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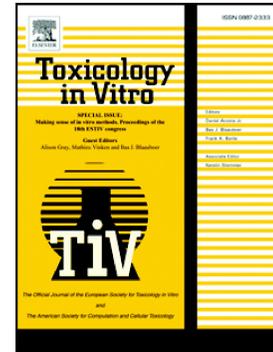
Please cite this article as: Elena Tatay, Silvia Espín, Antonio-Juan García-Fernández, María-José Ruiz , Oxidative damage and disturbance of antioxidant capacity by zearalenone and its metabolites in human cells, *Toxicology in Vitro* (2017), doi: 10.1016/j.tiv.2017.04.026

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

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List of acronyms

2',7'-dichlorodihydrofluorescein diacetate, H_2-DCFDA; 2',7'-dichlorofluorescein, DCF; adenosine binding cassette transporters, catalase, CAT; CCL13 (liver) cells; Chinese hamster ovary cells, CHO-K1 cells; deoxynivalenol, DON; dimethyl sulfoxide, DMSO; Dulbecco's Modified Eagle's Medium, DMEM; endocrine disruption, ER; fetal bovine serum, FBS; glutathione peroxidase, GPx; glutathione, GSH; hepatoma cell line, HepG2; human neuroblastoma cells, SH-SY5Y; L02 liver cells; low melting point agarose, LMP agarose; non-essential amino acids, NEAAs; phosphate buffered saline, PBS; proliferative effect, PE; propidium iodide, PI; reactive oxygen species, ROS; relative proliferative effect, RPE%; reporter gene assay, superoxide dismutase, SOD; tetrazolium salt, MTT; trichloroacetic acid, TCA; zearalenone, ZEA; α-zearalenol, α-ZOL; β-zearalenol, β-ZOL.

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) 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.

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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; Fenandez-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 tocell death (Dinu et al., 2011; Mallebrera et al., 2016; Tatay et al., 2016). Oxidative stress can result in an up-

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regulation of the antioxidant defense - i.e. enzymatic 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, insulin, glucose, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), trichloroacetic acid (TCA), 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, 37ºC, 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, 2×10^4 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 for were performed.

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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 x 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 Na₂EDTA, 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) x 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 x 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 substrate for Se-dependent peroxidase activity of GPx by following oxidation of NADPH at 340 nm during the first 2 min in a coupled enzymatic reaction with GR, as described by Maran et al. (2009). In 1-ml final volume, the reaction mixture contained 500 μl of 0.1 M phosphate buffer (pH 7.5, 1 mM EDTA, 2 mM NaN_3 and 0.1% Triton X-100), 250 μl of ultrapure water, 100 μl of 20 mM GSH, 20 μl of 0.2 mM NADPH, 2.5 U of freshly prepared GR and 50 μl of 5 mM H_2O_2). Fifty μl of homogenized cell samples were added to the reaction mixture. One unit of GPx will reduce 1 μmol of GSSG per min at pH 7.5. Assays were conducted at 25°C during 2 min with a spectrometer (Perkin Elmer UV/Vis Lambda 2 version 5.1). GPx activity was calculated by using the molar

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absorptivity of NADPH (6.22 mM^{-1} cm^{-1}) and expressed as nmol NADPH oxidized/min/mg of protein.

SOD activity was determined with the Ransod kit (Randox Laboratories, United Kingdom) adapted for 1.5 ml cuvettes. SOD activity was monitored at 505 nm during 3 min at 37ºC with a spectrometer (Perkin Elmer UV/Vis Lambda 2 version 5.1). SOD results were expressed as units of SOD per mg protein.

CAT activity was measured according to Espín et al. (2014) with slight modifications. Briefly, 50 μl of homogenized cell suspension was mixed with 950 μl of 0.05 M NaH$_2$PO$_4$ and 500 μl of 0.03 M H$_2$O$_2$. The rate of enzymatic decomposition of H$_2$O$_2$ was determined as absorbance decrements at 240 nm for 2 min with a spectrophotometer (Super Aquarius CECIL CE 9500). CAT activity was calculated by using the molar absorptivity of H$_2$O$_2$ (43.6 mM^{-1} cm^{-1}) and expressed as μmol H$_2$O$_2$/min/mg of protein. All the enzyme determinations were performed in triplicate.

2.7 Determination of total protein content

Cellular protein content was assayed using the Bio-Rad DC Protein Assay; catalog number 500-0116. Protein concentration was measured at 690 nm.

2.8 Statistical analyses

The statistical analysis of the data was carried out using the SPSS version 19 statistical package (SPSS, Chicago, IL, USA). All values are expressed as the mean ± standard error of the mean (SEM) of three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's HDS test for *post hoc* pairwise comparisons. A p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1 Intracellular ROS production

A previous study carried out on HepG2 cells demonstrated that the IC_{50} values obtained after ZEA, α-ZOL and β-ZOL exposure during 24 h by the MTT assay were >100, 27 ± 4 and >100 μM, respectively (Tatay et al., 2014).

In order to determine changes in redox status, HepG2 cells were exposed to different concentrations of ZEA, α-ZOL or β-ZOL (0, 6.25, 12.5 and 25 μM) in three different experiments for 120 min. The results obtained showed that HepG2 cells treated with ZEA and its metabolites increased the concentration of oxidizing species compared to the basal levels (Fig. 1). The highest fluorescence intensity in cells was observed at the lower exposure times. The ROS production detected at 0 min was 2-, 7- and 2.5-fold higher in HepG2 cells exposed to ZEA, α-ZOL and β-ZOL than in control cells, respectively (Fig. 1).

Significant differences in ROS production between the different treatment groups (6.25, 12.5 and 25 μM) for each mycotoxins were also observed (Fig. 1). Significant differences between 6.25 *vs.* 12.5 μM and between 6.25 *vs.* 25 μM were observed for ZEA and β-ZOL from 0 to 45 min exposure. Significant differences between 12.5 *vs.* 25 μM were also observed for these two mycotoxins from 0 to 15 min exposure (Fig. 1). However, α-ZOL showed significant differences between 6.25 *vs.* 12.5 μM, 12.5 *vs.* 25 μM, and between 6.25 *vs.* 25 μM at all times of exposure tested (0-120 min) (Fig. 1).

3.2 DNA damage by alkaline comet assay

Figure 2 shows the DNA strand breaks induced by ZEA, α-ZOL and β-ZOL (0-25 μM) in HepG2 cells. ZEA metabolites increased DNA damage in a dose-dependent manner. The greatest DNA migration was produced by α-ZOL (Fig. 2). Compared to untreated cells, the DNA damage was 9%, 45% and 30% higher in HepG2 cells exposed to ZEA, α-ZOL and β-ZOL, respectively. Significant differences in DNA migration between 6.25 *vs.* 12.5 μM, 6.25 *vs.* 25 μM and 12.5 *vs.* 25 μM treatment groups were observed for α-ZOL (Fig. 2); DNA damage increased by ca. 42%, 114% and 50%, respectively. Significant differences between 6.25 *vs.* 12.5 μM and between 6.5 *vs.* 25 μM were also found for β-ZOL (DNA damage increased by ca. 27% and 94%, respectively) (Fig. 2).

3.3 GSH levels

The HepG2 cells were exposed to 6.25, 12.5 and 25 μM ZEA, α-ZOL or β-ZOL for 24 h, and the alterations in GSH content were explored (Fig. 3). In HepG2 cells, the GSH levels (μg GSH/mg protein) decreased from 40% to 28%, from 62% to 46%, and from 40% to 25% for ZEA, α-ZOL and β-ZOL, respectively (Fig. 3). Significant differences in GSH concentrations (decreased by 20%) between 6.25 *vs.* 25 μM treatment group were observed for β-ZOL (Fig. 3).

3.4 Determination of enzymatic activities

GPx, CAT and SOD activities in HepG2 cells exposed to ZEA or its metabolites at different concentrations for 24 h are shown in Figures 4-6. GPx activity increased significantly when HepG2 cells were exposed to ZEA, α-ZOL and β-ZOL compared to control cells (Fig. 4). The greatest increase was observed in cells exposed to these compounds at a concentration of 25 μM. Overall, the GPx activity increased up to 50%,

95% and 90% in cells exposed to ZEA, α-ZOL and β-ZOL, respectively. Moreover, significant differences in GPx activity were observed between 6.25 and 25 μM ZEA-treated groups (increased by ca. 30%) and between 6.25 and 25 μM β-ZOL-treated groups (increased by ca. 25%).

CAT activity decreased in a dose-dependent manner from 95% to 25% in HepG2 cells exposed to ZEA, and from 95% to 15% for α-ZOL and β-ZOL. Significant differences in CAT activity between 6.25 *vs.* 12.5 μM, 6.25 *vs.* 25 μM, and 12.5 *vs.* 25μM groups were observed in cells exposed to ZEA and β-ZOL; CAT activity decreased by ca. 40%, 70% and 35% for these two mycotoxins, respectively. CAT activity did not differ between 6.25 *vs.* 12.5 μM groups in α-ZOL-treated cells. However, significant differences in CAT activity between 6.25 *vs.* 25 μM treatment groups (decreased by ca. 80%) and 12.5 *vs.* 25 μM treatment groups (decreased by ca. 60%) were observed (Fig. 5).

Mycotoxin treatment enhanced the SOD activity in HepG2 cells to values 1500- to 2100- fold higher (ZEA and β-ZOL-treated cells) or 2400- to 3400-fold higher (α-ZOL-treated cells) than those found in control cells. No significant differences in SOD activity were observed between 6.25 *vs.* 12.5 μM groups for α-ZOL-treated cells. However, significant differences in SOD activity were found between 6.25 *vs.* 25 μM treatment groups (increased by ca. 40%) and 12.5 *vs.* 25 μM treatment groups (increased by ca. 15%) for α-ZOL-treated cells. We also observed differences in SOD activity between 6.25 *vs.* 12.5 μM and 6.25 *vs.* 25 μM treatment groups for ZEA and β-ZOL-treated cells; SOD activity increased by 40% and 60% for ZEA-treated cells, respectively, and by 20% and 35% for β-ZOL-treated cells, respectively (Fig. 6).

4. Discussion

Small amounts of ROS are constantly generated within cells as a result of normal physiological processes. Toxic substances in the diet or environmental contaminants, when absorbed by mammalian cells, are also able to produce ROS. Overproduction of ROS can alter membrane permeability and structure and induce DNA mutations (Guo et al., 2013). Mitochondria contain their own DNA, which is particularly susceptible to ROS attack associated with oxidative stress (Guo et al., 2013). Acute ROS exposure can result in shut-down of mitochondrial energy production (Prosperini et al., 2013). In our study ZEA and its isomers induced oxidative stress through ROS generation in HepG2 cells (Fig. 1). ZEA and β-ZOL showed less ROS production than α-ZOL. Moreover, ZEA and β-ZOL induced ROS production in a dose-dependent manner during the first 45 min of exposure, whereas HepG2 cells exposed to α-ZOL showed significant ROS production in a dose-dependent manner during the entire exposure time (120 min). Taken together, these results suggest that α-ZOL may be the major contributor to the total ROS production. According to this, previous studies have reported increased ROS production in different cells exposed to ZEA at different concentrations (HepG2 cells, 100 μM ZEA, El Golli-Bennour et al., 2008; CHO-K1 cells, 1-50 μM ZEA, Ferrer et al., 2009; porcine granulosa cells, 15-60 μM ZEA, Qin et al., 2015; L02 liver cells, 10-40 μM ZEA, Wu et al., 2014). These authors also found that the production of ROS is time dependent (El Golli-Bennour et al., 2008; Ferrer et al., 2009). In this sense, Ferrer et al (2009) reported that ZEA-induced ROS generation is higher during the early stages of exposure (4-fold higher than controls), whereas its capacity to generate ROS decreases after 5 min (2-fold increase) when cells are exposed to 1, 5 and 50 μM ZEA.

The increased ROS generation in cells exposed to ZEA and its metabolites could consequently contribute to metabolic oxidative stress, genomic instability and cellular

injury. Chronic exposure to ROS can result in oxidative damage to DNA and RNA polymerase, histones, topoisomerase-II and other DNA-associated proteins. Thus, ROS generation can lead to DNA strand breaks and chromosome damage. Our results showed that ZEA and its metabolites increase DNA damage in HepG2 cells. Cells exposed to α-ZOL showed a higher increase in DNA damage compared to cells exposed to ZEA or β-ZOL at all concentrations tested. ZEA showed the lowest capacity to induce DNA damage, showing similar results for all concentrations tested, whereas α-ZOL and β-ZOL increased DNA strand breaks in a dose-dependent manner. These findings are consistent with those obtained by other authors using different types of cells (Vero cells, 10-40 μM ZEA, Ayed-Boussema et al., 2007; Caco-2 cells, $IC_{50}/2$, IC_{50} and 2 x IC_{50}) ZEA, α-ZOL and β-ZOL, Abid-Essefi et al., 2009; HepG2 cells, 100 μM ZEA, Gazzah et al., 2010; CCL13 liver cells, 25-200 μM ZEA, Kang et al., 2013; human neuroblastoma SH-SY5Y cells, 25-100 μM ZEA, Venkataramana et al., 2014). Therefore, our study supports that overproduction of ROS could contribute to DNA chain breakdown and cell damage. Our results suggest that α-ZOL may have a higher capacity to produce DNA damage than ZEA or β-ZOL.

Under aerobic living conditions, oxidative processes and the subsequent generation of ROS are normal in the cellular metabolism. Living beings are equipped with an antioxidant defense system able to inhibit ROS generation and reduce oxidation and the consequent damage. When the balance between the antioxidant defense and ROS production is disrupted, the cells try to survive by degrading their own protein aggregates or organelles, with the initiation of apoptosis (Qin et al., 2015). Particularly, cellular antioxidant enzymes play a major role in protecting cells from oxidative stress and damage. They are effective in scavenging ROS since they catalyze the breakdown of free radicals and support the antioxidant defense system by catalyzing the

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conjugation of toxic compounds with GSH (SOD, CAT, GPx, GR, GST) (Halliwell and Gutteridge, 1999). Regarding the non-enzymatic antioxidant defense system, the tripeptide GSH is one of the most abundant sulfhydryl-containing groups in most organisms and plays a basic role in binding with ROS. These antioxidants operate in association with each other forming an integrated antioxidant defense system (Halliwell and Gutteridge, 1999). ZEA, α-ZOL and β-ZOL have toxicological interest because of their potential to cause oxidative stress and damage.

In this study, HepG2 cells exposed to ZEA, α-ZOL and β-ZOL showed and up-regulation of the antioxidant defense system by increasing the activity of GPx and SOD as a possible protective response, while a reduction in CAT activity and GSH levels was found.

SOD enzyme catalyses the transformation of the superoxide anion radical into H_2O and O_2, while GPx enzyme reduces peroxides in cells, such as the transformation of H_2O_2 to H_2O by using GSH as a substrate (Halliwell and Gutteridge, 1999). While all the concentrations tested enhanced GPx activity to a similar level (only cells exposed to 25 μM ZEA or β-ZOL showed significantly higher GPx activity than cells exposed to 6.25 μM), the different treatments induced SOD activity in a dose-dependent manner. In our study, HepG2 cells exposed to ZEA and its metabolites increased the concentration of oxidizing species compared to the untreated cells. Thus, the increased GPx and SOD activities reported in our study can be a compensatory mechanism to scavenge ROS levels produced as a result of ZEA, α-ZOL and β-ZOL exposure. Our study shows that both enzymes play a major role in providing protection against damage induced by ZEA and its metabolites in HepG2 cells.

On the other hand, the reduction of GSH concentrations in HepG2 cells exposed to ZEA and its metabolites could be related to its requirement for conjugation reactions

of detoxification. In this sense, since GPx oxidizes GSH to reduce H_2O_2 (Halliwell and Gutteridge, 1999), the increased GPx activity due to mycotoxin exposure can promote a reduction in GSH levels. Both CAT and GPx catalyze the decomposition of H_2O_2, but CAT directly catalyses the transformation of H_2O_2 to H_2O and O_2 (Halliwell and Gutteridge, 1999). CAT enzymes are abundant in the peroxisomes of liver cells, while GPx is abundant in mitochondria and cytosol compartment. In our study, CAT activity significantly decreased in a dose-dependent manner in HepG2 cells exposed to ZEA, α-ZOL and β-ZOL. This marked effect can be due to high concentrations of H_2O_2 produced under mycotoxin exposure. In situations of high peroxide concentrations, a depression of CAT activity may occur, and it can be even inactivated. Moreover, an oxidation of the CAT enzyme by the excess of peroxide is also possible (Williams, 1928).

In general, the highest GPx and SOD activities and the lowest GSH concentrations were reached in cells exposed to α-ZOL. In addition, cells exposed to this metabolite and to β-ZOL showed higher ROS production and DNA damage levels compared to cells exposed to ZEA. These results suggest that α-ZOL and β-ZOL have a higher capacity to induce oxidative stress and damage in HepG2 cells at the tested concentrations.

Previous studies have found similar effects in different cell types exposed to ZEA or other *Fusarium* mycotoxins. However, to the best of our knowledge, there is no data available on the oxidative effects caused by α-ZOL and β-ZOL exposure. Lee et al. (2013) and Hassen et al. (2007) observed that ZEA (200 μM) decreased GSH levels in HepG2 cells (decreased by 71% and 33% compared to the control, respectively), while Qin et al. (2015) observed a decrease in CAT activity when porcine granulosa cells were exposed to ZEA (3-60 μM) for 24 h. Similar results were obtained by other

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authors, reporting reduced GSH levels and/or increased GPx, SOD and CAT activities in different cells (CHO-K1, Caco-2, PK15, U937, HeK-293 cells) exposed to different mycotoxins (beauvericin, BEA; fumonisin B1, FB1; deoxynivalenol, DON) (Mallebrera et al., 2014; Prosperini et al., 2013; Klaric et al., 2007; Costa et al., 2009; Dinu et al., 2011).

Based on our results, it can be concluded that mycotoxin-induced oxidative stress and damage may play a major role in the cytotoxic effects of ZEA and its metabolites. ZEA and its metabolites enhance ROS generation and DNA damage in HepG2 cell in a dose-dependent manner. HepG2 cells exposed to ZEA, α-ZOL and β-ZOL showed and up-regulation of the antioxidant defense system by increasing the activity of GPx and SOD, suggesting that these enzymes play a major role in providing protection against damage induced by these mycotoxins. In addition, a reduction in CAT activity and GSH levels was found, which could be related to the requirement of these antioxidants for H_2O_2 reduction and for conjugation reactions of detoxification, respectively. Our results suggest that ZEA has a lower capacity to induce oxidative stress and damage in HepG2 cells at the tested concentrations than its metabolites.

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Legend of figures

Figure 1. ROS-induced fluorescence in HepG2 cells exposed to ZEA (a), α-ZOL (b) and β-ZOL (c) for 120 min. HepG2 cells were exposed to H_2-DCFDA for 20 min before ZEA, α-ZOL and β-ZOL exposure. Results are expressed as mean ± SEM (n=3). (*)significant differences ($p \leq 0.05$) *versus* control. (&)significant differences between 6.25 and 12.5 µM treatment groups. (#)significant differences between 6.25 and 25 µM treatment groups. ($)significant differences between 12.5 and 25 µM treatment groups.

Figure 2. Percentage (%) of DNA in tail in HepG2 cells exposed to 6.25, 12.5 and 25 µM ZEA, α-ZOL and β-ZOL for 24 h. Data are expressed as mean ± SEM (n=3). (*) significant differences ($p \leq 0.05$) *versus* control. (&) significant differences between 6.25 and 12.5 µM treatment groups. (#) significant differences between 6.25 and 25 µM treatment groups. ($) significant differences between 12.5 and 25 µM treatment groups.

Figure 3. GSH levels (µg/mg protein) in HepG2 cells exposed to ZEA, α-ZOL or β-ZOL (6.25, 12.5 and 25 µM) for 24 h. Data are expressed as mean ± SEM (n=3) (*) significant differences ($p \leq 0.05$) *versus* control. (#) significant differences between 6.25 and 25 µM treatment groups.

Figure 4. GPx activity (nmol/min/mg protein) in HepG2 cells exposed to ZEA, α-ZOL and β-ZOL (6.25, 12.5 and 25 µM) for 24 h. Data are expressed as mean ± SEM (n=3). (*) significant differences ($p \leq 0.05$) versus control. (#) significant differences between 6.25 and 25 µM treatment groups.

Figure 5. CAT activity (µmol/min/mg protein) in HepG2 cells exposed to ZEA, α-ZOL and β-ZOL (6.25, 12.5 and 25 µM) for 24 h. Data are expressed as mean ± SEM (n=3). (*) significant differences ($p \leq 0.05$) *versus* control. (&) significant differences between 6.25 and 12.5 µM treatment groups. (#) significant differences between 6.25 and 25 µM treatment groups. ($) significant differences between 12.5 and 25 µM treatment groups.

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Figure 6. SOD activity (U/mg protein) in HepG2 cells exposed to ZEA, α-ZOL and β-ZOL (6.25, 12.5 and 25 μM) for 24 h. Data are expressed as mean ± SEM (n=3). (*) significant differences ($p \leq 0.05$) *versus* control. (&) significant differences between 6.25 and 12.5 μM treatment groups. (#) significant differences between 6.25 and 25 μM treatment groups. ($) significant differences between 12.5 and 25 μM treatment groups.

Figure 1

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Figure 3

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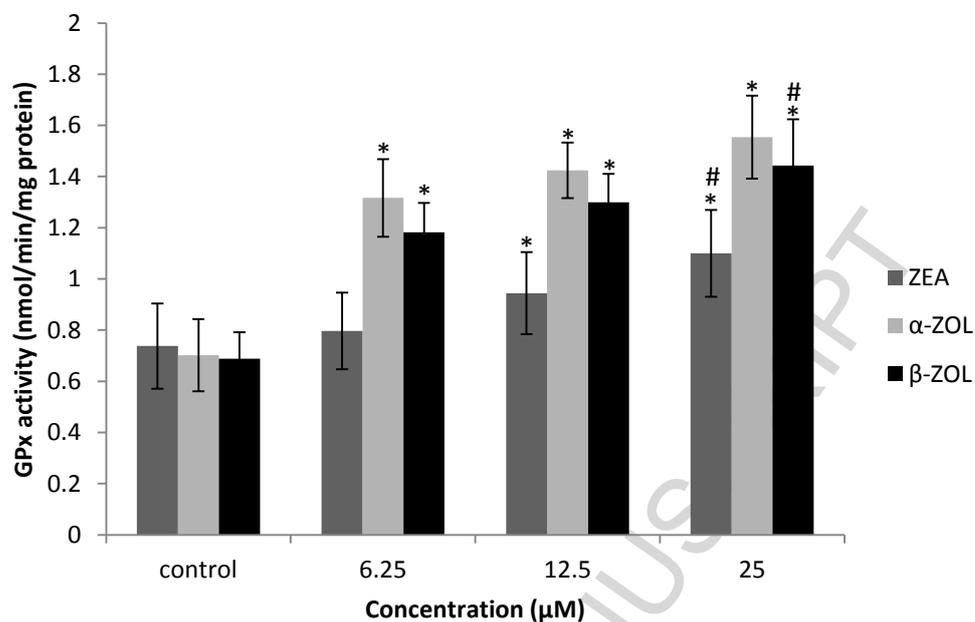
Figure 4

Figure 4. GPx activity (nmol/min/mg protein) in HepG2 cells exposed to ZEA, α-ZOL and β-ZOL (6.25, 12.5 and 25 μM) for 24 h. Data are expressed as mean ± SEM (n=3). (*) significant differences ($p \leq 0.05$) versus control. (#) significant differences between 6.25 and 25 μM treatment groups.

Figure 5

Figure 5. CAT activity (μmol/min/mg protein) in HepG2 cells exposed to ZEA, α-ZOL

and β-ZOL (6.25, 12.5 and 25 μM) for 24 h. Data are expressed as mean ± SEM (n=3).

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6.25 and 12.5 μM treatment groups. (#) significant differences between 6.25 and 25 μM

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Figure 6
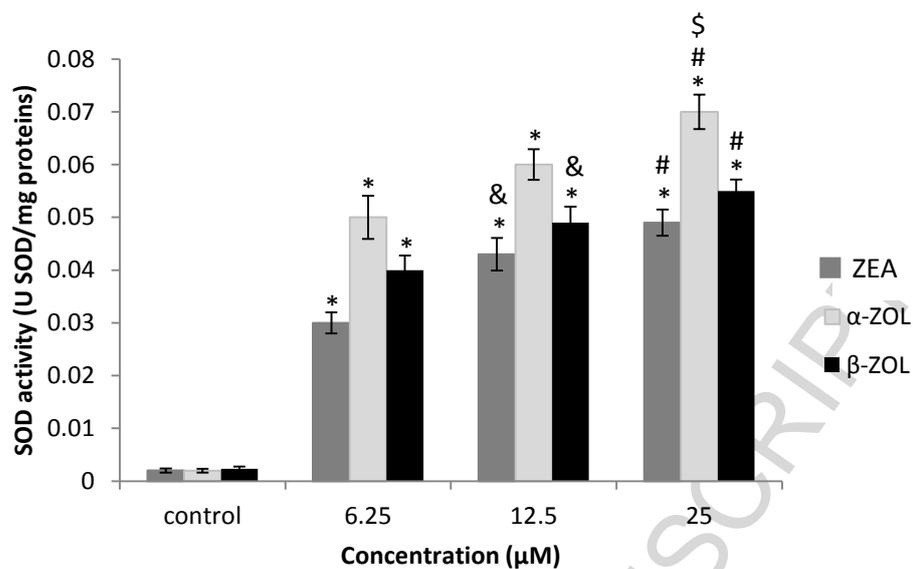
Figure 6. SOD activity (U/mg protein) in HepG2 cells exposed to ZEA, α-ZOL and β-ZOL (6.25, 12.5 and 25 μM) for 24 h. Data are expressed as mean ± SEM (n=3). (*) significant differences ($p \leq 0.05$) *versus* control. (&) significant differences between 6.25 and 12.5 μM treatment groups. (#) significant differences between 6.25 and 25 μM treatment groups. ($) significant differences between 12.5 and 25 μM treatment groups.

Graphical abstract
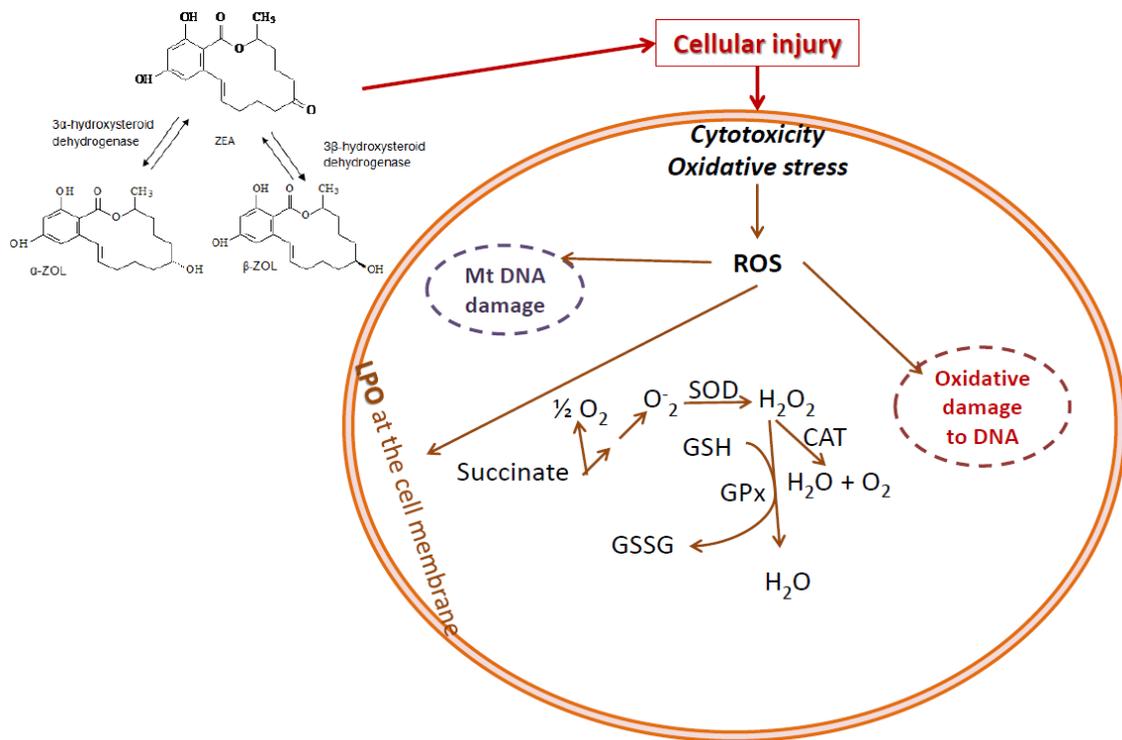
HIGHLIGHTS

- ZEA and its metabolites cause ROS generation and produced DNA damage in HepG2 cells.
- GSH levels and CAT activity decreased in HepG2 cells after ZEA and its metabolites exposure.
- ZEA and its metabolites increased SOD and GPx activities in HepG2 cells.
- ZEA has a lower capacity to induce oxidative stress and damage in HepG2 cells than its metabolites