Positive effect of methoxyflavones on pig intestinal inflammation diseases caused by bacteria and oxidative stress. An in vitro study.

Final report

Introduction

Polyphenols demonstrate various beneficial effects on our health – they have anti-cancer, anti-inflammatory… *etc.* activity. Many of these biological actions have been attributed to their antioxidant properties. However, there is an emerging view that these compounds do not act as conventional hydrogen-donating antioxidants but may modulate in cells through actions at protein kinase signalling pathways.

In the other hand, flavonoids show great potential as anti-inflammatory and cancer chemopreventive agents in cell culture studies. This does not translate well into *in vivo* activity, because of their extensive metabolism in intestine and liver. Methoxyflavones also show increased cancer chemopreventive properties and they have been of particular interest due to their anti-inflammatory properties too.

The gastrointestinal tract provides a physical barrier to the diffusion of foreign materials from the lumen into the circulatory systems. Impairment of the intracellular tight junction shield, which is the major determinant of intestinal barrier function, is associated with various diseases. Infections caused by pathogen bacteria and inflammation diseases developed because of oxidative stress are lead to serious economic and health problems.

The main goal of the work was to develop a functional model of the intestine mimicking oxidative stress and pathogen-induced inflammation. Furthermore, it was also an aim to test the effect of methoxyflavones, which are very promising but not extensively-studied compounds to prevent and cure inflammation diseases of the intestine. Complex and functional in vitro model was developed to study the above mentioned topics. In the *in vitro* system, modelling of inflammation and oxidative stress is possible, markers of stress, inflammation and paracellular permeability is also could be followed up. In order to understand the complex physiological effect of polyphenols, metabolism studies were also performed.

Anti-inflammatory and oxidative stress-reducing effect of polyphenols

In the experiments, IPEC-J2 cells were used. The IPEC-J2 cell line was isolated from jejunal epithelia of a neonatal, unsuckled piglet. The major advantage of IPEC-J2 cells compared to the most widely used colon carcinoma cell lines Caco-2 and HT-29, is that their glycosylation pattern, proliferation rate and colonisation ability characterize better the *in vivo* conditions in the gut ecosystem. Inflammation in IPEC-J2 cells was triggered by bacterial lipopolysaccharide (LPS) treatment. The protocol of LPS treatment on IPEC-J2 cells was developed. Viability of enterocytes after lipopolysaccharide treatment was tested. IPEC-J2 cells were seeded to 96-well plate and incubated with LPS derived from *Escherichia coli* and *Salmonella enterica* ser. Typhimurium, respectively. The applied LPS concentration range was between 1-50 μg/ml, incubation time changed from 1 h to 24 h. The viability of the cells was measured by Neutral Red Uptake assay. It could be stated, that 10 μg/ml 1 h long LPS treatment did not reduce the number of viable cells significantly.

For the experiments, IPEC-J2 cells were seeded onto six-well polyester membrane inserts and cultured for 21 days. Enterocytes were treated apically with LPS (1 and 10 μg/ml, respectively) for 1 h. RNA isolation was performed 1h after LPS treatment, while ELISA was performed 24 h after LPS exposure. The following pro-inflammatory cytokines were assayed as markers of the degree of inflammation: IL-1β, IL-6, IL-8 and TNF-α. Expression of IL-1β was not detectable in IPEC-J2 cells. There was no significant difference in the inflammation-inducing effects (changes in relative expression of IL-6, IL-8 and TNF-α genes) between *E. coli*- and *S. enterica*-derived LPS. After LPS treatment of enterocytes (1 and 10μg/ml), the IL-8 level was significantly higher in the basolateral culture medium than in the apical compartment ($P<0.001$). Level of TNF-α secretion was also determined by ELISA, but TNF-α could be not detected in LPS-treated IPEC-J2 cells.

Relative expression of heat shock protein 70 (Hsp70), TLR-4 and COX-2 in IPEC-J2 cells after LPS treatment was also determined. The mRNA level of Hsp70 did not changed after LPS exposure, while up-regulation of COX-2 was observed. Prostaglandin E2 was not detectable in IPEC-J2 cells tested by competitive EIA method. TLR-4 showed a slight up-regulation in IPEC-J2, opposite to the expected values, due the well-known function of TLR-4 as the LPS receptor. However, this result agrees with those found in human epithelial cell lines, which show a low level expression of TLR-4, explained by the intestinal epithelial cells

relative resistance to the permanent exposure to Gram-negative commensal bacteria (Arce et al. 2010 Microbiol Infect Dis. 2010; 33:161–174, Abreu 2010 Nature Reviews Immunology 10, 131-144).
In addition, transepithelial electric resistance values prior to and post treatment were compared to check if the polarized cell monolayer integrity was influenced by the presence of LPS. Lipopolysaccharide administration did not affect TEER values significantly.

The anti-inflammatory effect of two polyphenols was studied at first on IPEC-J2 cells in LPS-evoked stress. Either of them was apigenin, a well-studied trihydroxy-flavone, while the other was its trimethoxy-analogue (4', 5, 7- trimethoxyflavone or apigenin-trimethylether). These two compounds could be solved only in dimethyl-sulphoxide then diluted with culture medium. Viability of IPEC-J2 cells was monitored after apigenin and apigenin-trimethyether treatment, respectively. Neutral Red uptake assay showed that there was no significant difference in number of viable IPEC-J2 cells incubated with plain medium containing 0.1% DMSO. 25 μM treatments with both flavones for 1 h did not reduce the number of viable enterocytes. More than 40 % of the IPEC-J2 cells were killed by 1 h hour treatment when apigenin-trimethylether was applied at 50 μM concentration. After 1 h exposure of 50 μM apigenin, reduced viability of IPEC-J2 cells was detected (number of viable cells decreased 70%, compared to the control). Viability was tested also on a time-dependent manner. Number of living cells decreased to 75 % after 2 h 25 μM apigenin-trimethylether exposure, 4h and 24 h treatment widely reduced the number of living IPEC-J2 cells. Reduced viability was observed when enterocytes were treated with 25 μM apigenin for 4 and 24 h, respectively. According to the above mentioned results, it seemed to be safe to use both flavones in 25μM concentration and 1h incubation for further experiments.
Incubation of IPEC-J2 cells with LPS (10 μg/ml) and flavones in the same time was also performed. At 25 μM apigenin treatment dose, relative gene expression of IL-6 significantly decreased compared to LPS-treated cells. Apigenin-trimethylether in the same concentration did not influence the IL-6 mRNA level. Both apigenin and apigenin-trimethylether caused significant reduction in the IL-8 gene expression level. Relative expression of TNF-α was leveraged by 25 μM apigenin-trimethylether; mRNA level of TNF-α was significantly reduced compared to the LPS-treated enterocytes. Apigenin did not suppress TNF-α mRNA level. There was no significant difference in the level of tested genes, when IPEC-J2 cells were incubated with polyphenols without LPS.

Apigenin and apigenin-trimethylether treatment caused significant reduction in the mRNA level of COX-2. Effect of apigenin and apigenin-trimethylether was compared using one-way ANOVA (Fisher LSD test). It was shown, that there is a significant difference between the effect of hydroxy and methoxy-analogue, i.e. the methoxy-analogue decreased the COX-2 mRNA level more.

Anti-inflammatory activity of further flavones was also tested. Viability of IPEC-J2 cells was monitored after methoxyflavone tangeretin (5, 6, 7, 8-tetramethoxy-2-(4-methoxyphenyl)-4H-1-benzopyran-4-one) treatment by Neutral Red uptake assay. Treatment with 25 μM tangeretin for 1 h did not reduce the number of viable enterocytes significantly. However, treatment with 25 μM tangeretin for 2, 4 and 24 h caused the reduced number of viable IPEC-J2 cells. The same phenomena could be observed, when tangeretin was applied in higher concentrations (50, 100 μM). According to the above mentioned results, it seemed to be safe to use tangeretin in 25μM concentration and 1h incubation for further experiments.

Tangeretin treatment did not influence IL-6, IL-8 and TNF-α mRNA level. However, increased level of COX-2 mRNA was measured after tangeretin treatment in IPEC-J2 cells. Simultaneous incubation of IPEC-J2 cells with LPS (10 μg/ml) and tangeretin was also performed. At 25 μM tangeretin treatment dose, relative gene expression of IL-8 significantly decreased compared to LPS-treated cells, while tangeretin did suppress neither TNF-α, nor IL-6 mRNA level. The mRNA level of Hsp70 did not change after LPS exposure.

The anti-inflammatory activity of chlorogenic acid was also tested. At first, viability of IPEC-J2 cells after chlorogenic acid treatment was studied. It could be concluded, that 50 μM chlorogenic acid treatments for 1 h did not decrease the number of viable enterocytes significantly. Therefore, 25 μM and 50 μM chlorogenic acid treatment was applied in the following experiments. Chlorogenic acid (25 μM, 50 μM) significantly decreased the mRNA IL-6, IL-8, TNF- α and COX-2 level compared to the LPS-treated controls.

Metabolites of polyphenols are often responsible for more biological effects than their precursors. The gut microflora participate the flavone metabolism and could enhance the possible positive effects of polyphenols. In order to understand better the anti-inflammatory processes in the gut, *in vitro* intestinal models should be completed probiotic bacteria. Therefore, my aim was to develop a functional *in vitro* model of the small intestine mimicking pathogen-induced inflammation and to compare attenuating effect of hydroxy- and methoxyflavones, moreover, to study the role of gut microflora in the above mentioned

processes. *Lactobacillus plantarum* 2142 (*Lp* 2142) was applied as probiotic bacteria. The effect of sodium n-butyrate (formed by gut microbiota from dietary carbohydrates and used also as feed additive) was also studied.

IPEC-J2 cells were incubated with 25 μM apigenin and apigenin-trimethylether (the anti-inflammatory activity of these flavones was tested last year) respectively. The experiments were repeated using flavones and *Lp* 2142 (supernatant 13.3v/v %) / butyrate (2mM) at the same time. Relative gene expression of proinflammatory cytokines IL-8 and TNF-α was determined by quantitative real time PCR.

Relative gene expression of IL-8 and TNF-α was significantly higher ($p \leq 0.05$) in the enterocytes after LPS treatment. Both apigenin and apigenin-trimethylether reduced IL-8 level significantly, whereas TNF-α relative gene expression was attenuated by apigenin-trimethylether only. *Lp* 2142 and butyrate treatment also decreased IL-8 and TNF- α level, respectively. Simultaneous treatment reduced IL-8 gene expression levels in case of all treatment combinations except when butyrate was combined with apigenin-trimethylether. TNF-α expression was decreased in case of all treatment combinations. There was no significant difference between the efficiency of treatment combinations.

The experiments described above were repated using chlorogenic acid (25 μM). Simultaneous treatment of IPEC-J2 cells (chlorogenic acid combined with *Lp2142* supernatant) significantly reduced proinflammatory cytokine (IL-6, IL-8 and TNF-α) as well as COX-2 gene expression levels. When butyrate was combined with chlorogenic acid, only IL-6 and COX-2 levels were reduced significantly. Synergistic effect in anti-inflammatory activity could be observed, when chlorogenic acid was added with *Lp2142* supernatant. Fisher LSD test showed that there is a significant difference between the IL-8 values of chlorogenic acid and combined treatment.

Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites, referred to reactive oxygen species (ROS), and their elimination by protective mechanisms, so-called antioxidants. Oxidative stress could activate a variety of transcription factors including NF-κB, and lead to chronic inflammation. Flavonoid antioxidants could protect cells against the damaging effects of ROS.

Therefore, some oxidative stress markers were also followed. Fluorescent ROS measurement was based on the detection of H_2O_2 using the Amplex Red Hydrogen Peroxide keeping the IPEC-J2 cells on the 96-well plate. In the presence of horseradish peroxidase (HRP), Amplex Red reacts with H_2O_2 in a 1:1 stoichiometry producing a highly fluorescent resorufin.

IPEC-J2 cells were treated with LPS in phenol-red free DMEM and the H_2O_2 concentrations in the medium were determined using the working solution of 100 μM Amplex Red reagent and 0.2 U/ml HRP. H_2O_2 determination was also performed after 1 h LPS treatment immediately and 24 h incubation with phenol-red free DMEM. After 30 min incubation with the dye at room temperature the quantitative analyses of H_2O_2 contents were accomplished, the excitation wavelength was set at 560 nm and emission was measured at 590 nm.

It was found, that level of intracellular H_2O_2 was not affected by 10 μg/ml LPS treatment. LPS in higher concentration (50 μg/ml) increased intracellular H_2O_2 level significantly, however, IPEC-J2 cells are irreversible damaged. Tested flavonoids (apigenin, apigenin-trimethylether, chlorogenic acid) showed different effects on the rate of H_2O_2 production – depending the duration of incubation in DMEM after treatment. In case of short time effect measurements (1h incubation with flavonoids and detection immediately after the incubation period), neither apigenin nor its trimethylated analogue decreased mitochondrial H_2O_2 production rate effectively. Long-time effects of flavonoid treatment (Amplex Red measurement was performed 24 h after the 1 h flavonoid incubation) were also studied. Intracellular H_2O_2 level was significantly decreased both in case of lower (25 μM) and higher (50 μM) concentration apigenin treatment. The same reducing effect was found, when 25 μM and 50 μM apigenin-trimethylether was applied. Chlorogenic acid treatment (25 μM, 50 μM and 1h treatment) also significantly reduced intracellular H_2O_2 level, when measurement was performed 24 h after the 1 h flavonoid incubation.

Another fluorescent method has been in adapted in order to describe the redox status of enterocytes after flavonoid treatment under the condition of inflammation. IPEC-J2 cells were seeded onto 96-well plates and cultured for confluence. Enterocytes were treated with lipopolysaccharide (LPS) and flavonoids for 1 h. Fluorescence measurement was performed immediately after treatment and 24 h incubation with phenol-red free culture medium, in order to check short-and long term effects of polyphenols as well. 2', 7'-dichlorofluorescein-diacetate (DCFDA) was used as fluorescent dye. DCFDA is a cell permeant reagent, which measures hydroxyl, peroxyl and other reactive oxygen species activity in the cell. After diffusion in to the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2', 7' –dichlorofluorescein (DCF). DCF is a highly fluorescent compound which can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 529 nm respectively.

It was found in case of DCFDA measurements, that level of intracellular ROS in IPEC-J2 cells was increased by 10 μg/ml LPS treatment. Moreover, LPS treatment caused elevated level of ROS after 24 h when redox status of enterocytes was described using DCFDA method. Contrarily, no difference between the H_2O_2 levels of control and LPS-treated IPEC-J2 cell was found when Amplex Red method was applied. These results underline the significance the parallel usage of methods which characterize the reactive oxygen species in cells.

Continuing the research, effect of chlorogenic acid on redox status in enterocytes was measured using DCFDA fluorescent dye. The experimental system was completed with probiotic strain *Lp* 2142 in order to mimick functional *in vitro* model of the small intestine. In case of short time effect measurements (1h incubation with chlorogenic acid and detection immediately after the incubation period), neither chlorogenic acid nor *Lp* 2142 decreased ROS production rate effectively. Long-time effects of flavonoid treatment (DCFDA measurement was performed 24 h after the 1 h flavonoid incubation) were also studied. Intracellular ROS level was significantly decreased both in case of lower (25 μM) and higher (50 μM) concentration chlorogenic acid treatment compared to the LPS-treated samples. The same reducing effect was found, when chlorogenic acid (25 μM) was combined with *Lp* 2142 probiotic strain.

In the following, quercetin and its methylated analogues were studied. Quercetin-3, 5, 7, 3', 4'-pentamethylether, quercetin-3, 7, 3', 4' –tetramethylether, rhamnazin (3,5-dihydroxi-methylquercetin), tamarixetin (4'-o-methylquercetin), and 3'-o-methylquercetin are methylated quercetin derivatives. At first, influence of quercetin analogues on the viability of IPEC-J2 cells in different concentrations (10, 25 and 50 μM) was tested. Flavonoids were dissolved in DMSO and diluted in cell culture medium. It could be seen, that treatment with 10, 25 and 50 μM quercetin for 1 h did not reduce the number of viable enterocytes significantly. The same effect could be observed in case of quercetin-analogues. However, solubility of analogues with more than two methyl groups was very low in DMSO and ethanol as well. Therefore, in further studies quercetin, rhamnazin and 3-o-methylquercetin were used.

Quercetin analogues applied at 25 and 50 μM concentrations, significantly reduced the LPS-induced inflammation in IPEC-J2 cells. It was found, that level of inflammatory cytokines (IL-6 and IL-8) were significantly decreased in the supernatant of IPEC-J2 cell samples 24 h after combined treatment of LPS and quercetin compared to the LPS-treated samples. There

were no significant differences between the effect of quercetin and its methylated analogues in treatment LPS-induced inflammation. Effect of quercetin on the LPS-induced oxidative stress was also studied. In case of short time effect measurements (1h incubation with quercetin analogue and detection immediately after the incubation period), quercetin did not decreased ROS production rate effectively. Long-time effects of quercetin treatment (DCFDA measurement was performed 24 h after the 1 h flavonoid incubation) were also studied. Intracellular ROS level was significantly decreased both in case of lower (25 μM) and higher (50 μM) concentration quercetin treatment compared to the LPS-treated samples.

Effect of polyphenols on the metabolism of xenobiotics

Some flavonoids have toxic effects and can also interfere with essential biochemical pathways. Among the proteins that interact with flavonoids, cytochromes P450 monooxygenases metabolizing xenobiotics and endogenous substrates, play a prominent role. Flavonoid compounds influence these enzymes in several ways: they could induce the expression of several CYPs and modulate (inhibit or stimulate) their metabolic activity. However, the avaliable information about this research topic is incomplete. Activity of CYP1A1, CYP1A2 and CYP3A4 enzymes after flavonoid treatment was tested on hepatocyte cell culture in a preliminary study. Apigenin was used as test component; applied concentrations were 5 and 25 μM. Hepatocytes were seeded on 24-well plates and treated by apigenin for 2 h. Enzymatic activity was measured by luminescent method using the P450-Glo Assay Kit (Promega) following the instructions of the producer The hepatocytes were also treated with a known cytochrome inducer (phenobarbital) and inhibitors (alpha-naphtoflavone and ketoconazole). The induction and inhibition of CYP450 isoenzymes were found well characterized with the luminescence method. CYP1A1 activity values were not changed after flavonoid treatment. Contrarily, CYP1A2 activities decreased significantly, when hepatocytes were treated by apigenin and its trimethoxy-analogue, but no significant difference between the effects of the two polyphenols could be measured. Both apigenin and apigenin-trimethylether reduced the CYP3A4 activities in rabbit hepatocytes. Combination of polyphenols with known CYP450 inducers lead to decreased CYP450 activity values compared to the activities of inducer-treated cells in case of CYP3A4 and CYP1A2 as well.

CYP450 enzymes could be also found in the gut. Although the quantity of CYP450s in the intestinal tract is less than in the liver, the effect of these enzymes in the gut is very significant in case of oral drug administration and food consumption.

IPEC-J2 cells (non-transformed intestinal epithelial cells derived from pig) were seeded onto 24-well polyester plates and cultured for confluence. Enterocytes were treated with polyphenols for 24 and 48 h as well. CYP450 enzymatic activity was measured by luminescent method using the P450-Glo Assay Kit. Enzymatic activity could be measured only in case of CYP3A4 enzyme after 24 h treatment. Both apigenin and apigenin-trimethylether reduced the CYP3A4 activities in enterocytes, but there was no significant difference between the effects of the two analogues. When flavonoids were combined with the CYP inducer, the CYP activity values remained decreased in case of apigenin-trimethylether- and apigenin-treated samples as well. The same effects could be observed, when cells were treated with quercetin (25-50 μM).

Both hepatocytes and enterocytes were treated by antipyrine in order to model the possible interactions between flavones and a non-steroidal anti-inflammatory drug. Combined treatment with apigenin (50 μg) significantly reduced the CYP3A4 activity compared to the antipyrine-treated hepatocytes. The same phenomenon could be observed in case of CYP1A1 enzymatic activity. Combination with apigenin-trimethylether did not cause altered CYP450 enzymatic activities compared to the antipyrine-treated cells. When enterocytes were treated with antipyrine and flavonoids at the same time, CYP450 activities were found significantly reduced in case of both flavonoid-analogues compared to the antipyrine-treated cells.
There are multiple layers of CYP modulation; enzymatic activity could be influenced by direct enzymatic inhibition/induction and also at the level of transcription. Therefore, relative expression of CYP1A1, CYP1A2 and CYP3A4 genes were also measured after flavonoid treatment in hepatocytes and IPEC-J2 cells as well. In case of enterocytes, flavonoid treatment caused the down-regulation of CYP3A4 gene, while CYP1A1 relative gene expression was significantly increased after apigenin exposure. CYP1A2 relative gene expression levels were not altered after treatment with flavonoids. In hepatocytes, down-regulation of CYP1A1 gene could be observed after apigenin and apigenin—trimethylether treatment as well, while the relative gene expression remained unchanged in case of CYP1A2 and CYP3A4 genes.

Effect of polyphenols on the paracellular permeability in the gut

The gastrointestinal tract has a single contiguous layer of cells that separates the inside of the body from the external environment. Separation is important as there are a wide variety of environmental agents in the lumen of the bowel that can initiate or perpetuate mucosal

inflammation if they cross the epithelial barrier. The epithelial lining of the intestine plays a critical role in preventing access of these agents.

Paracellular permeability and effect of polyphenols were also studied. IPEC-J2 cells were plated to confluence on 6-well polyester membrane inserts without collagen coating and they were allowed to form differentiated monolayers. LPS was added at 10 μg/ml concentration for 1 h and TEER measurements were performed prior to and 2, 4, 24 h after LPS administration. In parallel, fluorescein isothiocyanate-dextran 4 kDa (FD4) was added at 1 mg/ml to the apical compartment of IPEC-J2 cell monolayer with different incubation times (3, 6 and 24 h) at 37°C. Samples of media from the basolateral chambers were collected and tracer concentration was quantified by fluorescence at excitation 485 nm and emission 535 nm (Victor X2 2030 fluorometer).

LPS-triggered partial disruption of cell monolayer integrity caused increased paracellular transport of the hydrophilic tracer, FD4 from apical chamber to basolateral compartment. Using 2, 4 and 24 h incubation time the measured fluorescence intensity of FD4 basolaterally in LPS-treated samples (1 and 10 μg/ml) was significantly higher compared to that of FD4 in controls. There was a significant interaction between treatment and incubation time. In the basolateral compartment of untreated IPEC-J2 cells only negligible amount of FD4 was detected indicating an intact cell monolayer before application of LPS. The LPS-elevated basolateral FD4 fluorescence intensity was altered significantly by simultaneous 1h treatment of IPEC-J2 cells with quercetin (25-50 μM) in addition to LPS administration. In accordance, TEER values prior to and post treatment were compared to check if the polarized cell monolayer integrity was influenced by LPS addition. LPS application did not affect TEER values significantly ($P>0.05$) at 1 μg/ml and 10 μg/ml concentrations.

In summary, it could be appointed, that the selected hydroxylated and methoxylated polyphenols could reduce the LPS-induced intestinal inflammation and oxidative stress as well. On the whole, no significant differences were found between the anti-inflammatory effect of hydroxylated and methoxylated analogues. They also have a positive effect on the intestinal barrier integrity. Therefore, they could improve and maintain gut health, which prevent gut pathogen invasion and inflammation. It is worthy to note, that polyphenols could influence the activity of intestinal CYP450 enymes, which could lead to drug-feed interactions. The results show, that both hydroxy- and methoxyflavones could be used in prevention as well as for therapeutic purposes in animal breeding.