of the male foetus in utero (Akingbemi et al., 2005). The dysregulation of testosterone biosynthesis in both foetal and adult Leydig cells can cause subsequent sub-fertility or infertility (Skakkebaek et al., 2016).

The present study has also shown that exposure of MA-10 cells treated with 8 µM and 16 µM of p,p'-DDT for 48 h significantly increased P4 synthesis. It is well established that p,p'-DDT and its metabolites act as endocrine disruptors through the alteration of steroidogenic pathway, receptor mediated changes in protein synthesis, and anti-androgenic and oestrogenic activity (Kelce et al., 1995; Crellin et al., 1999; Wójtowicz et al., 2007a, 2007b). However, p,p'-DDT did not significantly alter testosterone synthesis at 48 h of exposure. This is in accordance with several human epidemiological studies which reported non-significant reduction and/or no changes in testosterone levels in Swedish and Latvian men (Hagmar et al., 2001), in previous p,p'-DDT spray-workers (Cocco et al., 2004) and Swedish fishermen (Rignell-Hydbom et al., 2004). However, other human epidemiological studies reported that exposure to p,p'-DDT and

its metabolites was significantly associated with lower testosterone concentrations (Martin Jr et al., 2002; Haugen et al., 2011; Blanco-Muñoz et al., 2012).

4.3 Effects of binary combinations of mycotoxins and pesticide p,p′-DDT on Leydig cell steroidogenesis

Despite the fact that humans and animals are frequently exposed to mixtures of chemical contaminants (including mycotoxins and pesticides), very limited data is available on the combined toxic effects or the 'cocktail effect' of exposure to mycotoxins and/or persistent organochloride pesticides, thus the ability to accurately assess the risks to health of combined exposure is currently inadequate. This is the first study reporting the effects of mycotoxins and pesticide combinations on Leydig cell hormone production. A constant ratio of equimolar concentrations of mycotoxins and/or pesticides were used to ensure comparability between different exposures. We show that co-exposure of p,p'-DDT with ZEN, α-ZOL or β-ZOL caused alteration on Leydig cells steroidogenesis. Co-exposure of α-ZOL and β-ZOL strongly modulated P4 secretion which was significantly different compared with the P4 level after exposure to either α-ZOL or β-ZOL alone. This fact could lead to the conclusion that the effects observed after co-exposure to α-ZOL and β-ZOL are due to the synergistic interaction occurring between the two mycotoxins. This is possibly due to their ability to activate ER transcriptional activity and increase PR mRNA and protein expression (Frizzell et al., 2011) as the MA-10 cell line expresses both E2 and P4 receptor (Milon et al., 2017). In bovine small-follicle granulosa cells, co-treatment of α-ZOL and β-ZOL to similar concentrations used in our study had inhibitory effect on E2 production, but had no significant effect on P4 production (Pizzo et al., 2016). The combination of p,p'-DDT with ZEN, α-ZOL or β-ZOL significantly inhibited Leydig cell testosterone production when combined at equimolar concentrations (0.1 - 8 μM) compared to vehicle control, and this decline was also significantly different from the

level of testosterone generated after exposure to each of p,p'-DDT, ZEN, α-ZOL or β-ZOL, alone. This raises the potential for co-exposure to ZEN, α-ZOL or β-ZOL with p,p'-DDT to pose a health threat to male reproductive health.

4.4 Single mycotoxins and pesticides as well as their combinations exhibit biphasic hormonal response

A few biphasic hormonal responses were observed with an increase in either testosterone or progesterone production in low mycotoxin and/or pesticide exposure whereas at high exposures, the concentration of testosterone and progesterone declined. Low doses of α-ZOL, β-ZOL, p, p'-DDT/ α-ZOL, p, p'-DDT/ β-ZOL and α-ZOL/β-ZOL induced progesterone production whereas low doses of OTA and α-ZOL/β-ZOL induced the secretion of testosterone in MA-10 Leydig cells. However, a decrease in progesterone and testosterone concentrations were observed in high-dose exposure to these single chemicals and/or combinations. Kolle et al. (2012) also observed that ZEN had a biphasic effect on E2 production in H295R cells with increased production occurring at lower doses (0.03 μM - 10 μM) whereas E2 level decreased at 30 μM. It has been reported previously that chemicals can stimulate responses at low doses and block such responses at high doses resulting to biphasic effects (Welshons et al., 2003). There are possible mechanisms through which the biphasic hormonal effects occur, namely (i) activation of G-protein-coupled oestrogen receptor (GPER), instead of the classical nuclear ER receptors, (ii) dysregulation of the activity of the enzymes involved in steroidogenesis, and (iii) disruption of the gene expression of molecules that regulate lipid homeostasis and steroidogenesis. For instance, GPER activation is known to stimulate cAMP production through the activation of adenylyl cyclase enzyme (Filardo et al., 2002; Prossnitz and Barton, 2014), which in turn results in the stimulation of steroidogenesis (Dankers et al., 2013). Many xenoestrogens such as ZEN, p, p'-DDT and o, p'-DDT are known to readily bind and/or activate

GPER (Thomas and Dong, 2006). It is also known that ZEN mediate oestrogen feedback through GPER and not through the genomic pathway involving ER α/β (Zheng et al., 2019). In addition, ZEN increased intracellular cAMP levels in primary mouse Leydig cells at low doses and decreased cAMP levels in cells exposed to relatively high doses (Liu et al., 2014). Low doses of ZEN induced testosterone production whereas high dose ZEN inhibited testosterone secretion in mouse primary Leydig cells in vitro (Liu et al., 2014), indicating that the biphasic effects observed could be mediated through the GPER and cAMP.

In conclusion, the results presented here show that the MA-10 Leydig cell line is a useful model for complementing the H295R cell line in assessing the effects of chemical contaminants on testicular steroidogenesis. The findings of this study indicate that exposure to p, p'-DDT, ZEN, α-ZOL, β-ZOL, OTA and DON at concentrations relevant to human exposure could cause dysregulation of Leydig cell steroidogenesis, especially during the critical developmental periods. In addition, the toxicity of ZEN, α-ZOL and β-ZOL on Leydig cell steroidogenic function is enhanced by co-exposure with p,p'-DDT pesticide, further compounding the threat posed by these mycotoxins to male reproductive health.

Abbreviations

OTA: ochratoxin A; α-ZOL: alpha-zearalenol; β-ZOL: beta-zearalenol; p,p′-DDT: 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane; EDCs: endocrine disrupting chemicals; StAR: steroid acute regulatory protein; Cyp17a1: 17α-hydroxylase/17,20-lyase type 1; Cyp11a1: cytochrome P450 cholesterol side-chain cleavage enzyme; 3β-hsd1: 3β-hydroxysteroid dehydrogenase type 1; 3β-hsd2: 3β-hydroxysteroid dehydrogenase type 2; 17β-hsd3: 17β-hydroxysteroid dehydrogenase type 3; LH: luteinising hormone; FSH: follicle-stimulating hormone; PBS: Dulbecco's phosphate buffered saline; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-

dipenyltetrazolium bromide solution; SDS: sodium dodecyl sulfate; HCl: hydrochloric acid; DMEM/F-12: Dulbecco's Modified Eagle Medium/F-12 nutrient mixture; ANOVA: analysis of variance; DHT: 5β-dihydrotestosterone; DMSO: dimethyl sulfoxide; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid solution; 8-Br-cAMP: 8-Bromoadenosine 3′,5′-cyclic monophosphate; ELISA: Enzyme-linked immunosorbent assay; P4: Progesterone; E2: oestradiol/17β-oestradiol; IGF1: insulin-like growth factor 1; srd5α1: 5α-dihydrotestosterone; GPER: G-proten-coupled oestrogen receptor; ERα/β: Oestrogen receptor alpha/beta; mRNA: Messenger ribonucleic acid; RPM: Revolution per minute; TBBPA: tetrabromobisphenol A; T_3: triiodothyronine 3; T_4: thyroxine; LH: WHO: World Health Organisation

Conflict of interest statement

The authors declare that there are no conflict of interest.

Acknowledgements

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Appendix

Supplementary file: The interactive effects of combined mycotoxins and/or persistent organochloride pesticides on progesterone (P4) and testosterone production in MA-10 Leydig cells

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FIGURES

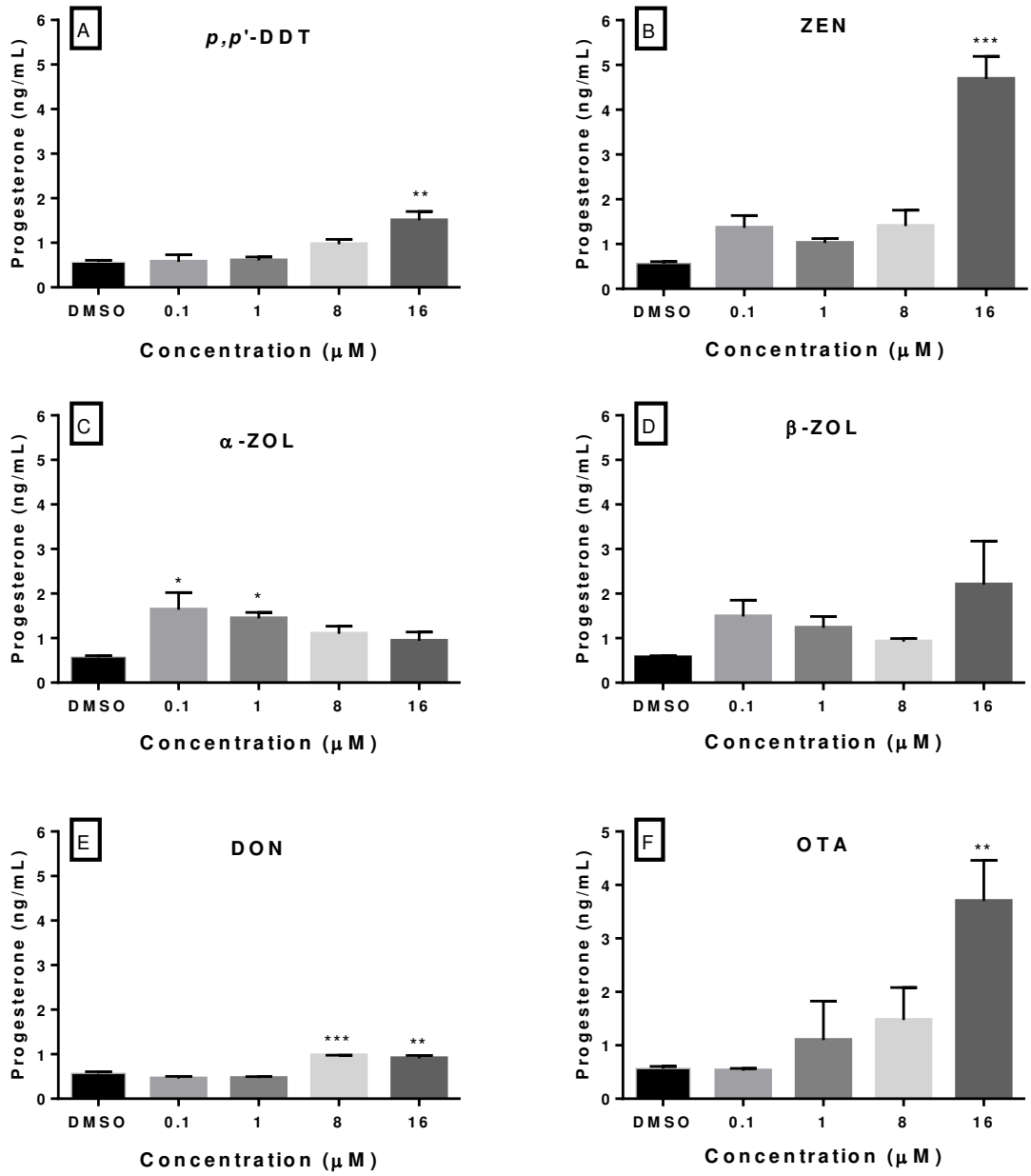

Fig. 1. The effects of single mycotoxins and pesticides exposure on the progesterone (P4) production in MA-10 Leydig cells. Cells were exposed to the compounds for 48 h and cell media supernatants were collected for P4 analysis using enzyme-linked immunosorbent assay (ELISA). Each value on the graph is the mean of two independent experiments with three technical replicates each and error bars show the standard deviation (SD) of two biological replicates. Data were analysed by comparing treatments and vehicle control using one-way ANOVA and corrected for multiple comparison with Dunnett's test. $p \leq 0.05$ (*), $p \leq 0.01$

(**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant effects. DON: deoxynivalenol; OTA: ochratoxin A; α-ZOL: alpha-zearalenol; β-ZOL: beta-zearalenol; p,p'-DDT: 1,1,1-trichloro-2,2-bis(**p**-chlorophenyl) ethane

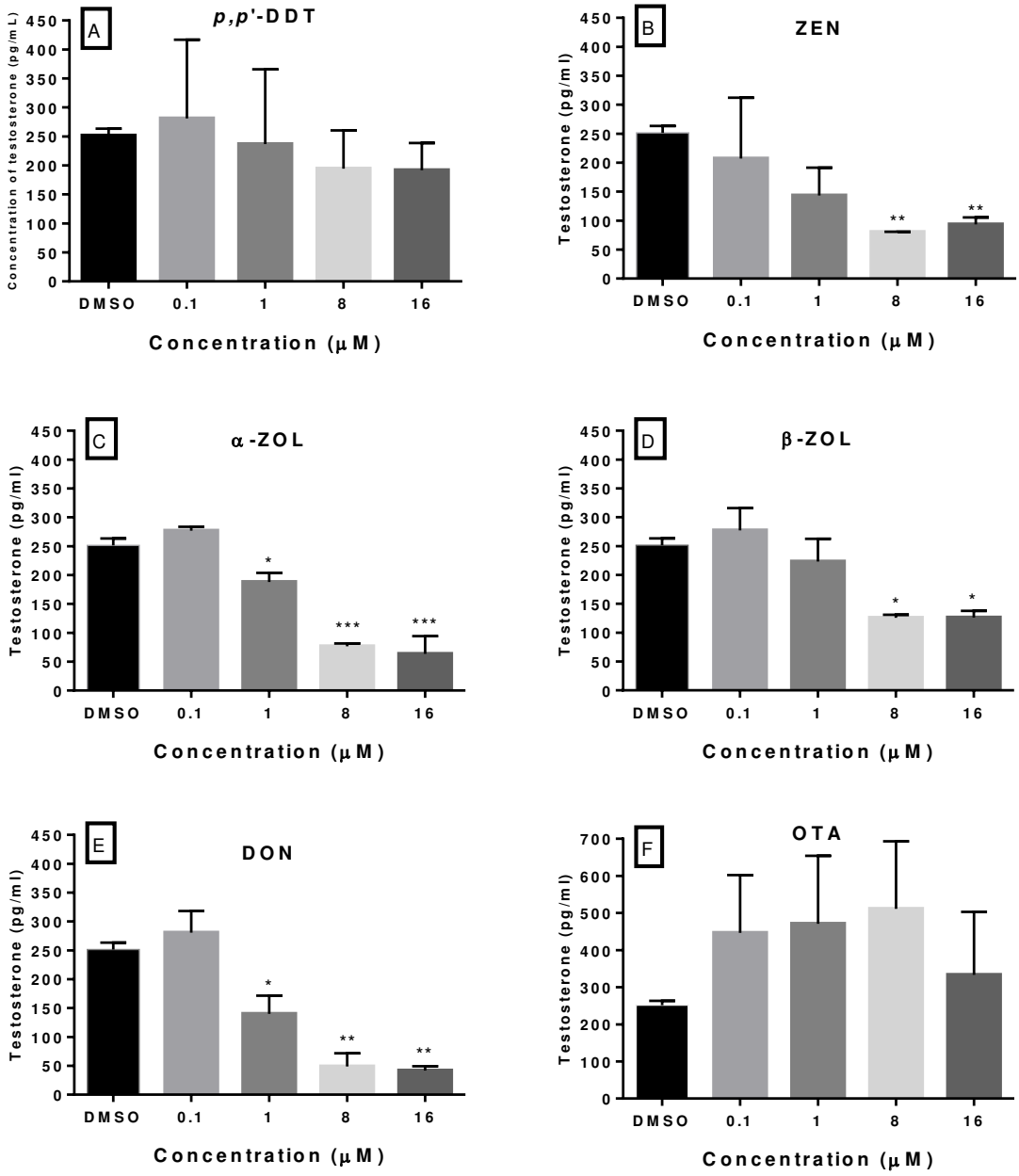

Fig. 2. The effects of single mycotoxins and pesticides exposure on the testosterone production in MA-10 Leydig cells. Cells were exposed to the compounds for 48 h and cell media supernatants were collected for testosterone analysis using enzyme-linked immunosorbent assay (ELISA). Each value on the graph is the mean of two independent

experiments with three technical replicates each and error bars show the standard deviation (SD) of biological replicates. Data were analysed by comparing treatments and vehicle control using one-way ANOVA and corrected for multiple comparison with Dunnett's test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant effects. DON: deoxynivalenol; OTA: ochratoxin A; α-ZOL: alpha-zearalenol; β-ZOL: beta-zearalenol; p,p'-DDT: 1,1,1-trichloro-2,2-bis(**p**-chlorophenyl) ethane

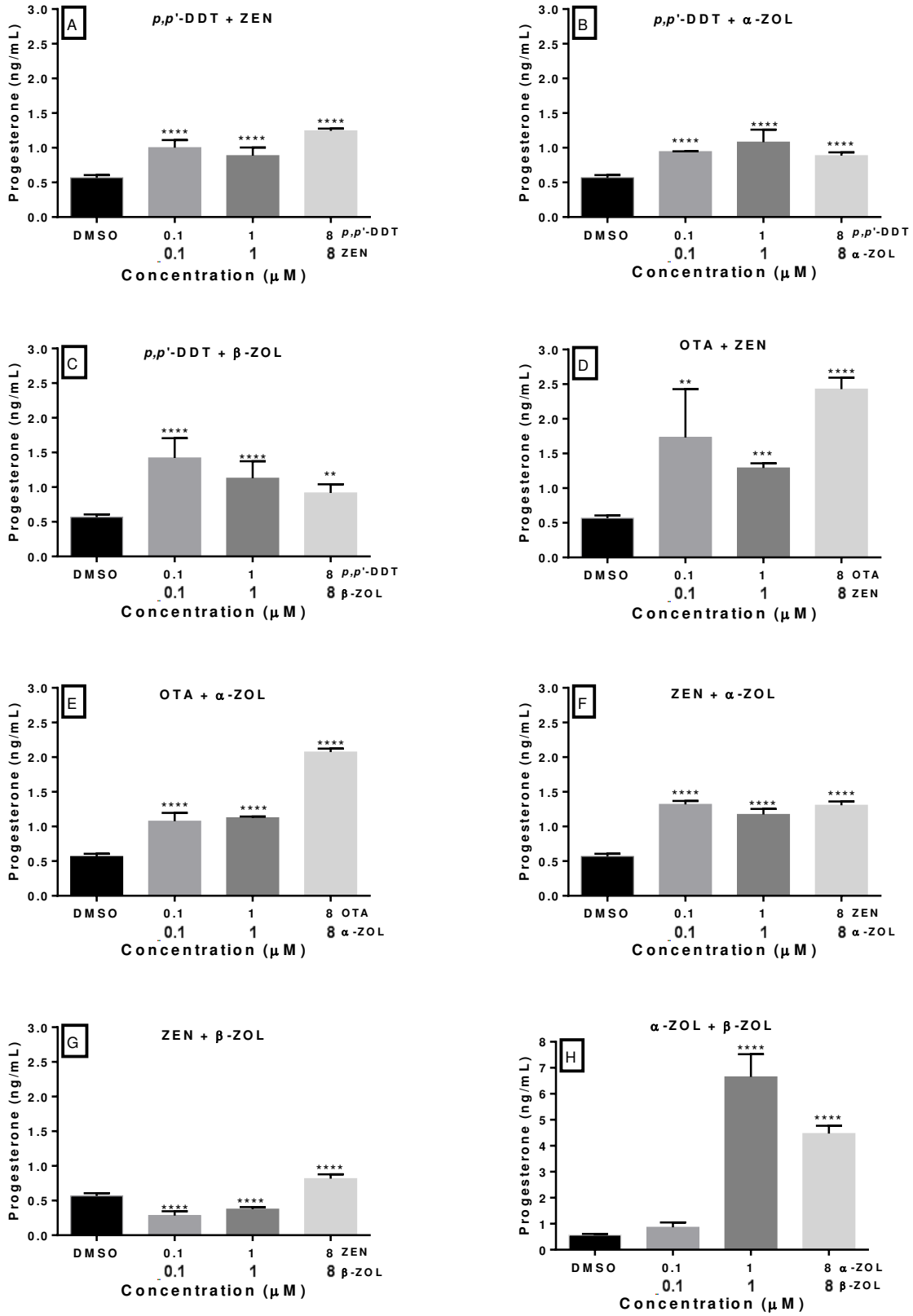

Fig. 3. The effects of mycotoxins and pesticide combinations on the progesterone (P4) production in MA-10 Leydig cells. Cells were exposed to the compounds for 48 h and cell media supernatants were collected for P4 analysis using enzyme-linked immunosorbent assay

(ELISA). Each value on the graph is the mean of at least 2 independent experiments and error bars show the standard deviation (SD) of biological replicates. Data were analysed by comparing treatments and vehicle control using one-way ANOVA and corrected for multiple comparison with Dunnett's test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant effects. OTA: ochratoxin A; α-ZOL: alpha-zearalenol; β-ZOL: beta-zearalenol; p,p'-DDT: 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane

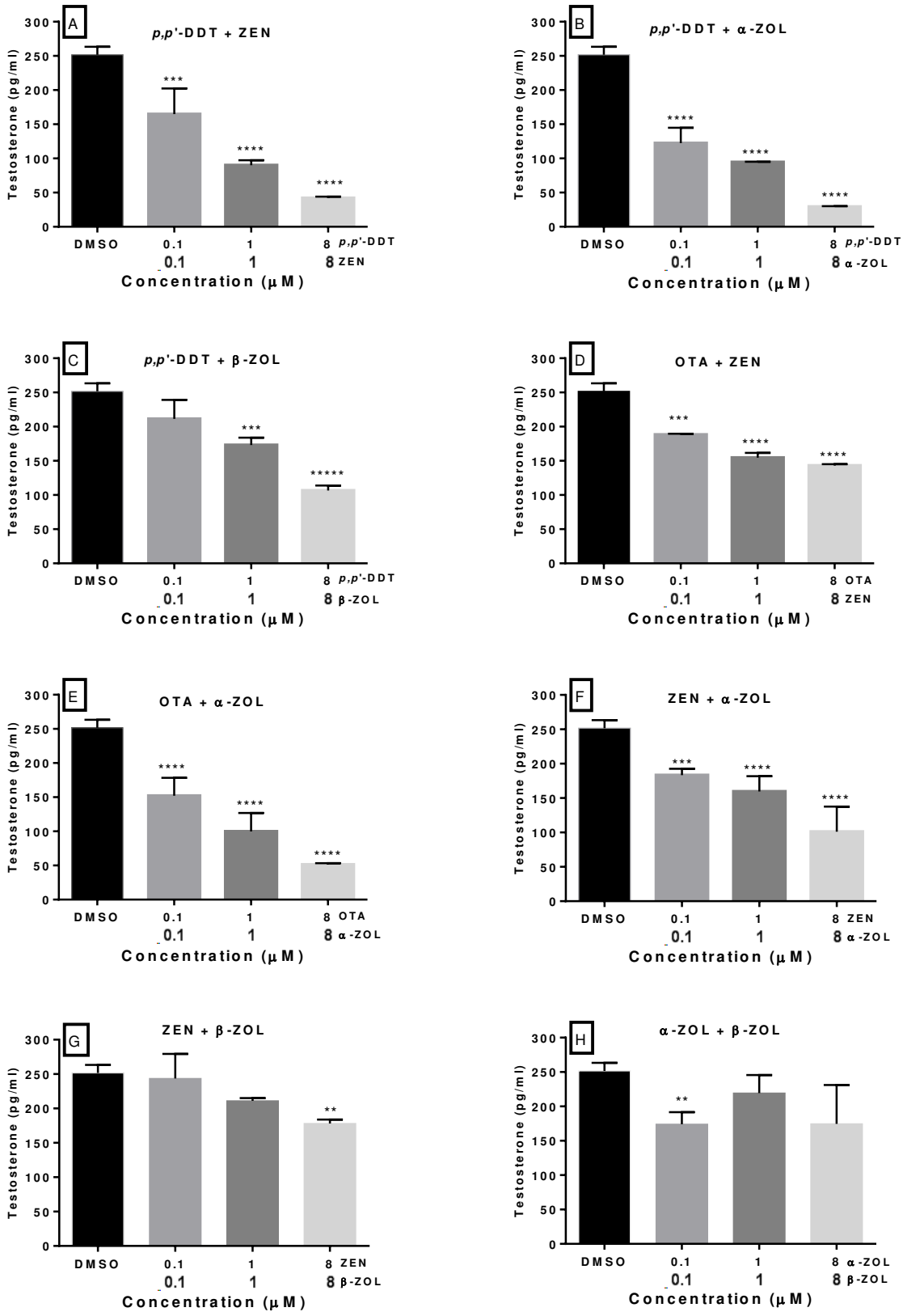

Fig. 4. The effects of mycotoxins and pesticide combinations on the testosterone production in MA-10 Leydig cells. Cells were exposed to the compounds for 48 h and cell media supernatants were collected for testosterone analysis using enzyme-linked immunosorbent

assay (ELISA). Each value on the graph is the mean of at least 2 independent experiments and error bars show the standard deviation (SD) of biological replicates. Data were analysed by comparing treatments and vehicle control using one-way ANOVA and corrected for multiple comparison with Dunnett's test. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant effects. OTA: ochratoxin A; α-ZOL: alpha-zearalenol; β-ZOL: beta-zearalenol; p,p'-DDT: 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane

In vitro effects of single and binary mixtures of regulated mycotoxins and persistent organochloride pesticides on steroid hormone production in MA-10 Leydig cell line

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Toxicology In Vitro

Supplementary file. The interactive effects of combined mycotoxins and/or persistent organochloride pesticides on progesterone (P4) and testosterone production in MA-10 Leydig cells

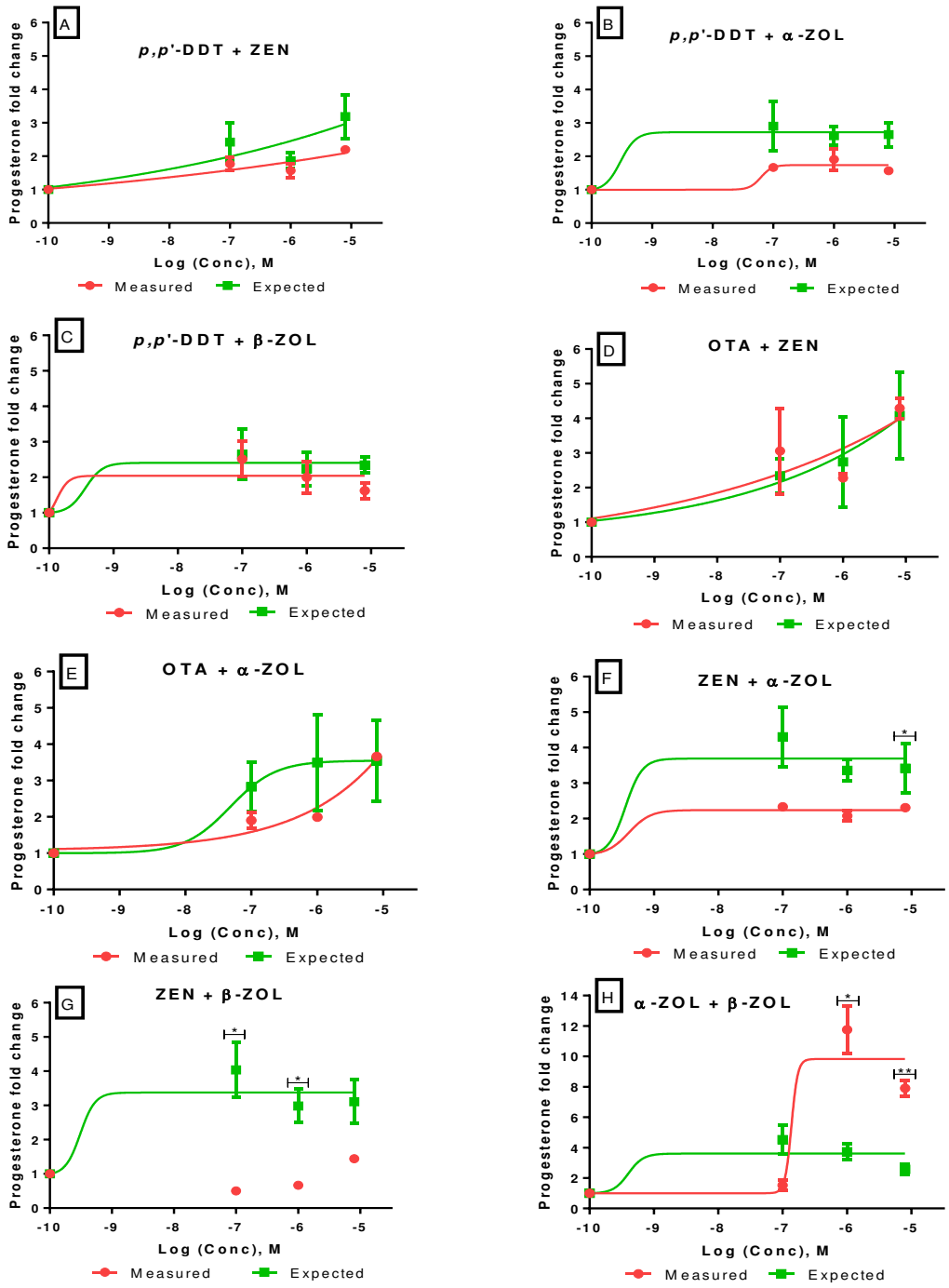

Fig. S1.1. Measured and expected fold change in progesterone (P4) production in MA-10 Leydig cells exposed to binary mixtures of p,p′-DDT or OTA with ZEN and its metabolites. Test substances were assayed in triplicate in two independent experiments. Data were analysed with multiple t-test and corrected for multiple comparison using the Holm-Šídák test method. Error bars represent standard deviation of two biological replicates. $p \leq 0.05$ (*),

$p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects.

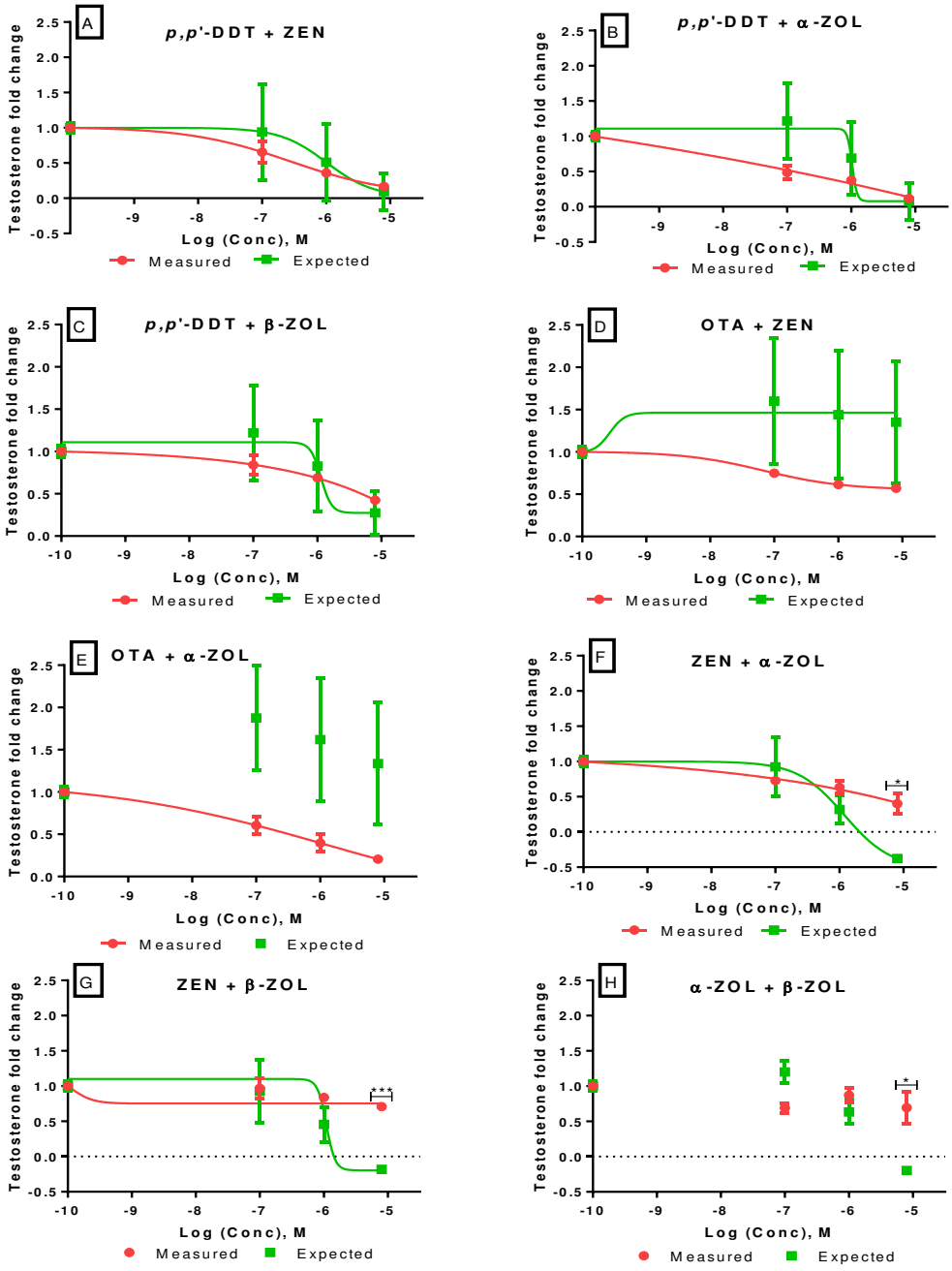

Fig. S1.2. Measured and expected fold change in testosterone production in MA-10 Leydig cells exposed to binary mixtures of p,p′-DDT or OTA with ZEN and its metabolites. Test substances were assayed in triplicate in two independent experiments. Data were analysed with multiple t-test and corrected for multiple comparson using the Holm-Šídák test method. Error bars represent standard deviation of two biological replicates. $p \leq 0.05$ (*), $p \leq 0.01$

(**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) represent significant deviation from additive effects.

Highlights

- The effects of single and binary mixtures of mycotoxins and pesticides on testicular steroidogenesis was evaluated
- MA-10 Leydig cell line secreted progesterone and testosterone both in unstimulated and stimulated condition
- MA-10 Leydig cell line is a useful model for testicular steroidogenesis
- Ochratoxin A modulated both progesterone and testosterone production
- Zearalenone and metabolites α-and β-zearalenol inhibited testosterone secretion
- Inhibitory effects of zearalenone, α-and β-zearalenol on testosterone release is enhanced by pesticides