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# Oxidative damage and disturbance of antioxidant capacity by zearalenone and its metabolites in human cells

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ABSTRACT

Mycotoxin contamination of foods and feeds represent a serious problem worldwide. Zearalenone (ZEA) is a secondary metabolite produced by *Fusarium* species. This study explores oxidative cellular damage and intracellular defense mechanisms (enzymatic and non-enzymatic) in the hepatoma cell line HepG2 after exposure to ZEA and its metabolites (α-zearalenol, α-ZOL; β-zearalenol, β-ZOL). Our results demonstrated that HepG2 cells exposed to ZEA, α-ZOL or β-ZOL at different concentrations (0, 6.25, 12.5 and 25 μM) showed: (i) elevated ROS levels (1.5- to 7-fold) based on the formation of the highly fluorescent 2′,7′-dichlorofluorescein (DCF), (ii) increased DNA damage measured by the comet assay (9–45% higher), (iii) decreased GSH levels and CAT activity (decreased by 54%–25% and by 62%–25% for GSH and CAT, respectively) and (iv) increased GPx and SOD activities (increased by 50%–90% and by 26%–70%, respectively), compared to untreated cells. Our results suggest that mycotoxin-induced oxidative stress and damage may play a major role in the cytotoxic effects of ZEA and its metabolites. GSH and endogenous enzymes function together in protecting cells from ROS and the consequent damage after mycotoxin exposure. ZEA has a lower capacity to induce oxidative stress and damage in HepG2 cells than its metabolites at the tested concentrations.

## 1. Introduction

Mycotoxins are biologically active products produced as secondary metabolites by certain fungal species. Fungal invasion of field crops and mycotoxin contamination of foods and feeds represent a serious problem worldwide. *Fusarium* is one of the main genus implicated in producing mycotoxins. It is known that different fungal species may produce the same mycotoxin and one species may produce different mycotoxins at the same time. Thus, field crops and derived processed food can be simultaneously contaminated with several mycotoxins and may enter the food chain, thus adversely affecting the health of both animals and humans (EFSA, 2011; Fernández-Blanco et al., 2014; Zinedine et al., 2007).

Zearalenone (ZEA) is a resorcylic acid lactone derivate produced by *Fusarium* fungi. The ZEA and its derivatives (α-zearalenol [α-ZOL]; β-zearalenol [β-ZOL]) have structural analogy to estrogen. The estrogenic activity of ZEA and its metabolites has been determined both *in vivo* and *in vitro* (Minervini et al., 2005; Parveen et al., 2009; Frizzell et al., 2011; Busk et al., 2012; Cortinovis et al., 2013). However, the toxicity by ZEA and its metabolites is not only due to the previously mentioned estrogenic effect, but other mechanisms such as oxidative stress and damage induced by these compounds may be important mediators involved in their toxicity (Wu et al., 2014). Previous studies have reported that mycotoxins exposure may lead to the production of reactive oxygen species (ROS), which can result in oxidative stress (Hassen et al., 2007; Prosperini et al., 2013; Fernández-Blanco et al., 2014). Oxidative stress is a state of imbalance between the antioxidant defense and ROS or radical production, so that an excess of ROS can cause oxidative damage to membrane lipids (lipid peroxidation, LPO), proteins, and DNA, which ultimately may lead to cell death (Dinu et al., 2011; Mallebrera et al., 2016; Tatay et al., 2016). Oxidative stress can result in an up-regulation of the antioxidant defense - *i.e.* enzymatic

*Abbreviations:* $H_2$-DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; DCF, 2′,7′-dichlorofluorescein; CAT, catalase; (liver) cells, CCL13; CHO-K1 cells, Chinese hamster ovary cells; DON, deoxynivalenol; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; GPx, glutathione peroxidase; GSH, glutathione; HepG2, hepatoma cell line; SH-SY5Y, human neuroblastoma cells; liver cells, L02; LMP agarose, low melting point agarose; NEAAs, non-essential amino acids; PBS, phosphate buffered saline; PI, propidium iodide; ROS, reactive oxygen species; SOD, superoxide dismutase; MTT, tetrazolium salt; ZEA, zearalenone; α-ZOL, α-zearalenol; β-ZOL, β-zearalenol

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activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and the antioxidant cellular component glutathione (GSH) - as a protective response to maintain cellular viability (Fernández-Blanco et al., 2014; Mallebrera et al., 2014).

Often more than one mycotoxin is found on a contaminated substrate (food and feed). Exposure to mycotoxins in humans and animals is mostly *via* ingestion of contaminated foods (Bennett and Klich, 2003). ZEA is rapidly absorbed after oral exposure (Minervini and Dell'Aquila, 2008; Pfeiffer et al., 2011) and it is metabolized to its reduced analogues (α-ZOL and β-ZOL) mainly *via* hepatic metabolism. The liver is the primary target organ (Bennett and Klich, 2003). Therefore, people can be exposed to these compounds by eating ZEA-contaminated food through a basic diet or food containing *Fusarium* fungi.

The main aim of the present study was to explore the cytotoxicity of ZEA and its major metabolites, α-ZOL and β-ZOL, with regards to oxidative stress in human hepatoma cells (HepG2 cells). For this purpose, HepG2 cells were exposed to ZEA, α-ZOL or β-ZOL standards at different concentrations (0, 6.25, 12.5 and 25 μM). Cell viability, ROS generation, DNA damage and a variety of antioxidants involved in protection against ROS and oxidative damage (*i.e.* GSH concentrations and GPx, SOD and CAT activities) were measured.

## 2. Materials and methods

### 2.1. Reagents and equipment

Dulbecco's Modified Eagle's Medium (DMEM), antibiotics (penicillin and streptomycin), methanol, HEPES, non-essential amino acids (NEAAs), phosphate buffered saline (PBS), glutamine, pyruvate, glucose, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), triton X-100, 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$-DCFDA), propidium iodide (PI), Na-EDTA, agarose, and low melting point (LMP) agarose were provided by Sigma Chemical Co. (St Louis, MO, USA). The mycotoxin standards ZEA (318.36 g/mol), α-ZOL (320.38 g/mol) and β-ZOL (320.38 g/mol) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Deionized water (resistivity < 18 MΩ cm) was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA). Stock solutions of mycotoxins were prepared in methanol and maintained at − 20 °C in darkness. The final concentrations tested were obtained by adding the culture medium with mycotoxins, and the final solvent concentration in medium was ≤ 1% (*v*/v).

### 2.2. Cell and culture conditions

The HepG2 (ATCC-HB-8086) cell line obtained from the American Type Culture Collection was used between passages 50 and 90. The HepG2 cells were grown in polystyrene tissue culture flasks at pH 7.4, 37 °C, 5% $CO_2$ and 95% relative humidity. The cells were grown in DMEM medium supplemented with 25 mM HEPES buffer, 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St. Louis, MO, USA).

The concentrations of ZEA and its metabolites were selected considering the previous data obtained in our laboratory in HepG2 cells. The inhibitory concentration 50% ($IC_{50}$) obtained after ZEA, α-ZOL and β-ZOL exposure during 24 h by the tetrazolium salt (MTT) assay was > 100, 27 ± 4 and > 100 μM, respectively (Tatay et al., 2014). Thus, the ZEA, α-ZOL and β-ZOL concentrations selected in this study (6.25, 12.5 and 25 μM) were below the $IC_{50}$ obtained.

### 2.3. Intracellular reactive oxygen species by $H_2$-DCFDA

Intracellular ROS production was monitored in HepG2 cells by adding $H_2$-DCFDA. This method is exceptionally sensitive and provides a direct measure of overall oxidative stress, with the detection of intracellular oxidants. 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) is taken up by the cells and then deacetylated by intracellular esterases, and the resulting 2′,7′-dichlorofluorescein (DCFH) becomes trapped inside the cell. The non-fluorescent DCFH is switched to highly fluorescent dichlorofluorescein (DCF) when oxidized by ROS. The method was developed according to Ruiz-Leal and George (2004). Briefly, $2 \times 10^4$ cells/well were seeded on a 96-well black microplate. After 24 h, the medium was removed and 20 μM $H_2$-DCFDA solution was added to each well. The cells were incubated for 20 min before removal of the supernatant, washed twice with PBS and 200 μl/well of medium, 1% MeOH (control), and medium with ZEA or its metabolites (6.25, 12.5 and 25 μM) was added. The increase in fluorescence was measured at intervals for up to 120 min at excitation and emission wavelengths of 485 and 535 nm, respectively. Eight replicates per concentration were developed. Results were expressed as the increase in fluorescence of the mycotoxin compared with solvent control. Three independent experiments were performed.

### 2.4. Alkaline comet assay

The alkaline comet assay was performed to determine the DNA strand breaks as described by Mallebrera et al. (2016). When an electric field is applied, intact DNA strands remain in the head, while the broken pieces of DNA migrate towards the anode forming a typical comet tail. Briefly, $3.4 \times 10^5$ cells were seeded in each well using 6-well plates. After confluence, cells were treated with ZEA or its metabolites at different concentrations (0, 6.25, 12.5 and 25 μM) for 24 h. Then, HepG2 cells were embedded in 0.8% LMP agarose, transferred to slides and lysed. The slides were then immersed in an alkaline solution (300 mM NaOH, 1 mM $Na_2$EDTA, pH 13) for 40 min at room temperature. The electrophoresis was run in the same solution at 0.7 V/cm (voltage across the platform) and 300 mA for 24 min. After electrophoresis, the slides were washed twice in neutralization buffer, dried in 96% ethanol and stained with 20 mg/ml propidium iodide (PI). The analysis was performed with a fluorescence microscope (NIKON Eclipse E800), equipped with camera (NIKON DXM1200F). Fifty cells/slide were processed by Comet-Score (Automatic Comet Assay), http://autocomet.com/index.php?id=cometscorepro. Results are expressed as the percentage of DNA in tail (%), calculated according to the equation: % DNA in tail = (total intensity of tail/total intensity of comet) × 100. Total intensity of comet = head length + tail length. Determinations were performed in three independent experiments.

### 2.5. Determination of GSH

For determining the GSH levels and GPx, SOD and CAT activities in HepG2 cells exposed to ZEA or its metabolites, $3 \times 10^5$ cells/well were seeded in 6-well culture plates. When cells achieved 65% confluence, the medium was removed and 200 μl of medium with ZEA, α-ZOL or β-ZOL (6.25, 12.5 and 25 μM) were added. Following the 24 h- treatment, the medium was removed and cells were washed twice with PBS. The cells were homogenized in 0.5 ml of 20 mM Tris and 0.1% Triton.

Determination of reduced GSH was made by adapting the method described by Maran et al. (2009). Briefly, 10 μl of each homogenized cell sample was placed in a 96-well black plate with 200 μl of GSH buffer (0.1 M $Na_2HPO_4$–0.005 M EDTA, pH 8.0) and 10 μl of o-phthaldialdehyde (OPT) solution, mixed and incubated in darkness at room temperature for 15 min. The concentration of GSH was determined using a microplate reader (Wallace Victor 2, 1420 Multilabel Counter, Perkin Elmer, Turku, Finland) at an excitation and emission wavelength of 345 nm and 425 nm, respectively. The GSH levels were expressed as μg GSH/mg protein. Determinations were performed in triplicate.

### 2.6. Determination of enzyme activities

GPx activity was assayed spectrophotometrically using $H_2O_2$ as

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