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ORIGINAL RESEARCH PAPER

The impact of quercetin on a porcine intestinal epithelial cell line exposed to deoxynivalenol

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ABSTRACT

Quercetin (Que) is present in many vegetables and fruits as a secondary antioxidant metabolite. Deoxynivalenol (DON) produced by various *Fusarium* mould species can induce cytotoxicity and oxidative stress in the gastrointestinal tracts of humans and farm animals. The aim of this study was to investigate the effects of Que on DON-induced oxidative stress in a non-tumourigenic porcine IPEC-J2 cell line. Two experimental designs were used in our experiments as follows: (a) pretreatment with 20 µmol/L Que for 24 h followed by 1-h 1 µmol/L DON treatment and (b) simultaneous application of 20 µmol/L Que and 1 µmol/L DON for 1 h. Cell cytotoxicity, transepithelial electrical resistance (TER) of cell monolayers and extracellular/intracellular redox status were studied. It was found that DON significantly decreased TER and triggered oxidative stress, while Que pretreatments were beneficial in maintaining the integrity of the monolayers and alleviated oxidative stress. However, co-treatment with Que was unable to preserve the integrity and redox balance of the cells exposed to DON. These results indicate that only the 24-h preincubation of cells with 20 µmol/L Que was beneficial in compensating for the disruption caused by DON in extracellular oxidative status.

KEYWORDS

quercetin, deoxynivalenol, IPEC-J2, transepithelial electrical resistance, oxidative stress

INTRODUCTION

Recently, the number of studies on the beneficial effects of plant-based dietary polyphenols has been growing continuously. Flavonoids can be further divided into six subclasses, namely flavonols, flavones, isoflavones, flavanones, anthocyanins and flavanols (Kroon et al., 2004; Kumar and Pandey, 2013). Flavonoids are known to have antibacterial (Xie et al., 2015), anticancer (Chahar et al., 2011; Abotaleb et al., 2018), anti-inflammatory (Serafini et al., 2010), and antioxidant properties (Galleano et al., 2010; Brunetti et al., 2013). Quercetin (Que) is a well-studied plant derived flavonol (D'Andrea, 2015) (Fig. 1). Vergauwen et al. (2016) reported that Que was beneficial in a concentration range of 25–800 µmol/L to reduce the levels of intracellular reactive oxygen species (ROSs) and strengthen the integrity of the monolayer of porcine non-tumourigenic IPEC-J2 cells. As indicated by Chen et al. (2018), Que protects IPEC-J2 cells from oxidation-induced apoptosis in 16.5 µmol/L concentration for 3 h.

It is extremely challenging to provide mycotoxin-free feedstuffs for livestock. Pigs are very sensitive to mycotoxin-contaminated feeds (Diekman and Green, 1992). In the temperate climate zone, *Fusarium* mould species occur frequently and are responsible for producing a wide variety of trichothecene mycotoxins such as deoxynivalenol (DON) (Placinta et al., 1999; Sundheim et al., 2013) (Fig. 1). In farm animals, dietary exposure to DON decreases growth performance. An important function of gastrointestinal epithelia is to provide a barrier against the penetration of food contaminants and pathogens present in the intestinal

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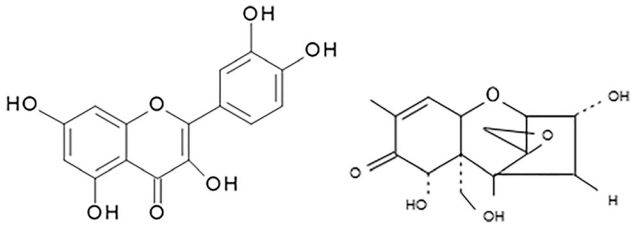

Fig. 1. The chemical structures of quercetin (Que) and deoxynivalenol (DON)

lumen. The disruption of the intestinal barrier allows increased penetration of normally excluded intraluminal substances that may promote intestinal disorders.

In general, DON interferes with the normal functions of the mitochondria as it generates ROS, which can lead to apoptosis. Oxidative stress is a phenomenon which occurs in a cell when the concentration of ROS exceeds the antioxidant capacity. ROS can initiate the process of lipid peroxidation causing damage to phospholipids and lipoproteins of the cell membrane and damage to DNA by propagating a chain reaction (AbdulSalam et al., 2016; Su et al., 2019). Moreover, oxidative stress may increase cell apoptosis (Chen et al., 2018). It is proven that the toxicity of trichothecene mycotoxins is mainly based on oxidative stress. Wan et al. (2019) studied oxidative stress in the human cancerous HT-29 cell line and it was found that 1 µmol/L DON significantly elevated the intracellular ROS after an incubation time of 6 h. The antioxidant properties of Que were previously proved in *in vitro* and *in situ* experiments. It is suggested that Que might scavenge ROS in two ways. One mechanism can be that Que directly acts on both intracellular and superoxide anion radicals or other free radicals and eliminates them. The other possible mode of action of Que seems to involve initiation of antioxidant pathways of cells via promoting the production of antioxidant enzymes (Ružić et al., 2010; Sak, 2014). Trichothecene mycotoxins inhibit protein synthesis in eukaryotic cells (Holladay et al., 1993), especially, as Pinton et al. (2009) and Li et al. (2011) have reported, in epithelial and immune cells, where the rates of cell replications are high.

Porcine intestinal epithelial IPEC-J2 cells are non-tumourigenic, intestinal columnar epithelial cells, which were isolated from the mid-jejunum of neonatal piglets. The IPEC-J2 cell line closely mimics *in vivo* conditions, which makes it a good model system (Zakrzewski et al., 2013) for studies on oxidative stress. It has been previously shown that H_2O_2 administration can lead to a weakened monolayer function in IPEC-J2 cells (Paszti-Gere et al., 2012a,b).

Vergauwen et al. (2016) conducted experiments with Que-treated IPEC-J2 cells, in which Que was effective against H_2O_2-induced oxidative stress and helped to strengthen the barrier functions. According to Goossens et al. (2012), DON caused a decreased in transepithelial electrical resistance (TER) and, at the same time, enhanced the permeability of the IPEC-J2 cell monolayers.

The goal of this study was to evaluate the effects of Que on IPEC-J2 cell line exposed to non-cytotoxic concentrations of DON. This study was focused on monitoring the TER values and determining the changes in extracellular H_2O_2 levels and intracellular ROS production in IPEC-J2 cells after Que and DON treatments.

MATERIALS AND METHODS

Cell line and culture conditions

IPEC-J2 cells were maintained in a complete culture medium made of a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F-12 nutrient medium (DMEM:F12, Merck, Darmstadt, Germany), 5% foetal bovine serum, 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium, 5 ng/mL epidermal growth factor and 1% penicillin–streptomycin solution. All substances were purchased from Thermo Fisher Scientific (Waltham, MA, USA). To remove the cells from the surface, 3 mL of trypsin-EDTA (0.05% trypsin, 0.6 mmol/L EDTA) were added to them for 10 min. IPEC-J2 cells forms polarised monolayers after seeded on 75-cm^2 cell culture flasks with filtered caps (Orange Scientific, Braine-l'Alleud, Belgium). The cells were cultured at 37 °C in a humidified atmosphere of 5% and the complete culture medium was changed every two days. Cells were used between passages 38 and 42.

Reagents

DON and Que were purchased from Merck (Darmstadt, Germany). These compounds were diluted in dimethyl sulphoxide (DMSO) and acetonitrile, which were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The final concentration of acetonitrile or DMSO in the cell culture medium was less than 0.5% (v/v). Dissolved substances were sterile filtered with syringe filters (Millex-GV, pore size: 0.2 µm, Merck, Darmstadt, Germany) before application on the IPEC-J2 cells.

Assessment of the viability of IPEC-J2 cells

Viability of differentiated IPEC-J2 cells was studied after an incubation time of 24 h with DON and Que by Neutral Red (NR) uptake assay (Merck, Darmstadt, Germany) (Repetto et al., 2008). The assay was used to evaluate the extent of uptaking the eurhodin dye, which is a well-established indicator for the amount of living cells.

The control cells were incubated with serum-free phenol red-free DMEM:F12 medium (Merck, Darmstadt, Germany). After the incubation time, the media were removed

and then the cells were washed with phosphate-buffered saline (PBS). A 45 mg/L NR solution was added to the IPEC-J2 cells in serum-free phenol red-free DMEM: F12 medium for 2 h. After this time period, the cells were washed with PBS, and a destaining solution (ethanol/demineralised water/glacial acetic acid, 7.5/7.4/0.15 v/v/v) was applied for 10 min. The viability of the IPEC-J2 cells was measured at 540 nm using an ELISA Plate Reader (EZ Read Biochrom 400, Cambridge, UK).

Experimental layout

For testing cell viability, the IPEC-J2 cells were seeded onto 6-well culture plates (Sigma, Merck, Darmstadt, Germany) at a density of 1×10^6 cells/well. Each concentration was tested with 5 parallel wells in the case of DON and 8 parallels with Que. For further experiments, the cells were seeded into a 6-well plate containing 6 membrane inserts (polyester membrane, cell growth area: 4.67 cm^2, pore size: 0.4 µm, Corning Costar Transwell®, Merck, Darmstadt, Germany) at a density of 1×10^6 cells/well. IPEC-J2 cells started to differentiate after 8 days; the status of polarisation was checked by TER measurement at each change of medium. TER determinations were carried out using 6 parallel measurements in the case of each concentration. For the measurement of the redox state of the IPEC-J2 cells, 8 parallel examinations were carried out.

Two experimental designs were used to test the combination of the two compounds: (1) Pretreatment with 20 µmol/L Que for 24 h followed by the addition of 1 µmol/L DON for 1 h (24-h 20 µmol/L Que + 1-h 1 µmol/L DON); (2) Co-treatment with 20 µmol/L Que and 1 µmol/L DON for 1 h (1-h 20 µmol/L Que + 1 µmol/L DON). Prior to the treatment, control medium was given to the IPEC-J2 cells.

Evaluation of transepithelial integrity after DON and Que treatments

The measurement of TER across epithelial monolayers is used to evaluate the integrity of the cell monolayer (Srinivasan et al., 2015). The barrier function of IPEC-J2 cells was evaluated after the cells reached confluent state on 6-well membrane inserts. The results were calculated as $k\Omega \times cm^2$ by multiplying the values by the surface area of the insert (4.67 cm^2).

TER measurements were carried out before treatment (0 h) to check the integrity values of the confluent, differentiated IPEC-J2 cells. Then TER was assessed after 24- and 25-h treatment of cells with DON, Que and their combination. TER values were measured using EVOM Epithelial Tissue Volt/Ohmmeter (World Precision Instruments, Berlin, Germany).

Determination of extracellular H_2O_2 production

The changes in H_2O_2 production were monitored in IPEC-J2 cells with the Amplex Red Hydrogen Peroxide Assay Kit (Invitrogen, Molecular Probes, Carlsbad, CA, USA) (Zhao et al., 2012). In the presence of horseradish peroxidase, the Amplex Red reagent reacts with H_2O_2 (in 1:1 stoichiometry) to produce fluorescent resorufin.

After an incubation time of 25 h the cell-free supernatants of IPEC-J2 cells were taken. The tests were carried out according to the manufacturer's instructions. The fluorescence intensity was measured with a fluorometer using 560 nm excitation and 590 nm emission wavelengths (Victor X2 2030, Perkin Elmer, Waltham, MA, USA).

Measurement of intracellular ROS in IPEC-J2 cells

The measurement of alteration in intracellular redox state of IPEC-J2 cells was carried out using 2,7-dichlorodihydrofluorescein-diacetate (DCFH-DA) dye (Merck, Darmstadt, Germany) (Aranda et al., 2013). DCFH-DA is oxidised into the highly fluorescent form, dichlorofluorescein (DCF) by the intracellular ROS.

After a treatment time of 25 h, the IPEC-J2 cells were taken and centrifuged at 1,000 rpm for 10 min at 5 °C. After that, the cell-free supernatant samples were collected. Fluorescence intensities of the supernatant were measured using 485 nm excitation and 530 nm emission wavelengths with a fluorometer (Victor X2 2030, Perkin Elmer, Waltham, MA, USA).

Statistical analysis

The statistical analysis of the results was performed by using the R Core Team (version of 2018). Differences between groups were analysed by one-way ANOVA coupled with the *post-hoc* Tukey's test for multiple comparisons. $^{*}P < 0.05$ and $^{***}P < 0.001$ were considered to be statistically significant.

RESULTS

Cytotoxicity of DON and Que

The effects of DON and Que on the viability of IPEC-J2 cells were evaluated after an incubation time of 24 h (Fig. 2). DON was applied in a concentration range of 0–50 µmol/L. DON caused significant cell death after 24 h of incubation at 50 µmol/L ($P < 0.001$). The effect of Que was tested in a concentration range of 0–100 µmol/L. Treatment with 75 µmol/L and higher concentrations of Que resulted in significant cell death rates compared to those in the controls after 24 h. For further investigations non-cytotoxic concentrations of DON (1 µmol/L) and Que (20 µmol/L) were applied.

Changes in transepithelial electrical resistance values after exposure to DON and Que

To determine the effect of 1 µmol/L DON and 20 µmol/L Que on the integrity of the IPEC-J2 cell monolayers, TER measurements were carried out (Fig. 3). After 24-h incubation and preincubation of the cells with 20 µmol/L Que, significant elevations in TER values compared to the control were found (Que: $P = 0.0323$; 24-h pretreatment: $P = 0.0191$). Exposure to 1 µmol/L DON significantly reduced

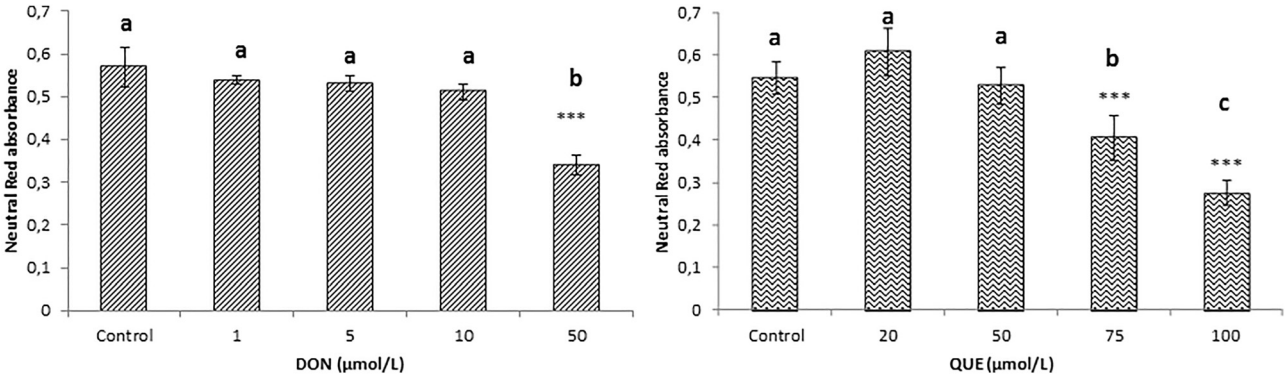

Fig. 2. Evaluation of the cytotoxicity of DON and Que on IPEC-J2 cells after an incubation time of 24 h. $^{***}P < 0.001$ compared to the control values. Data are presented as means ± standard deviations (DONs: $n = 5$; Que: $n = 8$). Different letters show significant differences between control and DON-treated groups (DONs at 50 µmol/L) and control and Que-treated groups (Que at 75 and 100 µmol/L) ($P < 0.05$)

the TER values in IPEC-J2 cells ($P < 0.001$). Beneficial effects of the pretreatment and the co-treatment with 20 µmol/L Que were observed after 24 h and 25 h, since significantly higher TER values were measured compared to those of cells treated only with DON.

Evaluation of extracellular H_2O_2 production after DON and Que treatments

After 25-h treatment with 1 µmol/L DON, 20 µmol/L Que and their combination the extracellular H_2O_2 concentrations were measured (Fig. 4). Exposure to 20 µmol/L Que did not alter the H_2O_2 concentration in the cell-free supernatant ($P = 0.4161$). Also, the 24-h 20 µmol/L Que + 1-h 1 µmol/L DON treatment of the cells did not change the extracellular H_2O_2 production ($P = 0.7006$). Treatment with 1 µmol/L DON for 25 h caused a significant increase in extracellular H_2O_2 production ($P < 0.001$); furthermore, DON-exposed IPEC-J2 cells co-treated with 20 µmol/L Que for 1 h showed significantly elevated H_2O_2 production ($P = 0.0003$) compared to the control values. There were significant differences between the 1-h 20 µmol/L Que + 1 µmol/L DON and the 1 µmol/L DON treatments ($P = 0.0072$).

Changes in intracellular ROS production after adding DON and Que to IPEC-J2 cells

To estimate the changes in intracellular ROS production the DCFH-DA assay was used after 25-h incubation (Fig. 5). Treatment with 20 µmol/L Que did not change the fluorescence intensity values significantly ($P = 0.9993$). In the pretreated cells, DON administration significantly increased intracellular ROS production compared to the control and to Que-treated IPEC-J2 cells (both $P < 0.001$). Exposure to 1 µmol/L DON significantly enhanced the fluorescence intensities after 25-h incubation ($P < 0.001$). After 1-h co-treatment with 20 µmol/L Que and 1 µmol/L DON the fluorescence intensities of intracellular ROS were

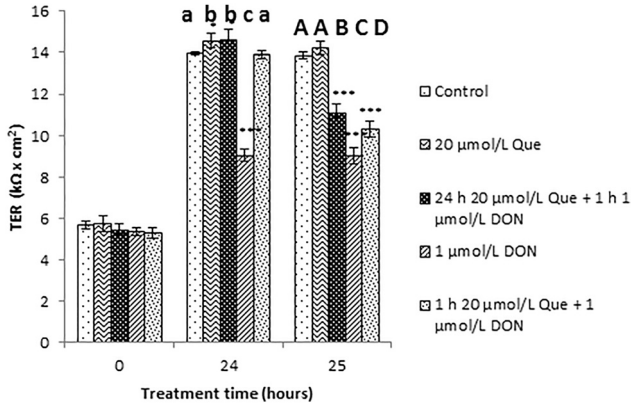

Fig. 3. The effects of DON and Que on the IPEC-J2 cell monolayer integrity. Prior to the experiments the TER values were measured (0 h). Cells were incubated with 1 µmol/L DON, 20 µmol/L Que or the combination of these two compounds for 24 and 25 h. $^{***}P < 0.001$ compared to the control values. Different small letters (24 h) and capital letters (25 h) show significant differences between the indicated groups. Data are presented as means ± standard deviations ($n = 6$)

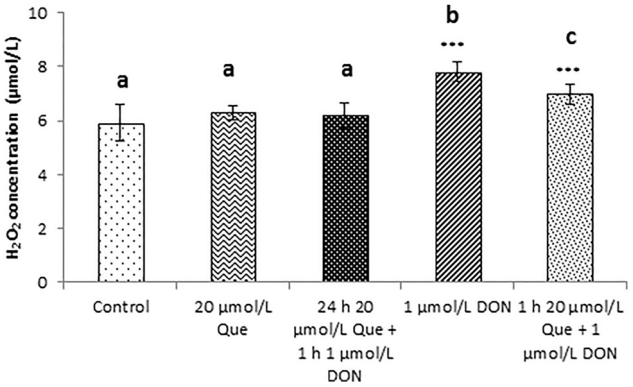

Fig. 4. The changes in H_2O_2 concentrations after DON and Que treatments. The results were obtained after 25 h incubation time, the IPEC-J2 cells were treated with 1 µmol/L DON, 20 µmol/L Que or their combinations. $^{***}P < 0.001$ compared to the control values. Different letters show significant differences between the 24-h 20 µmol/L Que + 1-h 1 µmol/L DON- and 1 µmol/L DON-treated groups and between the control and the 1-h 1 µmol/L DON-treated groups. Data are presented as means ± standard deviations ($n = 8$)

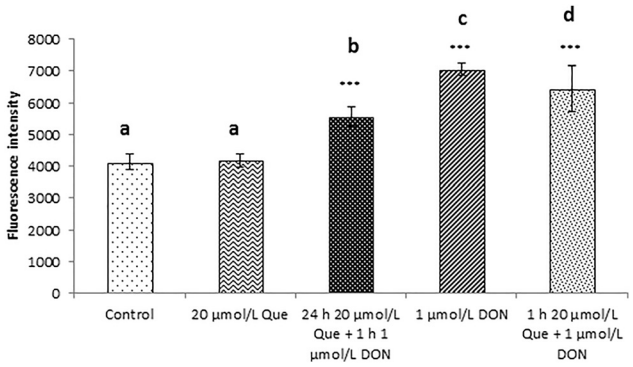

Fig. 5. The impact of DON and Que on the intracellular ROS levels in IPEC-J2 cells. Fluorescence intensities were measured in IPEC-J2 cells incubated with 1 µmol/L DON, 20 µmol/L Que or their combinations after 25 h. $^{***}P < 0.001$ compared to the control values. Different letters show significant differences between each indicated group. Data are presented as means ± standard deviations ($n = 8$)

significantly increased compared to the control values ($P = 0.001$). There were significant differences in intracellular redox status between the co-treatment and the treatment with 1 µmol/L DON (b, $P = 0.0315$), and also between the pretreatment with 20 µmol/L Que and the 1 µmol/L DON treatment ($P < 0.001$).

DISCUSSION

DON, as a major contaminant of cereals, has been implicated in various gastrointestinal problems in farm animals, such as vomiting, feed refusal, diarrhoea (Pestka, 2008), oesophageal perforation as well as malabsorption (Awad et al., 2010). Therefore, it is essential to find naturally occurring feed additives to restore the mycotoxin-perturbed digestion of animals. It was indicated by Chen et al. (2018) that Que protects IPEC-J2 cells from oxidative damage-mediated apoptosis and that the mechanism is related to the inhibition of the mitochondrial apoptosis pathway and lipid peroxidation. These findings indicate the effects of Que on proliferation enhancement of IPEC-J2 cells and their protection against H_2O_2-induced oxidative stress. The present study is the first research to investigate the potential protective ability of Que against DON-induced intestinal barrier dysfunction and oxidative stress in IPEC-J2 cells.

A great variety of cell viability assays can be used for evaluating the effect of foodborne compounds such as DON and the plant-derived Que. Vandenbroucke et al. (2011) reported that in the case of non-polarised IPEC-J2, cell death was significant upon 24-h addition of DON at concentrations as low as 0.8425–33.7 µmol/L. A 24-h treatment with Que at 16.5 µmol/L concentration resulted in significantly increased cell viability, while 33 µmol/L Que concentrations caused significant cell death in IPEC-J2 cells using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Chen et al., 2018). Our results were similar to those found in that study as we found that Que was cytotoxic at concentrations of 75 µmol/L or higher. In contrast, using the NR assay Vergauwen et al. (2016) found that IPEC-J2 cells showed cell death only from 800 µmol/L concentration of Que for 18 h. Yang et al. (2020) studied the effects of Que and DON on human, non-tumourigenic gastric epithelial cells (GES-1) and found that the treatment regimen involving 6.25 µmol/L Que pretreatment for 2 h was suitable for further investigations according to the results of CCK8 cytotoxicity and lactate dehydrogenase (LDH) release assays. Based on data of GES-1 cells which were pretreated with 6.25 µmol/L Que for 2 h followed by the administration of 5 µmol/L DON, it was confirmed that preincubation of the cells with 6.25 µmol/L Que led to better cell viability values compared to those observed in cells treated with 5 µmol/L DON.

Polarised cells form strong barriers through the development of tight junctions between them. The TER of the epithelial cell monolayers was found to be a good indicator of the degree of epithelial integrity. Pinton et al. (2009) observed a dose-dependent decrease in TER values in differentiated IPEC-1 following treatment with 5 µmol/L DON after an incubation time of 24 h. TER values were significantly reduced in IPEC-J2 cell monolayers after 24-h exposure to basolaterally added DON at 6.74 µmol/L concentration (Diesing et al., 2011). Springler et al. (2016) reported that DON reduced TER significantly at 5–20 µmol/L concentration after 24 h. Our results are consistent with these findings, as TER significantly dropped after 24-h incubation with 1 µmol/L DON in polarised IPEC-J2 cells. Our co-treatment experiment also showed that 20 µmol/L Que was not able to prevent the harmful effects of DON.

Several publications have assessed the influence of Que on a Caco-2 cell line. Suzuki and Hara (2009) found that Que in a concentration range of 10–100 µmol/L significantly increased TER after an incubation time of 24 and 48 h. Amasheh et al. (2008) reported that 200 µmol/L of Que was the most effective concentration in improving TER in Caco-2 cells after 24 h. Vergauwen et al. (2016) concluded that the preincubation of IPEC-J2 cells with 25–200 µmol/L Que for 18 h could strengthen the integrity of the cell monolayer. In our experiments, IPEC-J2 cells were pretreated with 20 µmol/L Que for 24 h and the TERs of cell monolayers were increased significantly. Carrasco-Pozo et al. (2013) observed that 33 µmol/L Que significantly elevated the TER values in Caco-2 cells when the non-steroidal anti-inflammatory drug indomethacin was given at a concentration of 250 µmol/L. Our results are in good correlation with these findings, as the TER values of cells co-treated and pretreated with 20 µmol/L Que were significantly higher than those measured in cells treated with 1 µmol/L DON after an incubation time of 25 h.

In the literature, there are only few publications in connection with the extracellular H_2O_2 production in IPEC-J2 cells. As Pászti-Gere et al. (2015) reported, IPEC-J2 cells are appropriate model systems to study the effects of different molecules on the redox status of cells. In our experiments it was found that extracellular H_2O_2 production was significantly elevated after 25-h treatment with 1 µmol/L DON. Pretreatment with 20 µmol/L Que followed by

exposure to 1 µmol/L DON for 1 h did not show significant changes in H_2O_2 production compared to control values. Co-treatment with 20 µmol/L Que and 1 µmol/L DON for 1 h significantly increased the H_2O_2 concentration. According to these findings, the pretreatment has a potential to prevent DON-induced extracellular H_2O_2 production, while a 1-h-long treatment with Que was not able to hinder the effect of DON-induced H_2O_2 overproduction.

Kang et al. (2019) reported that DON at a concentration of 6.7 µmol/L significantly elevated intracellular ROS levels in IPEC-J2 cells after 24-h mycotoxin exposure, as measured by the DCFH-DA assay. Vergauwen et al. (2016) carried out experiments on IPEC-J2 cells with 5-(and-6-)-chloromethyl-20,70-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) using various concentrations of Que, and confirmed that Que could reduce intracellular ROS. Using the DCFH-DA assay, Chen et al. (2018) demonstrated that the ROS content in IPEC-J2 cells was reduced dramatically by Que at 16.5 µmol/L concentration. In our study, 1 µmol/L DON significantly increased the intracellular ROS content. The cells treated with 20 µmol/L Que did not show differences compared to the control cells. While the Que-pretreated cells exposed to DON showed a significant increase in fluorescence intensity, these values were significantly lower compared to those of cells treated with DON only.

In conclusion, Que at a concentration of 20 µmol/L supports the cell monolayer to maintain optimal intestinal epithelial barrier integrity. It was also observed that pretreatment with Que displays a favourable effect against the DON-induced oxidative imbalance. In contrast, the simultaneous, short (1-h long) treatment with Que did not contribute to the restoration of the tipped redox homoeostasis induced by DON administration.

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