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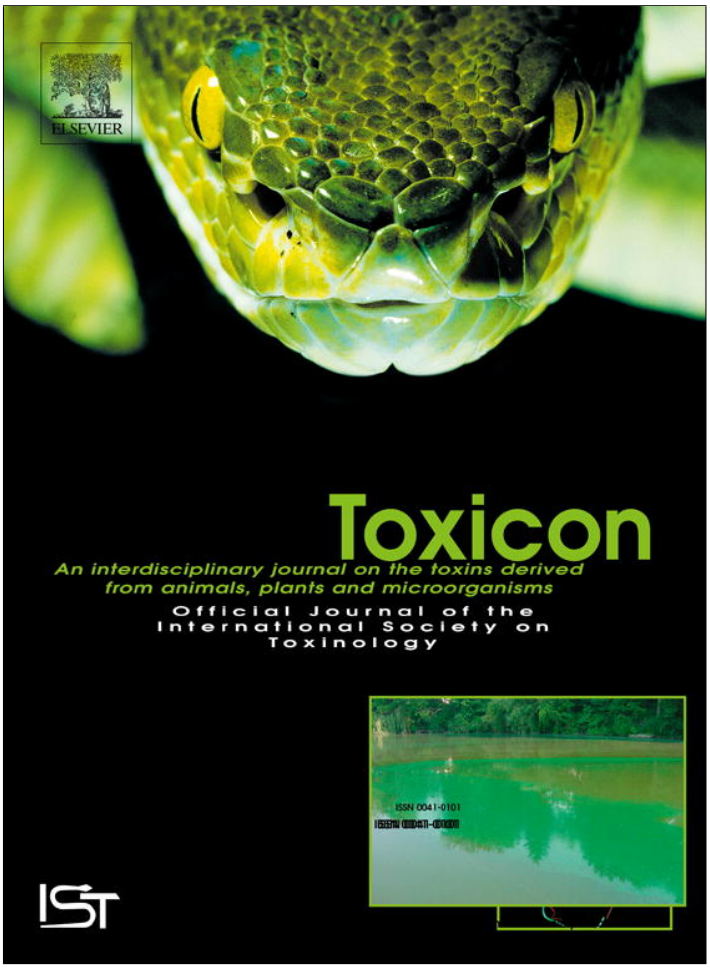


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# Effects of zearalenone and its derivatives on the innate immune response of swine

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ABSTRACT

Zearalenone (ZEN) is an estrogenic mycotoxin produced by several fungi of *Fusarium genera*. As it can contaminate food and feed it is a risk factor from both public health and agricultural perspectives. In this *in vitro* study, we compared the effects of zearalenone (ZEN) and some of its derivatives: α-zearalenol (α-ZOL); β-zearalenol (β-ZOL) and zearalanone (ZAN) on several neutrophil functions: proliferation, cytokine synthesis and oxidative stress in a porcine PMN model. The concentrations of toxins necessary to inhibit viability, in a MTT test, by 50% were: 73.4 μM for ZEN; 59.0 μM for α-ZOL; 56.8 μM for β-ZOL and 53.1 μM for ZAN, with ZEN being less toxic than its derivatives. A significant increase of $O_2^-$ synthesis compared to the control, as shown by NBT reduction, was observed at 1 μM concentration only for β-ZOL and ZAN, while at 10 μM, the ZEN derivatives (α-ZOL, β-ZOL, ZAN) induced a significant decrease of the IL-8 synthesis in swine PMNs with 49.2%, 45.6% and 45.1% respectively, compared to the control. Although, the precise mechanism of action of these toxins still remains unknown, the results of this study suggest that ZEN and its derivatives may have divergent effects on important parameters of swine innate immunity: cell proliferation, IL-8 and $O_2^-$ synthesis. Also ZEN derivatives are more toxic than ZEN.

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## 1. Introduction

Zearalenone (ZEN) is a mycotoxin that can be produced by several field fungi including *Fusarium graminearum* (*Gibberella zeae*), *F. culmorum*, *F. cerealis*, *F. equiseti* and *F. semitectum* (Richard, 2007; Bennett and Klich, 2003). It exists widely in many cereal crops such as: maize, barley, wheat, oats, sorghum and sesame seeds, as well as in hay and corn silage. These are all ingredients in many food products for human or animal nutrition (MacDonald et al., 2005; Engelhardt et al., 2006; Tabuc et al., 2009).

ZEA derivatives (α-zearalenol, β-zearalenol, zeranol, taleranol and zearalanone) can also be produced by *Fusarium* spp. in corn stems infected by fungi of this genus in the field (Mirocha et al., 1979; Bottalico et al., 1985) and in rice cultures (Richardson et al., 1985) (Fig. 1). Also, following oral exposure, zearalenone is metabolized in various tissues, particularly in the liver, the major metabolites being α-zearalenol and β-zearalenol (Dänicke et al., 2005; Malekinejad et al., 2005; Zinedine et al., 2007).

The reproductive system is the major target of ZEN toxicity (Tiemann and Dänicke, 2007; Minervini and Dell'aquila, 2008). ZEN causes alterations in the reproductive tract of laboratory animals (mice, rats, Guinea pigs) and farm animals. Decreased fertility, reduced litter size, changed weight of adrenal, thyroid and pituitary glands as well as a change in serum levels of progesterone and oestradiol have been observed, but no teratogenic effects were found (EFSA J., 2004). Occurrence in mixed feeds associated

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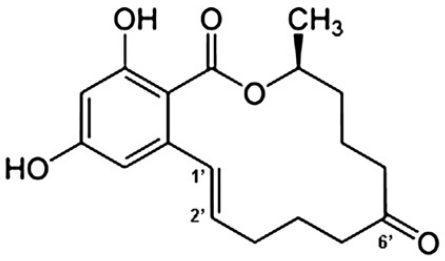


| C1' and C2' | C6' | Toxin |
|---|---|---|
| CH=CH | O | zearalenone (ZEN) |
| CH=CH | OH | α- and β- zearalenol (α- and β- ZOL) |
| $CH_2$-$CH_2$ | O | zearalanone (ZAN) |

**Fig. 1.** Chemical structure of zearalenone and derivatives used in this study.

with hyperoestrogenism has been reported in farm animals, particularly in pigs (Aucock et al., 1980; Richard, 2007). Numerous *in vivo* and *in vitro* studies have indicated that zearalenone binds to oestrogen receptors, generating an oestrogen-like response. Although, it has been thoroughly studied during the last couple of years, the pathway of ZEN toxicity is still unknown and remains unclear. At present, available data support besides the estrogenic activity, an epigenetic mechanism of ZEN carcinogenicity resulting from oxidative stress and cytotoxicity (Abid-Essefi et al., 2004).

In addition, ZEN has been shown to be immunotoxic (Atkinson and Miller, 1984; Pestka et al., 1987; Luongo et al., 2008), hepatonephrotoxic (Ouanes et al., 2003; Abbès et al., 2006; Bouaziz et al., 2009) and *an* enhancer of lipid peroxidation (Zourgui et al., 2008). It induces apoptosis (Minervini et al., 2006; Bouaziz et al., 2008), DNA fragmentation (Abid-Essefi et al., 2003; Ayed-Boussema et al., 2007), micronuclei production, chromosome aberrations (Ouanes et al., 2005; Malekinejad et al., 2007) and DNA adduct formation (Pfohl-Leszkowicz et al., 1995; Abid-Essefi et al., 2003).

However, to our knowledge few studies have investigated the effect of ZEN on innate and acquired immunity and none of them in swine. Moreover, the effect of ZEN and its derivatives has been rarely studied on neutrophils, a cell population with an important function in the achievement of the innate immune response. Neutrophils or polymorphonuclear (PMNs) cells are professional phagocytes, constituting the prime part of the innate defence against an extensive number of potentially harmful microorganisms in the environment (Witko-Sarsat et al., 2000). Their production of reactive oxygen species (ROS), including *superoxide* anion ($O_2^-$), hydrogen peroxide ($H_2O_2$) and nitric oxide, is considered to play a key role in the host defence against invading microorganisms, but can also be responsible for tissue damage (Miller and Britigan, 1995; Babior, 1984). To respond to tissue inflammation, the neutrophils migrate into the inflamed site where they produce and are also exposed to numerous environmental signals including cytokines.

In the present study we investigated the *in vitro* effects of ZEN and some of its derivatives (α-ZOL, β-ZOL, ZAN) on several neutrophil functions: proliferation, cytokine synthesis and oxidative stress in a porcine PMN model.

## 2. Materials and methods

### 2.1. Reagents

All chemicals, immunological reagents and media components were purchased from Sigma (Sigma–Aldrich, Steinheim, Germany) unless otherwise stated.

### 2.2. Toxin preparation

The toxins were dissolved in DMSO (Merck, Darmstadt, Germany) to a concentration of 1 mM and then further dilutions (50–0.1 μM) were made in PBS.

### 2.3. Biological samples

Porcine blood samples were obtained from commercial weaned piglets available in our laboratory. Animals were cared for in accordance with the National Institute of Health Guide and the Romanian Ministry of Agriculture Guide for care and use of Laboratory animals. They were acclimatized for at least 1 week prior to being used in experimental protocols and given *ad libitum* access to water and feed.

### 2.4. Cell preparation

Blood samples were aseptically collected from the left jugular vein of piglets in sodium heparin tubes (Vacutest®, Arzergrande, Italy). PMNs were isolated within three hours after blood collection. Briefly, 25 mL of blood collected in heparin tubes, was diluted with an equal volume of HBSS. PMNs were isolated by density gradient centrifugation on Hystopaque®-1077. The cell pellet was diluted in ice-cold hypotonic lysis solution (0.15 M $NH_4Cl$, 0.01 M $KHCO_3$, 0.1 mM $Na_2EDTA$), and kept on ice to disintegrate the erythrocytes. After centrifugation, neutrophils were washed two times with HBSS solution and re-suspended in DMEM, supplemented with 10% FCS, 2 mM L-glutamine, 100 U $mL^{-1}$ penicillin and 100 μg $mL^{-1}$ streptomycin and used for the assays. The purified cells consisted of a more than 95% pure population of viable neutrophils, as assessed by morphology and trypan blue exclusion test.

### 2.5. MTT-reduction assay

The cell viability was quantified by measurement of reduction of 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) produced by dehydrogenases in mitochondria of live cells, as described by Chung et al. (1993), with some modifications. Briefly, PMNs at a concentration of $3 \times 10^6$ cells/mL were cultivated with PMA (100 ng/mL) and different concentrations of toxins. After 1 h at 37 °C, 10 μl MTT solution in PBS (5 mg/mL) was added to each well and

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mixed thoroughly. After incubation for 2 h at 37 °C, 100 μl of MTT solvent (0.1 N HCl in anhydrous isopropanol) was added to each well and plates were read within 1 h after adding MTT solvent. The absorbance was measured at 570 nm using a microplate reader (TECAN SUNRISE, Austria) and the absorbance of the background at 650 nm was subtracted. All tests were performed in five independent replicates. The index of PMNs viability in the presence of the toxins was calculated with the following formula:

$$\textit{Index of viability}(\%) = \left(\frac{\textit{OD of stimulated cells treated with toxins}}{\textit{OD of stimulated and untreated cells}}\right) \times 100$$

### 2.6. Extracellular superoxide anion ($O_2^-$) release

The extracellular release of *superoxide* anion was evaluated by the cytochrome *c* reduction test (Livescu et al., 2003). Test samples containing 0.98 mg/mL cytochrome *c*, $3 \times 10^6$ cells/sample in the presence of stimuli (PMA 100 nM) and different concentration of toxins, were adjusted to a final volume of 500 μL with complete HBSS. Acellular (HBSS and cytochrome *c*) and unstimulated cellular (HBSS, cytochrome *c* and cells) samples were used as controls. Controls and test samples were incubated for 20 min at 37 °C. The reaction was stopped by centrifugation (5 min, 100 g at 4 °C) and the supernatants were collected and read at 550 nm. Data are expressed as nmol $O_2^-$ produced by $3 \times 10^6$ cells per 20 min. The calculation was made using the molar extinction coefficient of $21.1 \times 10^3$ $mol^{-1}$ $cm^{-1}$. All tests were performed in five independent replicates.

### 2.7. Intracellular $O_2^-$ synthesis

PMNs cultivated at a density of $3 \times 10^6$ were incubated at 37 °C in the presence of NBT (nitro-blue tetrazolium) (0.04%) (Fluka, Buchs, Switzerland), PMA (100 ng/mL) and different concentrations of toxins. After 1 h, the SN was replaced with a solution of 10% SDS in 0.08 N NaOH and the plates were incubated for 24 h at 37 °C. The absorbance was measured at 540 nm. All tests were performed in five independent replicates.

### 2.8. Cytokine synthesis

PMN cultivated at a density of $3 \times 10^6$ cells/well and stimulated with 100 ng/mL PMA, were incubated with different doses of toxins. Three hours later, culture supernatants were then collected and frozen at −20 °C, until analyzed for cytokines (IL-8; TNF-α; IL-1β) content by ELISA as already described by Taranu et al., 2005. Briefly, a purified fraction of anti-swine cytokines (R&D Systems, Minneapolis, USA): IL-8 (MAB5351), TNF-α (MAB6902) and IL-1β (MAB6811) were used as capture antibody in conjunction with biotinylated anti-swine cytokines: IL-8 (BAF 535), TNF-α (BAF 690) and IL-1β (BAF 681). Streptavidin-HRP (Biosource, Camarillo, USA) and TMB (tetramethylbenzidine) were used for detection.

Absorbance was read at 450 nm using the microplate reader. Recombinant swine IL-8, TNF-α and IL-1β were used as standards and results were expressed as picograms of cytokine/mL. All tests were performed in four independent replicates.

### 2.9. Phagocytosis

PMN cultivated at a density of $3 \times 10^6$ cells/well and stimulated with 100 ng/mL PMA, were incubated with the different doses of toxins. The latex beads (0.24 μM, 10% solids) were diluted to 1% aqueous suspension (in HBSS), pre-coated with porcine serum (30 min at 37 °C) and then washed twice with HBSS. 10 μL of this aqueous suspension was added to the cells together with the toxins and incubated for 90 min at 37 °C in 96 well plates. After the incubation period 20 μL of a 10% formaldehyde solution was added to each well. Control cultures with no addition of latex beads or PMA stimulation were also included. For each sample, plates were kept for 24 h at 4 °C and then washed twice with HBSS. One hundred cells were microscopically observed and the cells containing at least one latex bead were scored as positive. All tests were performed in three independent replicates.

The percentage of positive cells was calculated using the following formula:

$$\%\textit{of positive cells} = \left(\frac{\textit{Number of cells with at least one latex bead}}{\textit{Total number of cells}}\right) \times 100$$

### 2.10. Statistical analysis

Student's *t*-tests were used to analyze the differences in terms of cell proliferation, cytokine synthesis, intra- and extracellular superoxide anion production and phagocytosis between control cells and cells treated with various concentrations of ZEN, α-ZOL, β-ZOL, ZAN. The *P* values equal or less than 0.05 were considered significant. The

**Table 1**
The *index of viability* of the swine neutrophils using the MTT test.

| Toxins | Mycotoxin concentration (μM) | | | | |
|---|---|---|---|---|---|
| | 0 | 0.1 | 1 | 10 | 50 |
| ZEN | $100^{a,**}$ | $88.5 \pm 10^{a,b}$ | $91.1 \pm 10^{a,*}$ | $89.6 \pm 9.1^{a,*}$ | $71.2 \pm 19^{b}$ |
| α-ZOL | $100^{a,***}$ | $105 \pm 14^{a,**}$ | $94.7 \pm 13^{a,**}$ | $95.3 \pm 7.6^{a,**}$ | $65.5 \pm 17^{b}$ |
| β-ZOL | $100^{a,***}$ | $108 \pm 14^{a,***}$ | $98.3 \pm 13^{a,***}$ | $96.6 \pm 9.2^{a,***}$ | $62.6 \pm 15^{b}$ |
| ZAN | $100^{a,***}$ | $104 \pm 13^{a,**}$ | $98.4 \pm 10^{a,***}$ | $91.9 \pm 10^{a,**}$ | $51.1 \pm 10^{b}$ |

Results show means ± SD for five different experiments.
[a,b]Means within a row with different superscripts are significantly different.
*($p < 0.05$); **($p < 0.01$); *** ($p < 0.001$).

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**Table 2**
Cytotoxic effects, expresses as IC50 of zearalenone and its derivates on PMA stimulated swine neutrophiles.

| Toxin | IC50 (μM) |
|---|---|
| | Mean ± SD[a] |
| ZEN | 73.4 ± 26 |
| α-ZOL | 59.0 ± 9.5 |
| β-ZOL | 56.8 ± 9.6 |
| ZAN | 53.1 ± 8.8 |

IC50 is the concentration required to inhibit cell viability by 50%.
[a] Results are expressed as mean ± SD of five different experiments.

dose response curves were fitted to a non-linear regression equation and the inhibiting concentration of 50% (IC50) was defined by the regression procedure using Corel QUATTRO ProX3, Mathcad Professional 14.

## 3. Results

### 3.1. The effect of ZEN and selected ZEN derivatives on swine PMNs proliferation

The number of living cells after PMA stimulation and incubation with ZEN or ZEN derivatives was evaluated by the MTT-reduction assay (Table 1). Addition of increasing concentrations (1–50 μM) of mycotoxins in the PMA stimulated cultures inhibited PMNs viability in a dose dependent manner (Table 1). The effect was observed for all the toxins, the highest dose (50 μM) inducing a significant inhibition of viability ($p < 0.001$) compared to the PMA stimulated cell population (Table 1). The concentrations of toxins necessary to induce a 50% decrease of viability were: 73.4 μM for ZEN; 59.0 μM for α-ZOL; 56.8 μM for β-ZOL and 53.1 μM for ZAN. This shows that ZEN was less toxic than its derivatives (Table 2).

The low doses that do not induce a decrease of the PMNs viability were selected to be used in further assays as being non toxic for the cells. The lack of cytotoxicity of the selected doses of ZEN/ZEN derivatives was verified using the trypan blue exclusion method. Indeed, the doses of 1, 5 and 10 μM are not cytotoxic for the cells (data not shown).

### 3.2. Effect of ZEN and selected ZEN derivatives on the production of intracellular $O_2^-$ in swine PMNs

Neutrophil intracellular superoxide synthesis ($O_2^-$) under the action of the mycotoxins was assessed after 1 h of incubation using the NBT reduction test. As shown in Fig. 2, PMA-induced production of $O_2^-$, given as % of NBT reduction was increased compared to the control ($P < 0.0001$). At the lowest dose (1 μM) all the toxins induced an increase of $O_2^-$ synthesis, but only β-ZOL and ZAN induced a significant increase, 114.5 ± 14.7% and respectively 117.4 ± 14.4% versus 100% in the control (PMA stimulated cells).

### 3.3. Effect of ZEN and selected ZEN derivatives on extracellular $O_2^-$ production by swine PMNs

$O_2^-$ extracellular production induced by ZEA and its derivatives in PMNs was assessed by the cytochrome *c* reduction test after 20 min of incubation of cells at 37 °C. As expected, PMA stimulated the production of extracellular $O_2^-$ by porcine PMNs: 51.6 ± 7.5 nmol $O_2^-/3 \times 10^6$ cells/20 min versus 33.02 ± 3.95 nmol $O_2^-/3 \times 10^6$ cells/20 min, in control unstimulated cells (Table 3). There was no significant difference in neutrophil extracellular production between control and intoxicated cells, irrespective of the type of the toxin used.

### 3.4. Effect of ZEN and ZEN derivatives on swine PMNs phagocytosis

The influence of ZEN and ZEN derivatives on the phagocytic capacity of PMNs was assessed microscopically by counting the cells containing at least one latex bead. As shown in Table 3, when the cells were incubated with PMA, the phagocytic cells represent 46.6 ± 9.98% of the total PMNs versus 14.8 ± 1.17% in the unstimulated cells, as determined by their higher capacity to phagocyte the latex beads. When PMNs were incubated with increasing

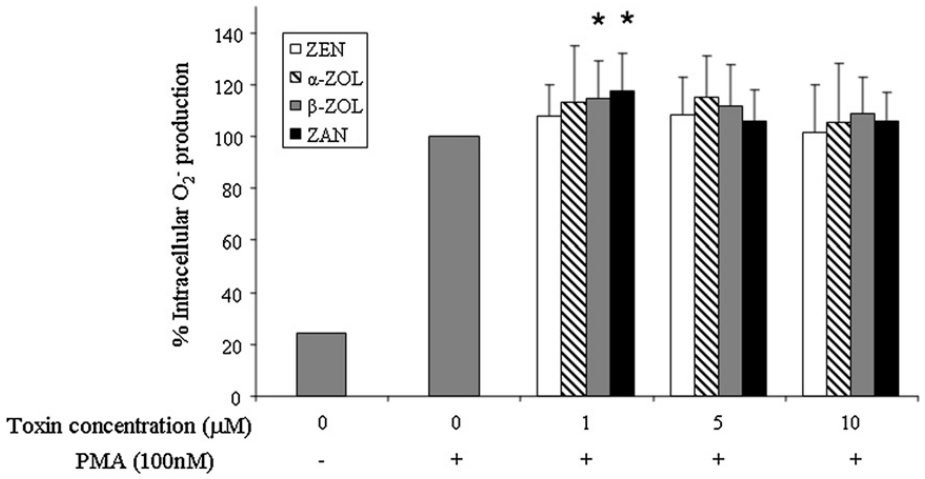


**Fig. 2.** Effect of ZEN and ZEN derivatives on ROS production by swine neutrophils, given as % of NBT reduction. Cells were incubated for 1 h in the presence of NBT (0.04%), PMA (100 ng/mL) and different concentrations of toxins. Data are means ± SD for five determinations.

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**Table 3**
The phagocytosis and the superoxide production by piglets neutrophils treated *in vitro* with ZEN and ZEN derivatives.

| Parameters/treatment[a] | C | PMA/iono | ZEN | | | α-ZOL | | | β-ZOL | | | ZAN | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 μM | 5 μM | 10 μM | 1 μM | 5 μM | 10 μM | 1 μM | 5 μM | 10 μM | 1 μM | 5 μM | 10 μM |
| Phagocytosis* (% of positive cells) | 14.8 ± 1 | 46.2 ± 9 | 46.1 ± 8 | 42.4 ± 5 | 43.1 ± 10 | 50.8 ± 6 | 53.7 ± 2 | 44.8 ± 3 | 51.0 ± 10 | 50.0 ± 7 | 49.3 ± 7 | 51.8 ± 3 | 45.9 ± 5 | 46.6 ± 4 |
| Superoxide concentration** (nmol $O_2^-$/3 × $10^6$ cells/20 min) | 33.0 ± 3 | 51.6 ± 7 | 52.6 ± 6 | 53.9 ± 9 | 52.3 ± 5 | 54.3 ± 9 | 53.5 ± 4 | 51.9 ± 4 | 54.3 ± 8 | 48.3 ± 4 | 47.7 ± 4 | 52.9 ± 3 | 50.6 ± 1 | 48.7 ± 2 |

[a] Results show means ± SD for three (*) or five (**) different experiments.

concentrations of ZEN and ZEN derivatives (1, 5, 10 μM) no variation in their ability to phagocyte latex beads was observed (Table 3).

### 3.5. Effect of ZEN and ZEN derivatives on cytokine production by swine PMNs

We next examined the ability of ZEN to modulate the synthesis of cytokines involved in the inflammatory response. PMNs were stimulated or not with PMA in the presence of non-cytotoxic concentrations of ZEN (0–10 μM) and the cell supernatant was analyzed for IL-8, TNF-α and IL-1β concentration by specific ELISA. PMA stimulation increased by 30 times the IL-8 synthesis comparative with the control (data not shown). The toxins induced a dose related decrease of the IL-8 synthesis (Fig. 3), but only the highest dose (10 μM) determined a significant decrease of the IL-8 synthesis in α-ZOL, β-ZOL and ZAN treated PMN with 49.2%; 45.6% and 45.1% respectively. No effect of ZEN on IL-8 synthesis was observed, irrespective of the used concentration. By contrast, the porcine PMNs were not able to synthesize detectable concentrations of TNF-α or IL-1β, even after PMA stimulation (data not shown).

## 4. Discussion

Pigs, and especially young gilts, seem to be the most sensitive animal species to ZEN effects, being even more sensitive than rodents (JECFA, 2000; SCF, 2002). Therefore, our experiments were designed to analyze the effects of ZEN and ZEN derivatives on porcine PMNs activity and to our knowledge it is the first study that investigates the involvement of ZEN in the modulation of the immune response in swine.

Knowing that neutrophils are immune cell with a short life span, we first investigated the effect of ZEN and ZEN derivatives on neutrophils viability. Our results showed that ZEN and ZEN derivatives decrease the number of living cells in response to mitogen stimulation *in vitro,* in a dose dependent manner (Table 1). To our knowledge, no other data are available concerning the in vitro effects of ZEN on neutrophil viability, irrespective of the species. However, the few data concerning the in vivo effects of mycotoxins on the number of neutrophils are controversial. For example, Abbès et al. (2006) observed that balb/c mice treated for 48 h with 40 and 500 mg/kg bw ZEN present a significant increase of the number of blood neutrophils, but 10 ppm ZEA administered to weanling female B6C3F1 mice for 8 weeks did not affect the number of circulating neutrophils (Forsell et al., 1986).

Other studies investigating the effects of ZEN derivatives on the production of ROS, which is considered to play a key role in the host defence against invading microorganisms, showed results similar with those obtained in the present study: (i) an induction in the percentage of intracellular $O_2^-$ synthesis (Fig. 2); (ii) no effect on the extracellular superoxide anion ($O_2^-$) synthesis by PMA stimulated PMNs under mycotoxins action (Table 3). Similarly, in a study that investigated the $O_2^-$ release by the use of the SOD-inhibitable reduction of cytochrome *c* assay, Richetti et al. (2003) showed that the dose of $10^{-2}$ mg/mL

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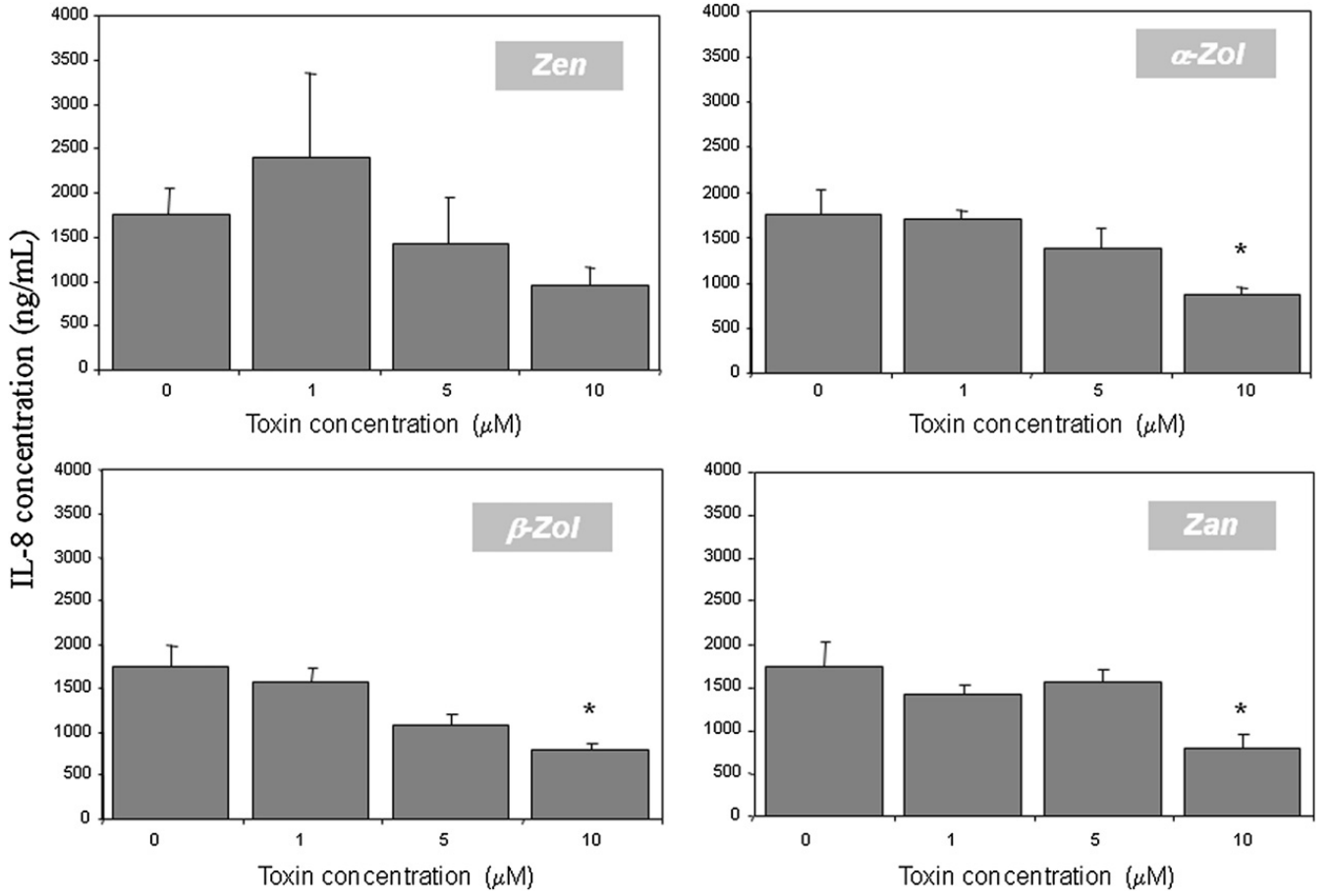


**Fig. 3.** Effect of ZEN and ZEN derivatives on IL-8 production by swine neutrophils. Cells stimulated with 100 ng/mL PMA, were incubated with different doses of toxins. Three hours later, culture supernatants were analyzed for cytokine content by ELISA. Data are means ± SD for four determinations.

(equivalent with the doses used in our study) of β-ZOL was not capable to induce a significant decrease of the $O_2^-$ release in the PMA or zymosan stimulated human neutrophils. The concentrations of ZEN and of the derivatives used in our experiment were not probably able to interfere with NADPH oxidase, enzyme that contributes to the killing of extracellular organisms taken up by phagocytosis (Delves et al., 2006), but rather with the mechanisms involved in the defence against microbes which invade the cytosol. The effect was significant only at lower concentrations (1 μM), suggesting that these mycotoxins are more immunomodulatory than immunotoxic.

Furthermore we analyzed the effect of ZEN and its derivatives on PMNs phagocytosis. We did not observe a reduction of the phagocytic activity after treatment with ZEN and ZEN derivatives (Table 3). Similarly, the bactericidal activity of the bovine PMN co-treated with PMA and toxins was not affected by ZEA (Murata et al., 2002, 2003). By contrast, in the bovine PMN intoxicated with ZEA and derivatives, before the PMA stimulation, the bactericidal activity of the cells was suppressed by $10^{-5}$ M ZEN, α-ZOL and β-ZOL. It seems that PMA priming (maybe through the PKC activation) triggers a chain of reaction that could not be affected by the toxin treatment anymore.

In the literature, data concerning the ZEN effects on the inflammatory cytokines is controversial. ZEN and its derivatives have been described as inductors (Ruh et al., 1998) as well as suppressors (Ben Salah-Abbès et al., 2008) of expression of proinflammatory cytokines. The chemokine IL-8 is often associated with inflammation and through the recruitment of inflammatory cells it induces a further increase in oxidant stress mediators, being thus a key parameter in localized inflammation (Peveri et al., 1988). Interestingly, in our study, ZEN derivatives decreased IL-8 synthesis in the porcine treated PMNs in a dose dependent manner. However, this decrease was not associated with the decrease of $O_2^-$ synthesis (Fig. 2).

Our results showed that ZEN derivatives altered several parameters of the innate immune response of swine PMNs, cell proliferation, IL-8 and $O_2^-$ synthesis in swine PMN and the toxic effects was higher than that produced by ZEN. In accordance with the amplitude of their toxic effect we classified the toxicity of the four mycotoxins investigated as follow: ZAN > β-ZOL > α-ZOL > ZEN. Zearalenols derived from ZEN through reduction of the C6′ ketone into hydroxide and ZAN posses a hydrogenated C1′–2′ bond instead of the double bond from the macrolide ring of ZEN (Fig. 1). In the case of β- and α-ZOL the presence of alpha-hydroxyl at the position C-6′ has been considered the factor involved in the toxicity in human lymphocytes (Forsell and Pestka, 1985). The results concerning ZAN toxicity are more controversial because the role of the C1′–2′ double bound (presence or absence) in the toxic activity of ZAN and respectively ZEN remains unclear (Murata et al., 2003). Pigs

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have been shown to be the most sensitive species to the estrogenic effects of ZEN. This seems to be due to the fact that in a hepatic biotransformation, pigs convert ZEA predominantly into α-ZOL (Malekinejad et al., 2006) that has a bigger affinity to bind oestrogen receptors (Fitzpatrick et al., 1989; Biehl et al., 1993). The α-ZOL is the most powerful oestrogen from the ZEN group, which was reported for oral administration in rats, the estrogenic potencies were as follows: α-ZOL > ZAN > ZEN > β-ZOL (Everett et al., 1987). This classification of the estrogenic potency of ZEN and its derivatives does not seem to be correlated with the ranking observed for their immunocellular toxicity as shown in our study or in other works (Murata et al., 2003; Forsell and Pestka, 1985).

To our knowledge, this is the first study that investigates the effects of ZEN and its derivatives on the swine cellular innate immune response. Although the precise mechanism of action of these toxins remains still unknown, the results of this study suggests that ZEN and its derivatives may have divergent effects on important parameters of swine innate immunity: cell viability, IL-8 and $O_2^-$ synthesis. Also ZEN derivatives manifest an effect more toxic than that of ZEN. Due to the crucial role of PMNs in the host innate immune response to bacterial infection and to the fact that the responses of these cells were modulated by ZEN and its derivatives, further studies are needed to elucidate the specific mechanisms by which these toxins influence PMNs functions.

## Conflict of interest statement

There are no conflicts of interest.

## References


Abbès, S., Ouanes, Z., Ben Salah-Abbès, J., Houas, Z., Oueslati, R., Bacha, H., Othman, O., 2006. The protective effect of hydrated sodium calcium aluminosilicate against haematological, biochemical and pathological changes induced by zeralenone in mice. Toxicon 5, 567–574.

Abid-Essefi, S., Baudrimont, I., Hassen, W., Ouanes, Z., Mobio, T.A., Anane, R., Creppy, E.E., Bacha, H., 2003. DNA fragmentation, apoptosis and cell cycle arrest induced by zearalenone in cultured DOK, Vero and Caco-2 cells: prevention by Vitamin E. Toxicology 192, 237–248.

Abid-Essefi, S., Ouanes, Z., Hassen, W., Baudrimont, I., Creppy, E., Bacha, H., 2004. Cytotoxicity, inhibition of DNA and protein syntheses and oxidative damage in cultured cells exposed to zearalenone. Toxicol. In Vitro 18, 467–474.

Atkinson, H.A., Miller, K., 1984. Inhibitory effect of deoxynivalenol, 3-acetyldeoxynivalenol and zearalenone on induction of rat and human lymphocyte proliferation. Toxicol. Lett. 23, 215–221.

Aucock, H.W., Marasas, W.F., Meyer, C.J., Chalmers, P., 1980. Field outbreaks of hyperoestrogenism (vulvo-vaginitis) in pigs consuming maize infected by *Fusarium graminearum* and contaminated with zearalenone. J. S. Afr. Vet. Assoc. 51, 163–166.

Ayed-Boussema, I., Ouanes, Z., Bacha, H., Abid, S., 2007. Toxicities induced in cultured cells exposed to zearalenone: apoptosis or mutagenesis? J. Biochem. Mol. Toxicol. 21, 136–144.

Babior, B.M., 1984. Oxidants from phagocytes: agents of defense and destruction. Blood 64, 959–966.

Ben Salah-Abbès, J., Abbès, S., Houas, Z., Abdel-Wahhab, M., Oueslati, R., 2008. Zearalenone induces immunotoxicity in mice: possible protective effects of radish extract (Raphanus sativus). J. Pharm. Pharmacol. 60, 761–770.

Bennett, J.W., Klich, M., 2003. Mycotoxins. Clin. Microbiol. Rev. 16, 497–516.

Biehl, M.L., Prelusky, D.B., Koritz, G.D., Hartin, K.E., Buck, W.B., Trenholm, H.L., 1993. Biliary excretion and enterohepatic cycling of zearalenone in immature pigs. Toxicol. Appl. Pharmacol. 121, 152–159.

Bottalico, A., Visconti, A., Logrieco, A., Solfrizzo, M., Mirocha, C.J., 1985. Occurrence of zearalenols (diastereomeric mixture) in corn stalk rot and their production by associated Fusarium species. Appl. Environ. Microbiol. 49, 547–551.

Bouaziz, C., Martel, C., Sharaf el dein, O., Abid-Essefi, S., Brenner, C., Lemaire, C., Bacha, H., 2009. Fusarial toxin-induced toxicity in cultured cells and in isolated mitochondria involves PTPC-dependent activation of the mitochondrial pathway of apoptosis. Toxicol. Sci. 110, 363–375.

Bouaziz, C., SharafEl Dein, O., El Golli, E., Abid-Essefi, S., Brenner, C., Lemaire, C., Bacha, H., 2008. Different apoptotic pathways induced by zearalenone, T-2 toxin and ochratoxin A in human hepatoma cells. Toxicology 254, 19–28.

Chung, W.B., Bäckström, L.R., McDonald, J., Collins, M.T., 1993. The (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) colorimetric assay for the quantitation of Actinobacillus pleuropneumoniae cytotoxin. Can. J. Vet. Res. 57, 159–165.

Dänicke, S., Swiech, E., Buraczewska, L., Ueberschär, K.H., 2005. Kinetics and metabolism of zearalenone in young female pigs. J. Anim. Physiol. Anim. Nutr. (Berl.) 89, 268–276.

Delves, P.J., Martin, S.J., Burton, D.B., Roitt, I.M., 2006. Roitt's Essential Immunology, eleventh ed. Blackwell Publishing, Massachusetts.

EFSA (European Food Safety Authority), 2004. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to Zearalenone as undesirable substance in animal feed. 89, 1–35.

Engelhardt, G., Barthel, J., Sparrer, D., 2006. Fusarium mycotoxins and ochratoxin A in cereals and cereal products: results from the Bavarian Health and Food Safety Authority in 2004. Mol. Nutr. Food Res. 50, 401–405.

Everett, D.J., Perry, C.J., Scott, K.A., Martin, B.W., Terry, M.K., 1987. Estrogenic potencies of resorcylic acid lactones and 17 beta-estradiol in female rats. J. Toxicol. Environ. Health 20, 435–443.

Fitzpatrick, D.W., Picken, C.A., Murphy, L.C., Buhr, M.M., 1989. Measurement of the relative binding affinity of zearalenone, alpha-zearalenol and beta-zearalenol for uterine and oviduct estrogen receptors in swine, rats and chickens: an indicator of estrogenic potencies. Comp. Biochem. Physiol. C 94, 691–694.

Forsell, J.H., Pestka, J.J., 1985. Relation of 8-ketotrichothecene and zearalenone analog structure to inhibition of mitogen-induced human lymphocyte blastogenesis. Appl. Environ. Microbiol. 50, 1304–1307.

Forsell, J.H., Witt, M.F., Tai, J.H., Jensen, R., Pestka, J.J., 1986. Effects of 8-week exposure of the B6C3F1 mouse to dietary deoxynivalenol (vomitoxin) and zearalenone. Food Chem. Toxicol. 24, 213–219.

JECFA, 2000. Joint FAO/WHO Expert Committee on Food Additives, 53rd Report. Safety Evaluation of Certain Food Additives. In: WHO Food Additives Series 44.

Livescu, A., Manda, G., Constantin, C., Neagu, M., Iordachescu, D., 2003. Plasma membrane potential interferes with the respiratory burst of peripheral granulocytes. J. Cell. Mol. Med. 7, 73–78.

Luongo, D., De Luna, R., Russo, R., Severino, L., 2008. Effects of four *Fusarium* toxins (fumonisin B(1), alpha-zearalenol, nivalenol and deoxynivalenol) on porcine whole-blood cellular proliferation. Toxicon 52, 156–162.

MacDonald, S.J., Anderson, S., Brereton, P., Wood, R., Damant, A.J., 2005. Determination of zearalenone in barley, maize and wheat flour, polenta, and maize-based baby food by immunoaffinity column cleanup with liquid chromatography: interlaboratory study. AOAC Int. 88, 1733–1740.

Malekinejad, H., Maas-Bakker, R.F., Fink-Gremmels, J., 2005. Enzyme kinetics of zearalenone biotransformation: pH and cofactor effects. Arch. Toxicol. 79, 547–553.

Malekinejad, H., Schoevers, E.J., Daemen, I.J., Zijlstra, C., Colenbrander, B., Fink-Gremmels, J., Roelen, B.A., 2007. Exposure of oocytes to the Fusarium toxins zearalenone and deoxynivalenol causes aneuploidy and abnormal embryo development in pigs. Biol. Reprod. 77, 840–847.

Malekinejad, H., Maas-Bakker, R., Fink-Gremmels, J., 2006. Species differences in the hepatic biotransformation of zearalenone. Vet. J. 172, 96–102.

Miller, R.A., Britigan, B.E., 1995. The formation and biologic significance of phagocyte-derived oxidants. J. Investig. Med. 43, 39–49.

Minervini, F., Dell'aquila, M.E., 2008. Zearalenone and reproductive function in farm animals. Int. J. Mol. Sci. 9, 2570–2584.

Minervini, F., Giannoccaro, A., Fornelli, F., Dell'Aquila, M.E., Minoia, P., Visconti, A., 2006. Influence of mycotoxin zearalenone and its derivatives (alpha and beta zearalenol) on apoptosis and proliferation of cultured granulosa cells from equine ovaries. Reprod. Biol. Endocrinol. 4, 62–75.

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Mirocha, C.J., Schauerhamer, B., Christensen, C.M., Kommedahl, T., 1979. Zearalenone, deoxynivalenol, and T-2 toxin associated with stark rot in corn. Appl. Environ. Microbiol. 38, 557–558.

Murata, H., Shimada, N., Yoshioka, M., Apr. 2002. Suppressive effect of zearalenone, an estrogenic mycotoxin, on bovine neutrophil chemiluminescence. Vet. Hum. Toxicol. 44, 83–86.

Murata, H., Sultana, P., Shimada, N., Yoshioka, M., 2003. Structure–activity relationships among zearalenone and its derivatives based on bovine neutrophil chemiluminescence. Vet. Hum. Toxicol. 45, 18–20.

Ouanes, Z., Abid, S., Ayed, I., Anane, R., Mobio, T., Creppy, E.E., Bacha, H., 2003. Induction of micronuclei by zearalenone in Vero monkey kidney cells and in bone marrow cells of mice: protective effect of Vitamin E. Mutat. Res. 538, 63–70.

Ouanes, Z., Ayed-Boussema, I., Baati, T., Creppy, E.E., Bacha, H., 2005. Zearalenone induces chromosome aberrations in mouse bone marrow: preventive effect of 17beta-estradiol, progesterone and Vitamin E. Mutat. Res. 565, 139–149.

Pestka, J.J., Tai, J.H., Witt, M.F., Dixon, D.E., Forsell, J.H., 1987. Suppression of immune response in the B6C3F1 mouse after dietary exposure to the Fusarium mycotoxins deoxynivalenol (vomitoxin) and zearalenone. Food Chem. Toxicol. 25, 297–304.

Peveri, P., Walz, A., Dewald, B., Baggiolini, M., 1988. A novel neutrophil activating factor produced by human mononuclear phagocytes. J. Exp. Med. 167, 1547–1559.

Pfohl-Leszkowicz, A., Chekir-Ghedira, L., Bacha, H., 1995. Genotoxicity of zearalenone, an estrogenic mycotoxin: DNA adduct formation in female mouse tissues. Carcinogenesis 16, 2315–2320.

Richard, J.L., 2007. Some major mycotoxins and their mycotoxicoses - an overview. Int. J. Food Microbiol. 119, 3–10.

Richardson, K.E., Hagler, W.M., Mirocha, C.J., 1985. Production of zearalenone, α- and β-zearalenol, and α- and β-zearalanol by Fusarium spp in rice culture. J. Agric. Food Chem. 33, 862–866.

Richetti, A., Cavallaro, A., Ainis, T., Fimiani, V., 2003. Effect of some mycotoxins on superoxide anion production of isolated human neutrophils and in whole blood. Immunopharmacol. Immunotoxicol. 25, 441–449.

Ruh, M.F., Bi, Y., Cox, L., Berk, D., Howlett, A.C., Bellone, C.J., 1998. Effect of environmental estrogens on IL-1beta promoter activity in a macrophage cell line. Endocrine 9, 207–211.

SCF (Scientific Committee on Food on Fusarium Toxins), 2002. Part 6: Group Evaluation of T-2 Toxin, HT-2 Toxin, Nivalenol and Deoxynivalenol. http://europa.eu.int/comm/food/fs/sc/scf/index_en.html.

Tabuc, C., Marin, D., Guerre, P., Sesan, T., Bailly, J.D., 2009. Molds and mycotoxin content of cereals in southeastern Romania. J. Food Prot. 72, 662–665.

Taranu, I., Marin, D.E., Bouhet, S., Pascale, F., Bailly, J.D., Miller, J.D., Pinton, P., Oswald, I.P., 2005. Mycotoxin fumonisin $B_1$ alters the cytokine profile and decreases the vaccinal antibody titer in pigs. Toxicol. Sci. 84, 301–307.

Tiemann, U., Dänicke, S., 2007. In vivo and in vitro effects of the mycotoxins zearalenone and deoxynivalenol on different non-reproductive and reproductive organs in female pigs: a review. Food Addit. Contam. 24, 306–314.

Witko-Sarsat, V., Rieu, P., Descamps-Latscha, B., Lesavre, P., Halbwachs-Mecarelli, L., 2000. Neutrophils: molecules, functions and pathophysiological aspects. Lab. Invest. 80, 617–653.

Zinedine, A., Soriano, J.M., Moltó, J.C., Mañes, J., 2007. Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. Food Chem. Toxicol. 45, 1–18.

Zourgui, L., Golli, E.E., Bouaziz, C., Bacha, H., Hassen, W., 2008. Cactus (Opuntia ficus-indica) cladodes prevent oxidative damage induced by the mycotoxin zearalenone in Balb/C mice. Food Chem. Toxicol. 46, 1817–1824.