THE EFFECTS AND MECHANISMS OF ACTION OF ZEARALENONE AND *E. COLI*-LPS CO-CONTAMINATION ON PORCINE INTESTINAL EPITHELIAL CELLS

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Abstract

Zearalenone (ZEA) is one of the most widespread fusariotoxins, which affects mainly the reproductive system, but it can also disturb the activity of the immune, nervous, and digestive system. Humans and animals are exposed to ZEA through the ingestion of contaminated food or feed, pigs being the most sensitive species, due to their rich diet in cereals and their native sensitivity to mycotoxins. In this in vitro study performed on the porcine intestinal cell line IPEC-1, the effects of ZEA on the oxidative stress and inflammation at intestinal level were studied. The gene expression of some antioxidant enzymes (CAT, SOD, Gpx) were analyzed, also pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8) and signaling molecules (NF-KB, p-38α, PPARγ, Nrf2, KEAP, HO1, NQO1) were quantified using real-time PCR, furthermore ROS levels were determined by flow cytometry. Our results showed that ZEA can reduce the gene expression of the pro-inflammatory cytokines IL-1β and IL-8, but also of NF-KB, p-38α and PPARγ, inflammatory mediator molecules, without inducing significant effects at the level of oxidative stress markers.

Key words: *Zearalenone, Intestine, Inflammation, Oxidative Stress, Pigs*

INTRODUCTION

The quality of life for both humans and animals is often influenced by the quality of the food and feed which they consume. In the recent times, digestive diseases caused by a diet contaminated with various chemical compounds have attracted global attention both at the level of scientists and the population [1]. Among the natural contaminants, mycotoxins have begun to be studied due to their widespread and increased resistance, but also to the fact that they directly affect one of the largest classes of raw materials in the food and feed industry, namely cereal, mycotoxins being able to contaminate crops such as wheat, barley, corn etc. [2]

Zearalenone (ZEA) is a mycotoxin produced by fungi of the genus *Fusarium* [3], which is known mainly for its toxicity in the reproductive system [4]. These effects are mainly attributed to the chemical structure similar to that of estrogen [5]. However, studies reported that it has also effects on other systems, even the digestive system considering the fact that the main way of exposure to ZEA is through the ingestion of contaminated food [6]. Humans are exposed both directly through the consumption of cereals and indirectly through the consumption of food of animal origin. Pigs are among the farm animals whose health is important mainly for the food industry. Its native sensitivity to mycotoxins, but also the alimentation very rich in cereals make it the main target of mycotoxicoses and digestive diseases [7]. At the digestive level, the intestinal barrier plays an important role in the body's defense against xenobiotics, being both a physical

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and an immunological barrier [8]. Studies show that ZEA toxicity could be induced by modulating the level of markers specific to oxidative stress like Reactive Oxygen Species (ROS)[9]. However, there are uncertainties regarding the direction of regulation of the activity of some markers such as antioxidant enzymes (CAT, SOD GPx), ZEA being able to increase or decrease the antioxidant activity, depending on the species, organ, concentration, and time of exposure[10,11]. Most of the time, the oxidative response leads further to the inflammatory response. Here, too, there are contradictory data regarding the effects of ZEA [12]. There is not much data regarding the mechanisms of ZEA toxicity either. Most studies indicate the involvement of signaling molecules such as Nrf2, NF-kB or MAPK-kinases [13–15] most of which are later involved in inflammation and oxidative stress.

In order to evaluate the effects produced by ZEA (10 and 20 μM) at the intestinal level, an *in vitro* experiment on the porcine intestinal epithelial cell line IPEC1 was carried out. At the end of the experiment, specific markers for inflammation (*TNF-α, IL-1β, IL-6, IL-8*) and oxidative stress (*CAT, SOD, Gpx*) were analyzed by qPCR and ELISA techniques. Furthermore, the gene expressions of some signaling molecules (*NF-KB, p-38α, PPARγ, Nrf2, KEAP, HO1, NQO1*) with an important role in inflammation and oxidative stress were evaluated, as well as the changes induced by ZEA at the ROS level by flow cytometry.

MATERIAL AND METHOD

Cell cultures and experimental design

The *in vitro* experiments were carried out in the Animal Biology Laboratory (IBNA Balotești). IPEC 1 passage 81+43 cells were thawed, washed with complete medium and cultured in T125 flasks. At confluence, cells were detached from the culture dish wall using a trypsin-EDTA mixture (Sigma). The recovered cells were counted using a Neubauer chamber and cultured in 3 experimental series. Cells were maintained in culture in 24-well plates, each well containing $1x10^5$ cells/ml, at a temperature of 37°C, in an atmosphere enriched with 5% CO_2. Complete culture medium was prepared using DMEM/F12 medium (Sigma) supplemented with 5% fetal bovine serum (Sigma), 1% penicillin/streptomycin (Sigma) 1% L - glutamine (Gibco BRL), 1% ITS (Insulin/Transferin/Selenium Gibco BRL) and 0.05% EGF (Epithelial Growth Factor).

To better simulate the *in vivo* conditions, where most of the time mycotoxin contamination occurs simultaneously with that of pathogens, the cells were stimulated with 10 μg/ml Lipopolysaccharide (LPS) (Sigma) for 2 hours, subsequently were treated with ZEA 10/20 μM for 24 hours. LPS and toxin were prepared according to the manufacturers' instructions, LPS was hydrated using sterile ultrapure water, and ZEA (Fermentek) was suspended in DMSO (dimethyl sulfoxide). ZEA concentrations were selected following a cell viability assay. At the end of the experiments there were four experimental groups: 1) Control (C) which did not receive any treatment, 2) Stimulated Control (LPS) which was treated with LPS 10 μM, 3) Stimulated Control + ZEA 10 μM (LPS 10 μM +ZEA 10 μM) and 4) Control Stimulated + ZEA 20 μM (LPS 10 μM +ZEA 20 μM). At the end of the experiments, samples of cell lysate and supernatant were taken for further analysis. All samples were stored at -80°C.

Optical microscopy

The microscopy images of cell culture were captured using the MShot Image Analysis System software and a Nikon Eclipse Ts2R inverted microscope.

Quantification of gene expression

Gene expression quantification was performed by real time PCR (qPCR) technique. After the incubation, the cells were resuspended in 700μl QIAzol® Lysis Reagent (QIAGEN, GmbH, Germany), later

the total RNA was isolated using miRNeasy Mini kit (QIAGEN, GmbH, Germany) according to manufacturer instructions. The quality of the isolated RNA was assessed for each sample by electrophoretic methods using the Agilent 4150 TapeStation bioanalyzer and the RNA ScreenTape Analysis kit (Agilent Technologies, Santa Clara, USA). The obtained data showed that the isolated RNA was of very good quality, the RIN of all samples being above 9.0. The next step was reverse transcription of RNA into complementary DNA (cDNA) using M-MLV Reverse Transcriptase Kit (Life Technologies, Carlsbad, USA). 500 ng of RNA was mixed with Oligo(dT)12-18 (0.5 µg/mL), dNTP (0.5 µg/mL) and nuclease-free water to a final volume of 26 µL. The mixture was incubated for 5 minutes at 65ºC, followed by a 5-minute incubation at 4ºC. Then, 12 µL of mix (2:1 of 5X reaction buffer: DTT solution) was added to each tube, followed by a 2-minute incubation at 37ºC. The last step was represented by the addition of 2 µL M-MLV Reverse Transcriptase Enzyme, incubation for 50 minutes at 37ºC, followed by enzyme inactivation for 15 minutes at 70ºC and cooling at 4°C.

To evaluate the gene expression of pro-inflammatory cytokines (*TNF-α, IL-1β, IL-6 and IL-8*), antioxidant enzymes (*CAT, SOD, GPx*) and some markers involved in the modulation of inflammation and oxidative stress (*NF-κB, Nrf2, p38α, PPARγ, KEAP, HO1* and *NQO1)* the Rotor-Gene-Q system (QIAGEN GmbH) was used. The reaction volume of 20 µL was obtained using 10 ng of cDNA sample, 0.3 µM primers for the genes of interest, 10 µl SYBR Green qPCR Master Mix (Life Technologies, Carlsbad, CA, USA) and nuclease-free water. In Table 1 are shown the nucleotide sequences of the primers used. The incubation steps followed were: 50°C (2 min); 95°C (10 min); 45 cycles of 95°C (15 sec) and 60°C (60 sec), finishing up with a elongation at 72°C (10 min). The normalization of the data was carried out with the Normfinder software, out of six reference genes being selected two. Using the $2^{(-\Delta\Delta C)q}$ calculation method, the obtained results were reported to the control group and expressed as Fold Change (FC).

Flow cytometry

The effects of ZEA on the porcine epithelial-intestinal cell line IPEC1 at the level of oxidative stress were analyzed by detecting reactive oxygen species using flow cytometry. Trypsin-detached cells were suspended in the buffer provided by the Muse Oxidative Stress kit (Cytek Biosciences, USA), using 10 µl of cells at a concentration of 1x106. The cells together with 190 µl working solution (Muse Oxidative Stress Working solution) were incubated for 30 minutes at a temperature of 37ºC. Results obtained using the Guava Muse Cell Analyzer (Cytek Biosciences, USA) were expressed as Negative Populations (ROS-) and Populations with ROS Activity (ROS+).

Statistical analysis

The data obtained were represented graphically using the mean ± SEM (Standard Error of the Mean). The statistical differences between the experimental groups were assessed using the one-way Anova test, followed by Fisher`s test using GraphPad Prism (9.3.0) and StatView software. The p values < 0.05 were considered statistically significant, and the p values < 0.1 were considered as trend.

Table 1 Nucleotide sequences of primers used in the qPCR reaction

Gene	*Nucleotide sequences of the primers*	*Primer Orientation*	*Ta (°C)*	*Amplicon length (bp)*
β-2 microglobulin	TTCTACCTTCTGGTCCACACTGA	Fw	50	162
	TCATCCAACCCAGATGCA	Rv	54	
GAPDH	ACTCACTCTTCTACCTTTGATGCT	Fw	49	100
	TGTTGCTGTAGCCAAATTCA	Rv	56	
Ciclofilin A	CCCACCGTCTTCTTCGACAT	Fw	54	92
	TCTGCTGTCTTTGGAACTTTGTCT	Rv	55	
β-actin	GGACTTCGAGCAGGAGATGG	Fw	60	230
	GCACCGTGTTTGCGTAGAGG	Rv	62	
HPRT-1	TGGAAAGAATGTCTTGATTGTTGAAG	Fw	58.57	93
	ATCTTTGGATTATGCTGCTTGACC	Rv	59.66	
RPL 32	TGCTCTCAGACCCCTTGTGAAG	Fw	61.93	106
	TTTCCGCCAGTTCCGCTTA	Rv	59.63	
TNF-α	ACTGCACTTCGAGGTTATCGG	Fw	60	118
	GGCGACGGGCTTATCTGA	Rv	60	
IL-8	GCTCTCTGTGAGGCTGCAGTTC	Fw	58	79
	AAGGTGTGGAATGCGTATTTATGC	Rv	54	
IL-6	GGCAAAAGGGAAAGAATCCAG	Fw	57	87
	CGTTCTGTGACTGCAGCTTATCC	Rv	61	
IL-1β	ATGCTGAAGGCTCTCCACCTC	Fw	62	89
	TTGTTGCTATCATCTCCTTGCAC	Rv	59	
NF-kB	CGAGAGGAGCACGGATACCA	Fw	55	62
	GCCCCGTGTAGCCATTGA	Rv	54	
Nrf2	CCCATTCACAAAAGACAAACATTC	Fw	57	72
	GCTTTTGCCCTTAGCTCATCTC	Rv	59	
p38α	TGCAAGGTCTCTGGAGGAAT	Fw	52	109
	CTGAACGTGGTCATCCGTAA	Rv	52	
HO-1	ATGTGAATGCAACCCTGTGA	Fw	57.71	89
	GGAAGCCAGTCAAGAGACCA	Rv	59.31	
NQO1	GTATCCTGCCGAGACTGCTC	Fw	59.97	134
	TAGCAGGGACTCCAAACCAC	Rv	59.31	
KEAP	ACGACGTGGAGACAGAAACGT	Fw	61.94	56
	GCTTCGCCGATGCTTCA	Rv	58.07	

RESULTS

Optical microscopy

As shown in Fig. 1, the IPEC-1 cells formed a monolayer, similar morphologically to intestinal epithelium. Stimulation of cells with LPS resulted in the formation of cell conglomerates, indicating disruption of cell activity. The addition of ZEA 10μM and ZEA 20μM seems to potentiate the effects produced by LPS stimulation, in the case of both concentrations of ZEA the cell conglomerates are larger and more numerous, the cell monolayer being visibly affected.

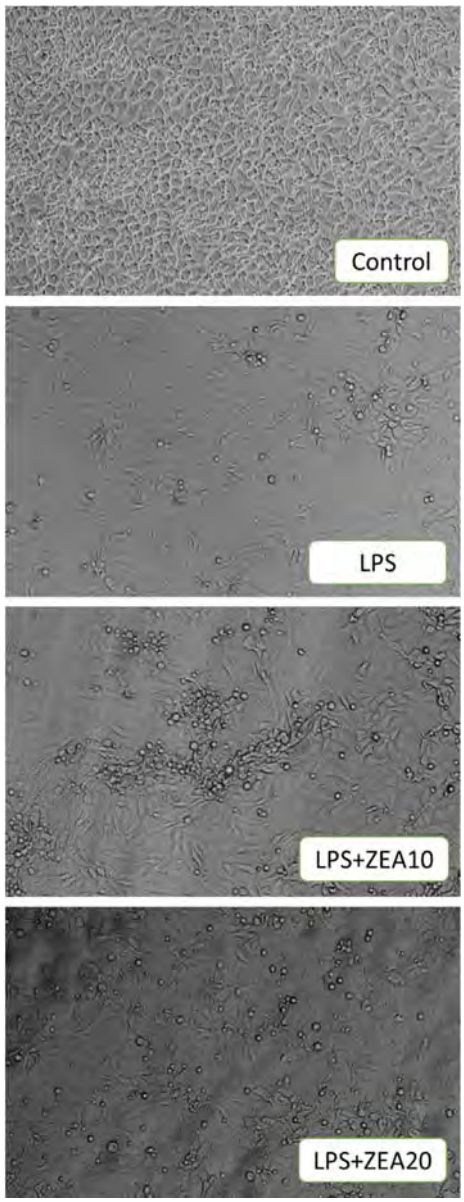

Fig. 1 Optical microscopy images of IPEC1 under the influence of experimental treatments

Effects of ZEA on the Inflammatory Response

The effects of ZEA on inflammation in the porcine intestinal epithelium were evaluated by analyzing the gene expression of the pro-inflammatory cytokines *TNF-α*, *IL-1β, IL-6* and *IL-8*, as well as markers involved in cell signaling pathway responsible for this process (*NF-κB, p38α* and *PPARγ*). As expected, (Fig.2) the results indicated an increase of the gene expression coding for pro-inflammatory cytokines induced by bacterial LPS. The combination of LPS with either ZEA10 μM or ZEA 20μM also increase the gene expression of pro-inflammatory cytokines comparing to the control except for *IL-1β* gene which decrease.

Regarding the gene expression of the key molecules involved in the inflammatory pathway (Fig.3) our results showed that *E. coli*-LPS significantly increased the *NF-kB* gene ($P<0.05$), and trend to increase that of PPAR-γ and p38α). The co-contamination between LPS and ZEA 20μM increased the gene expression of these markers above the level of LPS alone and control. By contrast, the combination of LPS with ZEA 10μM decreased the gene expression of analyzed signaling markers below the level induced by LPS alone, but increase their expression compared to control apart gene of *p38α*.

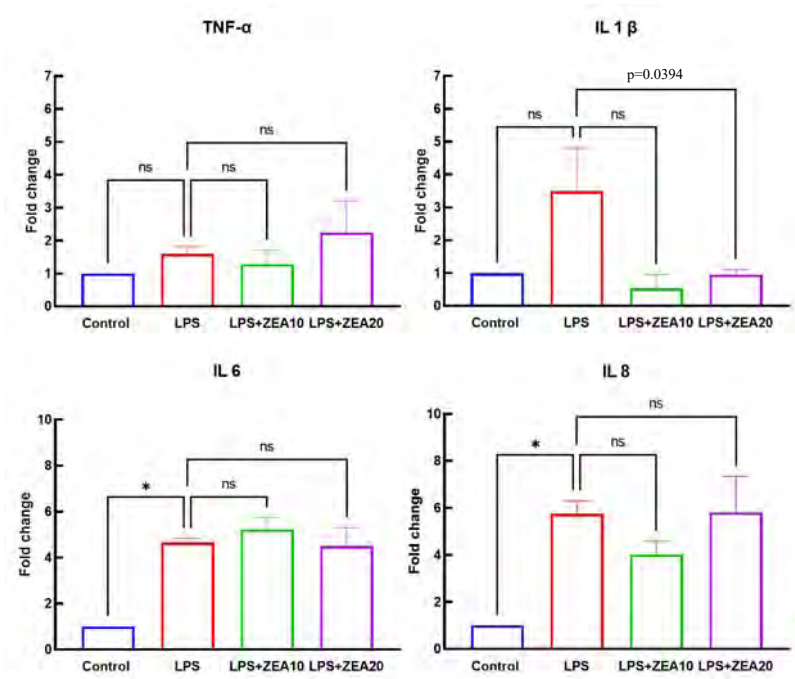

Fig. 2 Effects of ZEA on gene expression of pro-inflammatory cytokines *TNF-α*, *IL-1β*, *IL-6* and *IL-8*

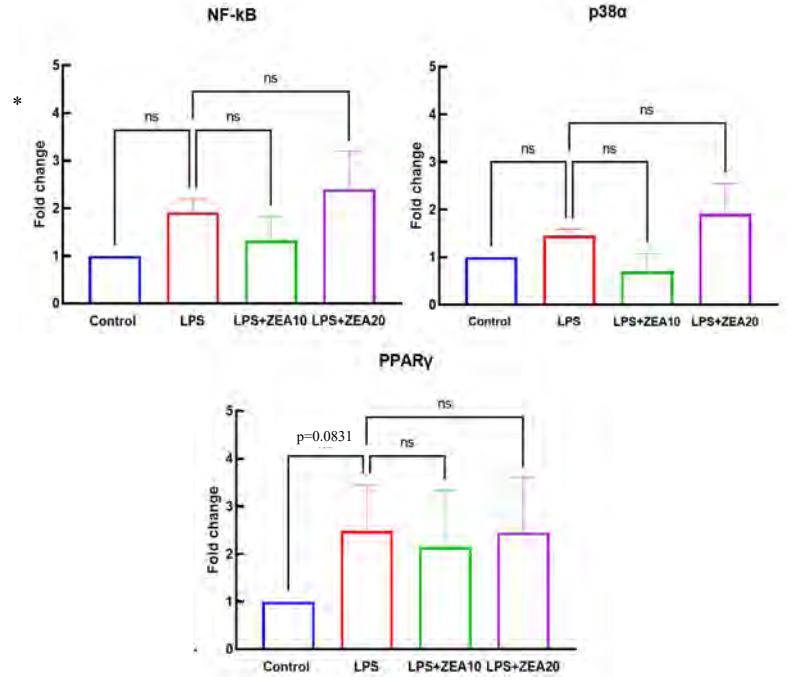

Fig. 3 Effects of ZEA on gene expression of NF-kB, p-38α and PPARγ -

Effects of ZEA on the Antioxidant Response

At the level of the antioxidant response, the effects of LPS and ZEA were evaluated by flow cytometry, but also by quantifying the gene expression of the nuclear receptor *Nrf2*, the antioxidant enzymes (*CAT, SOD, GPx)* whose activity is modulated by Nrf2 signaling molecule and those that interact with Nrf2 (*KEAP*, *HO1* and *NQO1*). Although LPS slightly decreased the gene expression of *Nrf2* it did not modify the genes of antioxidant enzymes when compared to control. A modulation of all markers responsible for antioxdants response was observed but the difference against control and LPS alone were not significant (Fig. 4 and Fig. 5)

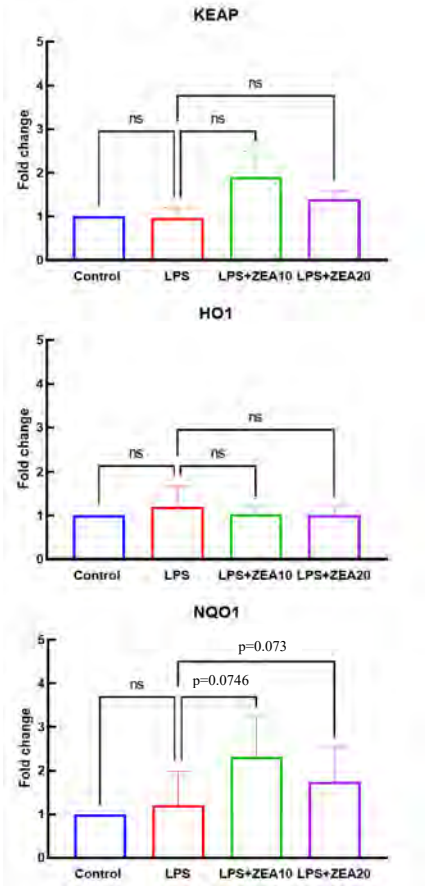

Fig. 4 Effects of ZEA on gene expression of the signaling molecules *KEAP, HO1* and *NQO1*

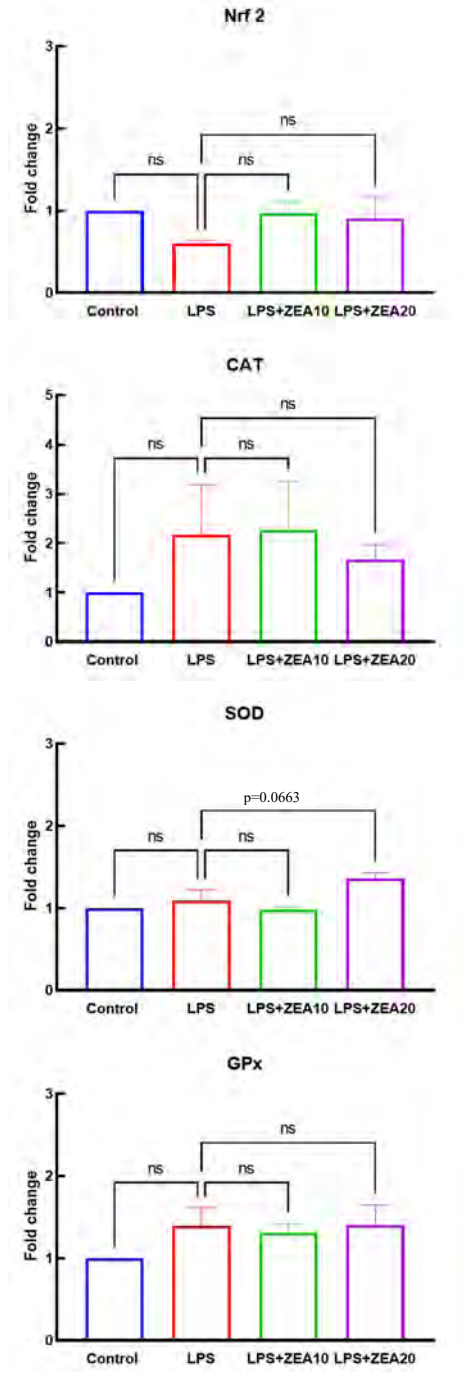

Fig. 5 Effects of ZEA on gene expression of transcription factor Nrf2 and antioxidant enzymes CAT, SOD and GPx

Regarding Reactive Oxygen Species (ROS), the results obtained by flow cytometry show no significant changes in the level of ROS+ and ROS-, the only significant effect being given by stimulation of cells with LPS.

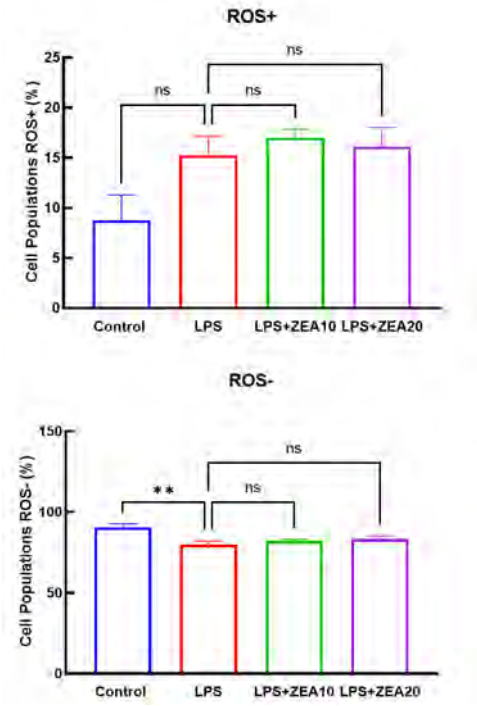

Fig. 7 Effects of ZEA on Reactive Oxygen Species (ROS)

DISCUSSIONS

Although there are currently studies on the effects and mechanisms of ZEA, most of them focus on its estrogenic effects, its effects on the intestine, although very important, are less studied.

Regarding oxidative stress, there are *in vitro* studies on porcine Granulosa cells that show that in low concentrations ZEA can lead to a decrease in the level of antioxidant enzymes [9], thus inducing oxidative stress, while *in vivo* studies show that ZEA would have a totally opposite effect, leading to an increase in CAT and Gpx gene expression in piglets' liver [10].

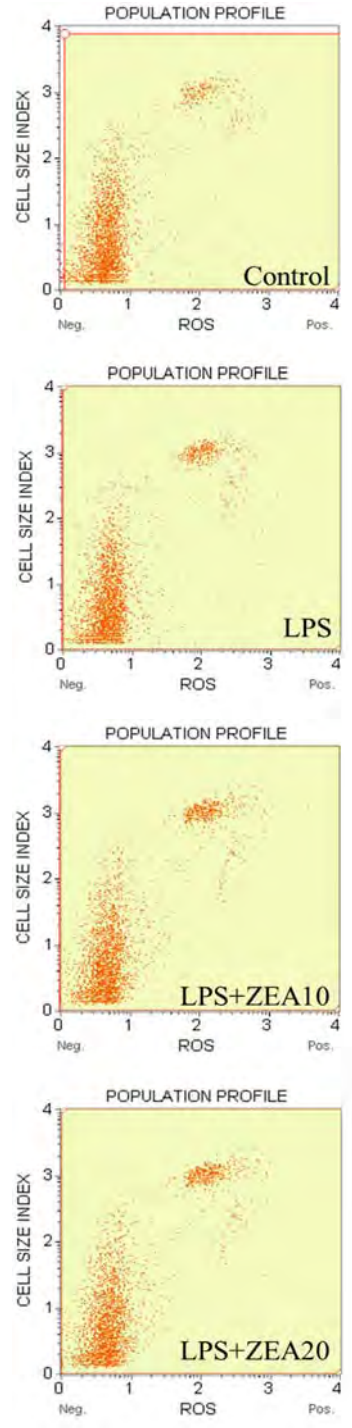

Fig. 8 Distribution of ROS+/ROS- cell populations by flow cytometry

In terms of inflammatory response, *in vitro* studies on porcine PBMCs show that ZEA can lead to a decrease in the level of inflammation markers such as IL-8 [16], while at the intestinal level it can lead to an increase in the secretion of IL-8 and IL-10 [17]. A study carried out by these authors, on the human intestinal epithelial cell line CaCO2, using the same concentration of ZEA as in the present study (10/20 μM) showed that ZEA can reduce gene expression and the protein concentration of the proinflammatory cytokines IL-1β, TNF-α, IL-6, IL-8 [18].

Data from the literature show that ZEA can affect the inflammatory process and oxidative stress, studies on the mechanisms triggered by ZEA indicate the involvement of key factors such as nuclear receptors NF-kB and Nrf2 [19]. The formation of a complex between these two molecules could later lead to the modulation of other signaling molecules such as ERK1, ERK2, JNK1, JNK2, p38α [20]. Regarding the intestinal epithelium, *in vitro* studies on the IPEC-J2 porcine cell line showed that ZEA could up-regulate the gene expression of p38α, without effects on JNK1/2 and ERK1/2 levels [21]. *In vitro* studies shown that the exposure of piglets to ZEA (316 ppb) lead to a decreased tendency of the NF-kB gene expression in spleen [22]. Other studies show similar effects, ZEA (250ppb) being able to decrease the level of NF-kB in the liver [19] and colon [23] of weaned piglets.

CONCLUSIONS

ZEA is one of the most widespread fusariotoxins worldwide, being able to trigger the immune response and oxidative stress at the intestinal level.

The results obtained showed that the exposure of IPEC-1 porcine cell line to bacterial LPS led to an increase in the gene expression of pro-inflammatory cytokines *IL-1β*, *IL-6* and *IL-8*, while the co-exposure to LPS and ZEA also increased the levels of this markers compared to the control group, except for the decrease in *IL-1β* gene expression induced by LPS and ZEA 10μM.

The modulation of these cytokines could be directly related to the level of signaling molecules NF-KB, p-38α and PPARγ, whose gene expression LPS was also able to increase compared to the control group. While the co-exposure to LPS and ZEA 20μM had similar effects, the co-exposure of IPEC-1 cells to LPS and ZEA 10μM decreased the gene expression of this signaling markers below the level induced by LPS.

However, the co-exposure to LPS and ZEA did not induce significant changes in the studied oxidative stress markers, except for an increasing trend of gene expression of *KEAP*, *NQO1* (LPS+ZEA10), SOD (LPS+ ZEA20) and a significant decrease in ROS- levels induced by LPS alone.

Considering the obtained data, additional studies are needed to establish the effects and mechanisms of ZEA toxicity.

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