Theses of doctoral (PhD) dissertation

Use of potentially protective substances to maintain the integrity of the intestinal barrier damaged by mycotoxins in *in vitro* models

Judit Mercédesz Bús-Pomothy

Supervisors: Dr. Erzsébet Pásztiné Dr. Gere

UNIVERSITY OF VETERINARY MEDICINE
Veterinary Doctoral School

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Supervisor:

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Dr. Erzsébet Pásztiné Dr. Gere PhD

Department of Pharmacology and Toxicology

University of Veterinary Medicine, Budapest, Hungary

Supervisor

.....

Judit Mercédesz Bús-Pomothy

Introduction

Secondary metabolites, mycotoxins, produced by moulds that infect crops, may be present in crop feed. Trichothecene mycotoxins produced by *Fusarium* species include deoxynivalenol (DON) and T-2 toxin, which can cause gastrointestinal dysfunction, necrotic and inflammatory responses. The European Union has set maximum levels for mycotoxin content in food and feed, which do not include glycosylated forms of mycotoxins, despite the fact that they have significant cytotoxic effects when hydrolysed in the gut. For feed, both physical and chemical methods are available to quantitatively reduce toxins. In recent decades, the use of phytobiotic feed supplements of plant origin, which can have a complex polyphenol pattern and antimicrobial, antioxidant and anti-inflammatory effects, has become more prominent.

Polyphenols can enter the human and animal body through plant-based foods. The biochemical properties of polyphenol aglycones include antioxidant activity due to the presence of a phenolic ring and hydroxyl groups, so these compounds may be able to neutralise free radicals. By studying plant antioxidants *in vitro*, it is possible to model the processes in the intestinal tract and to understand the specific effects of the compounds under mycotoxin stress.

In our studies, we investigated the effects of DON and T-2 toxin and a mixture of the two on cell viability and intestinal barrier integrity in two non-tumorigenic small intestinal epithelial cell lines, human HIEC-6 and porcine IPEC-J2 cells. Our studies may help to better understand how the co-occurrence of mycotoxins modifies the effects of mycotoxins that are already known but act only individually. Extensive testing of feed supplements will help identify the molecules responsible for the beneficial properties of phytobiotics. Our aim was to test aglycone formulations of two compounds with antioxidant properties, quercetin and rosmarinic acid, and a multi-ingredient fermented wheat germ extract in combination with mycotoxins, by analysing several experimental set-ups to understand their antioxidant and anti-inflammatory effects and their applicability in *in vitro* conditions.

Aims of the study

In our study, we wanted to investigate the effects of DON, T-2 toxin, quercetin, rosmarinic acid and fermented wheat germ extract alone and in combination in several experimental settings.

1. In this study, I aimed to determine the highest non-cytotoxic concentrations of DON and T-2 toxin and their combination DT2, as well as to determine the effects of DT2 on cell viability, oxidative status, inflammatory cytokine production and the distribution of claudin-1 and occludin proteins. HIEC-6 and IPEC-J2 non-tumorigenic intestinal epithelial cells were used for our experiments.

2. In the study of the effect of quercetin, we sought to answer the question at what concentrations it is appropriate to use quercetin to achieve the protective effect during 24 h pre-treatment and whether 24 h pre-treatment or 1 h co-treatment with quercetin can reduce the attenuating TEER and oxidative stress increasing effect of DON. I aimed to determine changes in redox status by measuring extracellular H_2O_2 concentration and intracellular ROS.

3. In the study with rosmarinic acid, we also determined the concentration required to achieve a protective effect during the 24-h pre-treatment. Our aim was to determine whether 24 h pretreatment with rosmarinic acid affects the potentially cell layer integrity-reducing, extracellular H_2O_2 and interleukin-6 and interleukin-8 cytokine concentration-increasing effects of DON, T-2 toxin and DT2. Furthermore, whether rosmarinic acid modifies possible changes in the presence of claudin-1 and occludin proteins in the cell membrane.

4. My research sought to determine whether 1% and 2% fermented wheat germ extracts affect the cell layer integrity-reducing and oxidative stress-increasing effects of the putative DON and T-2 toxin-induced cell layer integrity reduction and oxidative stress during co-treatment, and whether a beneficial effect of fermented wheat germ extract can be observed in the 24-h follow-up (regeneration) experimental design.

Materials and methods

Incubation of HIEC-6 cells with mycotoxins

In this experiment, the effects of DON, T-2 toxin and DT2, a combination of the two, were studied with an incubation time of 24 hours. We investigated the effect of the mycotoxins tested on the viability of human HIEC-6 cells of non-tumorigenic origin (MTS) (n =9), their extracellular hydrogen peroxide production (Amplex Red Hydrogen Peroxidase Assay Kit) (n =8) and the concentrations of inflammatory cytokines (interleukin-6 and interleukin-8) (sandwich ELISA Kit) (IL-6 n =8; IL-8 n =6). In our study, we determined the amount of intracellular ROS using cells (DCFH-DA) (n =10), immunofluorescence staining to visualise claudin-1 and occludin proteins, and the concentration of both proteins (Sandwich ELISA Kit) after disruption of cells by centrifugation (both n =4). Cells were plated on 96-well culture plates for cell viability monitoring and on 24-well culture dishes containing an inverted plate for sample collection and immunofluorescence staining. Assays were performed with at least 3 replicate measurements. Concentrations of 1 µM DON, 5 nM T-2 toxin and 1 µM DON + 5 nM T-2 toxin (DT2) were used in the experiments.

Pre-treatment and co-treatment of IPEC-J2 cells with quercetin under DON exposure

I investigated the effects of quercetin and DON on IPEC-J2 cells in two experimental set-ups: (I) after 24 h of quercetin pre-treatment, cells were incubated with DON for 1 h or (II) after 24 h of control treatment, cells were co-incubated with quercetin and DON treatment solution for 1 h. I determined the cytotoxic concentrations of DON and quercetin by Neutral Red staining after 24 h of treatment (DON n =8; quercetin n =5). Further studies were performed with 20 µM quercetin and 1 µM DON treatments in 6-well in culture for each experiment. Cell layer integrity was examined before addition of treatment solutions, 24 h after addition, and after an additional 1 h incubation with DON (n =6). At the 25 h time interval, samples were taken from the cell-free supernatant to determine the change in additional extracellular H_2O_2 concentration (Amplex Red Hydrogen Peroxidase Assay Kit) (n =8). After IPEC-J2 cells were plated, the amount of intracellular ROS (DCFH-DA) was measured (n =8).

Examination of the effect of 24 h rosmarinic acid pre-treatment prior to 48 and 72 h mycotoxin treatment in IPEC-J2 cells

The 24-hour protective effect of rosmarinic acid on the 48- and 72-hour DON, the T-2 toxin and a combination of both DT2 toxin. Cell culture was performed in a 6-well cell

culture dish. Cell viability was analysed using Neutral Red stain and based on the results, a mixture of 1 µM DON, 5 nM T-2 toxin, 1 µM DON and 5 nM T-2 (DT2) and a pre-treatment concentration of 50 µM rosmarinic acid was selected for further studies (n =8 for each). In the experimental setup, rosmarinic acid was added to one group, later DT2, for 24 h, after which the different mycotoxin treatment solutions were added to IPEC-J2 cells. The pre-treatment with rosmarinic acid for 24 h preceded the experimental starting point of 0 h indicated in the experimental results. Changes in cell layer integrity were measured at 48 and 72 h after application of mycotoxin treatment solutions (n =9). After both treatment times, cell-free supernatants were sampled for subsequent determination of extracellular H_2O_2 (Amplex Red Hydrogen Peroxidase Assay Kit) (n =8) and interleukin-6 and interleukin-8 (Sandwich ELISA Kit) (n =10 for both) concentrations. Cellular proteins claudin-1 and occludin were visualized by immunofluorescence staining in the membrane 72 h after treatments.

Study on the protective effect of fermented wheat germ extract when co-treated with DON and T-2 toxin

The effect of fermented wheat germ extract was tested on IPEC-J2 cells in combination with DON and T-2 toxin. In the experimental set-up, cells were incubated with the treatment solutions and their combination for 24 hours, and then control treatment was given to each treatment group for a further 24 hours. The cell viability was determined by MTS in 96-well dishes (n =8) using 8 µM DON, 5 nM T-2 toxin was tested in combination with 1% and 2% fermented wheat germ extract in 6-well culture dishes. TEER values were measured before the start of the experiment, 24 h after treatment and after regeneration, 48 h (n =9). At both time intervals, extracellular space was sampled to determine H_2O_2 concentration (Amplex Red Hydrogen Peroxidase Assay Kit) (n =8), followed by cellular and intracellular ROS quantification by addition of DCFH-DA stain (n =6).

Statistics

In the case of normally distributed samples, one-way ANOVA and post-hoc Tukey test were performed to determine the differences between the groups. Data from the experiments are presented as mean ± standard deviation (SD). Significant differences between control and treated groups are indicated by asterisks (*p <0.05; **p <0.01; ***p <0.001), while significant differences between groups are indicated by p values.

Results and discussion

Analysis of DON, T-2 and DT2 in HIEC-6 cells

Trichothecene mycotoxins produced by *Fusarium* species are present in food. Several species have been shown to be able to produce multiple mycotoxins, so the possibility of interactions between toxins cannot be excluded. Multitoxicity studies are becoming increasingly important, but there is still little literature on which toxins can act synergistically to amplify each other's effects. In studies based on human intestinal epithelial cells, several studies have so far used colon epithelial cell lines of tumour origin (Caco-2, HT-29). HIEC-6 cell lines can be used to more accurately model *in vivo* conditions. By investigating cell lines of non-tumour origin, a more complex picture of how mycotoxin exposure may affect cells still growing in the gut can be obtained.

In the present study, we investigated the effects of a mixture of 1 µM DON, 5 nM T-2 toxin, 1 µM DON and 5 nM T-2 administered alone and in combination, using a 24 h incubation time. We found that their effects significantly increased the concentration of hydrogen peroxide in the extracellular space and the level of intracellular ROS, and that all treatments increased the levels of the inflammatory cytokines IL-6 and IL-8. The presence and amount of claudin-1 were significantly reduced in the membrane, whereas the levels of occludin were unchanged.

The results suggest that the two fusariotoxins have a negative effect on the cells when administered at non-cytotoxic concentrations. We have also demonstrated that the HIEC-6 cell line is a suitable cell model for *in vitro* testing of the pro-inflammatory, intestinal epithelial damaging effects of certain xenobiotics in food.

Effect of DON and T-2 toxin combination on IPCE-J2 cells

Little information is available on the effects of mycotoxins in combination. While there is a large literature on the negative effect of DON alone, there are fewer experimental results with T-2 toxin. In this study, we investigated the effect of DON in combination with T-2 toxin (DT2) on cell viability, cell layer integrity changes, oxidative stress enhancement, inflammatory cytokine production, and the presence of claudin-1 and occludin proteins visualized by immunofluorescence staining in the porcine IPEC-J2 cell line.

The results of TEER measurements following DT2 treatment (1 µM DON + 5 nM T-2) showed that there was a significant difference between the 1 µM DON, 5 nM T-2 toxin and DT2 treatment groups after both 48 and 72 h, i.e. the cell layer integrity reducing effects of mycotoxins were prevailing. DT2 treatment significantly increased the concentration of IL-6 and IL-8 in the cell-free supernatant after both 48 and 72 h. Our results suggest that DT2 applied for 72 h affected the expression of claudin-1 in the

membrane but did not alter the localization pattern of occludin. Our results suggest that the TEER reduction induced by DT2 treatment may correlate, at least in part, with the altered amount of claudin-1.

Applicability of protective substances against the negative effects induced by DON, T-2 toxin and DT2

In vivo studies have shown that certain flavonoids in pig feed can reduce oxidative stress and inflammation and thus improve the overall well-being of pigs. However, there are not yet sufficient data on the amount, duration and farm animal species for which phenolic compounds can be used as feed additives. However, *in vitro* experiments show that polyphenolic compounds may have a beneficial effect in preventing adverse effects induced by mycotoxins due to their antioxidant activity.

Our results showed that the TEER values of IPEC-J2 cells incubated with 20 µM quercetin for 24 h were significantly higher compared to the control treatment. The pre-treatment with 20 µM quercetin was not able to prevent the TEER reducing effect of 1 µM DON. Co-administration of quercetin and DON for 1 hour also failed to attenuate the change in TEER compared to the control, but comparing the results of the two groups tested, we obtain that the cell layer integrity of the pre-treated cells remained higher compared to that measured with the co-administration of the compounds for 1 hour, i.e. the 20 µM quercetin pre-treatment helped to restore the DON-induced intestinal epithelial integrity damage. In our experiments, we found that co-treatment with 20 µM quercetin and 1 µM DON for 1 h significantly increased extracellular H_2O_2 concentrations, whereas 24 h pre-treatment prevented DON-induced extracellular H_2O_2 production. Cells pre-treated with 20 µM quercetin and then exposed to DON showed a significantly higher intracellular ROS increase compared to control cells, yet significantly lower ROS levels than co-treated cells.

In our experiments with rosmarinic acid, we obtained that a 24 h pre-treatment with 50 µM rosmarinic acid was effective in counteracting the TEER reducing effect of DT2 (1 µM DON + 5 nM T-2) treatment. Comparing the effects of pretreatment and DT2 treatment alone, we found that rosmarinic acid was able to attenuate the DT2-induced increase in extracellular H_2O_2 concentration in IPEC-J2 cells, i.e. 50 µM rosmarinic acid also had an effect on the oxidative state of the cells 72 h after incubation. There are few data in the literature on the anti-inflammatory effect of rosmarinic acid under *in vitro* conditions, our own results suggest that DT2 treatment significantly increased the cytokine concentrations of both IL-6 and IL-8 in IPEC-J2 cells, which were reduced by the 24 h 50 µM rosmarinic acid pre-treatment 48 and 72 h after incubation. The distribution of occludin and claudin-1 in control-treated IPEC-J2 cells appeared homogeneous after

immunofluorescence staining. The localization of the claudin-1 protein was altered by DT2 treatment, whereas rosmarinic acid added to IPEC-J2 cells at a concentration of 50 μM for 24 h promoted the presence of claudin-1 in the membrane after 48 and 72 h of incubation with DT2.

Fermented wheat germ extract contains several potentially protective active ingredients and may therefore be effective in reducing oxidative stress caused by the two most common mycotoxins. In our experiments, both 1% and 2% fermented wheat germ extracts increased the viability of IPEC-J2 cells, but the extract failed to attenuate cell death when added to cells in combination with 8 μM DON. In contrast, 1% and 2% fermented wheat germ extract enhanced the survival of IPEC-J2 cells co-treated with T-2 toxin compared to cells treated with 5 nM T-2 toxin alone. Based on literature data, the effect of fermented wheat germ extract on changes in cell layer integrity has not yet been investigated. Based on our data, 1% fermented wheat germ extract in combination with mycotoxins did not increase TEER values of IPEC-J2 cells. In contrast, 2% fermented wheat germ extract added with 5 nM T-2 toxin helped to maintain intestinal barrier integrity. The 2% fermented wheat germ extract reduced ROS after 24 h of treatment with both DON and T-2 toxin. In simultaneous 24 h treatment, both 1% and 2% fermented wheat germ extract significantly reduced the increase in ROS levels induced by DON and T-2 toxin. The fermented wheat germ extract had beneficial effects on the viability of IPEC-J2 cells and was able to reduce the oxidative stress induced by the applied DON and T-2 toxins.

Conclusion

In conclusion, non-tumorigenic human (HIEC-6) and porcine intestinal epithelial cells (IPEC-J2) are suitable for modeling intestinal barrier damage induced by *Fusarium* toxins such as DON and T-2. In our experimental work, plant compounds (quercetin and rosmarinic acid) and fermented wheat germ extract were tested and demonstrated to be involved in restoring the integrity of the damaged intestinal epithelial cell layer and mitigating oxidative stress caused by the xenobiotics applied. In all cases, pre-treatments with quercetin and rosmarinic acid, which are polyphenolic compounds, for 24 h increased the integrity of IPEC-J2 cells. Our experimental data showed that when the combination of DON and T-2 toxin was applied to co-existing mycotoxins in cereals infected with mould species, the levels of some inflammatory cytokines (IL-6 and IL-8) were elevated and cell layer resistance was significantly reduced, which deleterious lesions were effectively restored in IPEC-J2 cells by pre-treatment with rosmarinic acid. The new *in vitro* results may lay the foundation for the future practical application of several natural compounds with antioxidant and anti-inflammatory activities as dietary and feed additives.

New scientific results

Ad 1,

HIEC-6 of non-tumorigenic origin, human intestinal epithelial cells, were negatively affected by treatment with 1 μM DON, 5 nM T-2 and DT2 (1 μM DON + 5 nM T-2).After 24 h of incubation, there was a significant increase in extra- and intracellularly measured oxidative stress, inflammatory cytokine concentrations and a decrease in the amount of claudin-1 in the cell membrane.

Ad 2,

First, we investigated the effect of DON in combination with T-2 toxin (DT2) in a porcine non-tumorigenic IPEC-J2 cell line. DT2 (1 μM DON + 5 nM T-2) significantly increased oxidative stress and inflammatory cytokine concentrations. Immunofluorescence staining demonstrated that the localization pattern of claudin-1 was significantly reduced in the cell membrane after treatment.

Ad 3,

I compared the effects of 24 h pretreatment and 1 h co-treatment with 20 μM quercetin on 1 h 1 μM DON-induced cell layer integrity damage and oxidative stress increase in IPEC-J2 cells. Pre-treatment with 20 μM quercetin is able to attenuate TEER decrease and DON-induced H_2O_2 and ROS increase compared to 1 h co-treatment.

Ad 4,

The 24-hour 50 μM rosmarinic acid pre-treatment resulted in a 48- and 72-hour DT2 (1 μM DON + 5 nM T-2) treatment, TEER was higher compared to cells treated with DT2 alone. Rosmarinic acid pre-treatment significantly reduced DT2-induced increases in extracellular H_2O_2 and inflammatory cytokine concentrations in cells exposed to DT2 alone, and the localization pattern of claudin-1 protein in cell membranes was not altered by rosmarinic acid.

Ad 5,

Fermented wheat germ extract was first used in co-treatment with fusariotoxins. For the first time, fermented wheat germ extract was shown to increase the cell layer integrity of IPEC-J2 cells after 24 h of treatment. Our results showed that the effect of the T-2 toxin was significantly counteracted by the 2% fermented wheat germ extract during the treatment period. Oxidative stress induced by mycotoxins could be reduced by the co-treatment of 1% and 2% fermented wheat germ extract for 24 h. After an additional 24 h of regeneration, co-treatment with 1% fermented wheat germ extract was able to counteract the increase in intracellular ROS induced by both DON and T-2 toxin, while 2% fermented wheat germ extract reduced the production of intracellular ROS after co-incubation with T-2 toxin.

Own scientific publications

Publications related to the topic of the present dissertation

Pomothy J.M., Szabó O., Czimmermann Á.E., Babiczky Á., Jerzsele Á., Pászti-Gere E.: **Investigation of the inflammatory and oxidative stress-inducing effects of deoxynivalenol and T-2 toxin exposure in non-tumorigenic human intestinal cell model**, Toxicon, 200. 78−86, 2021. **Impact factor: 2,725**

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