The food contaminant deoxynivalenol activates the mitogen activated protein kinases in the intestine: Interest of ex vivo models as an alternative to in vivo experiments

Joelma Lucioli, Philippe Pinton, Patrick Callu, Joëlle J. Laffitte, François Grosjean, Martine Kolf-Klauw, Isabelle P. Oswald, Ana Paula F.R.L. Bracarense

The food contaminant deoxynivalenol activates the mitogen activated protein kinases in the intestine: interest of *ex vivo* models as an alternative to *in vivo* experiments

Joelma Lucioli[1, 2, 3+], Philippe Pinton[1, 2+], Patrick Callu[4], Joëlle Laffitte[1, 2], François Grosjean[4], Martine Kolf-Clauw[2, 5], Isabelle P. Oswald[1, 2], Ana Paula F. R. L. Bracarense[3*]

[1] INRA, UMR 1331, ToxAlim, Research Centre in Food Toxicology, F 31027, Toulouse, France
[2]Université de Toulouse, INP, UMR 1331, ToxAlim, F 31000, Toulouse, France
[3]Universidade Estadual de Londrina, Laboratory of Animal Pathology, Londrina, PR, Brazil
[4]Arvalis Institut du Végétal, Pouline, 41100 Villerable, France
[5]Université de Toulouse, École Nationale Vétérinaire, ENVT-INP, F 31076, Toulouse, France
[+] These authors equally contributed to this work.
*Corresponding author: Rodovia Celso Garcia Cid (PR 445 /Km 380) - Campus Universitário Cx. Postal 6001 - CEP 86051-980 - Londrina/PR - Brazil - telephone 55 43 33714766, fax 55 43 33714485 - e-mail: ana.bracarense@pq.cnpq.br,

Abstract

Trichothecenes induce changes in the intestinal barrier function through decreased expression of cell junction proteins and apoptosis of enterocytes. The mitogen activated protein kinases (MAPK) play an important role in the signaling pathways of cell turnover and differentiation. Using *ex vivo* and *in vivo* approaches, the purpose of this study was to investigate the ability of low doses of DON to induce histological changes in the intestine and to activate the MAPK ERK 1/2, p38 and JNK. Twelve weaning piglets received during four weeks a control diet or a DON-contaminated diet (2.3mg DON/Kg feed). Six weaning piglets were used to prepare jejunal explants (*ex vivo* model). Explants were exposed during 4 hours to vehicle, 5 or 10 µM DON. Intestinal changes were graded using a histological score. Pigs fed a DON-diet and explants exposed to DON showed a significant decrease in the jejunal score. In both models, the toxin significantly enhanced phosphorylation of ERK 1/2 and p38, whereas the increased phosphorylation of JNK was non significant. Taken together these results indicate that *in vivo* or *ex vivo* exposure of intestinal tissue to DON lead to similar intestinal lesions and activation of MAPK. These effects could impair the homeostasis of intestinal tissue in the aspects of barrier function and immune protection. The similarity of the *in vivo* and *ex vivo* results provides also strong evidence that the jejunal explant model is a good alternative for toxicological studies in intestinal tissue.

Key Words: trichothecenes, mycotoxins, pigs, MAPKinase, intestine.

1. Introduction

Mycotoxins are secondary metabolites produced by fungi and detected in various food commodities from many parts of the world. They are presently considered as one of the most hazardous contaminants of concern in food and feed, contaminating 25% of the world's crops each year (Cast, 2003).

The trichothecene deoxynivalenol (DON) contaminates cereals worldwide after grain infestation by *Fusarium* species fungi mainly in field before harvest (Pestka, 2010). DON is resistant to standard processes such as milling and baking and can be found in finished food or feed (Rotter et al., 1996). DON exhibits toxic effects in humans and all animal species investigated to date (Pestka and Smolinski, 2005). In pigs, ingestion of high doses of DON induces feed refusal, increased salivation and vomiting (Danicke et al., 2004), whereas chronic exposure to lower amounts causes reduced feed intake and weight gain, resulting in an increased incidence of infectious diseases and digestive disorders (Rotter et al., 1994; Pinton et al., 2008).

Surveys about contamination of raw materials and compound feed samples with DON reported different levels of contamination. Recently, 7049 samples sourced in North and South Americas, Europe and Asia were analyzed, and DON was present in 59% of the samples. Positive samples showed an average contamination level of 1 mg/Kg feed, with a maximum level of 49 mg/Kg feed (Rodrigues, Naehrer, 2012).

Trichothecenes inhibit protein synthesis by binding to the ribosomal peptidyltransferase resulting in a ribotoxic stress response that activates mitogen-activated protein kinases (MAPK). MAPK are components of the signaling cascade that regulates cell survival in response to stress (Zhou et al., 2005a). These kinases modulate numerous physiological processes including cell growth, differentiation and apoptosis (Raman et al, 2007; Pestka, 2008) and are crucial for signal transduction in the immune response (Dong et al., 2002). DON activates MAPK in *in vitro* assays with macrophages and intestinal cell lines *(*Moon and Pestka, 2002; Pinton et al., 2010). However, the capacity of DON to induce MAPK activation in the intestine of exposed pigs or in jejunal explants was never investigated. It is reasonable that changes in the phosphorylation of MAPK could impair intestinal nutrient absorption and cell functions affecting the barrier function of the intestine.

Intestinal explants represent a relevant and sensitive model to investigate the effects of food contaminants such as DON (Kolf-Clauw et al., 2009), nevertheless, there is no published data comparing the effects of *ex vivo* and *in vivo* models. Most toxicological *in vivo* data have used doses of DON above 5 mg/kg of feed, however such high levels are not frequent in cereals used for animal feed (Accensi et al., 2006). The objective of this study was to investigate the ability of DON to activate the MAPK after exposure to doses commonly seen in contaminated feed, using the *ex vivo* (jejunal explants) and *in vivo* models. The effects of DON on intestinal morphology were also evaluated.

2. Material and methods

2.1 *In vivo exposure of pigs to deoxynivalenol*

Twelve castrated male crossbred pigs, 4 week of age were acclimatized for 20 days, prior to being used in experimental protocols. Six pigs were allocated to receive a control

uncontaminated diet or a diet contaminated with 2.3 mg DON/Kg of feed. The experimental diets were prepared locally and formulated according to energy and amino acid requirements for piglets as already described (Accensi et al., 2006). Pigs were housed individually with free access to feed and water. After 35 days, the animals were submitted to electrical stunning, and euthanized by exsanguination. Samples of jejunum were collected and fixed in 10% buffered formalin for 24 h for histological analysis and scoring. Jejunal samples were collected, snap-frozen in liquid nitrogen and stored at – 80 °C for western blot analysis. All animal experimentation procedures were carried out in accordance with the European Guidelines for the Care and Use of Animals for Research Purposes (Directive 2010/63/EEC).

2.2 *Ex vivo exposure of pig intestine to deoxynivalenol*

Six crossbreed weaning piglets of 4 week-old were used for preparing jejunal explants. Piglets were acclimatized for 1 week with free access to feed and water, and then euthanized. The explants were obtained as described elsewhere (Kolf-Clauw et al., 2009). Briefly, 5 cm middle jejunum segments were collected in complete William's Medium E (Sigma, Saint Quentin Fallavier, France). Four to six washes were performed with William's Medium E. Each jejunum segment was opened longitudinally and pieces of 6 mm diameter were obtained with biopsy punches (Kruuse, Centravet, Dinan, France). Two explants/well were deposited villi upward on biopsy sponges in six well plates (Cellstar, Greiner Bio-One, Germany) containing control or DON-contaminated (5 and 10 µM) medium. All these operations were achieved in less than 1 h after the piglets were euthanized. The explants were exposed to different treatments at 37 °C under CO_2 controlled atmosphere with orbital shaking for 4 hours, being then fixed in 10% buffered formalin for histological analysis or stored at -80°C for western blot assay.

2.3. Histological analysis

Histological analysis were performed on both intestinal tissues obtained from piglets fed mycotoxin contaminated diet and from intestinal explants exposed *ex vivo* to the toxin. A tissue score was established based on the occurrence and severity of lesions as already described (Kolf-Clauw et al., 2009). The score system, representing a maximum of 12 points, includes both morphological and lesional data. The criteria included in tissue score were the number of villi and crypts, the length of villi, the morphology of enterocytes, the degrees of villi coalescence and autolytic changes of the tissue (oedema, necrotic debris, apoptotic cells).

2.3. Western blot assay

Frozen jejunal samples were washed on ice with PBS-EDTA (0.25 mol/L) with protease inhibitor cocktail (Roche Diagnostics, Meylan, France), lysed on ice in a potter tissue grinder with lysis buffer (20 mmol/L Tris–HCL pH 8, 5 mmol/L EDTA, 0.02% NaN3, 1%Triton X100) supplemented with protease inhibitor cocktail. Lysates were homogenized through a 26G needle

and sonicated for 30 s. Homogenates were diluted 1/2 with lysis buffer and heated at 100 °C for 10 min before protein quantification. Equal amounts of proteins were loaded a 12.5% acrylamide gel. Migration was conducted in a 250 mmol/L Tris buffer (pH7.6) containing 1% SDS and 1.92 mol/L glycine. After separation, proteins were transferred onto Optitran BA-S 83 membrane (Whatman, Germany). The primary antibodies used were phospho p44/42 ERK MAPK, phospho SAPK/JNK, phospho p38 MAPK (diluted 1:500) and ß-actin, used as control (diluted 1/1000) (Cell Signaling Technology, Danvers, MA). Membranes were then washed and incubated with secondary antibodies CFTM770 goat anti rabbit IgG or CFTM770 goat anti mouse IgG (diluted 1:10.000) obtained from Biotium (Hayward, CA, USA). Band densities were obtained by scanning the membranes using Odyssey Infrared Imaging System (LI-COR ScienceTec, Les Ulis, France). Fluorescent intensities were determined using LI-COR imaging software after correction for background. The expression of the protein was estimated after normalization calculated by the ratio of the intensity of the band of interest and of the ß-actin band.

2.4. Statistical analysis

The results are presented as means ± SD of independent experiments with different animals. The values of scores obtained in *ex vivo* and *in vivo* experiments were analyzed by ANOVA followed by multiple comparison-Tuckey test using the Systat software 10.0 (Systat, Chicago, IL, USA). *P* values < 0.05 were considered significant.

3. Results

3.1 DON induces histological lesions on the intestine

The intestinal tissue is a target for DON when contaminated feed is ingested. Our aim was first to evaluate the effects of DON on intestinal morphology in animals chronically exposed to the toxin, as well as in jejunal explants. The intestinal lesional and morphological scores were measured. The main lesional changes observed in both explants and intestine from animals exposed to DON were villi fusion and atrophy accompanied by focal apical necrosis of enterocytes. Morphological changes included a reduction in the number of villi and cuboid or flattened enterocytes. The changes were more severe in intestinal explants exposed *ex vivo* to 10 µM of DON (p=0.001). Ingestion of DON induced a significant decrease in the histological score in the jejunum (15%) in the *in vivo* model, whereas in the *ex vivo* assay, exposition to 5 and 10 µM of DON induced a score decrease of 26% and 49,4%, respectively (Figure 1).

3.2 DON activates the mitogen-activated protein kinases in vivo and ex vivo

MAPK are known to be important signaling modulators in cell proliferation and apoptosis (Pestka, 2008) and activation of this pathway by mycotoxins was reported in murine macrophages (Moon and Pestka, 2002) as well as in porcine intestinal epithelial cells (Pinton et al., 2010).

Therefore, western blot assay was used to evaluate the ability of DON to induce MAPK phosphorylation. Exposure of jejunal explants for 4h to 10 μM of DON induced a significant phosphorylation of ERK 1/2 and p38 compared to control group (2.61 fold increase, p=0.05 and 5.76 fold increase, p=0.001, respectively), whereas no changes were observed when explants were exposed to 5 μM of DON. Similar findings were observed in jejunal samples of animals fed 2.3 mg of DON/Kg for 35 days. As shown in Figure 2 an increase of p38 (61%, p= 0.01) and ERK (48%, p= 0.01) phosporylation was observed. Of note, in both experimental models, a slight but not significant increase of the expression of phosphorylated JNK was observed (Figure 3).

4. Discussion

The intestinal tract represents the first barrier against ingested food contaminants, as mycotoxins, and has also an important role in immune functions (Turner, 2009). Chronic exposure of intestinal tissues to low doses of DON induces changes in villi structure and cytokine expression in pigs (Bracarense et al., 2012). One of the proposed mechanisms of the deleterious effect of DON is the activation of the MAPK pathway via a mechanism called "r*ibotoxic stress response*" (Pestka, 2008).

To investigate the ability of DON to activate the MAPK, when administered at low doses, we used two experimental approaches: the *in vivo* exposure of pigs to DON contaminated feed and the *ex vivo* treatment of jejunal explants with the toxin. In the *in vivo* study, we demonstrated that MAPK activation occurs in the intestinal epithelium of piglets fed for 35 days a diet contaminated with low doses of DON. An activation of MAPK was also observed in jejunal explants exposed *ex vivo* to DON in a dose-dependent manner, since only the exposure to 10 μM of DON induced a significant increase in MAPK phosphorylation. It is difficult to correlate *in vitro* toxin concentration with *in vivo* exposure, however, the concentration of toxin used in both models are similar as 2.3 mg DON/Kg of feed corresponds to 7.7 μM (Sergent et al, 2006; Pinton et al., 2009).

It is interesting to observe that in both models, there is a good correlation in the increase of expression of phosphorylated MAPK. The extent of MAPK activation, lower in samples obtained from the in vivo experiment than in explants, could be explained by the mode of exposure to the toxin, in the culture medium or in ingested feed. A significant increase was observed only for ERK and p38. Following the same signaling arrangement, each individual MAPK pathway responds to specific stimuli and then regulates their specific substrates (Cui et al., 2007), which can explain the selective activation of MAPK.

JNK and ERK are involved in regulation of both cell survival and death depending on cell types and stimulus, whereas p38 can promote apoptosis via p53 activation (Bae and Pestka, 2008). ERK 1/2 is of particular importance because it can be involved in intestinal epithelial cell morphology and in the structure of tight junctions that regulate the barrier function of the intestinal tract (Oshima et al., 2008). Increase in MAPK phosphorylation was described in *in vitro* assays when the intestinal cell line IPEC-1 was exposed to DON, resulting in a decreased expression of

tight junction proteins (Pinton et al., 2010). In a previous study, we have also observed that piglets fed a diet contaminated with 3 mg/kg of DON, showed a significant decrease expression of occludin and E-cadherin in jejunum and ileum (Bracarense et al., 2012). Explants exposed to 10 µM of DON showed a decreased expression of E-cadherin in immunohistochemical assay (data not shown). All these data reinforce the role of DON in the activation of ERK which in turn induces changes in the expression of *adherens* and *occludens* junctions proteins.

In DON-stimulated RAW 264.7 cells competing apoptotic and survival cell pathway are induced by p38 and ERK activation, respectively (Zhou et al., 2005). In the present study, both *in vivo* and *ex vivo* exposure to DON induced a significant decrease in the total intestinal score in comparison to the control group. In addition, when immunohistochemical analysis for caspase-3 was performed in jejunal explants, a significant increase in immunostaining was verified in samples exposed to 10 µM of DON (data not shown). Probably, apoptosis of enterocytes was mediated by an activation of p38. DON and trichotecenes-related mycotoxins have shown to induce apoptotic changes *in vitro* and *in vivo* in several organs. *In vitro*, these changes were correlated to MAPKinases activation (Yang et al., 2000; Pinton et al, 2010). This correlation was also demonstrated with other stressors than trichotecenes, for example heat stress in intestinal cells ICE-6 (Yu et al., 2010). *In vivo*, to the best of our knowledge, there is no previous work demonstrating this correlation with mycotoxins. However, ERK1/2 phosphorylation was shown to be involved in apoptotic morphological changes induced by heat stress at jejunal level (Yu et al., 2010). Similarly, a recent paper as indicated a correlation between decreased intestinal barrier function, decresed expression of tight junction proteins and the intestinal activation of MAPK (He et al., 2013). So, the present results taken together with previous works allow to hypothesize that intestinal morphological alterations, such apical lyses of enterocytes and villi atrophy, were associated with changes in the tight junctions of the epithelium and the apoptosis induced by MAPK activation after exposure to DON.

In conclusion, we demonstrated that, in *in vivo* and *ex vivo* models, the histological changes induced by DON are similar as well as the response observed for the expression of MAPK in both models.This strongly suggest that intestinal toxicity of DON involve MAPK activation. In addition, using histological and protein expression analysis, we confirmed that the explant model is a good alternative for the studies focused on gastrointestinal toxicity following exposure to low doses of toxins.

Acknowledgements

This work was financially supported by the CAPES/COFECUB (593/08) international cooperation program, CNPq grant (474583/2010-4) and the French ANR Project DON & Co.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Accensi, F., Pinton, P., Callu, P., Abella-Bourges, N., Guelfi, J.F., Grosjean, F., Oswald, I.P., 2006. Ingestion of low doses of deoxynivalenol does not affect hematological, biochemical, or immune responses of piglets. J Anim Sci 84:1935–1942.

Bae, H.K., Pestka, J.J., 2008. Deoxynivalenol induces p38 activation with the ribosome in monocytes and macrophages. Toxicol Sci 105:59-66.

Bracarense, A.P.F.R.L., Lucioli, J., Grenier, B., Pacheco, G.D., Moll, W-D., Schatzmayr, G., Oswald, I.P., 2012. Chronic ingestion of deoxynivalenol and fumonisin, alone or in interaction, induces morphological and immunological changes in the intestine of piglets. Br J Nutr 107:1776-1786.

CAST Potential economic costs of mycotoxins in United States. In: Mycotoxins: Risks in plant, animal and human systems. Council for Agricultural Science and Technology. 2003. pp 136-142.

Cui, J., Zhang, M., Zhang, Y., Xu, Z., 2007. JNK pathway: diseases and therapeutic potential. Acta Pharmacol Sin 28:601-608.

Dänicke, S., Goyarts, T., Valenta, H., Razzazi, E., Böhm, J., 2004. On the effects of increasing deoxynivalenol (DON) concentrations in pig feed on growth performance, utilization of nutrients and metabolism of DON. J Anim Feed Sci 13:539-556.

Dong, C., Davis, R.J., Flavell, R.A., 2002. MAP kinases in the immune response. Annu Rev Immunol 20:55–72.

Hu, C.H., Xiao, K., Luan, Z.S., Song, J. 2013. Early weaning increases intestinal permeability, alters expression of cytokine and tight junction protein, and activates mitogen-activated protein kinases in pigs. J Anim Sci. in press.

Kolf-Clauw, M., Castellote, J., Joly, B., Bourges-Abella, N., Raymond-Letron, I., Pinton, P., Oswald, I.P. 2009. Development of a pig jejunal explant culture for studying the gastrointestinal toxicity of the mycotoxin deoxynivalenol: histopathological analysis. Toxicol in vitro 23:1580-1584.

Moon, Y., Pestka, J.J. 2002. Vomitoxin-induced cyclooxygenase-2 gene expression in macrophages mediated by activation of ERK and p38 but not JNK mitogen-activated protein kinases. Toxicol Sci 69:373–382.

Oshima, T., Miwa, H., Joh, T., 2008. Aspirin induces gastric epithelial barrier dysfunction by activation p38 MAPK via claudin-7. Am J Physiol Cell Physiol 295:C800-6.

Pestka, J.J., 2010. Deoxynivalenol: mechanisms of action, human exposure and toxicological relevance. Arch Toxicol 84:663-679.

Pestka, J.J., Smolinski, A.T., 2005. Deoxynivalenol: toxicology and potential effects on humans. J Toxicol Environ Health B Crit Rev 8: 39-69.

Petska, J.J., 2008. Mechanism of deoxynivalenol-induced gene expression and apoptosis. Food Add Contam Part A Chem Anal Control Expo Risk Assess 25:1128-1140.

Pinton, P., Accensi, F., Beauchamp, E., Cossalter, A. M., Callu, P., Grosjean, F., and Oswald, I. P., 2008). Ingestion of deoxynivalenol (DON) contaminated feed alters the pig vaccinal immune responses. Toxicol Lett 177, 215-222.

Pinton, P., Braicu, C., Nougayrede, J.P., Laffite, J., Taranu, I., Oswald, I.P., 2010. Deoxynivalenol impairs porcine intestinal barrier function and decrease the protein expression of claudin-4 through a mitogen-activated protein kinase-dependent mechanism. J Nutr 140:1956-1962.

Pinton, P., Nougayrede, J.P., Del Rio, J.C., Moreno, C., Marin, D., Ferrier, L., Bracarense, A.P., Kolf-Clauw, M., Oswald, I.P., 2009. The food contaminant, desoxinivalenol, decreases intestinal barrier function and reduces claudin expression. Toxicol Appl Pharmacol 237:41-48.

Raman, M., Chen, W., Cobb, M.H., 2007. Differential regulation and properties of MAPKs. Oncogene 26: 3100-3112

Rodrigues, I., Naehrer, K. 2012. A three-year survey on the worldwide occurrence of mycotoxins in feedstuffs and feed. Toxins 4:663-675.

Rotter, B.A., Prelusky, D.B., Pestka, J.J., 1996. Toxicology of deoxynivalenol (vomitoxin). J Toxicol Environ Health 48:1-34.

Rotter, B.A., Thompson, B.K., Lessard, M., Trenholm, H.L., Tryphonas, H., 1994. Influence of low level exposure to *Fusarium* mycotoxins on selected immunological and hematological parameters in young swine. Fundam Appl Toxicol 23:117-124.

Sergent, T., Parys, M., Garsou, S., Pussemier, L., Schneider, Y.J., Larondelle, Y., 2006. Deoxynivalenol transport across human intestinal Caco-2 cells and its effects on cellular metabolism at realistic intestinal concentrations. Toxicol Lett 164(2): 167-176.

Turner, J.R., 2009. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol 9: 799-809.

Yang, G.H., Jarvis, B.B., Chung, Y.J., Pestka, J. J., 2000. Apoptosis induction by satratoxins and other trichothecene mycotoxins: Relationship to ERK, p38 MAPK, and SAPK/JNK activation. Toxicol. Appl. Pharmacol. 164:149-160.

Yu J, Yin P, Yin J, Liu F, Zhu X, Cheng G, Guo K, Yin Y, Xu J., 2010. Involvement of ERK 1/2 signaling and growth-related molecule´s expression in response to heat stress-induced damage in rat jejunum and IEC-6 cells. Int J Hyperthermia, 26: 538-555.

Zhou, H.R., Jia, Q., Pestka, J.J., 2005a. Ribotoxic stress response to the trichothecene deoxynivalenol in the macrophage involves the Src Family Kinase Hck. Toxicol Sci 85:916-26.

Zhou, H.R., Islam, Z., Pestka, J.J., 2005b. Induction of competing apoptotic and survival signaling pathways in the macrophage by the ribotoxic trichotecene deoxynivalenol. Toxicol Sci 87:113-122.

Figure Legends

Figure 1-. Effect of DON exposure on jejunum histology. (A) and (B) Histological score. (A) Pigs received a control diet or a diet contaminated with 2.3 mg/kg DON. (B) Jejunal explants exposed to 5 µM or 10 µM DON. (C) Jejunum of a control pig. Normal villi. HE. (D) Jejunum of a pig fed a DON-diet. Villi fusion and atrophy (arrowhead) and apical lyses of enterocytes (arrow). HE. (E) Jejunal explant of control group. Villi covered by cylindrical enterocytes. HE. (F) Jejunal explant exposed to 10 µmol/L DON. Villi atrophy and fusion (arrow). HE. Bars 100 µm. Lesional score after histological examination according to the occurrence and severity of lesions. Values are mean scores ± SEM for 6 pigs. * =$P < 0.05$; ** = $P < 0.01$. AU= arbitrary units.

Figure 2 - Effect of DON exposure on intestinal expression of MAPK. (A) Pigs received a control diet or a diet contaminated with 2.3 mg/kg DON. (B) Jejunal explants exposed to 5 µM or 10 µM DON. A and B figures represent the expression of ERK 1/2, p38 and JNK estimated by densitometric analyses after normalization with ß-actin signal. The values are mean ± SEM for 6 pigs. * =$P < 0.05$; *** = $P < 0.01$.

Figure 3 - Effect of DON exposure on intestinal expression of MAPK. Pigs received a control diet or a diet contaminated with 2.3 mg/kg DON. (A) The figure represents the immunoblot from jejunum of pigs fed a diet contaminated with 2.3 mg/kg DON. (B) The figure represents the immunoblot from jejunal explants exposed to 10 µM DON.

The food contaminant deoxynivalenol activates the mitogen activated protein kinases in the intestine: interest of *ex vivo* models as an alternative to *in vivo* experiments

Joelma Lucioli[1, 2, 3+], Philippe Pinton[1, 2+], Patrick Callu[4], Joëlle Laffitte[1, 2], François Grosjean[4], Martine Kolf-Clauw[2, 5], Isabelle P. Oswald[1, 2], Ana Paula F. R. L. Bracarense[3*]

[1] INRA, UMR 1331, ToxAlim, Research Centre in Food Toxicology, F 31027, Toulouse, France
[2]Université de Toulouse, INP, UMR 1331, ToxAlim, F 31000, Toulouse, France
[3]Universidade Estadual de Londrina, Laboratory of Animal Pathology, Londrina, PR, Brazil
[4]Arvalis Institut du Végétal, Pouline, 41100 Villerable, France
[5]Université de Toulouse, École Nationale Vétérinaire, ENVT-INP, F 31076, Toulouse, France
[+] These authors equally contributed to this work.
*Corresponding author: Rodovia Celso Garcia Cid (PR 445 /Km 380) - Campus Universitário Cx. Postal 6001 - CEP 86051-980 - Londrina/PR - Brazil - telephone 55 43 33714766, fax 55 43 33714485 - e-mail: ana.bracarense@pq.cnpq.br,

Abstract

Trichothecenes induce changes in the intestinal barrier function through decreased expression of cell junction proteins and apoptosis of enterocytes. The mitogen activated protein kinases (MAPK) play an important role in the signaling pathways of cell turnover and differentiation. Using *ex vivo* and *in vivo* approaches, the purpose of this study was to investigate the ability of low doses of DON to induce histological changes in the intestine and to activate the MAPK ERK 1/2, p38 and JNK. Twelve weaning piglets received during four weeks a control diet or a DON-contaminated diet (2.3mg DON/Kg feed). Six weaning piglets were used to prepare jejunal explants (*ex vivo* model). Explants were exposed during 4 hours to vehicle, 5 or 10 µM DON. Intestinal changes were graded using a histological score. Pigs fed a DON-diet and explants exposed to DON showed a significant decrease in the jejunal score. In both models, the toxin significantly enhanced phosphorylation of ERK 1/2 and p38, whereas the increased phosphorylation of JNK was non significant. Taken together these results indicate that *in vivo* or *ex vivo* exposure of intestinal tissue to DON lead to similar intestinal lesions and activation of MAPK. These effects could impair the homeostasis of intestinal tissue in the aspects of barrier function and immune protection. The similarity of the *in vivo* and *ex vivo* results provides also strong evidence that the jejunal explant model is a good alternative for toxicological studies in intestinal tissue.

Key Words: trichothecenes, mycotoxins, pigs, MAPKinase, intestine.

1. Introduction

Mycotoxins are secondary metabolites produced by fungi and detected in various food commodities from many parts of the world. They are presently considered as one of the most hazardous contaminants of concern in food and feed, contaminating 25% of the world's crops each year (Cast, 2003).

The trichothecene deoxynivalenol (DON) contaminates cereals worldwide after grain infestation by *Fusarium* species fungi mainly in field before harvest (Pestka, 2010). DON is resistant to standard processes such as milling and baking and can be found in finished food or feed (Rotter et al., 1996). DON exhibits toxic effects in humans and all animal species investigated to date (Pestka and Smolinski, 2005). In pigs, ingestion of high doses of DON induces feed refusal, increased salivation and vomiting (Danicke et al., 2004), whereas chronic exposure to lower amounts causes reduced feed intake and weight gain, resulting in an increased incidence of infectious diseases and digestive disorders (Rotter et al., 1994; Pinton et al., 2008).

Surveys about contamination of raw materials and compound feed samples with DON reported different levels of contamination. Recently, 7049 samples sourced in North and South Americas, Europe and Asia were analyzed, and DON was present in 59% of the samples. Positive samples showed an average contamination level of 1 mg/Kg feed, with a maximum level of 49 mg/Kg feed (Rodrigues, Naehrer, 2012).

Trichothecenes inhibit protein synthesis by binding to the ribosomal peptidyltransferase resulting in a ribotoxic stress response that activates mitogen-activated protein kinases (MAPK). MAPK are components of the signaling cascade that regulates cell survival in response to stress (Zhou et al., 2005a). These kinases modulate numerous physiological processes including cell growth, differentiation and apoptosis (Raman et al, 2007; Pestka, 2008) and are crucial for signal transduction in the immune response (Dong et al., 2002). DON activates MAPK in *in vitro* assays with macrophages and intestinal cell lines *(*Moon and Pestka, 2002; Pinton et al., 2010). However, the capacity of DON to induce MAPK activation in the intestine of exposed pigs or in jejunal explants was never investigated. It is reasonable that changes in the phosphorylation of MAPK could impair intestinal nutrient absorption and cell functions affecting the barrier function of the intestine.

Intestinal explants represent a relevant and sensitive model to investigate the effects of food contaminants such as DON (Kolf-Clauw et al., 2009), nevertheless, there is no published data comparing the effects of *ex vivo* and *in vivo* models. Most toxicological *in vivo* data have used doses of DON above 5 mg/kg of feed, however such high levels are not frequent in cereals used for animal feed (Accensi et al., 2006). The objective of this study was to investigate the ability of DON to activate the MAPK after exposure to doses commonly seen in contaminated feed, using the *ex vivo* (jejunal explants) and *in vivo* models. The effects of DON on intestinal morphology were also evaluated.

2. Material and methods

2.1 *In vivo exposure of pigs to deoxynivalenol*

Twelve castrated male crossbred pigs, 4 week of age were acclimatized for 20 days, prior to being used in experimental protocols. Six pigs were allocated to receive a control

uncontaminated diet or a diet contaminated with 2.3 mg DON/Kg of feed. The experimental diets were prepared locally and formulated according to energy and amino acid requirements for piglets as already described (Accensi et al., 2006). Pigs were housed individually with free access to feed and water. After 35 days, the animals were submitted to electrical stunning, and euthanized by exsanguination. Samples of jejunum were collected and fixed in 10% buffered formalin for 24 h for histological analysis and scoring. Jejunal samples were collected, snap-frozen in liquid nitrogen and stored at – 80 °C for western blot analysis. All animal experimentation procedures were carried out in accordance with the European Guidelines for the Care and Use of Animals for Research Purposes (Directive 2010/63/EEC).

2.2 *Ex vivo exposure of pig intestine to deoxynivalenol*

Six crossbreed weaning piglets of 4 week-old were used for preparing jejunal explants. Piglets were acclimatized for 1 week with free access to feed and water, and then euthanized. The explants were obtained as described elsewhere (Kolf-Clauw et al., 2009). Briefly, 5 cm middle jejunum segments were collected in complete William's Medium E (Sigma, Saint Quentin Fallavier, France). Four to six washes were performed with William's Medium E. Each jejunum segment was opened longitudinally and pieces of 6 mm diameter were obtained with biopsy punches (Kruuse, Centravet, Dinan, France). Two explants/well were deposited villi upward on biopsy sponges in six well plates (Cellstar, Greiner Bio-One, Germany) containing control or DON-contaminated (5 and 10 µM) medium. All these operations were achieved in less than 1 h after the piglets were euthanized. The explants were exposed to different treatments at 37 °C under CO_2 controlled atmosphere with orbital shaking for 4 hours, being then fixed in 10% buffered formalin for histological analysis or stored at -80°C for western blot assay.

2.3. Histological analysis

Histological analysis were performed on both intestinal tissues obtained from piglets fed mycotoxin contaminated diet and from intestinal explants exposed *ex vivo* to the toxin. A tissue score was established based on the occurrence and severity of lesions as already described (Kolf-Clauw et al., 2009). The score system, representing a maximum of 12 points, includes both morphological and lesional data. The criteria included in tissue score were the number of villi and crypts, the length of villi, the morphology of enterocytes, the degrees of villi coalescence and autolytic changes of the tissue (oedema, necrotic debris, apoptotic cells).

2.3. Western blot assay

Frozen jejunal samples were washed on ice with PBS-EDTA (0.25 mol/L) with protease inhibitor cocktail (Roche Diagnostics, Meylan, France), lysed on ice in a potter tissue grinder with lysis buffer (20 mmol/L Tris–HCL pH 8, 5 mmol/L EDTA, 0.02% NaN3, 1%Triton X100) supplemented with protease inhibitor cocktail. Lysates were homogenized through a 26G needle

and sonicated for 30 s. Homogenates were diluted 1/2 with lysis buffer and heated at 100 °C for 10 min before protein quantification. Equal amounts of proteins were loaded a 12.5% acrylamide gel. Migration was conducted in a 250 mmol/L Tris buffer (pH7.6) containing 1% SDS and 1.92 mol/L glycine. After separation, proteins were transferred onto Optitran BA-S 83 membrane (Whatman, Germany). The primary antibodies used were phospho p44/42 ERK MAPK, phospho SAPK/JNK, phospho p38 MAPK (diluted 1:500) and ß-actin, used as control (diluted 1/1000) (Cell Signaling Technology, Danvers, MA). Membranes were then washed and incubated with secondary antibodies CFTM770 goat anti rabbit IgG or CFTM770 goat anti mouse IgG (diluted 1:10.000) obtained from Biotium (Hayward, CA, USA). Band densities were obtained by scanning the membranes using Odyssey Infrared Imaging System (LI-COR ScienceTec, Les Ulis, France). Fluorescent intensities were determined using LI-COR imaging software after correction for background. The expression of the protein was estimated after normalization calculated by the ratio of the intensity of the band of interest and of the ß-actin band.

2.4. Statistical analysis

The results are presented as means ± SD of independent experiments with different animals. The values of scores obtained in *ex vivo* and *in vivo* experiments were analyzed by ANOVA followed by multiple comparison-Tuckey test using the Systat software 10.0 (Systat, Chicago, IL, USA). *P* values < 0.05 were considered significant.

3. Results

3.1 DON induces histological lesions on the intestine

The intestinal tissue is a target for DON when contaminated feed is ingested. Our aim was first to evaluate the effects of DON on intestinal morphology in animals chronically exposed to the toxin, as well as in jejunal explants. The intestinal lesional and morphological scores were measured. The main lesional changes observed in both explants and intestine from animals exposed to DON were villi fusion and atrophy accompanied by focal apical necrosis of enterocytes. Morphological changes included a reduction in the number of villi and cuboid or flattened enterocytes. The changes were more severe in intestinal explants exposed *ex vivo* to 10 µM of DON (p=0.001). Ingestion of DON induced a significant decrease in the histological score in the jejunum (15%) in the *in vivo* model, whereas in the *ex vivo* assay, exposition to 5 and 10 µM of DON induced a score decrease of 26% and 49,4%, respectively (Figure 1).

3.2 DON activates the mitogen-activated protein kinases in vivo and ex vivo

MAPK are known to be important signaling modulators in cell proliferation and apoptosis (Pestka, 2008) and activation of this pathway by mycotoxins was reported in murine macrophages (Moon and Pestka, 2002) as well as in porcine intestinal epithelial cells (Pinton et al., 2010).

Therefore, western blot assay was used to evaluate the ability of DON to induce MAPK phosphorylation. Exposure of jejunal explants for 4h to 10 μM of DON induced a significant phosphorylation of ERK 1/2 and p38 compared to control group (2.61 fold increase, p=0.05 and 5.76 fold increase, p=0.001, respectively), whereas no changes were observed when explants were exposed to 5 μM of DON. Similar findings were observed in jejunal samples of animals fed 2.3 mg of DON/Kg for 35 days. As shown in Figure 2 an increase of p38 (61%, p= 0.01) and ERK (48%, p= 0.01) phosporylation was observed. Of note, in both experimental models, a slight but not significant increase of the expression of phosphorylated JNK was observed (Figure 3).

4. Discussion

The intestinal tract represents the first barrier against ingested food contaminants, as mycotoxins, and has also an important role in immune functions (Turner, 2009). Chronic exposure of intestinal tissues to low doses of DON induces changes in villi structure and cytokine expression in pigs (Bracarense et al., 2012). One of the proposed mechanisms of the deleterious effect of DON is the activation of the MAPK pathway via a mechanism called "r*ibotoxic stress response*" (Pestka, 2008).

To investigate the ability of DON to activate the MAPK, when administered at low doses, we used two experimental approaches: the *in vivo* exposure of pigs to DON contaminated feed and the *ex vivo* treatment of jejunal explants with the toxin. In the *in vivo* study, we demonstrated that MAPK activation occurs in the intestinal epithelium of piglets fed for 35 days a diet contaminated with low doses of DON. An activation of MAPK was also observed in jejunal explants exposed *ex vivo* to DON in a dose-dependent manner, since only the exposure to 10 μM of DON induced a significant increase in MAPK phosphorylation. It is difficult to correlate *in vitro* toxin concentration with *in vivo* exposure, however, the concentration of toxin used in both models are similar as 2.3 mg DON/Kg of feed corresponds to 7.7 μM (Sergent et al, 2006; Pinton et al., 2009).

It is interesting to observe that in both models, there is a good correlation in the increase of expression of phosphorylated MAPK. The extent of MAPK activation, lower in samples obtained from the in vivo experiment than in explants, could be explained by the mode of exposure to the toxin, in the culture medium or in ingested feed. A significant increase was observed only for ERK and p38. Following the same signaling arrangement, each individual MAPK pathway responds to specific stimuli and then regulates their specific substrates (Cui et al., 2007), which can explain the selective activation of MAPK.

JNK and ERK are involved in regulation of both cell survival and death depending on cell types and stimulus, whereas p38 can promote apoptosis via p53 activation (Bae and Pestka, 2008). ERK 1/2 is of particular importance because it can be involved in intestinal epithelial cell morphology and in the structure of tight junctions that regulate the barrier function of the intestinal tract (Oshima et al., 2008). Increase in MAPK phosphorylation was described in *in vitro* assays when the intestinal cell line IPEC-1 was exposed to DON, resulting in a decreased expression of

tight junction proteins (Pinton et al., 2010). In a previous study, we have also observed that piglets fed a diet contaminated with 3 mg/kg of DON, showed a significant decrease expression of occludin and E-cadherin in jejunum and ileum (Bracarense et al., 2012). Explants exposed to 10 µM of DON showed a decreased expression of E-cadherin in immunohistochemical assay (data not shown). All these data reinforce the role of DON in the activation of ERK which in turn induces changes in the expression of *adherens* and *occludens* junctions proteins.

In DON-stimulated RAW 264.7 cells competing apoptotic and survival cell pathway are induced by p38 and ERK activation, respectively (Zhou et al., 2005). In the present study, both *in vivo* and *ex vivo* exposure to DON induced a significant decrease in the total intestinal score in comparison to the control group. In addition, when immunohistochemical analysis for caspase-3 was performed in jejunal explants, a significant increase in immunostaining was verified in samples exposed to 10 µM of DON (data not shown). Probably, apoptosis of enterocytes was mediated by an activation of p38. DON and trichotecenes-related mycotoxins have shown to induce apoptotic changes *in vitro* and *in vivo* in several organs. *In vitro*, these changes were correlated to MAPKinases activation (Yang et al., 2000; Pinton et al, 2010). This correlation was also demonstrated with other stressors than trichotecenes, for example heat stress in intestinal cells ICE-6 (Yu et al., 2010). *In vivo*, to the best of our knowledge, there is no previous work demonstrating this correlation with mycotoxins. However, ERK1/2 phosphorylation was shown to be involved in apoptotic morphological changes induced by heat stress at jejunal level (Yu et al., 2010). Similarly, a recent paper as indicated a correlation between decreased intestinal barrier function, decresed expression of tight junction proteins and the intestinal activation of MAPK (He et al., 2013). So, the present results taken together with previous works allow to hypothesize that intestinal morphological alterations, such apical lyses of enterocytes and villi atrophy, were associated with changes in the tight junctions of the epithelium and the apoptosis induced by MAPK activation after exposure to DON.

In conclusion, we demonstrated that, in *in vivo* and *ex vivo* models, the histological changes induced by DON are similar as well as the response observed for the expression of MAPK in both models.This strongly suggest that intestinal toxicity of DON involve MAPK activation. In addition, using histological and protein expression analysis, we confirmed that the explant model is a good alternative for the studies focused on gastrointestinal toxicity following exposure to low doses of toxins.

Acknowledgements

This work was financially supported by the CAPES/COFECUB (593/08) international cooperation program, CNPq grant (474583/2010-4) and the French ANR Project DON & Co.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Accensi, F., Pinton, P., Callu, P., Abella-Bourges, N., Guelfi, J.F., Grosjean, F., Oswald, I.P., 2006. Ingestion of low doses of deoxynivalenol does not affect hematological, biochemical, or immune responses of piglets. J Anim Sci 84:1935–1942.

Bae, H.K., Pestka, J.J., 2008. Deoxynivalenol induces p38 activation with the ribosome in monocytes and macrophages. Toxicol Sci 105:59-66.

Bracarense, A.P.F.R.L., Lucioli, J., Grenier, B., Pacheco, G.D., Moll, W-D., Schatzmayr, G., Oswald, I.P., 2012. Chronic ingestion of deoxynivalenol and fumonisin, alone or in interaction, induces morphological and immunological changes in the intestine of piglets. Br J Nutr 107:1776-1786.

CAST Potential economic costs of mycotoxins in United States. In: Mycotoxins: Risks in plant, animal and human systems. Council for Agricultural Science and Technology. 2003. pp 136-142.

Cui, J., Zhang, M., Zhang, Y., Xu, Z., 2007. JNK pathway: diseases and therapeutic potential. Acta Pharmacol Sin 28:601-608.

Dänicke, S., Goyarts, T., Valenta, H., Razzazi, E., Böhm, J., 2004. On the effects of increasing deoxynivalenol (DON) concentrations in pig feed on growth performance, utilization of nutrients and metabolism of DON. J Anim Feed Sci 13:539-556.

Dong, C., Davis, R.J., Flavell, R.A., 2002. MAP kinases in the immune response. Annu Rev Immunol 20:55–72.

Hu, C.H., Xiao, K., Luan, Z.S., Song, J. 2013. Early weaning increases intestinal permeability, alters expression of cytokine and tight junction protein, and activates mitogen-activated protein kinases in pigs. J Anim Sci. in press.

Kolf-Clauw, M., Castellote, J., Joly, B., Bourges-Abella, N., Raymond-Letron, I., Pinton, P., Oswald, I.P. 2009. Development of a pig jejunal explant culture for studying the gastrointestinal toxicity of the mycotoxin deoxynivalenol: histopathological analysis. Toxicol in vitro 23:1580-1584.

Moon, Y., Pestka, J.J. 2002. Vomitoxin-induced cyclooxygenase-2 gene expression in macrophages mediated by activation of ERK and p38 but not JNK mitogen-activated protein kinases. Toxicol Sci 69:373–382.

Oshima, T., Miwa, H., Joh, T., 2008. Aspirin induces gastric epithelial barrier dysfunction by activation p38 MAPK via claudin-7. Am J Physiol Cell Physiol 295:C800-6.

Pestka, J.J., 2010. Deoxynivalenol: mechanisms of action, human exposure and toxicological relevance. Arch Toxicol 84:663-679.

Pestka, J.J., Smolinski, A.T., 2005. Deoxynivalenol: toxicology and potential effects on humans. J Toxicol Environ Health B Crit Rev 8: 39-69.

Petska, J.J., 2008. Mechanism of deoxynivalenol-induced gene expression and apoptosis. Food Add Contam Part A Chem Anal Control Expo Risk Assess 25:1128-1140.

Pinton, P., Accensi, F., Beauchamp, E., Cossalter, A. M., Callu, P., Grosjean, F., and Oswald, I. P., 2008). Ingestion of deoxynivalenol (DON) contaminated feed alters the pig vaccinal immune responses. Toxicol Lett 177, 215-222.

Pinton, P., Braicu, C., Nougayrede, J.P., Laffite, J., Taranu, I., Oswald, I.P., 2010. Deoxynivalenol impairs porcine intestinal barrier function and decrease the protein expression of claudin-4 through a mitogen-activated protein kinase-dependent mechanism. J Nutr 140:1956-1962.

Pinton, P., Nougayrede, J.P., Del Rio, J.C., Moreno, C., Marin, D., Ferrier, L., Bracarense, A.P., Kolf-Clauw, M., Oswald, I.P., 2009. The food contaminant, desoxinivalenol, decreases intestinal barrier function and reduces claudin expression. Toxicol Appl Pharmacol 237:41-48.

Raman, M., Chen, W., Cobb, M.H., 2007. Differential regulation and properties of MAPKs. Oncogene 26: 3100-3112

Rodrigues, I., Naehrer, K. 2012. A three-year survey on the worldwide occurrence of mycotoxins in feedstuffs and feed. Toxins 4:663-675.

Rotter, B.A., Prelusky, D.B., Pestka, J.J., 1996. Toxicology of deoxynivalenol (vomitoxin). J Toxicol Environ Health 48:1-34.

Rotter, B.A., Thompson, B.K., Lessard, M., Trenholm, H.L., Tryphonas, H., 1994. Influence of low level exposure to *Fusarium* mycotoxins on selected immunological and hematological parameters in young swine. Fundam Appl Toxicol 23:117-124.

Sergent, T., Parys, M., Garsou, S., Pussemier, L., Schneider, Y.J., Larondelle, Y., 2006. Deoxynivalenol transport across human intestinal Caco-2 cells and its effects on cellular metabolism at realistic intestinal concentrations. Toxicol Lett 164(2): 167-176.

Turner, J.R., 2009. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol 9: 799-809.

Yang, G.H., Jarvis, B.B., Chung, Y.J., Pestka, J. J., 2000. Apoptosis induction by satratoxins and other trichothecene mycotoxins: Relationship to ERK, p38 MAPK, and SAPK/JNK activation. Toxicol. Appl. Pharmacol. 164:149-160.

Yu J, Yin P, Yin J, Liu F, Zhu X, Cheng G, Guo K, Yin Y, Xu J., 2010. Involvement of ERK 1/2 signaling and growth-related molecule´s expression in response to heat stress-induced damage in rat jejunum and IEC-6 cells. Int J Hyperthermia, 26: 538-555.

Zhou, H.R., Jia, Q., Pestka, J.J., 2005a. Ribotoxic stress response to the trichothecene deoxynivalenol in the macrophage involves the Src Family Kinase Hck. Toxicol Sci 85:916-26.

Zhou, H.R., Islam, Z., Pestka, J.J., 2005b. Induction of competing apoptotic and survival signaling pathways in the macrophage by the ribotoxic trichotecene deoxynivalenol. Toxicol Sci 87:113-122.

Figure Legends

Figure 1-. Effect of DON exposure on jejunum histology. (A) and (B) Histological score. (A) Pigs received a control diet or a diet contaminated with 2.3 mg/kg DON. (B) Jejunal explants exposed to 5 µM or 10 µM DON. (C) Jejunum of a control pig. Normal villi. HE. (D) Jejunum of a pig fed a DON-diet. Villi fusion and atrophy (arrowhead) and apical lyses of enterocytes (arrow). HE. (E) Jejunal explant of control group. Villi covered by cylindrical enterocytes. HE. (F) Jejunal explant exposed to 10 µmol/L DON. Villi atrophy and fusion (arrow). HE. Bars 100 µm. Lesional score after histological examination according to the occurrence and severity of lesions. Values are mean scores ± SEM for 6 pigs. * =$P < 0.05$; ** = $P < 0.01$. AU= arbitrary units.

Figure 2 - Effect of DON exposure on intestinal expression of MAPK. (A) Pigs received a control diet or a diet contaminated with 2.3 mg/kg DON. (B) Jejunal explants exposed to 5 µM or 10 µM DON. A and B figures represent the expression of ERK 1/2, p38 and JNK estimated by densitometric analyses after normalization with ß-actin signal. The values are mean ± SEM for 6 pigs. * =$P < 0.05$; *** = $P < 0.01$.

Figure 3 - Effect of DON exposure on intestinal expression of MAPK. Pigs received a control diet or a diet contaminated with 2.3 mg/kg DON. (A) The figure represents the immunoblot from jejunum of pigs fed a diet contaminated with 2.3 mg/kg DON. (B) The figure represents the immunoblot from jejunal explants exposed to 10 µM DON.

Figure 1 - Lucioli et al.

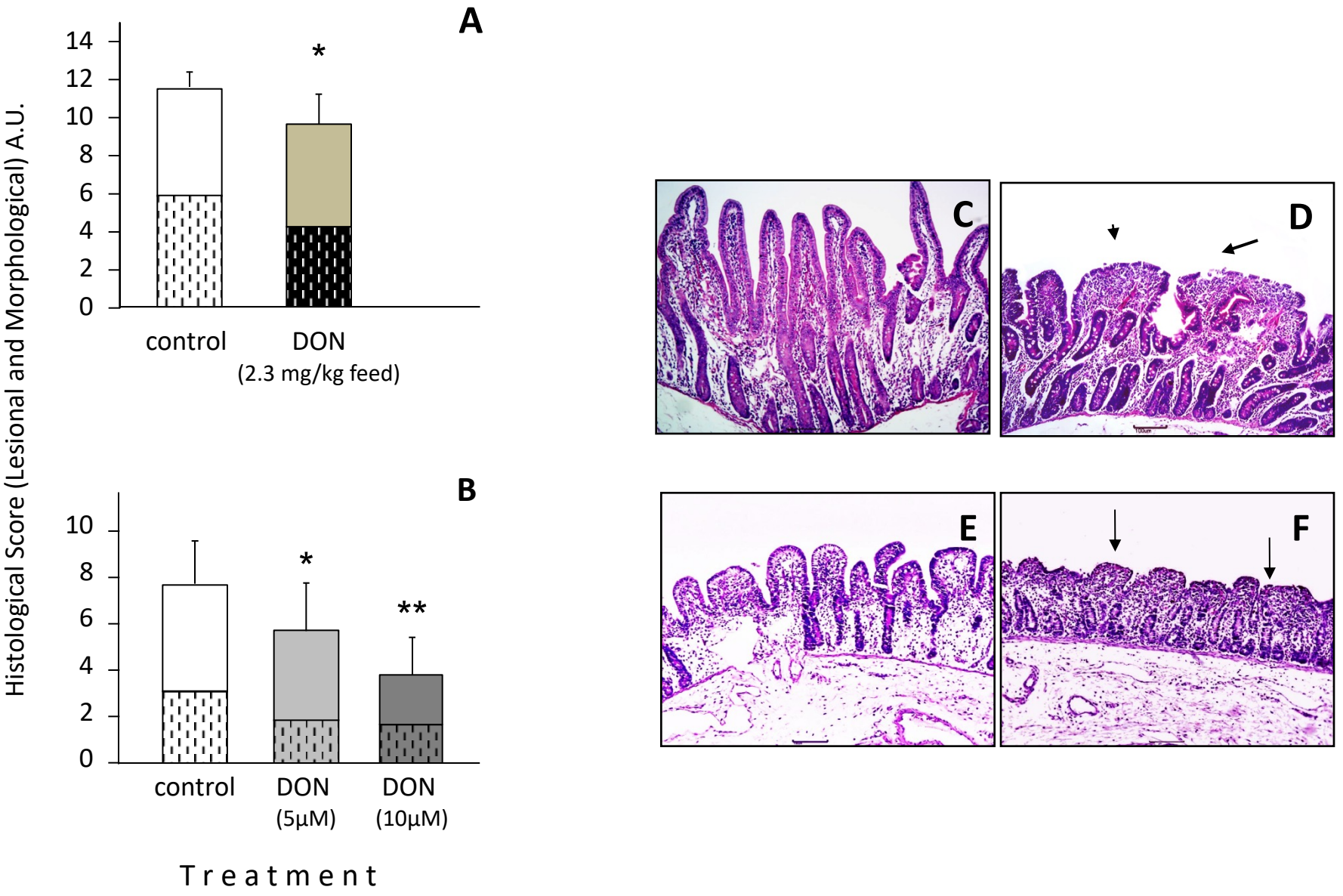
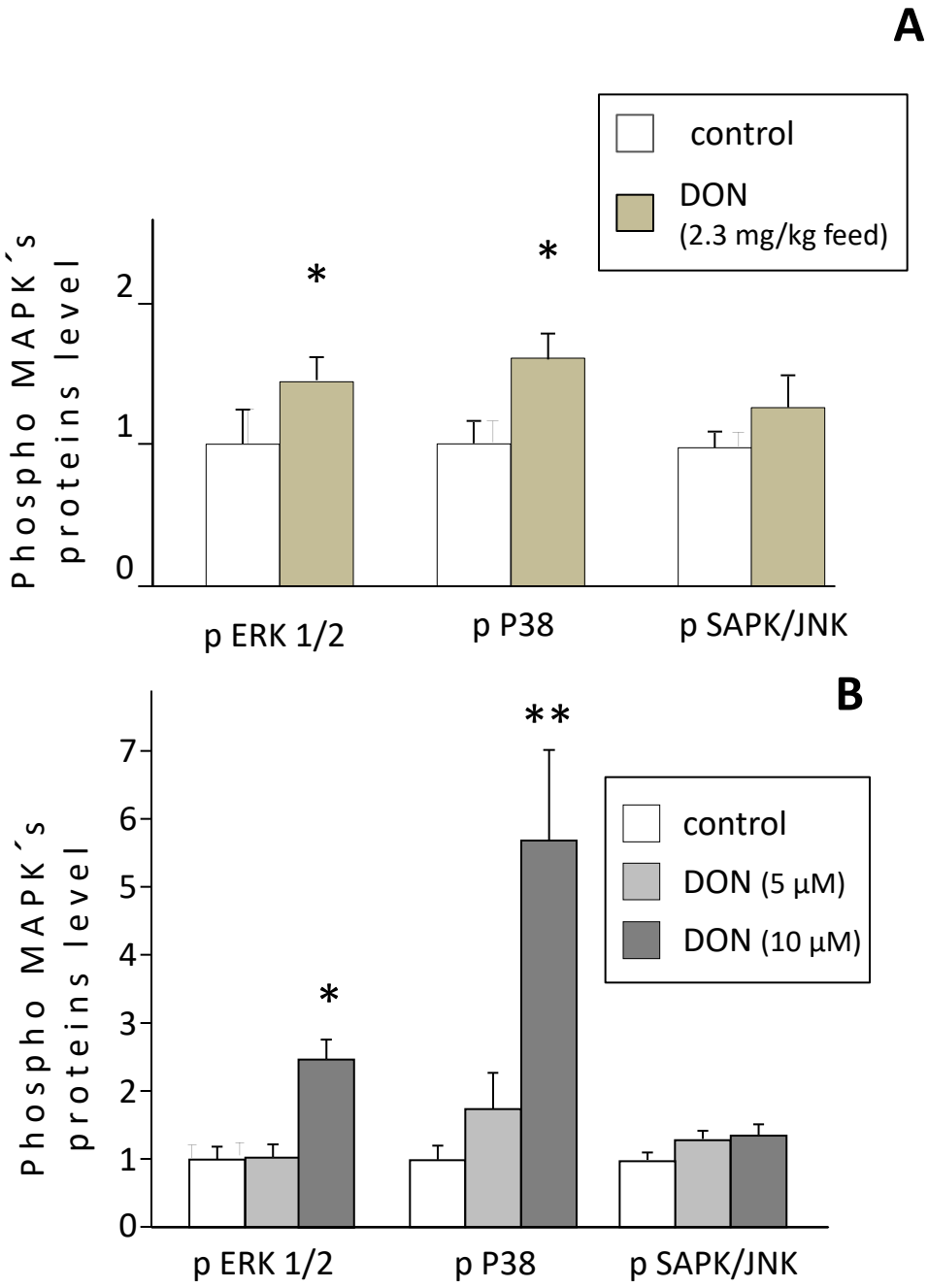


Figure 2 - Lucioli et al.



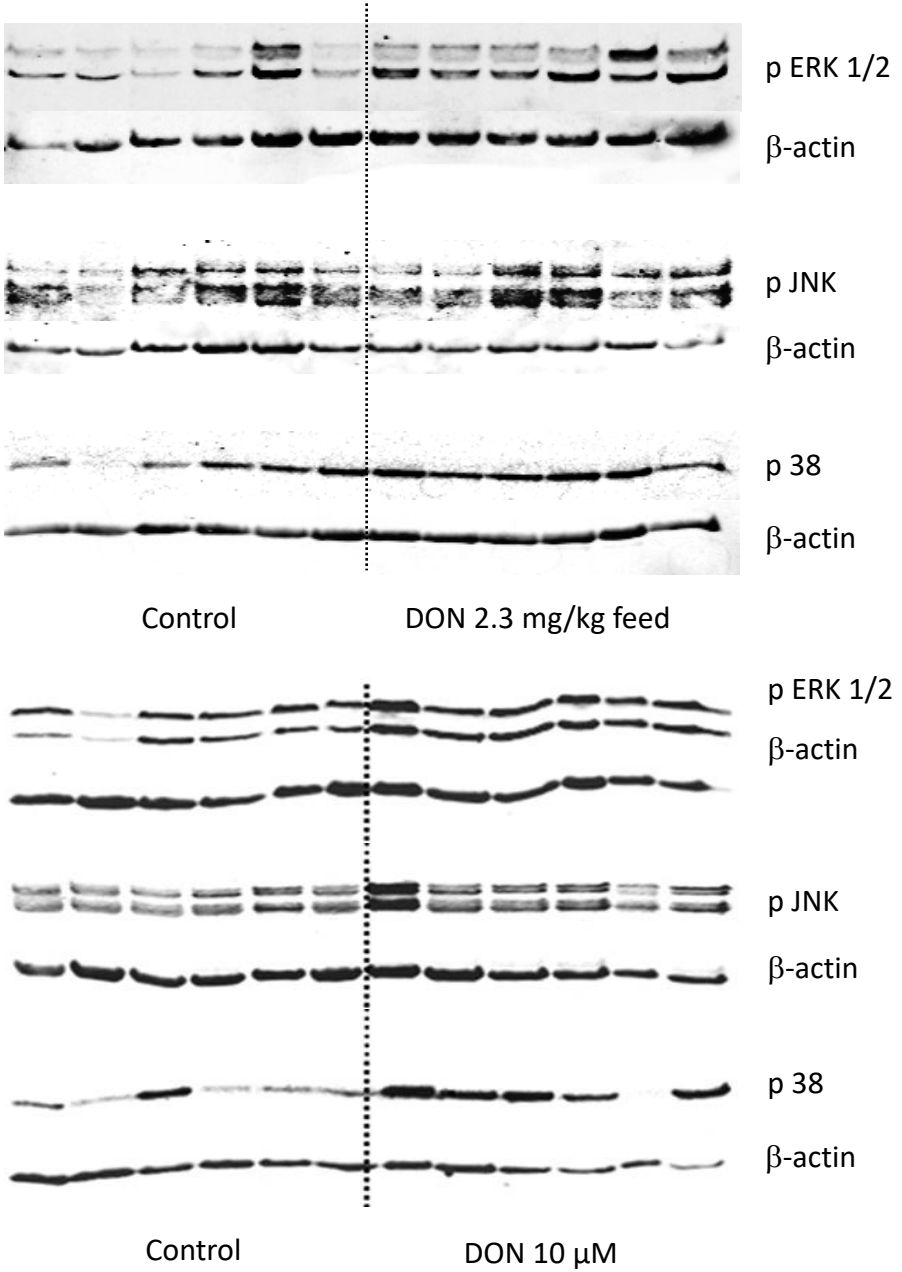


Figure 3 Lucioli et al.