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RESEARCH ARTICLE

Abstract

Deoxynivalenol (DON) is one of the most prevalent type B trichothecenes present in food inducing adverse effects, including intestinal changes and immunosuppression. The aim of the present study was to investigate the effects of DON on rats exposed for 7, 14 and 28 days to mycotoxin-contaminated diets, using histological and immunohistochemical analyses on liver and lymphoid organs. Fifty rats received a control diet, or a diet contaminated with 1.75 mg/kg of DON for 30 days, or a diet contaminated with 11.4 mg/kg of DON for 7, 14 or 30 days. Ingestion of contaminated feed induced a significant increase in the lesional score in the liver, spleen, and lymph nodes. The main histological findings observed in the liver were cytoplasmic vacuolisation and hepatocelular megalocytosis. A significant increase in hepatocyte proliferation was observed in rats that received 1.75 mg/kg of DON. Lymphoid depletion was the main histological alteration observed in lymphoid organs, resulting in a significant increase in the lesional score in all groups that received the contaminated diets. The histological changes and lymphocyte apoptosis were more severe in lymph nodes of rats fed 11.4 mg/kg of DON during 30 days. The results of the morphological and immunohistochemical analyses suggest that the ingestion of DON can induce functional hepatic impairment and immunosuppression in a dose- and time-dependent manner.

Keywords: apoptosis, cell proliferation, histology, mycotoxins

1. Introduction

Mycotoxins are secondary metabolites produced by fungi that commonly contaminate food and feed. Deoxynivalenol (DON) is the most frequent mycotoxin of the trichothecenes group worldwide (Escrivá *et al.*, 2015). Contamination level of grains and cereals with DON varies between 0.250 to 50.289 mg/kg with a mean level of 0.967 mg/kg (Streit *et al.*, 2013). DON has been associated with a spectrum of adverse effects that includes anorexia, growth suppression, emesis, nausea, neuroendocrine changes, and immunosuppression (Pestka, 2010; Pinton and Oswald, 2014). Surprisingly, there are few studies focusing on the histological parameters due to the effects of DON in rats (Abdel-Wahhab *et al.*, 2015; Arnold *et al.*, 1986a; Wang *et al.*, 2014), and no reports have analysed the immunohistochemical changes.

Lymphoid depletion and apoptosis are the main histological changes reported in animals exposed to DON (Mikami *et al.*, 2010). Considering that DON affects cells with high turnover, both *in vitro* and *in vivo* studies have reported mainly the effects of DON on lymphoid cells or tissues (Choi *et al.*, 2013; Forsell *et al.*, 1987). Further, *in vitro* studies have demonstrated that DON inhibits human and rat granulomonocytic progenitors in a concentration-dependent manner (Lautraite *et al.*, 1997), and also blood cell lines (Baltriukiene *et al.*, 2007). However, data relative to the toxic effects of DON on tissues with low turnover, such as liver, are conflicting. Liver cell lines from different species (rat, mouse and human) exposed to increasing concentrations of DON showed a dose-dependent hepatotoxicity and different species sensitivity (Sahu *et al.*, 2010). Moreover, DON hepatotoxicity was related to an increase in oxidative stress and a decrease in mitochondrial function (Sahu *et al.*, 2008).

Toxicological differences between species are well known. High acute doses of mycotoxins are frequently used in *in vivo* studies with murine models (Morrisey *et al.*, 1985; Zhou *et al.*, 2000). However, natural contamination of feed and food presents mainly low doses and animals are chronically exposed (Zain, 2011). Considering the few reports focusing on the multiple histological alterations and the interest in discerning the effects of exposure to different doses of DON and duration, we investigated these effects in rats exposed for 7, 14 and 28 days to low and high DON-contaminated diets as well as the effects in low (hepatocytes) and high (lymphocytes) renewing cells in an *in vivo* model, using a combination of histological and immunohistochemical assays.

2. Material and methods

Animals

Fifty Wistar rats of 56 days old were used in this study. All animals were housed in plastic cages at 22 °C with periods of 12 h of dark-light, had free access to water and food, and were weighed weekly until the end of the experimental period. All animal experiments were performed at the Toxalim animal facility (INRA, UMR 1331, Toulouse), licensed by the French Ministry of Agriculture (agreement no. B31.555.13). This study complied to the usage of animal in experiments as stipulated by the European Union regulation, reviewed by the regional ethics committee (CNREEA no. 1; MP/03/62/11/11).

Purified DON was purchased from Sigma (Saint-Quentin Fallavier, France), and included in the diet of all animals. Control and contaminated diets were manufactured at the INRA UPAE (Unité de Préparation des Aliments, Jouy-en-Josas, France), and formulated according to requirements for rats. Feed was supplemented with DON at 1.75 and 11.4 mg/kg and checked for the absence of other mycotoxins (3- and 15-acetyldeoxynivalenol, nivalenol, fusarenon X, diacetoxyscirpenol, T2 and HT2 toxins, zearalenone, fumonisin B_1 and B_2, moniliformin, ergot alkaloids, aflatoxins and ochratoxins). The animals were distributed in five groups: a control group, a group fed 1.75 mg/kg of DON for 30 days, a group fed 11.4 mg/kg of DON for 7 days, a group fed 11.4 mg/kg of DON for 14 days and a group fed 11.4 mg/kg of DON for 30 days.

Histopathological and immunohistochemical assays

At the end of the experimental periods, all rats were euthanised by cervical dislocation. Samples of mesenteric lymph nodes, spleen, and liver were fixed in 10% buffered formalin solution, dehydrated trough graded alcohols and embedded in paraffin. Sections of 5 µm were stained with the haematoxylin-eosin method for histological evaluation. A morphological score was designed to compare possible histological changes in the liver and lymphoid organs (Gerez *et al.*, 2015). The frequency and severity of each criterion were considered by using the predetermined score: 0 (absent), 1 (discrete), 2 (moderate), and 3 (severe). The following criteria were included in the score analysis: lymphoid depletion of follicular area, necrosis of lymphoid follicles, medullary histiocytosis, eosinophilic infiltrate, and number of mitotic figures and apoptotic corpuscles. The severity factor considered for necrosis was 3; lymphoid depletion was estimated at 2, and all other criteria diagnostic were considered as 1. The mitotic figures and apoptotic corpuscles were determined by randomly counting five fields (400× magnification), considering the following score: 1 (0 to 5 mitotic figures or apoptotic bodies/field), 2 (6 to 10), and 3 (>10) (Gerez *et al.*, 2015).

Apoptosis was evaluated by an immunohistochemical assay using anti-caspase-3 (CCasp3) (anti-Asp 175, 1:200 dilution, Cell Signaling Technology, Beverly, MA, USA) and cell proliferation by anti-proliferating cell nuclear antigen (PCNA) (Clone PC10, 1:200 dilution, Zymed). The immunohistochemical procedures were described previously (Gerez *et al.*, 2015). The immunoexpression of CCasp3 in the liver, lymph nodes, and spleen was estimated by counting the cytoplasm of cells that were immunoreactive in five fields at 400× magnification. The immunoexpression of PCNA in the liver was estimated by counting the nucleus of cells that were immunoreactive in five fields at 400× magnification.

Statistical analysis

The values of scores were presented as means ± standard error of the mean; all scores were statistically analysed using normality (Shapiro-Wilk) and homogeneity (Bartlett) tests. When these two assumptions were verified, the analysis of variance (ANOVA) was applied, followed by Tukey's test. The alpha level was set at 0.05.

3. Results

Effects of deoxynivalenol on weight gain

Animals were weighed at the end of each experimental period to assess weight gain. Ingestion of 11.4 mg/kg for 30 days induced a significant decrease in weight gain compared to the control and other contaminated diets. Rats fed the low dose of DON and the highest dose for shorter periods (7 and 14 days) showed no difference in weight gain when compared to the control group (Figure 1).

Effects of deoxynivalenol on liver

The histological alterations were not observed in the liver of animals from the control group (Figure 2A). The main histological findings observed in the liver of rats fed 1.75

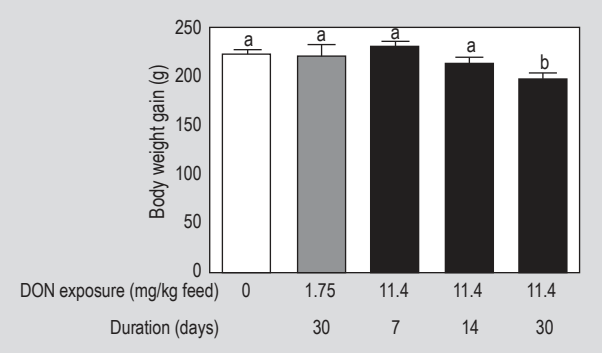

Figure 1. Body weight. Effects of deoxynivalenol (DON) on the body weight (g) of rats submitted to different treatments with DON. Mean ± standard error of the mean. Different letters show significant differences by Tukey's test (*P*<0.05).

mg/kg of DON were mild trabecular disorganisation, moderate hepatocyte vacuolisation, and inflammatory infiltrate (Figure 2B). The groups that received 11.4 mg/kg of DON for 7 and 14 days showed similar changes in addition to mild hepatocyte megalocytosis. Rats fed the higher dose of DON for 30 days showed the greatest lesional score, characterised by the lesions observed in the other groups accompanied by nuclear vacuolisation and moderate megalocytosis (Figure 2C).

Ingestion of contaminated diets induced hepatic histological changes in a dose and time dependent manner (Figure 2G). Both groups that received contaminated diets for 30 days showed a significant increase in lesional score when compared to the control ($P \leq 0.001$). A more elevated

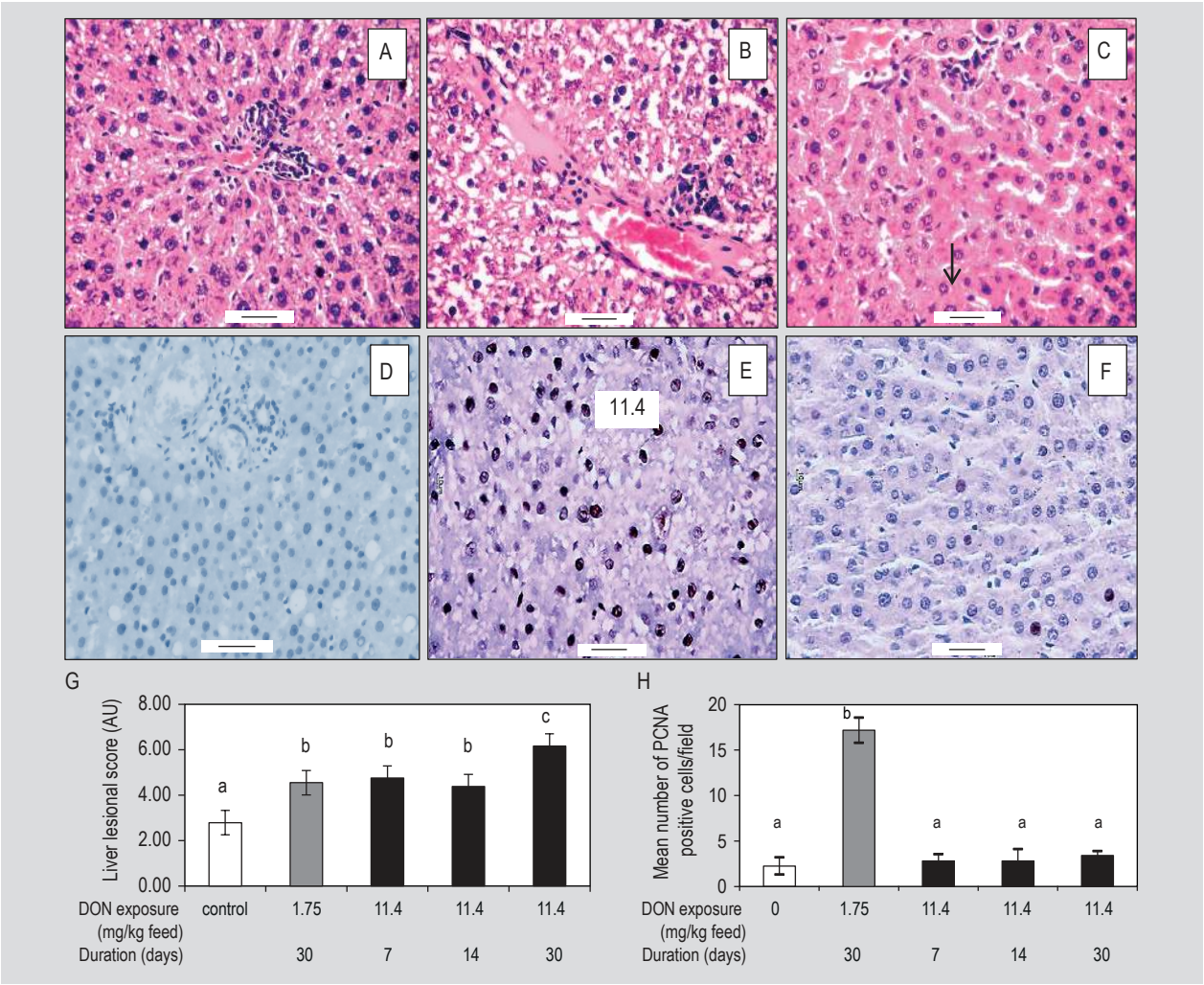

Figure 2. Effects of deoxynivalenol (DON) on the liver of rats submitted to different treatments with DON. (A) Normal histological aspect (control). (B) Disorganisation of hepatic cords and cytoplasmic vacuolisation (1.75 mg/kg of DON/30 days). (C) Hepatocyte megalocytosis (arrow) (11.4 mg/kg of DON/30 days). (D) Hepatocytes showing no immunostaining for proliferating cell nuclear antigen (PCNA) (control); (E) Strong and diffuse immunostaining for PCNA in the liver of rats exposed to DON (1.75 mg/kg of DON/30 days); (F) Mild immunostaining for PCNA in the liver of rats exposed to DON (11.4 mg/kg of DON/ 7 days). (G) Mean lesional score on liver. (H) Mean number of immunoreactive hepatocytes/field for PCNA. A-C = haematoxylin-eosin staining; D-F = immunoperoxidase staining; bar 50 µm; AU = arbitrary units; different letters show significant differences by Tukey's test (*P*<0.05).

increase in hepatic lesions were observed after the ingestion of 11.4 mg/kg of DON for 30 days when compared to the groups fed the same diet for shorter periods (7 and 14 days).

A significant increase in cell proliferation was observed in the liver of rats fed 1.75 mg/kg of DON (Figure 2E) when compared to control (Figure 2D) (17.16 versus 2.25 immunoreactive cells/field, $P \leq 0.001$, Figure 2H). By contrast, the ingestion of the higher dose of DON showed no significant difference on hepatocyte proliferation when compared to control (Figure 2F). However, no immunoreactivity to caspase-3 was observed in the liver of all experimental animals.

Effects of deoxynivalenol on lymphoid organs

The main histological findings of the lymphoid organs of rats fed the contaminated diets were lymphoid depletion and lymphocyte apoptosis. Lymphoid depletion was characterised by scanty or the absence of lymphatic nodules, and small and few germinal centres. A significant increase in the lesional score of the spleen was observed in all DON-contaminated groups compared to control animals (Figure 3A). However, there was no significant difference between the contaminated diets. In the control group, immunostaining for caspase-3 varied from no staining to mild immunostaining (Figure 3C). A mild apoptotic index was observed in groups fed 1.75 mg/kg (Figure 3D) and 11.4 mg/kg for 7 and 14 days. Lymphocyte apoptosis showed a significant increase only in the spleen of rats fed 11.4 mg/kg of DON for 30 days (Figure 3B).

The lymph nodes of all animals that received contaminated diets (1.75 and 11.4 mg/kg) showed a significant increase in the lesional score compared to control animals. In addition, the combination of the highest dose and longer duration induced more changes in lymphoid tissue when compared to that of other contaminated groups (Figure 4A). Similarly, the apoptotic index showed a significant increase ($P \leq 0.001$) in rats that received 11.4 mg/kg DON-contaminated diets for 30 days compared to other contaminated groups (Figure 4B, 4D). All groups receiving DON-contaminated diets showed higher levels of lymphocyte apoptosis when compared to control (Figure 4B, 4C).

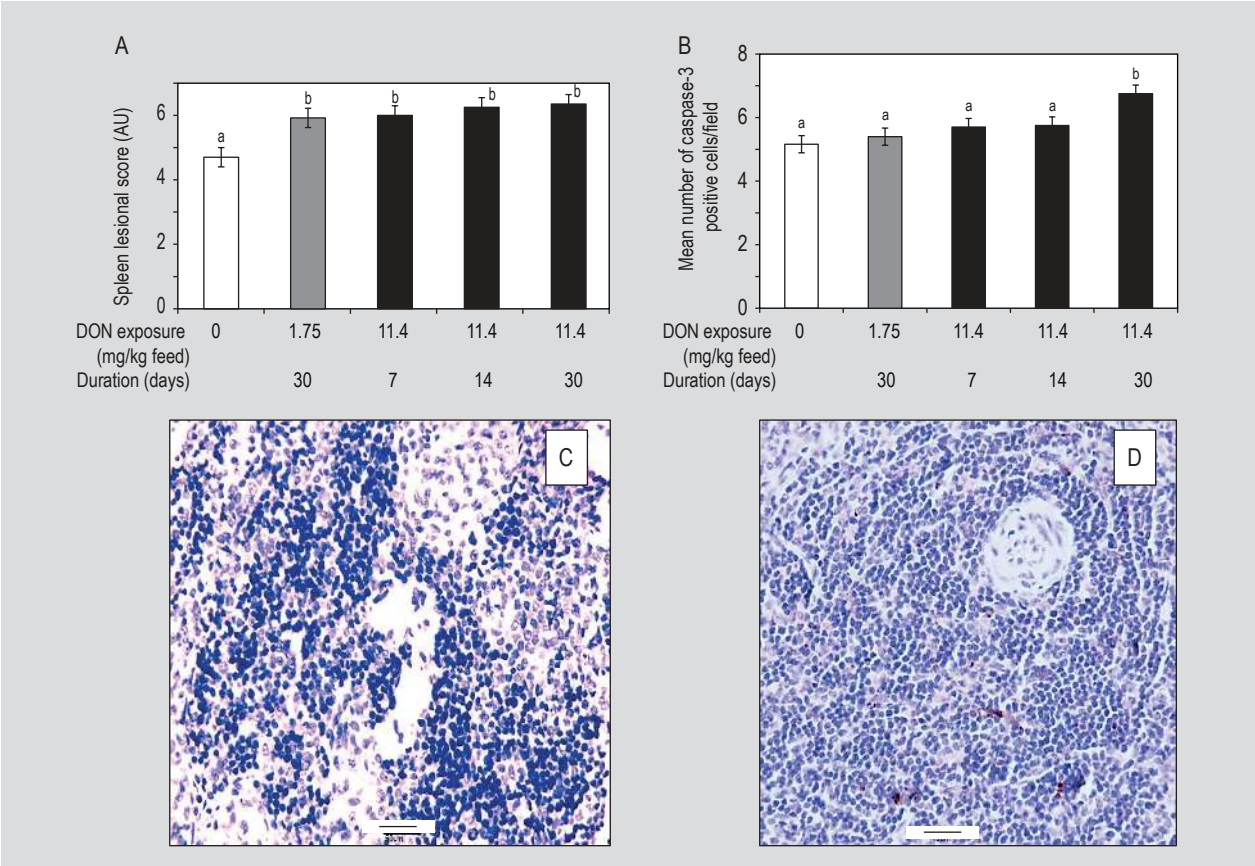

Figure 3. Effects of deoxynivalenol (DON) on spleen of rats submitted to different treatments with DON. (A) Mean lesional score on spleen. (B) Mean number of immunoreactive cells for caspase-3 in spleen/field. (C) Lymphoid follicle with absence of immunoreactivity to caspase-3 (control); (D) Mild immunostaining for caspase-3 in the spleen (1.75 mg/kg of DON/30 days). C and D = immunoperoxidase staining; bar 50 µm; AU = arbitrary units; different letters show significant differences by Tukey's test ($P<0.05$).

4. Discussion

Trichothecene toxicity differs among species, and pigs were considered one of the most sensitive species. Despite this characteristic, most of the experimental mycotoxins procedures use rodents for toxicological studies (Pestka *et al.*, 2008). Data relative to DON hepatoxicity and immunotoxicity in rats and mice are controversial. Moreover, studies focusing on the histological and immunohistochemical aspects in rats are scarce. The present study investigated the effects of DON in rats that received feed contaminated with low (1.75 mg/kg) and high (11.4 mg/kg) doses of toxins during 7 to 30 days. These doses were chosen to include the lowest observed adverse effect level (LOAEL) for chronic toxicity in rats (Pestka and Smolinski, 2005) and a fivefold higher dose. In this study, we have demonstrated by using an *in vivo* model that DON induced a dose and time-dependent effect on weight gain and also on liver and lymphoid tissues. We also showed that the deleterious effects on lymphoid tissues are modulated by the activation of caspase-3.

DON induces a reduction in feed ingestion that impairs weight gain in several species of animals (Maresca, 2013). In this study, a significant decrease in body weight gain was observed only in rats fed 11.4 mg/kg of DON for 30 days. Previous studies (28 to 90 days) have reported reductions in body weight gain in rats fed diets with low (0.12 to 2.5 mg/kg of body weight) and high doses of DON (6 to 20 mg/kg) (Arnold *et al.*, 1986b; Morrisey *et al.*, 1985). It is interesting to note that in the present study only the high dose associated with the longest exposure (30 days) affected weight gain. The decrease in weight gain in this diet may be related to a decrease in feed ingestion since DON can induce an anorectic effect (Clark *et al.*, 2015), or to a less efficient conversion of feed into body mass. In this latter case, an increased metabolic rate is expected due to the process of elimination of DON or repair of damaged tissues.

In previous studies, rats chronically fed with diets containing 1.69 to 15.72 mg/kg of DON showed no lesions in the liver and lymphoid organs (Arnold *et al.*, 1986a,b). Similar results were reported in mice fed various doses (0.5 to 100 mg/kg feed) of DON during 1 to 8 weeks (Forsell *et*

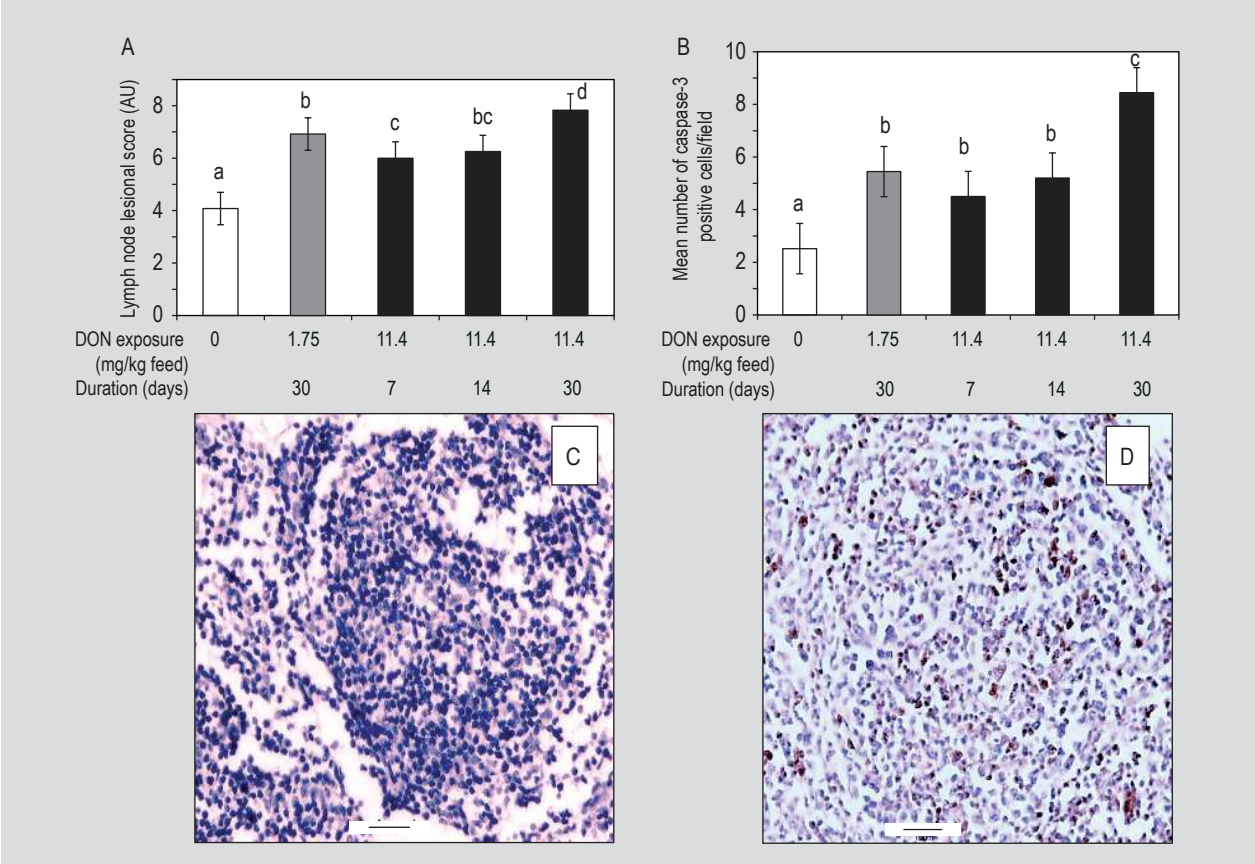

Figure 4. Effects of deoxynivalenol (DON) on mesenteric lymph nodes of rats submitted to different treatments with DON. (A) Mean lesional score on mesenteric lymph nodes. (B) Mean number of immunoreactive cells for caspase-3 in lymph nodes/field. (C) Lymphoid follicle with absence of immunoreactivity to caspase-3 (control); (D) Diffuse immunostaining for caspase-3 in the lymph node (11.4 mg/kg of DON/30 days). C and D = immunoperoxidase staining; bar 50 µm; AU = arbitrary units; different letters show significant differences by Tukey's test (P<0.05).

al., 1986; Robbana-Barnat *et al.*, 1988). In this study, DON-hepatotoxicity was observed in all groups irrespectively of the dose and duration of exposition. Moreover, the higher dose (11.4 mg/kg) of DON combined with the longer period of exposition (30 days) induced a significant increase in the histological lesions (a 2.2-fold increase) of the liver when compared with other contaminated groups. Hepatocellular necrosis was reported in rats exposed to a single intra-peritoneal dose of 10 mg/kg of DON 3 h after inoculation (Sahu *et al.*, 2008). Liver injury indicated by increased function of hepatic enzymes, lipid peroxidation and decreased hepatic glutathione content was also reported in rats fed DON-contaminated diets (5 mg/kg body weight) during 21 days. Furthermore, increased expression of tumour necrosis factor-α (TNF-α), DNA fragmentation, up regulation of mRNA Fas and TNF-α genes were associated with DON cytotoxicity (Abdel-Wahhab *et al.*, 2015). Alternatively, rats fed contaminated diets with DON (20 mg/kg) during 90 days did not demonstrate significant tissue lesions or changes in hepatic detoxification systems (Morrisey *et al.*, 1985). In *in vitro* studies using hepatocytes culture cytotoxicity was reported in a dose-dependent manner (Bradlaw *et al.*, 1985; Taranu *et al.*, 2010), with toxicity being related to an increase in oxidative stress and a decrease in protein and DNA syntheses (Rizzo *et al.*, 1994; Sahu *et al.*, 2008), that can result in cell death. In addition, rat liver cells BRL 3A treated with DON for 12, 24, and 48 h showed an increased production in oxygen reactive species resulting in apoptosis. This effect was modulated by increased expression of stress and apoptotic genes (Sun *et al.*, 2015). However, during this study hepatocelular apoptosis was not observed. This difference in hepatocelular apoptosis can probably be related to the different methods (*in vitro* and *in vivo*) and doses used in each experiment, suggesting that the doses used during this study were not sufficient to induce hepatocelular apoptosis.

The cell proliferation index in the liver was significantly increased in rats fed 1.75 mg/kg of DON for 30 days (a 7.16-fold increase) compared to the control group. In a previous study, we observed that in pigs fed DON-contaminated diets (3 mg/kg) there was a significant increase in hepatocyte proliferation (Grenier *et al.*, 2011). However, *in vitro* studies reported an inhibitory effect on the proliferation of intestinal cells exposed to DON (Bianco *et al.*, 2012). It is interesting to note that during this experiment, only the lowest dose of DON induced a significant increase in hepatocyte proliferation, suggesting that this difference is probably dose-dependent. The actual hypothesis regarding DON effects associates the type of activation of mitogen-activated protein kinases (MAPK) with the doses of DON administered. Low doses of DON activate ERK signalling pathway resulting in cell survival and gene expression, while elevated doses activate p38 with consequent inhibition of protein synthesis (Maresca, 2013). The results of the present study reinforce this hypothesis, since higher doses, irrespective of the period of exposure, had no effect on cell proliferation.

Modulation of the immune system due to exposition to DON was reported in different species of animals and in experimental models (Choi *et al.*, 2013; Mikami *et al.*, 2010). During the present study, an increase in the lesional score was observed both in the spleen and lymph nodes of animals in all contaminated groups, with alterations being more severe in rats fed the highest dose for 30 days (a 1.35 and 1.92 fold increase when compared to the spleen and lymph nodes of control animals, respectively). The main histological change identified during this study was lymphoid depletion; similar findings were reported in the thymus of rats exposed to 5 mg DON/kg body weight for 28 days (Sprando *et al.*, 2005). In addition, there was a decrease in thymidine labelling in the spleen of rats fed a diet with 1 mg/kg of DON (Arnold *et al.*, 1986a). DON also reduced the peripheral blood lymphocytes of rats and humans exposed to 90 ng/ml and 220 ng/ml of the mycotoxin (Atkinson and Miller, 1984). Further, we have recently demonstrated that lymphoid depletion was related to an increase in lymphocyte apoptosis in pigs exposed to DON (1.5 mg/kg) for 28 days (Gerez *et al.*, 2015). However, to the best of our knowledge data relative to DON-induced apoptosis in rats are limited to *in vitro* studies (Bianco *et al.*, 2012; Sun *et al.*, 2015).

In this study, a significant increase in lymphocyte apoptosis (assessed by caspase-3 expression) was observed in the mesenteric lymph nodes of all animals in the contaminated groups, whereas in the spleen this increase occurred in rats that received the highest dose for 30 days. This difference could be related to the variation in the response of immune cells to DON-induced toxicity (Islam *et al.*, 2003; Zhou *et al.*, 1998). Supporting evidence was also demonstrated in an *in vitro* investigation that demonstrated that the effect of DON was cell origin- and dose-dependent, affecting primarily blood cell lines, while hepatocytes were considered less sensitive to DON (Baltriukiene *et al.,* 2007). Moreover, it must be highlighted that the mesenteric lymph nodes are responsible for drainage of the intestine (the first exposed system to xenobiotics) and as a consequence are more likely to be exposed to mycotoxin effects than the spleen.

The apoptotic signalling pathway may occur by modulating the expression of TNF-α and caspase-3 activation (Mikami *et al.*, 2010; Zhou *et al.*, 2000). The deleterious mechanism caused by DON is the activation of the MAPK pathway, also called 'ribotoxic stress response' (Pestka *et al.*, 2008). This mechanism affects the cytoskeleton organisation, lymphocyte activation and contribute to dysregulation of the immune system (Pan *et al.*, 2013). A pro-apoptotic way is proposed for DON-induced apoptosis: (1) Bax induction; (2) Bcl-2 inhibition; and (3) caspase-3 activation as a result of increased reactive oxygen species (Bianco *et al.*, 2012; Sun *et*

al., 2015). Our results showed that in lymphoid tissues DON induce an increase in the expression of caspase-3. Taken together, the results of the present study suggest that the immune system is a primary target for caspase-3 activation.

DON is known to target the intestine in various species including the mice, pig and poultry (Akbari *et al.,* 2014; Cano *et al.*, 2013; Lucioli *et al.,* 2013). An alteration of human intestinal epithelial cells has also been described at the transcriptomic and molecular levels (Akbari *et al.,* 2014; Pierron *et al.,* 2016). Although beyond the scope of this study, we can anticipate that rats exposed to DON, especially at the highest dose, have an intestinal inflammation associated with an impaired intestinal integrity (Cano *et al.*, 2013; Pinton and Oswald, 2014).

In conclusion, the exposition of rats to low and high doses of DON during 7, 14, and 28 days induced toxic effects on the liver and lymphoid organs. The toxic effect associated with lymph nodes is probably related to caspase-3 activation; however, the mechanism of the hepatocelular lesion requires further investigation. In addition, the modulation of cytokines and chemokines combined with cell death can predispose animals to vaccination failures and infectious diseases.

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