TDK THESIS

Simon Hebenstreit

2019

Department of Pharmacology and Toxicology

University of Veterinary Medicine, Budapest

Effect of quercetin and its methylated derivatives on porcine intestinal epithelial cells

By

Simon Hebenstreit

Supervisor:

Dr. Farkas Orsolya, senior research fellow

Dr. Karancsi Zita, assistant lecturer

Budapest, Hungary

2019

Table of contents

1. Abbreviations

5,7-DMF = 5,7-dimethoxyflavone

AP-1 = activator protein-1

AR = Amplex Red

BSβG = broad-specific β-glucosidase enzyme

DCFH-DA = dichlor-dihydro-flurescein diacetate

DMEM = Dulbecco's Modified Eagle's Medium

DR = death receptor

EC = extracellular

EGF = epidermal growth factor

EGFR = epidermal growth factor receptor

EM = electron microscope

FBS = foetal bovine serum

FG = flavonoid glucosides

GM-CSF = granulocyte macrophage colony-stimulating factor

GSH = glutathione

H_2O_2 = hydrogen peroxide

HRP = horse radish peroxidase

IARC = International Agency for Research on Cancer

IC = intracellular

IL = interleukin

IPEC-J2 = intestinal porcine enterocytes isolated from the jejunum of a neonatal piglet

JAM-1 = junctional adhesion molecule

LPH = lactase phlorizin hydrolase

LPS = lipopolysaccharide

MAPK = mitogen-activated protein kinase

MHC-1 = major histocompatibility complex 1

MRP2 = multidrug resistance-associated protein 2

NADH = nicotinamide-adenin-dinukleotid-hydrogen

NADPH = nicotinamid-adenin-dinukleotid-phosphat-hydrogen

NOX = NADPH oxidase

NFκB = nuclear factor-kappa B

PBS = phosphate buffered saline

PDGF = platelet-derived growth factor

PDGFR = platelet-derived growth factor receptor

PS = porcine serum

PTK = protein tyrosine kinase

PTP = protein tyrosine phosphatase

Q = quercetin

Q3GA = 3-O-β-D-glucuronide

QM = 3-O-methyl-quercetin

QQ = quercetin-quinone

R = rhamnazin (3’7-dimethylquercetin)

Ras = rat sarcoma

ROS = reactive oxygen species

Scr = selecticve catalytic reduction

SGLT1 = sodium dependent glucose transporter 1

TEER = transepithelial electrical resistance

TNF-α = tissue necrosis factor α

VSMC = vascular smooth muscle cells

ZO-1 = zonula occludens 1

2. Introduction

Flavonoids are a part of naturally occurring polyphenolic compounds that possess a C_6-C_3-C_6 carbon framework. They can be found in fruits, vegetables and teas and their pharmacological properties range from antioxidant and anti-inflammatory to anti-cancer activities. One of the most frequently consumed and used flavonoid is the quercetin which has a potent antioxidant effect. Even though the classical hydrogen-donating antioxidant ability is thought to be the reason for its positive effects (Rice-Evans, Miller and Paganga, 1996; Rice-Evans, 2001), there are conceptions that it is not the only explanation for the antioxidant and anti-inflammatory effects (J. P. E. Spencer *et al.*, 2001a; J. P. E. Spencer *et al.*, 2001b; Schroeter *et al.*, 2001). However, quercetin has numerous positive effects according to *in vitro* studies, the *in vivo* examinations show moderated effects probably due to the low oral bioavailability. Therefore, the methylated forms of quercetin, for example 3-O-methylquercetin or rhamnazin (3'7-dimethylquercetin) come into view, because these substances can be absorbed by the intestine more easily and can be more stable against the metabolization in the liver.

Using the *in vitro* cell lines to model the healthy intestinal barrier and to investigate the differences between the hydroxyl and methylated flavonoids is a good opportunity. However, several cell lines are used in the science, more of them are tumorous and could not mimic the physiological circumstances properly. Hence in our experiments we used a non-transformed intestinal epithelial cell line (IPEC-J2), which is derived from piglet jejunal cells. This cell line can be grown *in vitro* for a longer time period and allows us to study antioxidant effects of the flavonoids. Our aims for this study was to compare the effects of quercetin and its methylated derivatives, 3-O-methylquercetin and rhamnazin. In the first investigation we checked their effects on the cell morphology of the IPEC-J2 cells using transmission electron microscope. In the second part of the experiment we examined the antioxidant effects of these flavonoids with luminescent method than we compared our results to a more frequently used fluorescent assay.

3. Literature review

3.1. The importance of *in vitro* studies

Although most relevant results can be obtained by well-designed *in vivo* studies, to immediately provide human or animal experiments for new substances can be dangerous. This is the reason why *in vitro* studies are important, due to their ability to isolate certain parameters and specifically examine them. These have the advantage of being very specific to the circumstances during the investigations. The *in vitro* studies help with the isolating, but this is also their biggest disadvantage, because the effects on the whole system cannot be seen (Sadan, 2007). Additionally, cell models can provide and model a lot of tissue types and these can be more readily available than their *in vivo* counterparts (Hartung and Daston, 2009).

Unfortunately, there are a few disadvantages concerning *in vitro* studies. The fundamental problem is that the artificial non-physiological conditions, that the cells are not able to modelling the total and actual physiological system (e.g. body temperature, blood electrolyte concentration, the extracellular matrix or the extent of cell contacts, which is maximally 15% of a normal monolayer) (Hartung and Daston, 2009). This kind of tunnel vision can lead to results far from the actual clinical parameters of an *in vivo* experiment (Sadan, 2007). Most of the *in vitro* cell layers are grown with a rapid speed, which is opposing the cell differentiation (Hartung and Daston, 2009). Probably the best-known limitation is the lack biotransformation capabilities (Coecke *et al.*, 2006) and the lack of defence mechanisms has also a big impact in the estimations of the precision of toxic effects (Hartung and Daston, 2009).

In vitro studies are very important to find out the basic effects and mechanism of actions of active substances before the study can go into *in vivo* experiments. Additionally, *in vitro* studies reduce the cost of experiments compared to *in vivo* experiments (Polli, 2008) and they are also an opportunity to follow the 3R guideline (replacement, reduction and refinement), because it reduces the amount of lab animals that have to be used in order to test specific properties of possible medications.

3.2. IPEC-J2 cell line

The IPEC-J2 cell line is comprised of intestinal porcine enterocytes isolated from the jejunum of a neonatal unsuckled piglet (IPEC-J2) (Vergauwen, 2015). The cells are unique due to that these are neither transformed nor tumorigenic. IPEC-J2 cells can model the swine

intestine and beside that these can mimic the human intestine more closely than any other cell line of non-human origin due to the similarities between the human and swine intestine (Vergauwen, 2015). IPEC-J2 cells can be even better than human origin tumorigenic cell lines like Caco-2 or HT-29 (Ferraretto *et al.*, 2018).

The IPEC-J2 cells during the culturing on semipermeable membranes undergo a process of spontaneous differentiation that leads to the formation of a polarized monolayer (Figure 1) with low or high transepithelial electrical resistance (TEER) within a few weeks. The TEER depends on the serum that will be used. Porcine serum will lead to low resistance and normal active transport rates, whereas bovine serum will lead to higher resistance. The cells cultured with porcine serum can be used for more reliable *in vivo* comparisons, while the cells cultured with bovine serum can be used for more aggressive procedures or procedures where the negative effect on the monolayer and tight junctions is more severe (Vergauwen, 2015).

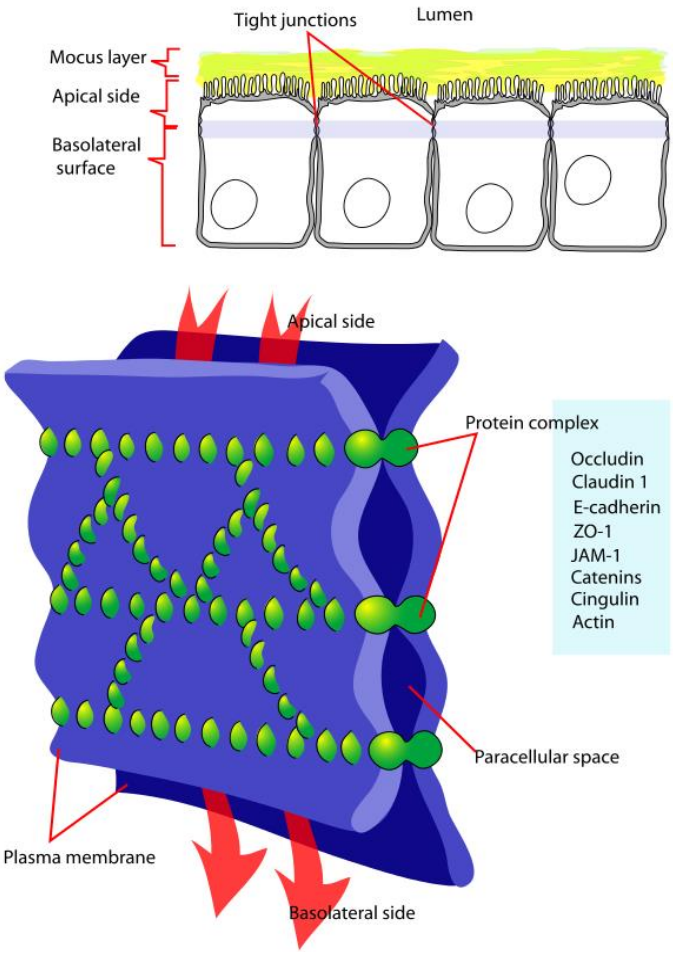

Figure 1 Tight Junctions (protein complexes) connecting the cell membranes of two neighbouring cells.

ZO-1 = zonula occludens 1

JAM-1 = junctional adhesion molecule 1

3.2.1. Morphology and function of IPEC-J2 cells

The IPEC-J2 cell line, cultured on a 0,4 µm pore-size semipermeable membrane filter with 50% Dulbecco's Modified Eagle's Medium (DMEM) and 50% Ham's F12 Nutrient Mixture containing foetal bovine serum (FBS) and antibiotics can form a monolayer with apical and basolateral compartments (Brosnahan and Brown, 2012). Although this is the most commonly used composition, there are more culturing protocol that will lead to other properties. In these culturing methods DMEM is used as the base medium, but the serum can differ. In species specific experiments, adult pig serum (PS) mixed with antibiotics, insulin and epidermal growth factor (EGF) was used (Zakrzewski *et al.*, 2013). On semipermeable membrane the IPEC-J2 cells form microvilli on the apical side and the connection between the neighbouring cells is achieved by tight junctions (Vergauwen, 2015). The confluent cell monolayer on semipermeable membrane creates a polarised layer with apical and basolateral compartments, which can be characterized with the TEER. The height of the cells can increase the tight junction ultrastructure, specially under 10% porcine serum culturing (Schierack *et al.*, 2006; Vergauwen, 2015).

The protein expression of claudin-1, -3, -4, -5, -7, -8, -12, tricellulin, occluding, E-cadherin and zonula occludens-1 was also confirmed in IPEC-J2 cells (Schierack *et al.*, 2006; Vergauwen, 2015). These cells also produce cytokins, defensins, toll-like receptors, Mucin-1 and -3. The missing of Mucin-2 results, that IPEC-J2 cells cannot be compared totally to human *in vivo* cells because Mucin-2 is the main mucus producer in humans. Although in bovine serum culturing the IPEC-J2 cells have a very thin layer of mucus and in porcine serum the layer will be thicker (Vergauwen, 2015).

Major histocompatibility complex 1 (MHC-1) proteins are expressed by IPEC-J2 cells and further cytokines like granulocyte and macrophage colony-stimulating factor (GM-CSF) and tissue necrosis factor α (TNF-α) are also can be detected. Additionally, the mRNA expression of TLRs and interleukin (IL) has been confirmed (Brosnahan and Brown, 2012; Vergauwen, 2015).

Since IPEC-J2 cells express the F4 fimbria receptor the cell line is an interesting *in vitro* model to investigate pathogenesis of zoonotic enteric infections affecting humans (Vergauwen, 2015).

3.2.2. Vacuolisation of IPEC-J2 cells during drug exposure

Cellular vacuolisation is frequently observed upon exposure of chemicals or pharmaceutical substances (Aki, Nara and Uemura, 2012). The mechanism was first described by Christian de Duve in 1974, who identified the lysosome, the digestive acidic compartment of the cell (De Duve *et al.*, 1974).

There are three main points in the vacuole formation. The first is, that the membrane of the cell needs to be permeable to lipophilic substances, like amines in their uncharged form. Secondly the membrane is not permeable to the lipophilic substances in their charged form and thirdly the pH gradient between the cytoplasm and the lysosome needs to be high enough, so that the protonation of the substances takes place only in the lysosome (Aki, Nara and Uemura, 2012).

The first case is important for the passive diffusion of the substances and their absorption into the lysosomes. During the protonation the substances get charged therefore these ionised form are unable to transverse the lipid bilayer and get trapped in the lysosomes (Aki, Nara and Uemura, 2012).

Vacuolisation can derive from different parts, not only the lysosomes. There are also endosomes and the Golgi apparatus, which are acidic (6.0-6.7 pH), and the endoplasmic reticulum (around 7.1 pH) and mitochondria (6.7-7.0 pH), which were believed to only have a slight acidic milieu (Aki, Nara and Uemura, 2012).

Additionally, there are many substances (chloroquine, neutral red, propranolol, etc.) which will induce the formation of lysosome accumulation causing a substantial increase of intra-lysosomal pH that leads to lysosomal dysfunction (Aki, Nara and Uemura, 2012).

At this time there was no available literature examining the connection between flavonoids and vacuolization, although these natural compounds are frequently used.

3.3. Flavonoids

Flavonoids are an important class of natural products; they belong to a class of plant secondary metabolites polyphenols (Figure 2). These polyphenols have been examined for their positive health benefits like antioxidant properties, mediated by their functional hydroxyl groups (Pizzino *et al.*, 2017). The *in vitro* examinations have proven benefits of polyphenols, but the results in *in vivo* investigations were not as prominent, because of the relatively low oral bioavailability (Wen and Walle, 2006). The free hydroxyl groups, which are the main component for the antioxidant effects, is the reason why polyphenols are conjugated very quickly (Wen and Walle, 2006).

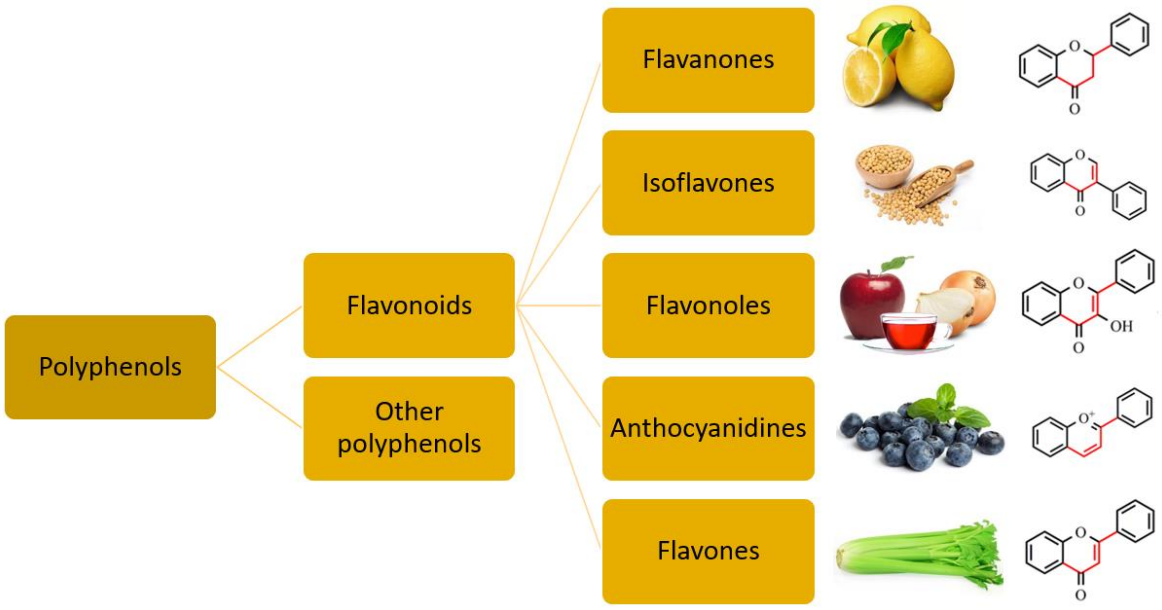

Figure 2 Classification of polyphenols and their general structures.

Since the flavonoids belong to polyphenols, they play a major part in the dietary antioxidant capacity of fruits and vegetables and are also found in nuts, wine and tea. In human and animal studies it has been shown that those compounds have cancer chemopreventive properties, beneficial effects in cardiovascular diseases, osteoporosis (Adlercreutz and Mazur, 1997) and strokes (Ness and Powles, 1997). The basic structure of the flavonoids consists of the molecular formula 2-phenyl-1,4-benzopyrone (Figure 3). This allows for a multitude of hydroxyl and methyl substitution patterns in the benzene rings. The effects of flavonoids can be related to the antioxidant properties due to the presence of the hydroxyl groups (Ribeiro *et al.*, 2013), however more recent findings indicate that modulation the essential signal transduction pathways could be of a higher importance.

The oral bioavailability of flavonoids in their aglycon form is very low, therefore the biological effects *in vivo* were only partially fulfilled. In the natural sources (fruits, vegetables and beverages) flavonoids are found as glucoside forms. It was first believed that the fairly large and highly polar molecules of these flavonoid glucosides (FG) had to be hydrolysed to aglycones by bacterial enzymes before they were absorbed from the lower intestines (Murota and Terao, 2003).

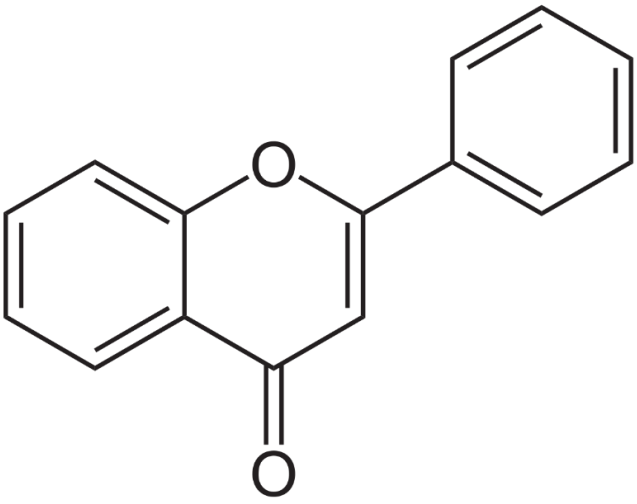

Figure 3 Basic molecular structure (2-phenyl-1,4-benzopyrone) of a flavone

However, in 1995 Hollman described that it was indeed possible for the intact FGs to be absorbed in the small intestine using the sodium dependent glucose transporter 1 (SGLT1) (Hollman *et al.*, 1995), although the efficiency of the absorption was dramatically suppressed by the apical transporter multidrug resistance-associated protein 2 (MRP2). There was also an evidence shown that some FGs could be hydrolysed in the small intestine (Kurowska and Manthey, 2004). It was also detected that, if the FG entered the epithelial cells, they could be hydrolysed by a broad-specific β-glucosidase enzyme (BSβG). Additionally, lactase phlorizin hydrolase (LPH) showed hydrolysis for at least some FGs. The disadvantage of FGs is still the low bioavailability and the major problem is that only a minimal amount of the FGs can reach the systemic circulation (Walle, 2004). Their oral bioavailability depends on their structure. Flavonoles like quercetin, chrysin, curcumin and resveratrol bioavailability have very low bioavailability while isoflavonoids have better absorption (Erlund, 2004; Walle, 2007). The methylated flavonoles, like 3-O-methylquercetin and rhamnazin, seem to be more metabolically stable than their non-methylated counterparts (Murota and Terao, 2003; Wen and Walle, 2006a; Walle, 2007).

Since hydroxyflavonoids have poor oral bioavailability in humans, their methylated derivatives are frequently tested in *in vitro* studies. Comparing chrysin with its methylated counterpart 5,7-dimethoxyflavone (5,7-DMF), the methylated version of the flavonoid was much more metabolically stable than the unmethylated one (Wen and Walle, 2006). Furthermore, studies showed that even the absorption of the methylated flavonoids were better than the hydroxyflavonoids (Artursson and Karlsson, 1991; Wen and Walle, 2006).

These results lead to the continuation of studies performed on rats, the findings were similar to the *in vitro* examinations (Erlund, 2004; Walle, 2007).

Besides having antioxidant properties, flavonoids also have prooxidant features, which can cause oxidative stress by reacting with various biomolecules (Walle, 2007; Tsuji and Walle, 2008). In 2008 Tsuji and Walle described that methylated and un-methylated flavones had a toxic effect in hepatocytes of a rainbow trout (Tsuji and Walle, 2008).

3.3.1. Quercetin

Quercetin represents the most abundant dietary flavonoid found in a broad range of fruits, vegetables and beverages like tea, wine and beer. The aglycon structure of quercetin contains four hydroxyl groups (Figure 4). Quercetin has a relatively high bioavailability compared to other phytochemicals. The daily intake of quercetin ranges from 5-40 mg but can be elevated to 200-500 mg in individuals who consume high quantities of fruit and vegetables rich in

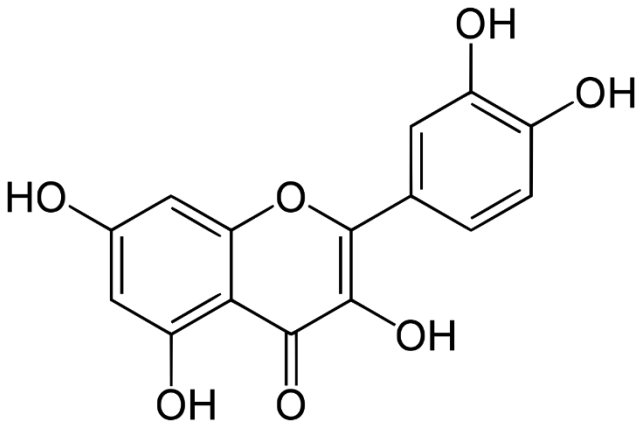

Figure 4 Structure of quercetin
(5,7,3',4'-flavon-3-ol)

flavonols like apples, onions or tomatoes. Quercetin in food is presents in differently glycosylated forms. Therefore, its bioavailability depends on the type of glycosides found in the food source. Formerly it was believed that only the free form of quercetin was able to be absorbed through passive diffusion, but in later studies it was shown, that quercetin glycoside almost doubled the corresponding aglycons absorption (Hollman *et al.*, 1995; Russo *et al.*, 2012). This ability of higher bioavailability could be attributed either due to the deglycosylation processes at the intestinal level or carrier-mediated transport (Russo *et al.*, 2012). Quercetin also has been shown to undergo cellular oxidation by peroxidases (M. Awad *et al.*, 2003) or non-enzymatic chemical reactions in the epithelial cells (Walle, Vincent and Walle, 2003; van der Woude *et al.*, 2005).

The metabolization happens in the liver or the kidney where the molecule will be conjugated to small compounds such as methyl and sulphate groups or glucuronic acid. Its major conjugates in humans are 3'-O-methylquercetin, quercetin-3-O-glucuronide, 3'-O-methylquercetin-3-O-glucuronide and quercetin-3'-O-sulfate (Russo *et al.*, 2012). In an *in vivo* model with rats the absorption of quercetin is heavily influenced by intestinal microflora, which converts more than 95% of the quercetin-4'-glucoside to phenolic acids (Mullen *et al.*, 2008), while in humans, half of the quercetin-3-rutinoside is metabolized to phenylacetic acids by colonic microflora (Olthof *et al.*, 2003). As a result, the quercetin concentration in the blood plasma is in nanomolar range, but it can be significantly increased by further supplementation up to micromolar level (Conquer *et al.*, 1998; Manach *et al.*, 2005). This can be achieved due to the half-life of quercetin is between 11 to 28 hours range, which allows a significant increase of the plasma concentration upon continuous supplementation. There are several factors, like drug interactions, dose, administration frequency or duration of treatment, which can influence quercetin's effects (Russo *et al.*, 2012). *In vitro* quercetin has shown great preventive properties both at initiation and the promotion stages of chemically induced carcinogenesis, additionally the antioxidant and anti-inflammatory properties of quercetin have been associated with the prevention and therapy of cardiovascular diseases and cancer (Russo *et al.*, 2012).

3.3.1.1. Cardiovascular effects of quercetin

Due to the anti-inflammatory and antioxidant properties of quercetin, it is assumed that these can have beneficial activities in cardiovascular risk patients. Although its effects on healthy people is questionable, earlier studies showed reduced risk of death from coronary heart disease (Hertog *et al.*, 1993, 1995; Russo *et al.*, 2012). Quercetin in cardiac failure patients caused significant reduction of systolic, diastolic and mean arterial pressure the in cardiovascular risk patients (Edwards *et al.*, 2007; Russo *et al.*, 2012).

The cardioprotective activity of quercetin was also examined in different cellular or animal models (Chuang *et al.*, 2010; Prince and Sathya, 2010; Youl *et al.*, 2010; Kleemann *et al.*, 2011; Overman, *et al.*, 2011). Overman performed on human macrophages and adipocytes, that quercetin reduced inflammation (Overman *et al.*, 2011). In human endothelial cells quercetin protected against H_2O_2-induced lipid peroxidation and in human hepatocytes reduced nuclear factor-kappa B (NFκB) transcriptional activity (Kleemann *et al.*, 2011). It was described, that quercetin's metabolites (isorhamnetin, 3-methyl-quercetin) and quercetin 3-O-β-D-glucuronide (Q3GA), show preventive effects against arteriosclerosis. Isorhamnetin is able to produce a vasodilator effect in vascular tissue like aorta, mesenteric

arteries, portal vein, or coronary arteries while Q3GA inhibits platelet derived growth factor (PDGF) induced cell migration and proliferation in vascular smooth muscle cells (VSMC) and attenuated angiotensin II-induced VSMC hypertrophy (Yoshizumi and Matsumura, 2011; Russo *et al.*, 2012).

3.3.1.2. Quercetin in cancer treatment

Quercetin has a special role as a naturally occurring chemopreventive agent, because of its activities on all stages of carcinogenesis (initiation, invasion and metastasis). Hanahan and Weinberg found that there are six "hallmarks of cancer" and quercetin can interfere with different targets of these hallmarks (Cavallo *et al.*, 2011).

In vitro and *in vivo* studies have shown, that quercetin has an inhibiting effect on the growth and proliferation of different cancer cell lines (Lamson and Brignall, 2000; Gee, *et al.*, 2002; Boots, *et al.*, 2008). *In vivo* studies were done in animal models, where the cancer was induced by carcinogens and dietary amounts of quercetin were able to inhibit intestinal crypt cell proliferation and suppressed aberrant crypt formation (Gee, *et al.*, 2002). Jeong found that a relatively low dose of quercetin was administered to breast cancer cells resulted in cell cycle arrest at the G0/G1 phases (Jeong *et al.*, 2009). Due to the interference with different signalling pathways in the cells, quercetin can act as a potent inhibitor of epidermal growth factor receptor, which plays a significant role in cell proliferation.

Quercetin and other polyphenols also have an effect on the telomerase length, telomeric structure and oncogene activation of immortalized cells, like human tumour cells (Hanahan and Weinberg, 2011). Telomerase enzymes are specialized DNA polymerases that attach telomere repeat sequence to the ends of the DNA. Activating all the telomerases leads to lack of the senescence and apoptosis, forming tumour cells. Quercetin acts by inducing senescence on those long telomere chains in the DNA and inhibiting the activity of the telomerase.

Apoptosis is another way that quercetin can interact with cancer cells. There are two main ways of apoptosis, the extrinsic and the intrinsic pathway (Mantovani *et al.*, 2008). The extrinsic pathway is regulated by cytokines which will bind to the tumour necrosis factor (TNF) receptor and induce the cell death. Those receptors are also called "death receptors" (DR). The mechanism how tumour cells acquire DR resistance is still unknown. The intrinsic pathway is controlled by the mitochondria and is activated by genotoxic damage, which stops the replication of the cells. Quercetin is able to activate apoptosis with bypassing those DR resistances through multiple mechanisms (Russo *et al.*, 1999, 2010) on human acute lymphocytic leukaemia cell lines, human leukaemia of lymphoid or myeloid origin, prostate

cancer cells and *in vivo* in a mouse model of pancreatic cancer. This mechanism was not due to the antioxidant properties of quercetin because other dietary antioxidant flavonoids were not able to mimic this apoptogenic activity of quercetin (Spencer, *et al.*, 2003; Russo *et al.*, 2012).

Autophagy is a mechanism that the body uses to prevent malignant transformation autophagosomes of the cells. This process is induced with oncogenic stress. Quercetin has been studied to be able to induce autophagy by down regulating the levels of oncogenic Ras proteins (Psahoulia *et al.*, 2007). Another way of inducing autophagic cell death is by endoplasmic reticulum stress, which quercetin can also induce (Russo *et al.*, 2012).

3.3.1.3. Antioxidant effect of quercetin

Quercetin has a very potent antioxidant effect due to its ability to react with free radicals to form phenoxy radicals which are considered less reactive (Lamson and Brignall, 2000). These radicals then rapidly oxidise with GSH and NADH, which results in an extensive oxygen uptake and superoxide radical anion formation (Galati *et al.*, 2002). This reaction is used as treatment for oxidative stress.

The reason of quercetin can react with the free radicals is its hydroxyl groups, which bind to the free radicals. This simplifies the process of decreasing free radicals and thus decreasing oxidative stress in the cells (Wen and Walle, 2006). However, the methylated quercetin derivatives with less or without hydroxyl groups can also act against the free radicals through different pathways.

3.3.1.4. Potential negative properties of quercetin

In 1970 quercetin was found to be a genotoxic substance, but this *in vitro* mutagenicity was not confirmed by *in vivo* tests in animal models, where there were no significant changes seen (Harwood *et al.*, 2007). In 1999, the International Agency for Research on Cancer (IARC) concluded that quercetin is not carcinogenic to humans (Harwood *et al.*, 2007; Russo *et al.*, 2012). During a two-year study by NTP (National Toxicity Program), male F344/N rats fed daily 2 g/kg of body weight of quercetin, showed severe chronic nephropathy, hyperplasia and neoplasia of the renal tubular epithelium. However, a lower dose of quercetin (50-500 mg/kg/day) did not cause significant changes (Harwood *et al.*, 2007). Parallel studies, that performed with the same rat model, failed to confirm the renal histopathological effects of quercetin (Harwood *et al.*, 2007; Russo *et al.*, 2012). The recommended daily dose of quercetin, for a unique phase one clinical trial, is 1400 mg/m^2, which corresponds to 2.5 g of quercetin for a 70 kg of body weight person since at a higher

doses (3.5 g for a 70 kg person), renal toxicity was detected without signs of nephritis or obstructive uropathy (Ferry *et al.*, 1996). However adverse effects after oral administration were not seen even after a single dose of 4 g or with 500 mg doses twice daily for one month (Ferry *et al.*, 1996; Lamson and Brignall, 2000; Russo *et al.*, 2012). The toxicity of quercetin might be due to its prooxidant properties, which arise from its chemical properties and position of its hydroxyl substitution functional groups and its catechol-type B-ring. When quercetin is used as an antioxidant it gets oxidized and generates quercetin-quinone (QQ) (Boots, *et al*., 2008; Russo *et al.*, 2012). In *in vitro* and *in vivo* experiments has been shown that this quinone form is toxic for the human body. The normal protection comes from glutathione (GSH) which binds QQ and builds glutathionyl-quercetin (GSQ). If the concentration of GSH is too low, it cannot bind all the QQ and the toxic effects become apparent (Boots, *et al*., 2008; Russo *et al.*, 2012).

3.3.1.5. Methylated quercetin derivatives

Quercetin has a low oral bioavailability and gets metabolized fast in the body. This is the reason why scientific researches are looking for possibilities to use the positive properties in the best way. A way to solve this issue is to study and using methylated derivatives of the quercetin. The methylated derivatives of quercetin show more pronounced lipophilicity that

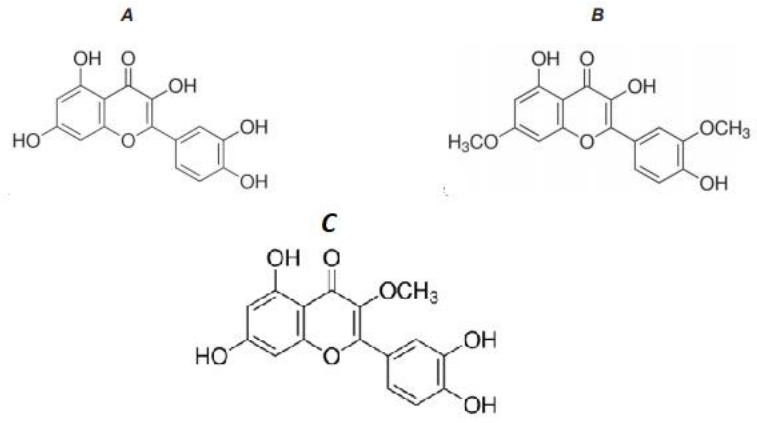

Figure 5 The structural formulas of quercetin (A), rhamnazin (B) and 3-O-methylquercetin (C)

promotes the access of the flavonoids into the cell. Two of those derivatives are 3-O-methylquercetin and rhamnazin (Figure 5). Both can have similar antioxidative properties as quercetin. Additionally, the methylation of the free hydroxyl groups results in a more metabolically stable form with better membrane penetration and therefore better bioavailability, which improves the ability of the actions of these substances (Kuhnle *et al.*, 2000; Novo Belchor *et al.*, 2017).

Novo Belchor examined the anti-inflammatory effects through the phospholipase A2 inhibiting ability of rhamnetin, 3-O-methylquercetin and rhamnazin compared to quercetin. The results showed that from the three derivatives, rhamnazin had the highest inhibiting ability followed by 3-O-mehtylquercetin, while rhamnetin had the weakest effect and showed similar results as quercetin itself (Novo Belchor *et al.*, 2017).

Additionally, the same study examined the cytotoxic effects of the 4 substances on the J774 macrophage cell lineage. The results revealed that quercetin and 3-O-methylquercetin had a higher cell toxicity level, on the other hand rhamnetin and rhamnazin showed no toxicity in this specific cell line (Novo Belchor *et al.*, 2017).

3.4. Oxidative Stress and reactive oxygen species

Oxidative stress is a phenomenon caused by the imbalance between production and accumulation of reactive oxygen species (ROS) and the ability of the tissues to eliminate these products. The ROS are play several important physiological roles in cell signalling and they are by-products of the oxygen metabolism. Presence of environmental stressors and xenobiotics can greatly increase the ROS production. This will cause an imbalance which will lead to cell and tissue damages. Oxidative stress is normally described as harmful to the body, but it also has a therapeutic effect in the cancer treatment (Pizzino *et al.*, 2017). ROS are constantly generated from oxygen in all aerobic organisms during intracellular metabolism. Their formation is due to the incomplete one-electron reduction of oxygen of small molecules or ions. They include free radicals such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), peroxyl radical ($RO_2^{\bullet}$) and alkoxyl radical ($RO^{\bullet}$). Additionally, non-radical species which are oxidizing agents or easily converted into radicals, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), and singlet oxygen ($^1O_2$) are also part of ROS (Kelly, Dennis and Anthony, 2002; Leonarduzzi, Sottero and Poli, 2010).

ROS are generated in a lot of places and by numerous enzymes in a cell. The major source of their productions seems to be the mitochondrial electron transport chain, but monoamine oxidase might contribute as well. During the inflammatory process, a burst in ROS production can be identified in different inflammatory cell types, which is caused by a specialised NADPH-dependent oxidase system. NADPH oxidase (NOX) is an enzyme consisting of a catalytic subunit (pg91phox aka NOX2) and a regulatory subunit (p45phox, p67phox, p22phox and Rac). All these oxidases are transmembrane electron carriers that use NADPH as a source of electrons and molecular oxygen as acceptors. They function as a transfer mechanism for electrons from the cytoplasm into the extra-cytoplasmic compartments to generate $O_2^{\bullet-}$ and other downstream ROS. There are some additional

sources of ROS including cytochrome P450 enzymes, cyclooxygenase, lipoxygenase and one-electron reduction of quinones by NADPH-cytochrome P450 reductase (Leonarduzzi, *et al*., 2010).

Because of the evolution of protective mechanisms, the production and release of ROS can be limited. These defence systems include antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase and several other non-enzymatic antioxidants and small proteins including glutathione, vitamins A, E and C, carotenoids and polyphenols like flavonoids.

The excessive production of ROS without adequate defence leads to oxidative stress and cell damage which underlies numerous diseases, developmental abnormalities and aging. However, excessive amounts of ROS are harmful to nucleic acids, proteins or lipids, a small amount of ROS can be used for cell signalling, particularly at the level of redox modulation. The role of ROS seems to be more complex; they play a central role in the key of intracellular signal transduction pathways for a variety of pathophysiological cellular responses, such as inflammation, proliferation, migration, differentiation, angiogenesis, aging and apoptosis (Leonarduzzi, *et al*., 2010).

ROS production is not only involved in receptor signal intracellular pathways, but also induces phosphorylation, and therefore it activates several receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR). These receptors play an important role in the transmission of information from extracellular into the cytoplasm and the nucleus. This information is transmitted through activation of the mitogen-activated protein kinase (MAPK) signalling pathways (Leonarduzzi, *et al*., 2010).

Moreover, there was a discovery (Adler *et al.*, 1999; Dröge, 2002) about specific genes and pathways affected by oxidants, which confirmed that ROS serve as subcellular messengers in redox sensitive gene regulatory and signal transduction pathways. H_2O_2 is the most common messenger, because of its constant production in the mitochondria, it being a relatively stable molecule, strong oxidant and a molecule small enough to diffuse across most biomembrane barriers (Leonarduzzi, *et al*., 2010).

Protein containing cysteine residues, signalling enzymes and protein-bound metals, including hem iron, are primary targets of ROS. These include phospholipase C (Zhao *et al.*, 2005; Cheng *et al.*, 2006), phospholipase A_2 (Akiyama *et al.*, 2006; Muralikrishna Adibhatla and Hatcher, 2006) and phospholipase D (Tappia, *et al*., 2006). Ion channels including calcium channels and signalling mechanisms that respond to a change in the disulphide redox

state, including selective catalytic reduction (Src) family kinases and MAPKs, can also be targets and directly activated by ROS. This is explained by the reversible or irreversible oxidation of the sulphur in cysteine to either a disulphide bond (-SSR), sulfenic acid (-SOH), sulfinic acid ($-SO_2H$) or sulfonic acid ($-SO_3H$). It is unlikely that the latter two are involved in the signalling, because they are irreversible forms, while disulphide bonds and protein sulfenic acid can easily be reduced and are often considered to be mediators of redox signalling (Leonarduzzi, *et al.*, 2010).

Protein phosphorylation plays a critical role in regulating many cellular responses and cellular transduction sites. These processes are regulated by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). ROS are supposedly able to modulate those PTKs and PTPs by changing their activity, which can regulate their ability of phosphorylation. Several reports show, that ROS, in particular H_2O_2, activate MAPKs which lead to the modulation of gene expression (Torres and Forman, 2003; Dasari *et al.*, 2006; Lu, *et al*., 2007; Leonarduzzi, *et al*., 2010).

The main defence mechanism against oxidative stress is the scavenging of ROS. Flavonoids can eliminate ROS involving of their hydroxyl groups and via their modulatory effect in different cell pathways. The ability of the inhibition of mitochondrial respiration by flavonoids was also seen in toxicity tests (Hodnick, *et al*., 1994). This may be related to the ability of the flavonoids to undergo autooxidation catalysed by transition metals *in vitro*, but this was not observed in *in vivo* experiments (Miyoshi *et al.*, 2007). Flavonoid toxicity can caused by peroxidases catalysing the oxidation of the polyphenols (O'Brien, 2000). Plasma myeloperoxidase catalyses the production of prooxidant phenoxyl radicals which catalyse lipoprotein oxidation and protein crosslinking, thereby contribute to atherosclerotic plaque formation (Heinecke *et al.*, 1993). Intracellular phenoxyl radicals formed by myeloperoxidase also induce lipid peroxidation and co-oxidize GSH to form thiyl radicals with concomitant oxygen activation (Goldman *et al.*, 1999). Dietary polyphenols with phenol rings will be metabolized by peroxidases to form prooxidant phenoxyl radicals. These radicals then rapidly oxidised with GSH and NADH, resulting in extensive oxygen uptake and superoxide radical anion formation (Chan, *et al.*, 1999; Galati *et al.*, 1999, 2002).

4. Aims

During our *in vitro* investigation our aim was to test and described first the effects of quercetin, 3-O-methyl-quercetin and rhamnazin on the intestinal epithelial cells (IPEC-J2) and examine the changes in the structural morphology using a transmission electron microscope (TEM). We would like to find out if the treatments of the quercetin and the methylated derivatives can alter the cell size, the number and size of the vacuoles or the density of the microvilli in the enterocytes.

Our other aim was to check the antioxidant effect of these flavonoids after *Salmonella enterica* serovar Typhimurium lipopolysaccharide (LPS) induced oxidative stress. To examine the extracellular H_2O_2 concentration we used a luminescent assay (ROS-Glo). Furthermore, we would like to compare the above-mentioned luminescent assay to a fluorescent method (Amplex Red) which was previously applied in the Department of Pharmacology and Toxicology and also frequently used in scientific studies.

5. Materials and Methods

5.1. Cell line culture conditions and treatments

The porcine intestinal epithelial cell line (IPEC-J2) was a kind gift from Dr. Jody Gookin, Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA. These cells are stored in liquid nitrogen. For our experiments we used 1 ml of frozen IPEC-J2 cells and put them into a cell culture flask of 25 cm^3 containing 10 ml of DMEM/F12, which is a complete medium containing a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture (DMEM/F12) supplemented with 5% fetal bovine serum (FBS), 5µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 5 ng/ml epidermal growth factor (EGF) and 1% penicillin-streptomycin (Biocenter Kft., Szeged, Hungary). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and the cell culture medium was changed every second day.

For transmission electron microscopy (TEM) cells were seeded onto a 24-well cell culture plate containing Aclar film (Electron Microscopy Sciences, PA, USA). Each well contained 500 µl cell culture suspension and cells were cultured until confluency was reached. After washing with phosphate-buffered saline (PBS), we treated the cells for 1 hour with quercetin (Q), 3-O-methyl quercetin (QM) and rhamnazin (R) in 25 µM concentrations using plain DMEM/F12 as a solvent agent. For control group plain DMEM/F12 was used.

For measure the extracellular H_2O_2 concentration with the luminescent ROS-Glo assay, cells were seeded onto a 96-well cell culture plate, each well containing 200 µl of cell suspension and they were cultured for two days to reach the confluent monolayer. After that, we washed the cells with PBS and to induce oxidative stress LPS from *Salmonella enterica* serovar Typhimurium was used for 1 hour in 10 µg/ml concentration dissolving in plain DMEM/F12. Quercetin (Q), 3-O-methyl quercetin (QM) and rhamnazin (R) in 50 µM concentration were used for 1 hour *per se* to check the influence on the extracellular H_2O_2 concentration. In addition, simultaneous treatments were also applied with the LPS and flavonoids to investigate the potential protective effects of the quercetin and its derivatves. Effect of 50 µM flavonoid concentration on the IPEC-J2 cell viability was tested earlier in the department. Quercetin (≥97%), 3-O-methyl quercetin (≥97%), rhamnazin (≥97%) and LPS (derived from *Salmonella enterica* ser. Typhimurium) were purchased from Sigma-Aldrich (Steinheim, Germany).

5.2. Fixation and embedding for TEM

After the treatments with the quercetin and its methylated quercetin derivatives DMEM/F12 culture medium was aspirated from the cells and 1000 µl of buffered fixative was added to them. For TEM assays the fixative contained 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1M phosphate buffer. The fixation was performed at 4°C for 20 minutes followed by two washing steps with PBS (pH 7.4).

For TEM examination, cells were postfixed with 1% OsO_4 in 0.1M phosphate buffer, washed with 0.1 M phosphate buffer, and dehydrated in an increasing series of ethanol (50-100%) and embedded in epoxy resin (Durcupan, Sigma). After polymerization at 60°C for 36 hours, the samples were scrutinized at medium magnification in a light microscope, and areas rich in cells were cut out from the Aclar samples and mounted on plastic blocks. Sixty nm thin sections were cut on a Reichert ultramicrotome, mounted on 300 mesh copper grids, contrasted with lead citrate (Ultrostain II, Leica) and examined with a JEM-1011

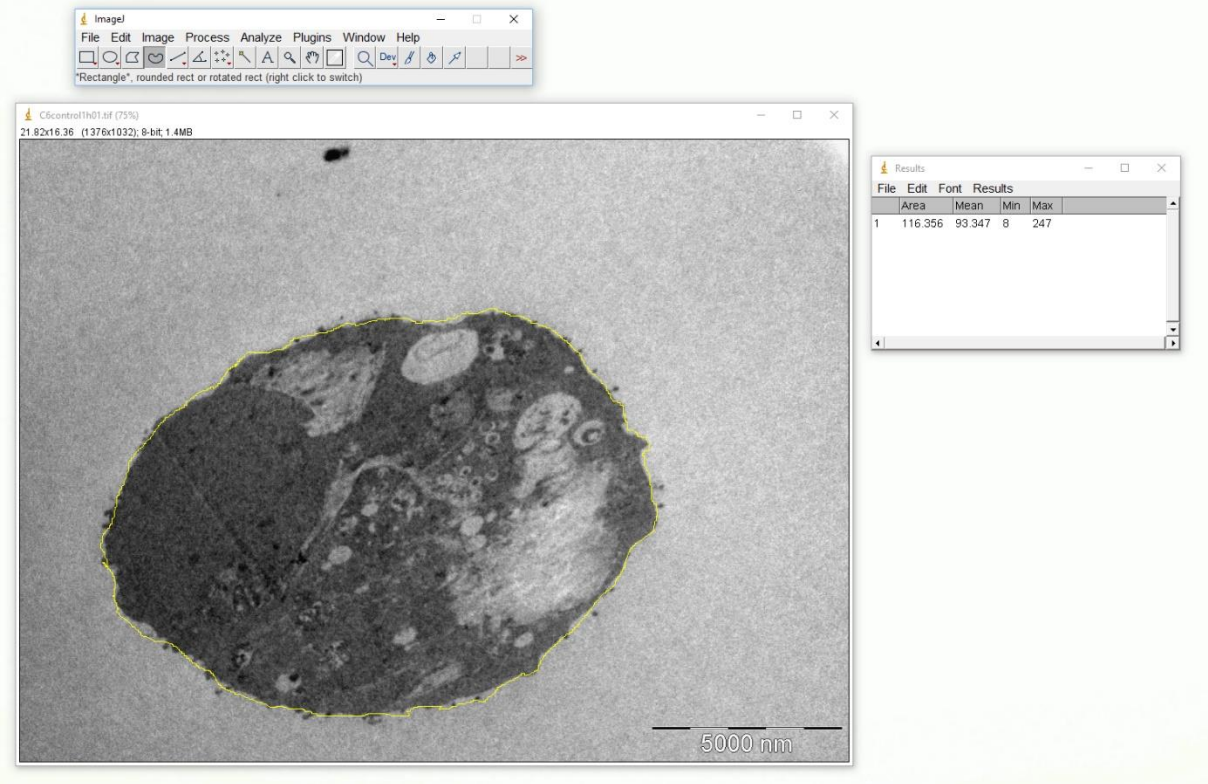

Figure 6 IPEC-J2 cell in the program Image J with the size measurement

transmission electron microscope (JEOL, Tokyo, Japan) equipped with a Mega-View-III digital camera and a Soft-Imaging-System (SIS, Münster, Germany). From the images made by the TEM, Image J 1.52a software was used to further analyse the features of the epithelial cells (Figure 6). We measured the size of the intact cell profiles, the number and size of their

nucleus and their vacuoles and the number of the microvilli than we further analysed the data with statistical methods.

5.3. Redox state measurements

After the treatments we washed the cells with PBS and replaced with 80 µl of plain DMEM/F12 medium. After 20 hours we added 20 µl ROS-Glo H_2O_2 Substrate to the samples and they were incubated for more 4 hours at 37°C. Thereafter 50 µl of the samples were

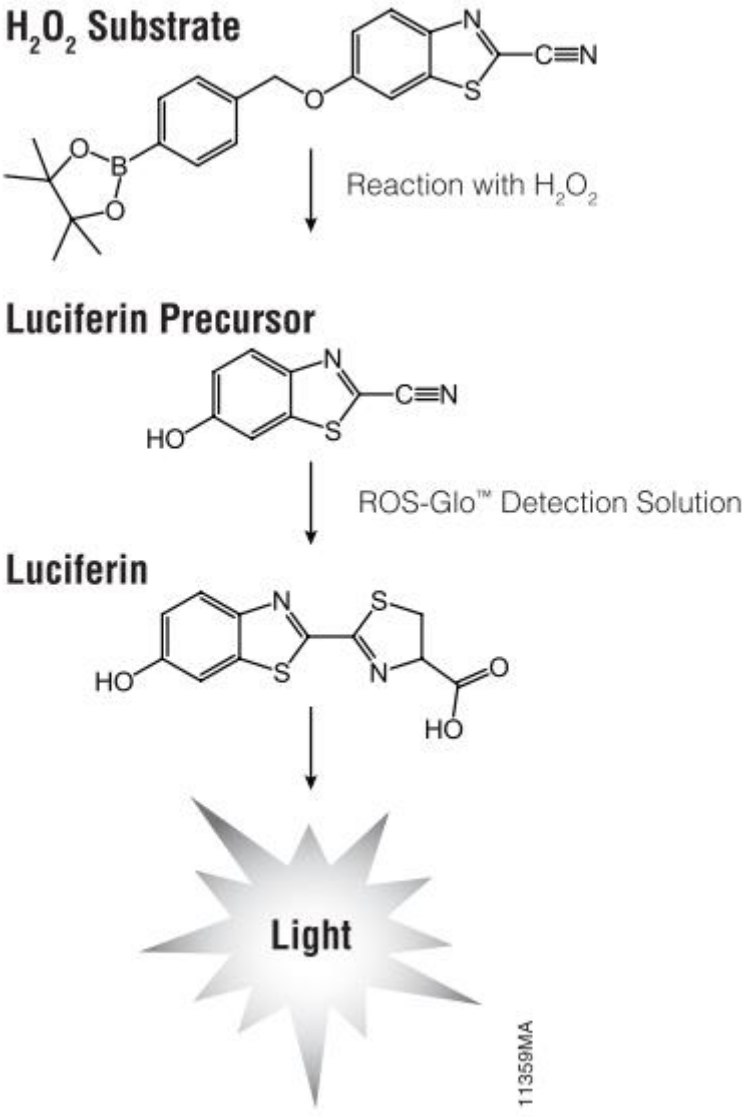

Figure 6 Detection with ROS-Glo H2O2 Kit (Promega, 2012).

transferred in a white opaque 96-well plate for the luminescence measurements and 50 µl of Detection Reagent was added to them. The samples were incubated for another 20 minutes, then luminescence was measured by Perkin Elmer Victor X2 spectrophotometer.

The ROS-Glo Assay (Promega, 2012) works by directly reacting with the extracellular H_2O_2 to generate a luciferin precursor, which will be turned into a luciferin by adding ROS-Glo Detection Reagent (Figure 7). The luciferin generates a luminescent signal which correlates proportional to the H_2O_2 concentration.

Another method, which also measures the extracellular H_2O_2 concentration is the Amplex Red assay. Amplex Red is a non-fluorescent and colourless compound that will be oxidized by H_2O_2 to resorufin which is highly light absorbing and fluorescing compound (Ehman *et al.*, 2017). Extracellular H_2O_2 concentration after *Salmonella* Typhimurium derived LPS and quercetin derivatives was previously measured in the Pharmacology and Toxicology Department. Results from Amplex Red assay were compared with the data extracted from ROS-Glo measurements.

5.4. Statistical analysis

Statistical analysis of the data was performed with R 3.3.2 (2016) software (R Foundation, Vienna, Austria). Differences between means were evaluated by two-way ANOVA, with data of normal distribution, and homogeneity of variances was confirmed. To compare treated groups to controls Dunnett post-hoc test was used. For the comparison of different treatments, we used Fisher LSD test. Level of significance was set at $p < 0.05$. All values were expressed as means ± standard deviations.

To compare the results of the ROS-Glo and the previously used Amplex Red, the data were converted to control percentages and paired t-test was performed.

6. Results

6.1. Extracellular redox state

In our experiment we compared the results of the different treatments with the quercetin (Q), 3-O-methyl quercetin (QM), rhamnazin (R) and the LPS to the control group.

6.1.1. ROS-Glo assay

The results of the ROS-Glo measurements showed that the quercetin, the 3-O-methyl quercetin and the rhamnazin significantly decreased ($p < 0.05$) the extracellular H_2O_2 concentration applied *per se*. The LPS from *Salmonella* Typhimurium did not alter the H_2O_2 concentration compared to the control group, however the simultaneous treatments caused significantly lower ($p < 0.05$) luminescence intensity values (Figure 8).

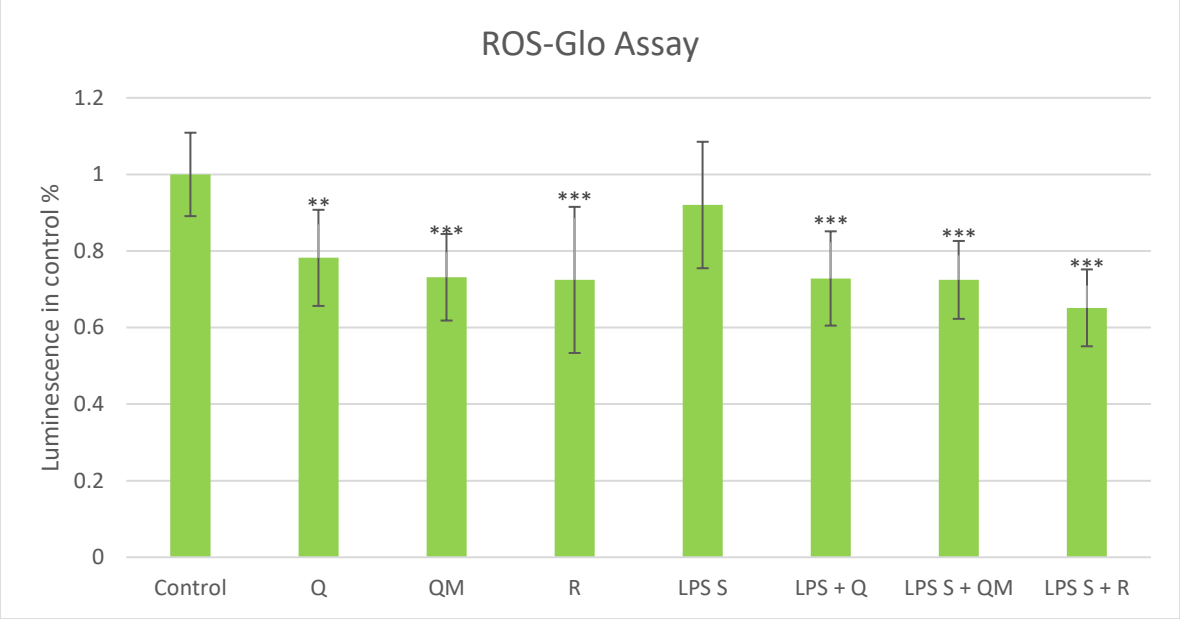

Figure 7 Results of the ROS-Glo Assay measurements. Average luminescence values with standard deviation in control percentages. (n= 8, **: $p < 0.01$, *** $p < 0.001$; Q: quercetin 50 µM, QM: 3-O-methyl quercetin 50 µM, R: rhamnazin 50 µM, LPS S: lipopolysaccharide of *Salmonella* Typhimurium 10 µg/ml).

6.1.2. Amplex Red assay

The results of the Amplex Red assay showed that the quercetin and the rhamnazin significantly increased ($p < 0.05$) the extracellular H_2O_2 concentration *per se* and in the combination with the LPS compared to the control group. However, the 3-O-methylquercetin and the LPS treatment did not cause changes in the extracellular H_2O_2 concentration compared to the control group (Figure 9).

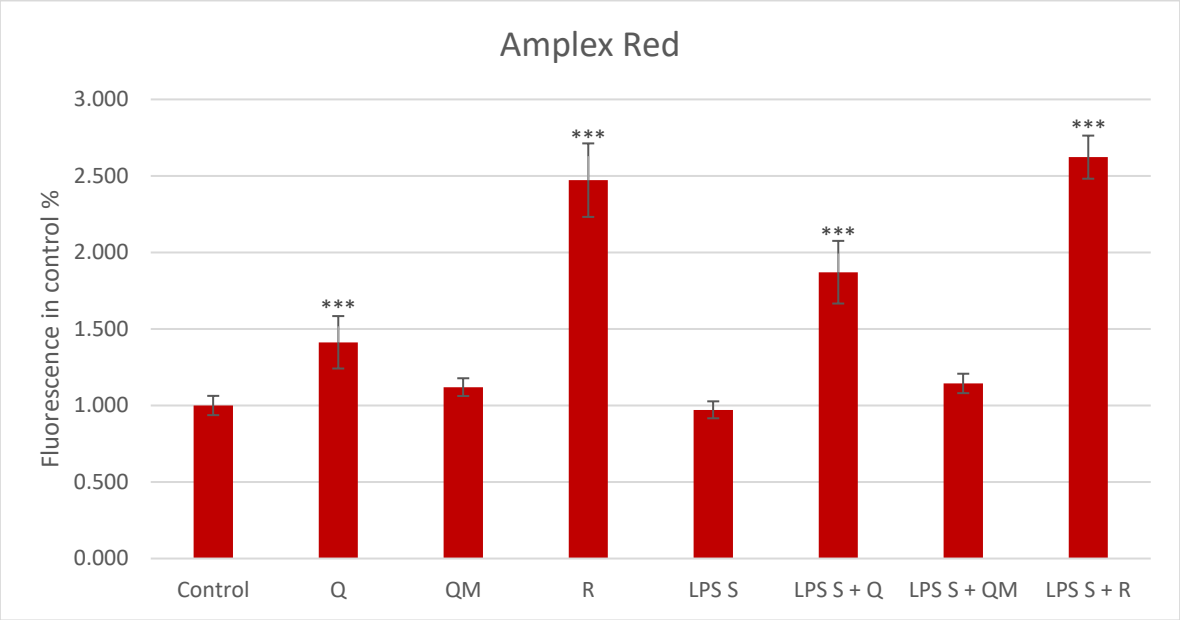

Figure 8 Results of the Amplex Red Assay measurements. Average fluorescence values with standard deviation in control percentages. (n= 6, *** $p < 0.001$; Q: quercetin 50 µM, QM: 3-O-methyl quercetin 50 µM, R: rhamnazin 50 µM, LPS S: lipopolysaccharide of *Salmonella* Typhimurium 10 µg/ml).

6.1.3. Comparison of Amplex Red and ROS-Glo assay

However, both methods are used to detect the extracellular H_2O_2 concentration, we found high-contrast differences. Regarding to the Amplex Red assay, quercetin, 3-O-methyl quercetin and rhamnazin treatments increased the extracellular H_2O_2 concentration significantly ($p < 0.05$) while using the ROS-Glo assay these flavonoid treatments resulted in significantly ($p < 0.05$) lower H_2O_2 concentration. The LPS treatment showed the same in case of both methods, although the results of combination treatments were different. Simultaneous treatments with the LPS and the quercetins caused significantly ($p < 0.05$) lower H_2O_2 concentration in case of the ROS-Glo assay whereas the Amplex Red

measurement resulted in significantly ($p < 0.05$) higher extracellular H_2O_2 concentration after LPS treatment in combination with quercetin and rhamnazin (Figure 10).

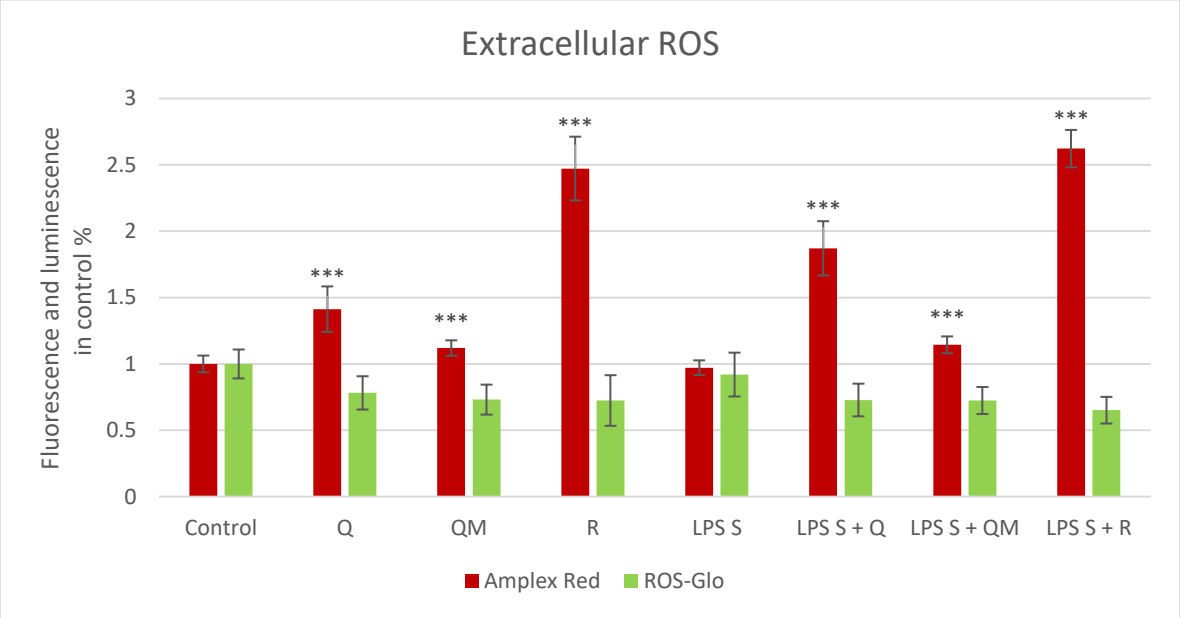

Figure 9 Comparison of Amplex Red and ROS-Glo assay measurements. Average fluorescence (red) and luminescence (green) values with standard deviations in control percentages. (n=6; 8, *** $p < 0.001$; Q: quercetin 50 µM, QM: 3-O-methyl quercetin 50 µM, R: rhamnazin 50 µM, LPS S: lipopolysaccharide of *Salmonella* Typhimurium 10 µg/ml).

6.2. Cell morphology

When EM images have analysed, cell areas, cell nucleus areas, vacuole areas and the number of microvilli (microvilli density) after the treatment with different flavonoids (quercetin, 3-O-methyl quercetin and rhamnazin) have been measured. These structures can be seen in Figure 11.

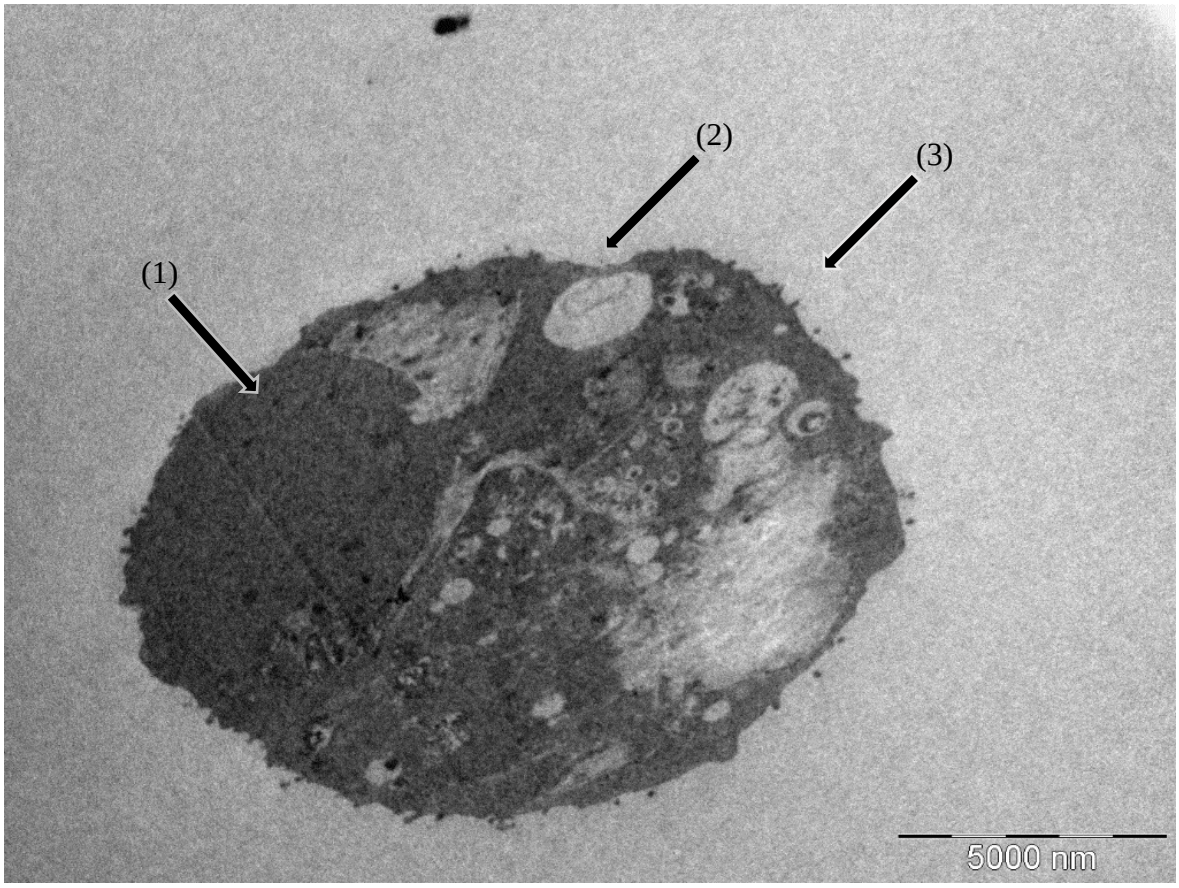

Figure 10 Control of IPEC-J2 cell under the electron microscope

(1) = Nucleus

(2) = Vacuole

(3) = Microvilli

To receive more comparable results and data we calculated the ratio of cell nuclei versus total cell cytoplasmic area, vacuoles area versus whole cell profile area and density of microvilli per cell surface area. The amount of data we gathered did not allow us to draw a quantitative statistical conclusion, but it can demonstrate directions of potential changes.
The different quercetin treatments in 25 µM concentration showed no differences between the cell nucleus area and cell area ratio (Figure 12).

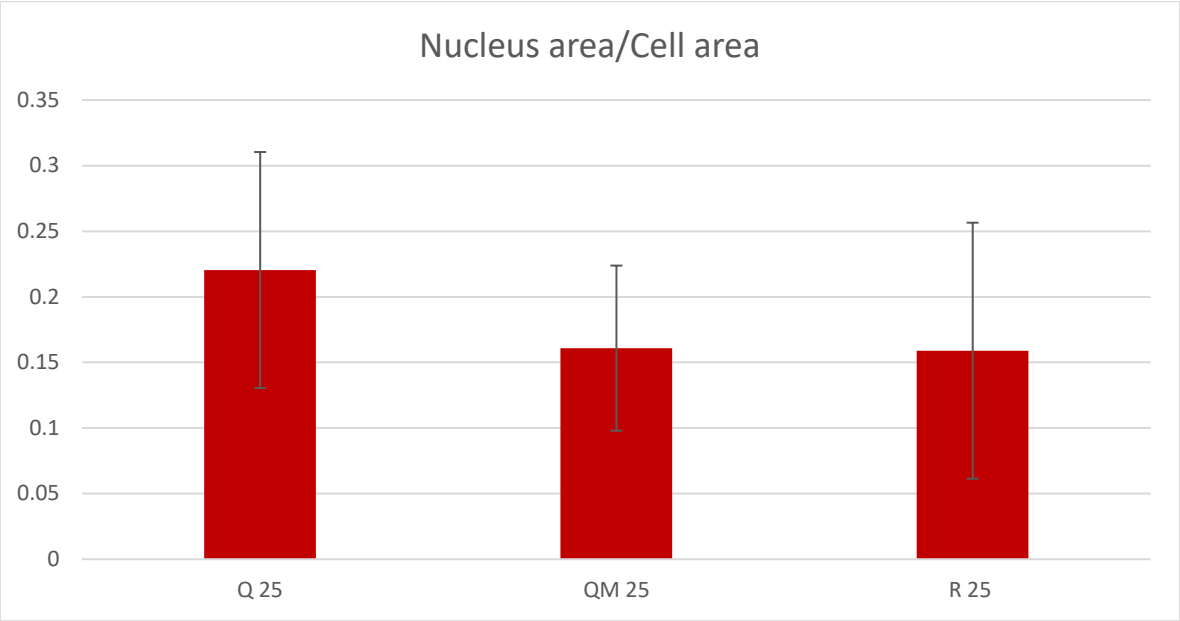

Figure 11 Average of the ratio of the cell nucleus area and the cell area with standard deviation. (n= 3-10; Q 25: quercetin 25 µM, QM 25: 3-O-methyl quercetin 25 µM, R 25: rhamnazin 25 µM).

However, rhamnazin, containing two methyl group, in 25 µM concentration increases the mean area of vacuoles of the cells because it showed increased vacuole area and cell area ratio, while the treatment of quercetin remained low in vacuole area and cell area ratio (Figure 13).

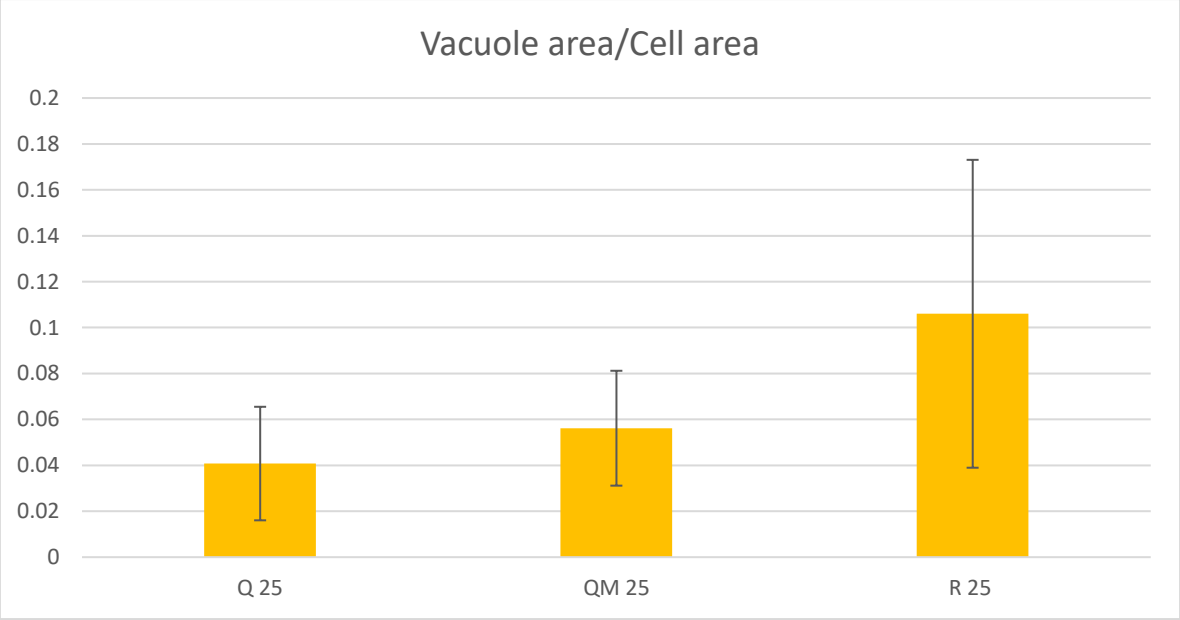

Figure 12 Average of the ratio of the vacuole area and the cell area with standard deviation. (n= 3-10; Q 25: quercetin 25 µM, QM 25: 3-O-methyl quercetin 25 µM, R 25: rhamnazin 25 µM).

Regarding to the microvilli density and cell area ratio, the microvilli number was the highest after the treatment of 3-O-methyl quercetin in 25 µM concentration, meanwhile the lowest ratio was resulted after quercetin treatment (Figure 14).

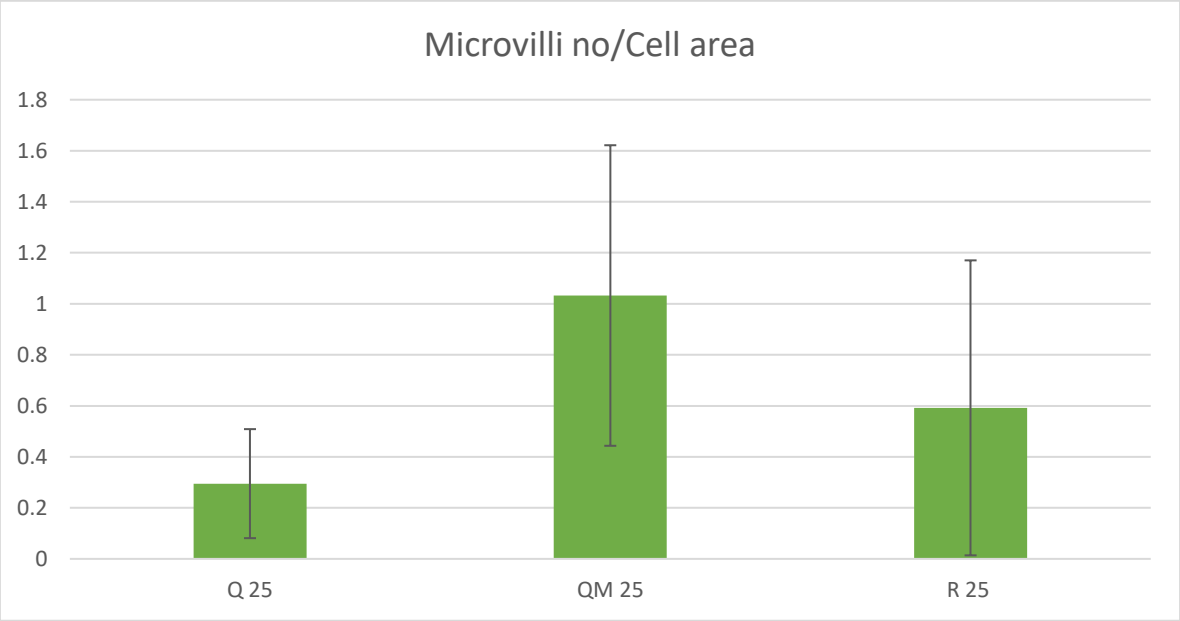

Figure 13 Average of the ratio of the number of the microvilli (density) and the cell area with standard deviation. (n= 3-10; Q 25: quercetin 25 µM, QM 25: 3-O-methyl quercetin 25 µM, R 25: rhamnazin 25 µM).

In the meantime, we are making every effort to gather more electron micrographs in order to get enough data that can be used to quantitative statistical analysis.

7. Discussion

Flavonoids have become common supplements in the everyday diet of humans. There are increasingly used in the small- and farm animal veterinary praxis as well. With further research of flavonoid compounds, a wider choice of prophylactic and therapeutic options is available, and reduction of excessive antibiotic usage can be achieved.

Hydroxylated flavonoids such as quercetin are known as positive compounds which have anti-inflammatory, anti-bacterial, anti-carcinogenic…etc. effect. Earlier, these biological actions have been attributed to their antioxidant properties. In the last decades, there is an emerging view that flavonoids do not act as conventional hydrogen-donating antioxidants but may modulate in cells through actions at different protein kinase signalling pathways. In the other hand, flavonoids often show great potential in cell culture studies, but this does not translate well into *in vivo* activity, because of their extensive metabolism in intestine and liver. Methylated flavonoids do not have such a pronounced radical-scavenging effect as their hydroxylated analogues. Nevertheless, these compounds also show increased anti-cancer effect and they have been of interest due to their anti-inflammatory properties, and they can decrease the ROS level as well. Farkas et al. found that intracellular H_2O_2 level in intestinal epithelial cells was significantly decreased 24 h after treatment both in case of apigenin and apigenin-trimethylether (Farkas *et al.*, 2015).

In this work, we examined the effect of three flavonoid compounds in the IPEC-J2 jejunal epithelial cells. Beside the well-known quercetin the effect of its methylated analogues, which are very promising but not extensively studied compounds, were performed. Flavonoid treatments were also combined with LPS treatment in order to model intestinal inflammation and check the anti-inflammatory effect of quercetin derivatives.

On one hand, the antioxidant effect on the above-mentioned molecules was studied. There are many different methods to determine antioxidant capacity of biomolecules or to measure the level of different ROS in biological samples. Extracellular H_2O_2 concentration is often determined by the fluorescent Amplex Red assay; this method is applied at the Department of Pharmacology and Toxicology as well. Our goal was to compare the Amplex Red method with the ROS-Glo method which is based on luminescent detection. It was discovered that in case of Amplex Red the extracellular level of H_2O_2 was significantly increased after quercetin and rhamnazin treatment compared to the controls. In contrast, H_2O_2 level was not changed significantly after quercetin and rhamnazin treatment when measurement was performed with the ROS-Glo method. The possible reason for that is because Amplex Red has a higher low false hit rate than ROS-Glo. The ROS-Glo reagent reacts directly with the

H_2O_2 reagent, thus minimizing this false hit rate significantly compared to Amplex Red. Most of the ROS reagents require horseradish peroxidase (HRP) and this will lead to a higher false hit rate either through inhibiting the HRP or by competing with H_2O_2 for the HRP enzyme. Nevertheless, further studies are needed to elucidate the underlying mechanisms of this phenomenon.

Our other main goal was to study the effect of quercetin derivatives on the structure of IPEC-J2 cells. Structural and functional studies on enterocytes were carried out using TEM. The morphological dimensions of the enterocytes, presence and size of vacuoles, number of microvilli were analysed. Our results with TEM and immunohistochemistry suggest that the treatment with quercetin and its methylated derivatives may influence the ultrastructure of IPEC-J2 cells, but further evaluation of our TEM samples is required to draw a quantitative conclusion. This work is still in progress. Processing *in vitro* cells for TEM is extremely challenging and requires significantly more time than light microscopy. Therefore, we plan process more samples in the near future, and also will make a systematic analysis of the profiles and morphological features of these IPEC-2 cells. We also would like to know, if the effect of hydroxylated quercetin and its methylated analogues differs each other or not, therefore analysis of flavonoid-treated samples were performed. Evaluation of sections are being processed, so in the future, statistical probes are going to be made to compare the effect on the cell structure of different flavonoid compounds.

In general, morphology of cells was well preserved. The average cell areas of the enterocytes were approximately the same in case of all treatment. It seems that the number of microvilli increased after QM treatment, compared to the Q and R treated samples. Cell vacuolization in the sections can be observed. Cytoplasmic vacuolization is a well-known morphological phenomenon observed in mammalian cells after exposure to pathogens as well as to various natural and artificial compounds. Cell vacuolization can be transient or irreversible. Most known inducers of transient vacuolization are weakly basic amine-containing lipophilic compounds (Shubin *et al.*, 2016). Until now in the literature vacuole formation after application of natural compound like flavonoids is not examined, however these substances frequently used as a feed additive of feed supplementary.

Changes in structure of tight junction proteins are planned also to be studied by confocal laser microscopy method. Further studies will focus on the effect of quercetin and its derivatives when IPEC-J2 cells are treated by bacterial lipopolysaccharides or pathogenic bacteria.

8. Summary

Flavonoids display various beneficial effects in the human and animal body. Their anti-inflammatory, anti-cancer, anti-bacterial properties have been shown in several studies. The above-mentioned positive effects of these compounds are often attributed to their antioxidant properties which is in close correlation to the number and position of hydroxyl groups in the molecules. Besides hydroxyl flavonoids, their methylated analogues have also significant antioxidant effect because these can modulate different protein kinase signalling pathways in the cells. Nevertheless, the information regarding their effects on intestinal epithelium is quite limited. The oxidative stress developed by intestinal infections and inflammation diseases caused by pathogen bacteria leads to serious economic and health problems in animal breeding. Alternative food and feed additives, such as quercetin and its methylated analogues, could be also effective to prevent the above-mentioned diseases.

In this study, the *in vitro* effect of quercetin, 3-O-methylquercetin and rhamnazin (3',7-dimethylquercetin) has been investigated in order to evaluate whether these flavonoids could attenuate lipopolysaccharide (LPS) induced oxidative stress in IPEC-J2 non-transformed porcine intestinal epithelial cells. Moreover, effect of the flavonoids on the morphology of the enterocytes was examined.

The cells have been grown in 24-well cell culture plates until they formed a confluent monolayer. Oxidative stress has been induced by LPS from *Salmonella* Typhimurium (10 µg/ml). Quercetin, 3-O-methylquercetin and rhamnazin have been administered to the cells *per se* in different concentrations (25, 50 µM) and simultaneous treatment of cells with LPS and quercetin derivatives has been also performed. For the measurement of the extracellular H_2O_2 the luminescent ROS-Glo method has been used. Other cells have been cultured on Aclar film than treated with the flavonoids (25 µM) and prepared for electron microscopy (EM) examination. The EM pictures were evaluated with the ImageJ program.

The ROS-Glo measurements showed that quercetin and its derivatives significantly decreased the extracellular H_2O_2 level compared to the controls. The results of the EM measurements showed that flavonoid treatment decreased the number and size of vacuoles and increased the number of microvilli on the surface of the cells. In the future we would like to examine the cell morphology with EM after simultaneous treatment with LPS and the flavonoids.

9. Összefoglaló

A flavonoidok jótékony hatása az állati és emberi szervezetre régóta ismert. Számos tanulmány bizonyítja, hogy gyulladáscsökkentő, daganatellenes, antibakteriális hatással is rendelkeznek. Korábban elfogadott volt, hogy a fent említett pozitív hatások antioxidáns kapacitásukkal vannak kapcsolatban, ami szoros összefüggésben áll a hidroxilcsoportok számával és elhelyezkedésével a molekulákon. A hidroxiflavonoidokon kívül a metilcsoportokkal rendelkező flavonoidok is jelentős antioxidáns hatással rendelkeznek, ami annak tulajdonítható, hogy képesek különféle protein kináz jelátviteli utakat módosítani a sejtekben. A metilezett flavonoidok bélrendszerre gyakorolt hatásáról azonban nem áll rendelkezésünkre sok információ. A baktériumok okozta fertőzések és az oxidatív stressz következményeképpen létrejött bélrendszeri megbetegedések jelentős gazdasági károkhoz vezetnek. Az alternatív táplálék és takarmány kiegészítők, mint pl. a kvercetin és metilezett származékai, hatékonyak lehetnek a fent említett betegségek megelőzésében.

Vizsgálataink során a kvercetin, valamint származékainak (3-O-metilkvercetin és 3',7-dimetilquercetin, más néven ramnazin) hatását vizsgáltuk lipopoliszacharid (LPS) által kiváltott oxidatív stressz esetén IPEC-J2 nem-transzformált sertés bélhámsejteken. Továbbá vizsgáltuk a flavonoidok hatását a bélhámsejtek morfológiájára.

A sejteket 24-lyukú edényben tenyésztettük, míg egy rétegben benőtték a rendelkezésükre álló felületet. Az oxidatív stresszt *Salmonella* Typhimurium LPS kezeléssel (10 µg/ml) váltottuk ki. A sejteket a kvercetinnel és származékaival önmagukban (25, 50 µM), illetve LPS-sel együtt is kezeltük. Az extracelluláris H_2O_2 meghatározásához a lumineszcens ROS-Glo módszert alkalmaztuk. A sejtek másik részét Aclar filmen tenyésztettük, majd a flavonoidokkal (25 µM) történő kezelés után előkészítettük őket az elektronmikroszkópos (EM) vizsgálathoz. Az EM képeket az ImageJ programmal értékeltük.

Megállapítottuk, hogy a kvercetinnel és származékaival történő kezelés jelentősen csökkentette az extracelluláris H_2O_2 mennyiségét a kontroll sejtekhez képest. Az EM eredmények alapján úgy látszik, hogy a flavonoidok hatására csökkent a sejtekben a vakuólumok száma és mérete, a mikrovillusok száma pedig növekedett. Jövőbeli terveink közt szerepel, hogy vizsgáljuk a flavonoidok hatását a morfológiára LPS által kiváltott oxidatív stressz esetében is.

10. Bibliography

Adler, V. *et al.* (1999) 'Role of redox potential and reactive oxygen species in stress signaling', *Oncogene*, 18(45), pp. 6104–6111. doi: 10.1038/sj.onc.1203128.

Adlercreutz, H. and Mazur, W. (1997) 'Phyto-oestrogens and Western Diseases', *Annals of Medicine*. Taylor & Francis, 29(2), pp. 95–120. doi: 10.3109/07853899709113696.

Aki, T., Nara, A. and Uemura, K. (2012) 'Cytoplasmic vacuolization during exposure to drugs and other substances', *Cell Biology and Toxicology*, 28(3), pp. 125–131. doi: 10.1007/s10565-012-9212-3.

Akiyama, N. *et al.* (2006) 'Up-regulation of cytosolic phospholipase A2α expression by N,N-diethyldithiocarbamate in PC12 cells; involvement of reactive oxygen species and nitric oxide', *Toxicology and Applied Pharmacology*. Academic Press, 215(2), pp. 218–227. doi: 10.1016/J.TAAP.2006.02.013.

Artursson, P. and Karlsson, J. (1991) 'Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells', *Biochemical and Biophysical Research Communications*. Academic Press, 175(3), pp. 880–885. doi: 10.1016/0006-291X(91)91647-U.

Boots, A. W., Haenen, G. R. M. M. and Bast, A. (2008) 'Health effects of quercetin: From antioxidant to nutraceutical', *European Journal of Pharmacology*. Elsevier, 585(2–3), pp. 325–337. doi: 10.1016/J.EJPHAR.2008.03.008.

Brosnahan, A. J. and Brown, D. R. (2012) 'Porcine IPEC-J2 intestinal epithelial cells in microbiological investigations', *Veterinary Microbiology*. Elsevier B.V., 156(3–4), pp. 229–237. doi: 10.1016/j.vetmic.2011.10.017.

Cavallo, F. *et al.* (2011) '2011: The immune hallmarks of cancer', *Cancer Immunology, Immunotherapy*, 60(3), pp. 319–326. doi: 10.1007/s00262-010-0968-0.

Chan, T., Galati, G. and O'Brien, P. J. (1999) 'Oxygen activation during peroxidase catalysed metabolism of flavones or flavanones', *Chemico-Biological Interactions*. Elsevier, 122(1), pp. 15–25. doi: 10.1016/S0009-2797(99)00103-9.

Cheng, Y. *et al.* (2006) 'Phosphatidylcholine-specific phospholipase C, p53 and ROS in the association of apoptosis and senescence in vascular endothelial cells', *FEBS Letters*, 580(20), pp. 4911–4915. doi: 10.1016/j.febslet.2006.08.008.

Chuang, C. C. *et al.* (2010) 'Quercetin is equally or more effective than resveratrol in attenuating tumor necrosis factor-α-mediated inflammation and insulin resistance in primary human adipocytes', *American Journal of Clinical Nutrition*, 92(6), pp. 1511–1521. doi: 10.3945/ajcn.2010.29807.

Coecke, S. *et al.* (2006) 'Metabolism: A Bottleneck in In Vitro Toxicological Test Development: The Report and Recommendations of ECVAM Workshop 541', *Alternatives to Laboratory Animals*. SAGE Publications Ltd STM, 34(1), pp. 49–84. doi: 10.1177/026119290603400113.

Conquer, J. A. *et al.* (1998) 'Supplementation with Quercetin Markedly Increases Plasma Quercetin Concentration without Effect on Selected Risk Factors for Heart Disease in Healthy Subjects', *The Journal of Nutrition*, 128(3), pp. 593–597. doi: 10.1093/jn/128.3.593.

Dasari, A. *et al.* (2006) 'Oxidative stress induces premature senescence by stimulating caveolin-1 gene transcription through p38 mitogen-activated protein kinase/Sp1-mediated activation of two GC-rich promoter elements', *Cancer Research*, 66(22), pp. 10805–

10814. doi: 10.1158/0008-5472.CAN-06-1236.

Dröge, W. (2002) ‘Free radicals in the physiological control of cell function’, *Physiological Reviews*, 82(1), pp. 47–95. doi: 10.1152/physrev.00018.2001.

De Duve, C. *et al.* (1974) ‘Lysosomotropic agents’, *Biochemical Pharmacology*. Elsevier, 23(18), pp. 2495–2531. doi: 10.1016/0006-2952(74)90174-9.

Edwards, R. L. *et al.* (2007) ‘Quercetin Reduces Blood Pressure in Hypertensive Subjects’, *The Journal of Nutrition*, 137(11), pp. 2405–2411. doi: 10.1093/jn/137.11.2405.

Ehman, E. C. *et al.* (2017) ‘Mechanism of oxidative conversion of Amplex Red to resorufin: pulse radiolysis and enzymatic studies’, *Free Radical Biology and Medicine*, 46(5), pp. 1247–1262. doi: 10.1002/jmri.25711.PET/MRI.

Erlund, I. (2004) ‘Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology’, *Nutrition Research*, 24(10), pp. 851–874. doi: 10.1016/j.nutres.2004.07.005.

Farkas, O. *et al.* (2015) ‘Polymethoxyflavone apigenin-trimethylether suppresses lps-induced inflammatory response in nontransformed porcine intestinal cell line IPEC-J2’, *Oxidative Medicine and Cellular Longevity*, 2015. doi: 10.1155/2015/673847.

Ferraretto, A. *et al.* (2018) ‘Morphofunctional properties of a differentiated Caco2/HT-29 co-culture as an in vitro model of human intestinal epithelium’, *Bioscience Reports*, 38(2), pp. 1–16. doi: 10.1042/BSR20171497.

Ferry, D. R. *et al.* (1996) ‘Phase I clinical trial of the flavonoid quercetin: Pharmacokinetics and evidence for in vivo tyrosine kinase inhibition’, *Clinical Cancer Research*, 2(4), pp. 659–668.

Galati, G. *et al.* (1999) ‘Glutathione-Dependent Generation of Reactive Oxygen Species by the Peroxidase-Catalyzed Redox Cycling of Flavonoids’, *Chemical Research in Toxicology*. American Chemical Society, 12(6), pp. 521–525. doi: 10.1021/tx980271b.

Galati, G. *et al.* (2002) ‘Prooxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics | Omid Sabzevari - Academia.edu’, 177, pp. 91–104. Available at: http://www.academia.edu/1006810/Prooxidant_activity_and_cellular_effects_of_the_phenoxyl_radicals_of_dietary_flavonoids_and_other_polyphenolics.

Gee, J. M., Hara, H. and Johnson, I. T. (2002) ‘Suppression of Intestinal Crypt Cell Proliferation and Aberrant Crypt Foci by Dietary Quercetin in Rats’, *Nutrition and Cancer*. Routledge, 43(2), pp. 193–201. doi: 10.1207/S15327914NC432_10.

Goldman, R. *et al.* (1999) ‘Myeloperoxidase-catalyzed redox-cycling of phenol promotes lipid peroxidation and thiol oxidation in HL-60 cells’, *Free Radical Biology and Medicine*. Pergamon, 27(9–10), pp. 1050–1063. doi: 10.1016/S0891-5849(99)00140-9.

Hanahan, D. and Weinberg, R. A. (2011) ‘Hallmarks of cancer: The next generation’, *Cell*. Elsevier Inc., 144(5), pp. 646–674. doi: 10.1016/j.cell.2011.02.013.

Hartung, T. and Daston, G. (2009) ‘Are in vitro tests suitable for regulatory use?’, *Toxicological Sciences*, 111(2), pp. 233–237. doi: 10.1093/toxsci/kfp149.

Harwood, M. *et al.* (2007) ‘A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties’, *Food and Chemical Toxicology*. Pergamon, 45(11), pp. 2179–2205. doi: 10.1016/J.FCT.2007.05.015.

Heinecke, J. W. *et al.* (1993) ‘Tyrosyl radical generated by myeloperoxidase catalyzes the

oxidative cross-linking of proteins', *Journal of Clinical Investigation*, 91(6), pp. 2866–2872. doi: 10.1172/JCI116531.

Hertog, M. G. *et al.* (1993) 'Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study.', *Lancet (London, England)*. Elsevier, 342(8878), pp. 1007–11. doi: 10.1016/0140-6736(93)92876-u.

Hertog, M. G. L. *et al.* (1995) 'Flavonoid Intake and Long-term Risk of Coronary Heart Disease and Cancer in the Seven Countries Study', *JAMA Internal Medicine*, 155(4), pp. 381–386. doi: 10.1001/archinte.1995.00430040053006.

Hodnick, W. F., Duval, D. L. and Pardini, R. S. (1994) 'Inhibition of mitochondrial respiration and cyanide-stimulated generation of reactive oxygen species by selected flavonoids', *Biochemical Pharmacology*. Elsevier, 47(3), pp. 573–580. doi: 10.1016/0006-2952(94)90190-2.

Hollman, P. C. *et al.* (1995) 'Absorption of dietary quercetin healthy ileostomy volunteers^1-3', *American Journal of Clinical Nutrition*, 62(February), pp. 1276–1282.

Jeong, J.-H. *et al.* (2009) 'Effects of low dose quercetin: cancer cell-specific inhibition of cell cycle progression', *Journal of cellular biochemistry*, 106(1), pp. 73–82. doi: 10.1002/jcb.21977.

Kelly, E. H., Dennis, J. B. and Anthony, R. T. (2002) 'Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships', *Journal of Nutritional Biochemistry*, 13, pp. 572–584.

Kleemann, R. *et al.* (2011) 'Anti-inflammatory, anti-proliferative and anti-atherosclerotic effects of quercetin in human in vitro and in vivo models', *Atherosclerosis*. Elsevier, 218(1), pp. 44–52. doi: 10.1016/j.atherosclerosis.2011.04.023.

Kuhnle, G. *et al.* (2000) 'Epicatechin and Catechin are O-Methylated and Glucuronidated in the Small Intestine', *Biochemical and Biophysical Research Communications*. Academic Press, 277(2), pp. 507–512. doi: 10.1006/BBRC.2000.3701.

Kurowska, E. M. and Manthey, J. A. (2004) 'Hypolipidemic Effects and Absorption of Citrus Polymethoxylated Flavones in Hamsters with Diet-Induced Hypercholesterolemia', *Journal of Agricultural and Food Chemistry*. American Chemical Society, 52(10), pp. 2879–2886. doi: 10.1021/jf035354z.

Lamson, D. W. and Brignall, M. S. (2000) 'Antioxidants and cancer, part 3: quercetin.', *Alternative medicine review : a journal of clinical therapeutic*, 5(3), pp. 196–208. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10869101.

Leonarduzzi, G., Sottero, B. and Poli, G. (2010) 'Targeting tissue oxidative damage by means of cell signaling modulators: The antioxidant concept revisited', *Pharmacology and Therapeutics*. Elsevier Inc., 128(2), pp. 336–374. doi: 10.1016/j.pharmthera.2010.08.003.

Lu, D., Chen, J. and Hai, T. (2007) 'The regulation of ATF3 gene expression by mitogen-activated protein kinases', *Biochemical Journal*, 401(2), pp. 559–567. doi: 10.1042/BJ20061081.

M. Awad, H. *et al.* (2003) 'Quenching of Quercetin Quinone/Quinone Methides by Different Thiolate Scavengers: Stability and Reversibility of Conjugate Formation', *Chemical Research in Toxicology*, 16(7), pp. 822–831. doi: 10.1021/tx020079g.

Manach, C. *et al.* (2005) 'Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies.', *The American journal of clinical nutrition*, 81(1 Suppl), pp. 230–242. doi: 10.1093/ajcn/81.1.230s.

Mantovani, A. *et al.* (2008) 'Cancer-related inflammation', *Nature*, 454(7203), pp. 436–

444. doi: 10.1038/nature07205.

Miyoshi, N. *et al.* (2007) 'Dietary flavonoid apigenin is a potential inducer of intracellular oxidative stress: The role in the interruptive apoptotic signal', *Archives of Biochemistry and Biophysics*, 466(2), pp. 274–282. doi: 10.1016/j.abb.2007.07.026.

Mullen, W. *et al.* (2008) 'Bioavailability of [2-14C]Quercetin-4′-glucoside in Rats', *Journal of Agricultural and Food Chemistry*, 56(24), pp. 12127–12137. doi: 10.1021/jf802754s.

Muralikrishna Adibhatla, R. and Hatcher, J. F. (2006) 'Phospholipase A2, reactive oxygen species, and lipid peroxidation in cerebral ischemia', *Free Radical Biology and Medicine*. Pergamon, 40(3), pp. 376–387. doi: 10.1016/J.FREERADBIOMED.2005.08.044.

Murota, K. and Terao, J. (2003) 'Antioxidative flavonoid quercetin: Implication of its intestinal absorption and metabolism', *Archives of Biochemistry and Biophysics*, 417(1), pp. 12–17. doi: 10.1016/S0003-9861(03)00284-4.

Ness, A. R. and Powles, J. W. (1997) 'Fruit and vegetables, and cardiovascular disease: a review.', *International Journal of Epidemiology*, 26(1), pp. 1–13. doi: 10.1093/ije/26.1.1.

Novo Belchor, M. *et al.* (2017) 'Evaluation of Rhamnetin as an Inhibitor of the Pharmacological Effect of Secretory Phospholipase A2', *Molecules (Basel, Switzerland)*, 22(9), pp. 1–13. doi: 10.3390/molecules22091441.

O'Brien, P. J. (2000) 'Peroxidases', *Chemico-Biological Interactions*. Elsevier, 129(1–2), pp. 113–139. doi: 10.1016/S0009-2797(00)00201-5.

Olthof, M. R. *et al.* (2003) 'Chlorogenic Acid, Quercetin-3-Rutinoside and Black Tea Phenols Are Extensively Metabolized in Humans', *The Journal of Nutrition*, 133(6), pp. 1806–1814. doi: 10.1093/jn/133.6.1806.

Overman, A., Chuang, C. C. and McIntosh, M. (2011) 'Quercetin attenuates inflammation in human macrophages and adipocytes exposed to macrophage-conditioned media', *International Journal of Obesity*. Nature Publishing Group, 35(9), pp. 1165–1172. doi: 10.1038/ijo.2010.272.

Pizzino, G. *et al.* (2017) 'Oxidative Stress: Harms and Benefits for Human Health', *Oxidative Medicine and Cellular Longevity*. Hindawi, 2017. doi: 10.1155/2017/8416763.

Polli, J. E. (2008) 'In Vitro Studies are Sometimes Better than Conventional Human Pharmacokinetic In Vivo Studies in Assessing Bioequivalence of Immediate-Release Solid Oral Dosage Forms', *The AAPS Journal*, 10(2), pp. 289–299. doi: 10.1208/s12248-008-9027-6.

Prince, P. S. M. and Sathya, B. (2010) 'Pretreatment with quercetin ameliorates lipids, lipoproteins and marker enzymes of lipid metabolism in isoproterenol treated cardiotoxic male Wistar rats', *European Journal of Pharmacology*. Elsevier, 635(1–3), pp. 142–148. doi: 10.1016/J.EJPHAR.2010.02.019.

Promega (2012) 'ROS-Glo™ H2O2 Assay', *Promega Technical Bulletin*, pp. 4–5. doi: 10.1002/kin.

Psahoulia, F. H. *et al.* (2007) 'Quercetin mediates preferential degradation of oncogenic Ras and causes autophagy in Ha-RAS-transformed human colon cells', *Carcinogenesis*, 28(5), pp. 1021–1031. doi: 10.1093/carcin/bgl232.

Ribeiro, D. *et al.* (2013) 'Modulation of human neutrophils' oxidative burst by flavonoids', *European Journal of Medicinal Chemistry*. Elsevier Masson SAS, 67, pp. 280–292. doi: 10.1016/j.ejmech.2013.06.019.

Rice-Evans, C. (2001) ‘Flavonoid Antioxidants’, *Current Medicinal Chemistry*, pp. 797–807. doi: http://dx.doi.org/10.2174/0929867013373011.

Rice-Evans, C. A., Miller, N. J. and Paganga, G. (1996) ‘Structure-antioxidant activity relationships of flavonoids and phenolic acids’, *Free Radical Biology and Medicine*. Pergamon, 20(7), pp. 933–956. doi: 10.1016/0891-5849(95)02227-9.

Russo, M. *et al.* (1999) ‘Quercetin and anti-CD95(Fas/Apo1) enhance apoptosis in HPB-ALL cell line’, *FEBS Letters*, 462(3), pp. 322–328. doi: 10.1016/S0014-5793(99)01544-6.

Russo, M. *et al.* (2010) ‘Exploring death receptor pathways as selective targets in cancer therapy’, *Biochemical Pharmacology*. Elsevier, 80(5), pp. 674–682. doi: 10.1016/J.BCP.2010.03.011.

Russo, M. *et al.* (2012) ‘The flavonoid quercetin in disease prevention and therapy: Facts and fancies’, *Biochemical Pharmacology*. Elsevier Inc., 83(1), pp. 6–15. doi: 10.1016/j.bcp.2011.08.010.

Sadan, A. (2007) ‘The significance of in vitro studies.’, *Quintessence international (Berlin, Germany : 1985)*, 38(1), p. 13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17216902.

Schierack, P. *et al.* (2006) ‘Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine.’, *Histochemistry and cell biology*. Germany, 125(3), pp. 293–305. doi: 10.1007/s00418-005-0067-z.

Schroeter, H. *et al.* (2001) ‘Flavonoids protect neurons from oxidized low-density-lipoprotein-induced apoptosis involving c-Jun N-terminal kinase (JNK), c-Jun and caspase-3’, *Biochemical Journal*, 358(3), pp. 547–557. doi: 10.1042/0264-6021:3580547.

Shubin, A. V. *et al.* (2016) ‘Cytoplasmic vacuolization in cell death and survival’, *Oncotarget*, 7(34), pp. 55863–55889. doi: 10.18632/oncotarget.10150.

Spencer, J. P. . *et al.* (2001) ‘Contrasting influences of glucuronidation and O-methylation of epicatechin on hydrogen peroxide-induced cell death in neurons and fibroblasts’, *Free Radical Biology and Medicine*. Pergamon, 31(9), pp. 1139–1146. doi: 10.1016/S0891-5849(01)00704-3.

Spencer, J. P. E. *et al.* (2001) ‘Epicatechin and its in vivo metabolite, 3′-o-methyl epicatechin, protect human fibroblasts from oxidative-stress-induced cell death involving caspase-3 activation’, *Biochemical Journal*, 354(3), pp. 493–500. doi: 10.1042/0264-6021:3540493.

Spencer, J. P. E., Rice-Evans, C. and Williams, R. J. (2003) ‘Modulation of pro-survival Akt/protein kinase B and ERK1/2 signaling cascades by quercetin and its in vivo metabolites underlie their action on neuronal viability’, *Journal of Biological Chemistry*, 278(37), pp. 34783–34793. doi: 10.1074/jbc.M305063200.

Tappia, P. S., Dent, M. R. and Dhalla, N. S. (2006) ‘Oxidative stress and redox regulation of phospholipase D in myocardial disease’, *Free Radical Biology and Medicine*. Pergamon, 41(3), pp. 349–361. doi: 10.1016/J.FREERADBIOMED.2006.03.025.

Torres, M. and Forman, H. J. (2003) ‘Redox signaling and the MAP kinase pathways’, *BioFactors*. John Wiley & Sons, Ltd, 17(1-4), pp. 287–296. doi: 10.1002/biof.5520170128.

Tsuji, P. A. and Walle, T. (2008) ‘Cytotoxic effects of the dietary flavones chrysin and apigenin in a normal trout liver cell line’, *Chemico-Biological Interactions*, 171(1), pp. 37–44. doi: 10.1016/j.cbi.2007.08.007.

Vergauwen, H. (2015) ‘In vitro intestinal tissue models: General introduction’, *The Impact*

of Food Bioactives on Health: In Vitro and Ex Vivo Models, pp. 239–244. doi: 10.1007/978-3-319-16104-4.

Walle, T. (2004) 'Absorption and metabolism of flavonoids', *Free Radical Biology and Medicine*, 36(7), pp. 829–837. doi: 10.1016/j.freeradbiomed.2004.01.002.

Walle, T. (2007) 'Methoxylated flavones, a superior cancer chemopreventive flavonoid subclass?', *Seminars in Cancer Biology*, 17(5), pp. 354–362. doi: 10.1016/j.semcancer.2007.05.002.

Walle, T., Vincent, T. S. and Walle, U. K. (2003) 'Evidence of covalent binding of the dietary flavonoid quercetin to DNA and protein in human intestinal and hepatic cells', *Biochemical Pharmacology*. Elsevier, 65(10), pp. 1603–1610. doi: 10.1016/S0006-2952(03)00151-5.

Wen, X. and Walle, T. (2006a) 'Methylated flavonoids have greatly improved intestinal absorption and metabolic stability', *Drug Metabolism and Disposition*, 34(10), pp. 1786–1792. doi: 10.1124/dmd.106.011122.

Wen, X. and Walle, T. (2006b) 'Methylated Flavonoids Have Greatly Improved Intestinal Absorption and Metabolic Stability', *Drug Metabolism and Disposition*, 34(10), pp. 1786 LP – 1792. doi: 10.1124/dmd.106.011122.

van der Woude, H. *et al.* (2005) 'Formation of Transient Covalent Protein and DNA Adducts by Quercetin in Cells with and without Oxidative Enzyme Activity', *Chemical Research in Toxicology*, 18(12), pp. 1907–1916. doi: 10.1021/tx050201m.

Yoshizumi, M. and Matsumura, Y. (2011) 'Pharmacology in health foods: Preface', *Journal of Pharmacological Sciences*, 115(4), pp. 459–460. doi: 10.1254/jphs.10R35FM.

Youl, E. *et al.* (2010) 'Quercetin potentiates insulin secretion and protects INS-1 pancreatic β-cells against oxidative damage via the ERK1/2 pathway', *British journal of pharmacology*. Blackwell Publishing Ltd, 161(4), pp. 799–814. doi: 10.1111/j.1476-5381.2010.00910.x.

Zakrzewski, S. S. *et al.* (2013) 'Improved cell line IPEC-J2, characterized as a model for porcine jejunal epithelium', *PLoS ONE*, 8(11). doi: 10.1371/journal.pone.0079643.

Zhao, J. *et al.* (2005) 'Upregulating of Fas, integrin β4 and P53 and depressing of PC-PLC activity and ROS level in VEC apoptosis by safrole oxide', *FEBS Letters*, 579(25), pp. 5809–5813. doi: 10.1016/j.febslet.2005.09.051.

Picture reference:

Mariana Ruiz (LadyofHats) Figure 1 author: https://commons.wikimedia.org/wiki/File:Cellular_tight_junction-en.svg

11. Acknowledgement

I would like to thank my supervisors Dr. Farkas Orsolya and Dr. Karancsi Zita for their help and support throughout my laboratory work and thesis writing and for their patient guidance throughout the complete project.
I would also like to thank Palkovicsné Pézsa Nikolett and Dr. Rácz Bence who helped during the cell culturing and the electron microscope examination. Furthermore, I am grateful to the department of Pharmacology and Toxicology and the Department of Anatomy and Histology for supporting the facilities for this investigation.
And last but not least, I want to thank my family for giving me the opportunity to study at this university and for their constant support during the years and I also would like to thank all my friends that kept me motivated throughout the years and while writing my thesis.