XENOBIOTICS-INDUCED INTESTINAL DAMAGE IN SWINE

Final report

1. Main tasks of the project

Different xenobiotics such as drug candidate capable of transmembrane serine protease inhibition, proliferative enteropathy-causing intracellular bacterium, *Lawsonia intracellularis* and trichotechene mycotoxins in the feedstuffs can cause severe enterohepatic damage in swine. These putative deteriorative effects can be mimicked successfully using appropriate cell-based models. Our aims in this research project were twofold: Firstly functional *in vitro* model of the intestine was developed to mimick oxidative stress- and pathogen-induced inflammation upon administration of xenobiotics. In this research work porcine intestinal cells were used to map the cross-talks between intestinal epithelium of swine hosts and selected xenobiotics. Secondly, bioactive molecules and their mixtures such as polyphenol rosmarinic acid and quercetin, fermented wheat germ extract were screened to evaluate their potential beneficial effects in case of fusariotoxin-induced redox imbalance and inflammation. In addition, the efficacy of sodium- butyrate (formed by gut microbiota from dietary carbohydrates and used also as feed additive) and spent culture supernatants (SCS) of probiotic *Bacillus licheniformis* were applied to reduce damage to intestinal epithelial cells exposed to *Lawsonia intracellularis*.

2. Main results of the project

2.1. *In vitro* characterization of matriptase/TMPRSS2 inhibitor

Matriptase and TMPRSS2 belong to the group of type II trypsin -like transmembrane serine protease (TTSP) and they are expressed in both non-tumorigenic and cancerous epithelial cells. Some of the selective matriptase/TMPRSS2 inhibitors seem to be potential drug candidates in the treatment of osteoarthritis, human influenza, coronavirus-caused respiratory disease (COVID-19) and tumor metastases, thus determination for safety profiles of these compounds is of key importance.

In this project the effects of 3- aminidophenylalanine (3-AphA) structure-based inhibitor, matriptase inhibitor (MI)- 432 on the barrier integrity of IPEC-J2 monolayer was investigated.

Firstly, our aim was to establish a cell modell suitable for tracing transport processes between the apical and basolateral compartments mimicking physiological and pathological processes in the small intestine *in vitro*. We used non- tumorgenic, porcine jejunal cell monolayer, IPEC-J2 cells cultured on membrane insert. IPEC-J2 cells were treated with 3-AphA matriptase/TMPRSS2 inhibitor, MI-432. Viability of IPEC-J2 cells was measured 24 and 48 h after treatment with inhibitor MI-432 versus control at 540 nm by Neutral red uptake assay using a EZ Read Biochrom 400 microplate reader.

Transepithelial electrical resistance (TER) measurements of cell layers were performed prior to and immediately after MI-432 administration using an EVOM Epithelial Tissue Volt/Ohmmeter (World Precision Instruments, Berlin, Germany). Fluorescein isothiocyanate–dextran 4 kDa (FD4) at 1 mg/ml was added to the apical compartment of IPEC-J2 cell layer with 4, 24 and 48 h incubation times at 37 °C in the presence of the matriptase inhibitors at 50 µM. Samples of media from the basolateral chambers were collected and the tracer concentrations were then quantified using Victor X2 2030 fluorometer at λ_{ex} = 480 nm and λ_{em} = 530 nm.

Measurement of perturbances in intracellular redox state of IPEC-J2 cells was carried out using 2', 7'-dichlorodihydrofluorescein-diacetate (DCFH-DA) dye. In this form, the probe can penetrate the cell membrane before being deacylated by cellular esterases to another non-fluorescent form, DCFH. DCFH is then oxidised to the highly fluorescent form dichlorofluorescein (DCF) by the intracellular ROS. IPEC-J2 cells were treated with matriptase inhibitor MI-432 at 10, 25 and 50 µM for 2, 24 and 48 h in phenol red free DMEM. A working solution of 10 µM DCFH-DA was added and incubated for 30 min after which a quantitative analysis of the intracellular ROS activity was carried out using the Victor X2 2030 fluorometer (λ_{ex} = 480 nm, λ_{em} = 530 nm). Extracellular H_2O_2 measurement of cell supernatant was based on the application of Amplex red method, in which Amplex red reacts with H_2O_2 in a 1:1 stoichiometry producing a highly fluorescent resorufin In the presence of horseradish peroxidase (HRP). After 30 min incubation with the dye at room temperature the H_2O_2 contents was measured using a Victor X2 2030 fluorometer (λ_{ex} = 560 nm, λ_{em} = 590 nm).

It was found that treatment with inhibitor MI-432 at the highest dose of 50 µM used in this study over a period 48 h did not affect IPEC-J2 cell viability compared to that of untreated cells. The rate and reversibility of decrease in TER was dependent on the inhibitor concentration and the cell differentiation. After 2 h and 24 h incubation time it can be seen that TER is decreased significantly by addition of 50 µM MI-432. A significant decrease in TER was also found when IPEC-J2 cells were treated with MI-432 for longer periods of time (48 h). Based on findings from apico-basolateral

transport of FD4, significant increase in paracellular permeability of cell monolayer exposed to matriptase inhibition was confirmed as a result of barrier dysfunction.

During monitoring the changes in ROS levels in IPEC-J2 cells after a 2 h incubation time with MI-432 at 10 μM, 25 μM, and 50 μM fluorescence intensities of both the control and treated samples were measured. It was found that there was an acute decrease in the intracellular ROS activity with the decrease being more pronounced as the concentration of the inhibitor was increased. All three inhibitor concentrations caused a significant change, which may be representative of putative outflow of hydrogen peroxide from the cells. For this reason it was important to compare these findings with the corresponding extracellular oxidative stress using Amplex red method. The results showed that there was an increase in the extracellular H_2O_2 amounts leading to a significant elevation in fluorescence when MI-432 was applied at a concentration of 50 μM for 2 h. After a 24 h incubation time with MI-432 at 10 μM, 25 μM, 50 μM, a significant increase of intracellular ROS can be seen with the 50 μM concentration of MI-432 while no significant changes were found in the extracellular compartment for any concentrations of the inhibitor. This measurement shows an almost two fold increase in the intracellular ROS activity compared to the control group, which indicates that 24 h inhibition of matriptase caused marked disturbances in the ROS production inside the cells. The extracellular fluorescence was, however, not significantly elevated. After a 48 h incubation time with the MI-432 at 10 μM, 25 μM and 50 μM fluorescence values of DCF were determined in control and in treated IPEC-J2 cells. The results show that there were not any perturbations in redox balance in IPEC-J2 cells for the 10 μM and 25 μM concentrations of the inhibitor and less significant relative increase after treatment with 50 μM inhibitor than was present after 24 h. In the extracellular compartment, there were not any significant increases in H_2O_2 levels.

In conclusion, it was found that the matriptase inhibitor MI-432 decreased TER and simultaneously increased the apico-basolateral transport of FD4. It was also concluded that acute administration of MI-432 provoked elevated H_2O_2 levels detected extracellularly, which can be a result of ROS leakage from the intracellular production pool due to some kind of membrane barrier dysfunction. As for a longer timeframe the concentration of extracellularly detected peroxide was not different in control and in MI-432-treated IPEC-J2 cells. In the future it can be predicted that the in-depth understanding of matriptase effect may contribute to establishment of new therapeutical options in the treatment of Crohn-disease, colitis ulcerosa or to introduction of defensive strategies into veterinary practice to restore barrier dysfunction in infectious diseases in livestock.

2.2. The effects of feed-additive sodium- butyrate and probiotic *Bacillus licheniformis* supernatant in *Lawsonia intracellularis* -caused infection

The project work involved *in vitro* investigation of *Lawsonia intracellularis (L. intracellularis*)- caused enteropathy and selection of beneficial antibiotic alternatives in IPEC-J2 cell model of porcine origin. The major advantage of IPEC-J2 cell line used for infection experiment is that its glycosylation pattern, proliferation rate and colonisation ability characterize better the *in vivo* conditions in the gut ecosystem compared to widely used cancerous Caco-2 and HT-29 cell lines. Proliferative enteropathy of weaning pigs has significant economical importance in the swine industry. The causative agent, *L. intracellularis* is a Gram-negative, microaerophilic, obligate intracellular bacterium, that can replicate in cytoplasm of enterocytes of swine and several other species. Administration of antibiotics against *L. intracellularis* results in drug residues in the meat, resistance formation among pathogenic bacteria and cause further economic losses. Antibiotic alternatives, like organic acids and probiotics have an increasing importance in the prevention and treatment of several bacterial diseases. To avoid the usage of antibiotics in proliferative enteropathy caused by intracellularly acting enteropathogen, *L. intracellularis*, SCS of *Bacillus licheniformis* (*B. licheniformis*) CECT 4536 and sodium-n-butyrate (SB) were used to test their potential beneficial effects *in vitro*. Non-tumorigenic, intestinal porcine epithelial IPEC-J2 cell line was infected with $5x10^5$ CFU *L. intracellularis* (strain PHE/MN1-00) two times with each incubation for 1 day. *B. licheniformis* CECT 4536 was grown in lysogeny broth (10 g peptone, 5 g yeast extract, 10 g NaCl suspended in distilled water) at 37 °C for 24 hour. SCSs were prepared by centrifugation of the bacterial suspension at 1500 g at 5°C for 15 min. Centrifuged SCSs were then passed through a sterile 0.22 µm pore size filter unit. The cells were treated with either SB solution at 2 mM or SCS of *B. licheniformis* at 0.5 % for 2 hours prior to *L. intracellularis* infection. The extent of *L. intracellularis*-provoked inflammatory responses were measured using porcine specific interleukin (IL)-8 ELISA technique and the changes in barrier integrity were monitored with determination of TER values.

The altered barrier integrity of IPEC-J2 monolayers exposed to *L. intracellularis* were significantly improved when cells were pretreated with SCS of *B. licheniformis* at 0.5%. No beneficial effects were observed upon preincubation of the cells with SB at 2 mM. Increases in IL-8 levels in *L. intracellularis*- infected cells were lowered significantly when SCS of *B. licheniformis* was preadministered at 0.5% in constrast to addition of SB, which could not exert IL-8-reducing effects. These findings suggest that SCS of *B. licheniformis* could improve the barrier integrity and it was capable of exerting anti-inflammatory effect in IPEC-J2 cell model of *L. intracellularis*-caused porcine proliferative enteropathy. Based on our results we concluded that SCS of *B. licheniformis* at 0.5% improved barrier function of *L. intracellularis*- infected IPEC-J2 cell monolayers in contrast to SB at 2 mM, which could not contribute to the restoration of weakened barrier integrity *in vitro*.

We also proved that IPEC-J2 cell line cultured on membrane inserts acts as a reliable model for investigation of porcine-specific *L. intracelluris* infection-induced inflammatory processes involving regulatory changes in cytokine network. This cell system enables the screening for potential protective immunmodulatory substances operating on preventing intestinal epithelium from certain bacterial invasion or on restoration of physiological microbial communities disturbed by exogenous or endogenous agent-provoked inflammatory processes.

2.3. Evaluation of the cellular effects of fusariotoxins and antioxidant/anti-inflammatory substances on IPEC-J2 cells

Recently, the amounts of studies about the beneficial effects of plant- based dietary polyphenols have been continuously growing. Flavonoids are known to have antibacterial, anticancer, anti-inflammatory and antioxidant properties. Quercetin (Que), a well-studied plant-derived flavonol appears to be beneficial to reduce the levels of intracellular reactive oxygen species (ROS) and strengthen the integrity of the monolayer of porcine non-tumorigenic IPEC-J2 cells. It is extremely challenging to provide mycotoxin-free feedstuff for livestock. Pigs are very sensitive to feed contaminated with trichothecene mycotoxins such as deoxynivalenol (DON). In farm animals, dietary exposure to DON decreases growth performances via disruption of the intestinal barrier thus enabling increased penetration of normally excluded luminal substances that may promote intestinal disorders.

The goal of this study was to evaluate the effects of Que on IPEC-J2 cell line exposed to non-cytotoxic concentration of DON. This study was focused on measuring the TER values and determining the changes in extracellular H_2O_2 levels and intracellular ROS productions in IPEC-J2 cells after Que and DON treatments. Two experimental designs were used in our experiments as follows: Pretreatment with 20 µmol/L Que for 24 h followed by 1 h 1 µmol/L DON treatment and simultaneous application of 20 µmol/L Que and 1 µmol/L DON for 1 h. Cell cytotoxicity, TER of cell monolayers and extracellular/intracellular redox status were studied.

It was found that DON decreased significantly TERs and triggered oxidative stress, while Que pretreatments were beneficial in maintaining the integrity of the monolayers and could alleviate oxidative stress. However, co-treatment with Que was unable to preserve the integrity and redox balance of the cells exposed to DON. These results indicated that only pre-incubation of cells with Que at 20 µmol/L for 24 h was beneficial in compensating the DON-disrupted extracellular oxidative status.

Fermented wheat germ extract contains several bioactive ingredients, such as flavonoids (apigenin, luteolin), dietary fibers, as well as lignins, oligosaccharides, and vitamins and is rich in the glycosylated form of 2,6 -dimethoxy-p-benzoquinone (DMBQ). Fermented wheat germ extract (FWGE) is available in both human (*Avemar*®) and veterinary medicine (*Immunovet*®). FWGE is applied as a supplement in human cancer therapy, because benzoquinones have anti-metastatic, anti-metabolic, anti-angiogenic and anti-proliferative properties. Furthermore, FWGE can enhance the cellular immune response and has confirmed antioxidant effect.

The effect of FWGE was evaluated with co-treatments with deoxynivalenol (DON) and another fusariotoxin, T-2 toxin (T-2). The effects of FWGE on IPEC-J2 contaminated with DON and T-2 have not been studied until now. The cells were treated for 24 h with the selected solutions, then the IPEC-J2 cells were allowed to regenerate in culture medium for an additional 24 h. Our study found that 8 µmol/L DON and 5 nmol/L T-2 significantly reduced the TER values during and after the treatments, while 1% and 2% FWGE significantly increased them *in vitro*. To our knowledge, these are the first findings of determination for the impact of FWGE on the barrier integrity of the IPEC-J2 cell monolayer exposed to mycotoxins. FWGE used in 1% and 2% concentrations appeared to be beneficial to IPEC-J2 exposed to fusariotoxins since the aqueous extract significantly decreased the ROS levels. In conclusion, the results demonstrate that FWGE is more effective in barrier integrity reinforcement and redox homeostasis maintenance when cells were treated with the extract during contamination with mycotoxins.

2.4. Impact for combined deoxynivalenol and T-2 toxin administration and screening for potential beneficial antioxidant compounds

Mycotoxin contamination in feedstuffs is a worldwide problem that causes serious health problems in human and in animals and contributes to serious economic losses. DON and T-2 toxin are major trichothecene mycotoxins and they are known to challenge mainly intestinal barrier functions. Polyphenol-rich rosmarinic acid (RA) was used in our experiments with IPEC-J2 cells. Our results demonstrated that moderate to low concentrations of RA improved cell viability significantly using 24 h exposure time. However, high concentrations of RA (above 200 µM) for 24 h caused cell death to great extent. It was also proven that 50 µM RA significantly improved the integrity of the IPEC-J2 cell monolayer after 24 h treatment and did not disturb basal extracellular H_2O_2 production. It can be concluded that RA at 50 µM has the capacity to increase cell viability and the membrane integrity while it does not cause significant changes in physological extracellular H_2O_2 level. Application of low

concentration of RA seems to be an effective way to strengthen epithelial barrier integrity. However, further *in vivo* studies are needed for confirming *in vitro* observed beneficial effects of RA is to establish daily application schedule of RA as porcine feed additive in the future.

Our research work also focused on protective effects of RA against DON and T-2 or combined mycotoxin-induced intestinal damage in non-tumorigenic porcine cell model, IPEC-J2 cell line. The aims of our study were (i) to elucidate cytotoxicity of DON, T-2, DT2 and RA (ii) to determine the impact of combination of DON and T-2 toxin (DT2) and RA on intestinal barrier integrity (iii) to assess IL-6 and IL-8-regulating and oxidative stress- inducing properties of mycotoxin combination and RA using intestinal epithelial IPEC-J2 cells. Moreover, immunofluorescent study was also performed to monitor the changes in localization pattern of two TJ proteins, occludin and claudin-1 in IPEC-J2 cells exposed to DT2 in the absence and in the presence of RA.

The cytotoxic effects of DON, T-2, DT2 and RA on IPEC-J2 cells were evaluated over 48 and 72 h. Significant cell death was observed upon exposure of cells exposed to DON at 50 µmol/L after 48 and 72 h incubation. After 48 and 72 h treatments at 20 nmol/L and at higher concentrations T-2 showed cytotoxic effects. It was also found that when IPEC-J2 cells were treated for 72 h with DON and T-2 simultaneously 5 µmol/L DON + 10 nmol/L T-2, the 10 µmol/L DON + 5 nmol/L T-2 and the 10 µmol/L DON + 10 nmol/L T-2 treatments were toxic to the cells significantly. As for RA administration, RA at 500 and 1000 µmol/L significantly decreased the cell viability after 48 h treatment, however, RA did not deteriorate cell viability at lower concentrations (50 and 100 µmol/L). For further investigations we applied non-cytotoxic 1 µmol/L DON + 5 nmol/L T-2 mycotoxin combination and RA at 50 µmol/L.

To determine the barrier disrupting effects of DON, T-2, DT2 and the putative barrier reinforcing effect of RA, TER measurements were carried out using 48 h and 72 h treatment times. 48 h lasting DON and DT2 administration caused significant decrease in TER values. TERs of IPEC-J2 cells treated with DT2 + 50 µmol/L RA showed lower TERs compared to those of controls, but they were significantly elevated to decreased TERs of DT2-treated samples.

The detrimental effect of DT2 could be restored partially with the application of RA after 48 and 72 h. Based on the results, after 48 h incubation of cells with 1 µmol/L DON and DT2, the extracellular H_2O_2 levels were significantly increased. The H_2O_2 contents produced by DT2 + RA-treated IPEC-J2 cells did not differ from the H_2O_2 levels from control cells. DON, T-2 and DT2 induced significant increase in extracellular H_2O_2 levels after 72 h treatment. DT2-caused oxidative stress could be quenched effectively with the pretreatment of cells with RA at 50 µmol/L for 24 h.

The IL-6 levels were elevated after DT2 exposure and overproduction was completely inhibited by RA treatments for. There were no significant differences in IL-6 levels between control samples and the DT2 + RA- treated cells. The IL-8 levels were also increased significantly in DT2- treated IPEC-J2 cells using 48 h and 72 h incubation times. DT2 + RA could normalize perturbed IL-8 levels

compared to DT2- induced cytokine concentrations for both incubation times. There were no significant differences in IL-8 levels between RA protected- DT2 samples and control treated cells.

Localization of claudin-1 and occludin in TJ assembly was assessed in untreated control and in DT2- treated IPEC-J2 cells using immunofluorescence staining. In controls, occludin localized in membranes of polarized IPEC-J2 cells and when cells were exposed to DT2 (1 µmol/L DON + 5 nmol/L T-2) occludin maintained cell membranous presence. The localization pattern of claudin-1 significantly changed when DT2 was continuously administered. The loss of membranous claudin-1 proteins from TJ was observed in the form of discontinuous membrane pattern in contrast to the distribution of those in control cells. This phenomenon might be an explanation to TER changes observed when DT2 was administered to the IPEC-J2 cells for 72 h. RA given at the same time with DT2 seemed to preserve the integrity of TJ protein assembly with maintenance of belt-like structures of occludin and claudin-1. DT2 + RA treatment (1 µmol/L DON + 5 nmol/L T-2 + 50 µmol/L RA) resulted in similar continuous lining of occludin and claudin-1 around each cell similarly to that detected in untreated cells.

In conclusion, this study demonstrated that binary mixture of DT2 at non-cytotoxic concentration could deteriorate barrier integrity of IPEC-J2 cells and it could elevate levels of inflammatory cytokines, IL-6 and IL-8. These harmful effects could be alleviated by 24 h preadministration of polyphenolic RA. It was also shown that DT2-promoted oxidative stress could be effectively quenched by RA application. Moreover, changes in protein TJ assembly including claudin-1 relocalization were detected in DT-treated cells which could be restored by RA addition. Finally RA appeared to have anti-inflammatory, antioxidant and barrier-reinforcing potential in the prevention of DT-2-caused detrimental intestinal effects *in vitro*.

3. Other relevant information

In spring 2017 I gave birth to my third child. Due to this circumstance 1.5 year extension for finishing the project was requested and obtained in 2017.

SUPPLEMENTARY MATERIAL

TDK AND THESIS WORKS DONE IN CONNECTION WITH THE PROJECT:

Gatt Katrina: *In vitro* evaluation of DON-induced intestinal barrier damage 2019.

Prokoly Dorottya: Study on the effect of fermented wheat germ extract on intestinal epithelial cells exposed to fusariotoxins 2019.

Kiss Zsófia: Modelling of intestinal damage caused by zearalenone using IPEC-J2 cells 2017.

Szombath Gergely: 3D physiological and inflammatory hepatic *in vitro* models for screening drug candidates 2016.

National TDK Conference 1st place 2017.

Czimmermann Ágnes Eszter: *In vitro* pharmacological characterization of selective TMPRSS2 inhibitor 2015.

National TDK Conference 3rd place 2017.

McManus Seamus: New insights for bowel dysfunctions via targeted matriptase modulation 2016.

Rokonál Patrik: Effects of matriptase-2 inhibition on hepatocytes and hepatocyte-Kuffer cell co-cultures 2016.

Larissa Gardner: The impact of altered matriptase activity on cell viability and spheroid growth in 2 and 3D cell cultures 2015.

Barna Réka Fanni: The effect of selective matriptase inhibitors on paracellular permeability 2015. National TDK Conference 2015.

TWO OTHER TDK WORKS WILL BE PRESENTED ON TDK CONFERENCE:

Szóládi Áron: Beneficial effects of rosmarinic acid on IPEC-J2 cells exposed to combination of deoxynivalenol and T-2 toxin

Paréj Zsuzsanna: Pharmacological and toxicological characterization of S1P as a treatment option in anaemias

PUBLICATIONS OF THE PROJECT RESULTS

presented on requested site in chronological order