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Deoxynivalenol, but not fumonisin B1, aflatoxin B1 or diesel exhaust particles disrupt integrity of the horse's respiratory epithelium and predispose it for equine herpesvirus type 1 infection

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ABSTRACT

The horse's respiratory tract daily encounters a plethora of respirable hazards including air pollutants, mycotoxins and airborne pathogens. To date, the precise effect of air pollution and mycotoxins on respiratory epithelial integrity and subsequent pathogen invasion in the horse has not been studied. Here, diesel exhaust particles (DEP) and three major mycotoxins (deoxynivalenol [DON], aflatoxin B1 [AFB1] and fumonisin B1 [FB1]) were applied to the apical surfaces of both *ex vivo* respiratory mucosal explants and *in vitro* primary equine respiratory epithelial cells (EREC) cultivated at the air-liquid interface, prior to inoculation with equine herpesvirus type 1 (EHV1). DON, but not AFB1, FB1 and DEP affected epithelial integrity in both *ex vivo* and *in vitro* systems, as demonstrated by histological changes in respiratory epithelial morphology and a drop in transepithelial electrical resistance across the EREC monolayer. Further, DON-pretreated explants showed on average 6.5 ± 4.5-fold more EHV1 plaques and produced on average 1 $\log_{10}$ more extracellular virus particles compared to control diluent- and FB1-pretreated respiratory mucosal explants. Similarly, EHV1 infection was greatly enhanced in EREC upon pretreatment with DON. Based on our findings, we propose that inhalation of DON predisposes horses for EHV1 infection by affecting respiratory epithelial integrity.

1. Introduction

In modern equestrian society, the air breath by horses is inevitably filled with respirable hazards, ranging from dust and ammonia to mycotoxins, pollens and air pollutants. Despite this daily struggle, the well-armed respiratory tract of most horses seems to cope efficiently with these constant threats. Still, a vast number of horses develop asthma or other chronic respiratory diseases during their life-span (Bracher et al., 1991; Robinson et al., 2006; Winder and Von Fellenberg, 1987; Wood et al., 2005). In addition, (subclinical) respiratory disease is one of the mainly recognized causes of poor performance in competing horses and thus, has serious economic impacts on the horse industry worldwide (Allen et al., 2006; Couetil and Denicola, 1999; Pirrone et al., 2007). Besides imposing direct damage to the respiratory tract's mucosa, these threats might predispose the horse for subsequent pathogen invasion. Indeed, we previously showed that pollen proteases disrupt integrity of the horse's respiratory epithelium and therefore predispose it for subsequent infection with equine herpesvirus type 1 (EHV1) (Van Cleemput et al., 2019). So far, the precise effect of dust, ammonia, mycotoxins and air pollution on the horse's respiratory mucosa and its role in subsequent EHV1 infection remains obscure. Since mycotoxins and air pollutants have been shown to alter epithelial intercellular junctions, this study examined their effect on the horse's respiratory epithelium using well-established *ex vivo* and *in vitro* equine models (Bracarense et al., 2012; Gao et al., 2017; Gerez et al., 2015; Heussen and Alink, 1992; Lehmann et al., 2009; Quintana et al., 2011; Van Cleemput et al., 2017; Van De Walle et al., 2010; Vandekerckhove et al., 2009).

Mycotoxins are toxic secondary metabolites produced by fungi mainly belonging to the genera *Fusarium*, *Aspergillus* and *Penicillium*. Aflatoxins, ochratoxins, fumonisins, trichothecenes (*e.g.* deoxynivalenol) and zearalenone are five frequently encountered mycotoxins in equine feeds such as green forages, hay, silages and grains (Liesener et al., 2010; Ogunade et al., 2018). Upon inhalation in the

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horse's respiratory tract, these toxins or potentially toxin-producing moulds can cause acute respiratory disease, such as guttural pouch mycosis and/or a more chronic asthma-like syndrome, recurrent airway obstruction (RAO) (Laan et al., 2006; Lepage et al., 2004). Deoxynivalenol, aflatoxin and fumonisin have already been shown to alter epithelial integrity in intestinal epithelia *in vivo* and *in vitro* through inhibition of intercellular junction protein synthesis (Bracarense et al., 2012; Gao et al., 2017; Gerez et al., 2015; Van De Walle et al., 2010). Here, we elucidated their impact on respiratory epithelial integrity and on subsequent infection with the alphaherpesvirus EHV1.

Since equestrian enterprises are often located in (sub)urban regions, horses are regularly exposed to air pollution. Air pollution majorly consists of diesel exhaust, a mixture of particulate matter, hydrocarbons (*e.g.* benzene), gases (*e.g.* nitrogen oxide or NOx), sulfur and particulate matter, produced during the combustion of diesel fuel in engines from vehicles, as well as industrial plants. Especially the particulate matter, mainly comprised of soot, contributes to the toxic effects of diesel exhaust as these small particles (ranging from 0.1 µm to 10 µm) can easily reach the deeper structures of the respiratory tract upon inhalation (Mohankumar and Senthilkumar, 2017; Reis et al., 2018). Diesel exhaust particles (DEP) are therefore commonly used to study the toxic effects of air pollution. Upon inhalation, these DEP cause oxidative stress with accompanying inflammatory processes and DNA damage (Grevendonk et al., 2016; Ito et al., 2000; Ma and Ma, 2002; Shukla et al., 2000; Zhao et al., 2009). High concentrations of DEP have been shown to alter tight junctions in 16HBE cells, a human bronchial epithelial cell line (Lehmann et al., 2009). By facilitating antigen delivery to subepithelial antigen-presenting cells, epithelial barrier dysfunction is involved in the initiation or progression of allergic diseases (Xiao et al., 2011). In addition, diesel exhaust-induced inflammatory processes typically promote the expression of a T helper 2 immunologic response, stimulate mucus secretion and induce bronchial smooth muscle contractions (Meldrum et al., 2017; Totlandsdal et al., 2015; Zhao et al., 2009). These symptoms are hallmarks of the development of allergic diseases and asthma. Finally, tumorigenesis is promoted through the induction of DNA damage and the alteration of gap junction intercellular communication by diesel exhaust compounds (Heussen and Alink, 1992; Rivedal and Witz, 2005; Song and Ye, 1997).

Together, this study is the first to examine the effect of mycotoxins and diesel exhaust compounds on the barrier function of the equine respiratory epithelium and its susceptibility to subsequent EHV1 infection.

2. Material and methods

2.1. Reagents

Mycotoxins aflatoxin B1 (AFB1) from *Aspergillus flavus* (A6636), fumonisin B1 (FB1) from *Fusarium moniliforme* (F1147) and deoxynivalenol (DON, D0156) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were dissolved in ethanol (1 mM AFB1, 10 mM FB1, 50 mM DON). Diesel exhaust particles (DEP) were purchased from Sigma-Aldrich (DEP NIST® SRM® 2975) and stock solutions were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/mL.

2.2. Tissue collection and processing

The tracheae from different healthy horses were collected at the slaughterhouse and transported in phosphate-buffered saline (PBS) with calcium and magnesium, supplemented with 0.1 mg/mL gentamicin (ThermoFisher Scientific, Waltham, MA, USA), 0.1 mg/mL kanamycin (Sigma-Aldrich), 100 U/mL penicillin, 0.1 mg/mL streptomycin (ThermoFisher Scientific) and 0.25 µg/mL amphotericin B (ThermoFisher Scientific).

2.2.1. Respiratory mucosal explant isolation and cultivation

Trachea mucosal explants were prepared as previously described (Van Cleemput et al., 2017; Vandekerckhove et al., 2009).

2.2.2. EREC isolation and cultivation

Primary equine respiratory epithelial cells (EREC) were isolated and cultured as described by Quintana et al. (2011) and Van Cleemput et al. (2017).

2.3. Mycotoxin treatment of respiratory mucosal explants and EREC

2.3.1. Respiratory mucosal explants

Explants were cultured 24 h for adaptation before thoroughly washing and embedding them in agarose diluted in MEM, to mimic *in vivo* conditions, as previously published (Vairo et al., 2013; Van Cleemput et al., 2017). Next, the apical surface of the epithelium was exposed for 3 h at 37 °C to 2 nM AFB1, 10 µM FB1, 50 µM DON or 1 µg/mL DEP, dissolved in serum-free medium (DMEM/RPMI [ThermoFisher Scientific], supplemented with 0.1 mg/mL gentamicin, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 µg/mL amphotericin B). An ethanol- and DMSO-based diluent was used as solvent control. Explants were removed from the agarose and washed three times in PBS and fixed in a phosphate-buffered 3.5% formaldehyde solution for 24 h, either immediately after the last wash or after an additional 24 h incubation on metal gauzes. Explants were then stored in 70% alcohol until further processing. Cell viability in the explants was evaluated by means of terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labelling (TUNEL) and was not significantly affected upon different treatments.

2.3.2. EREC

Cells were grown to confluence and the transepithelial electrical resistance (TEER) was measured daily until a steady TEER of ˜500-700 $\Omega \cdot cm^{-2}$ was attained. Mycotoxins, DEP, ethanol (control) or DMSO (control) were dissolved in EREC medium (DMEM/F12, containing 2% Ultroser G [Pall Life Sciences; Pall Corp., Cergy, France], 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 1.25 µg/mL amphotericin B) at the above-described concentrations and were applied onto the apical EREC surfaces. Cells were incubated for 3 h and cell viability was evaluated by means of ethidium monoazide bromide (EMA) staining and was not significantly affected upon different treatments.

2.4. Assessment of epithelial cell integrity

2.4.1. Respiratory mucosal explants

Integrity of respiratory epithelial intercellular junctions was verified by examining the intercellular space herein on haematoxylin-eosin stained paraffin coupes, as previously described (Van Cleemput et al., 2017).

2.4.2. EREC

To assess epithelial integrity of the EREC, the transepithelial electrical resistance (TEER) was measured using an epithelial voltohmmeter (Millipore corporation, Bedford, MA, USA). The net resistance was calculated by subtracting the background resistance and multiplying the resistance by the surface area of the membrane.

2.5. Viral infection assays

2.5.1. Virus

A Belgian EHV1 isolate (03P37) was used in this study and originates from the blood of a paralytic horse collected during an outbreak in 2003 (van der Meulen et al., 2003a). The virus was propagated on rabbit kidney (RK13) cells and used at the 6th passage.

2.5.2. Respiratory mucosal explants

Explants were cultured at the air-liquid interface for 24 h, prior to extensive washing and embedment in agarose. Next, explants were exposed to the reagents or control diluents for 3 h, as described above. Following a washing step for removal of the reagents, the apical surface of the epithelium was inoculated with $10^{6.5}$ $TCID_{50}$ of the 03P37 EHV1 strain for 1 h at 37 °C. Explants were removed from the agarose and washed 3 times in PBS to remove non-adherent virus particles. Finally, explants were placed back onto their gauzes and serum-free medium was added. Twenty-four hours post-inoculation, explants were placed in methylcellulose-filled plastic tubes and frozen at −80 °C until further processing.

2.5.3. EREC

EREC were fully grown in a transwell cell culture system prior to treatment with the reagents or control diluents. Following a thorough washing step, cells were exposed for 1 h to 100 µL EHV1 03P37 strain (MOI of 1) at the apical surface. Non-adsorbed virus particles were removed by washing the EREC three times with DMEM/F12. Fresh EREC medium was added to the platewells and cells were further incubated at the air-liquid interface. Ten hours post inoculation, cells were fixed in methanol for 20 min at −20 °C and stored dry at −20 °C until further processing.

2.6. Immunofluorescence staining and confocal microscopy

2.6.1. Respiratory mucosal explants

Explants were embedded in methylcellulose and snap-frozen for subsequent cryosectioning. Sixteen µm thick cryosections were cut using a cryostat at −20 °C and loaded onto 3-aminopropyltriethoxysilane-coated (Sigma-Aldrich) glass slides. Slides were then fixed in 4% paraformaldehyde for 15 min and subsequently permeabilized in 0.1% Triton-X 100 diluted in PBS. Non-specific binding sites were blocked by 15 min incubation with avidin and biotin (ThermoFisher Scientific) at 37 °C. To label late viral glycoproteins, a polyclonal biotinylated horse anti-EHV1 antibody was used for 1 h at 37 °C (van der Meulen et al., 2003b), followed by incubation with streptavidin-FITC® (ThermoFisher Scientific) for 1 h at 37 °C. The basement membrane of the respiratory mucosa was stained with monoclonal mouse anti-collagen VII antibodies (Sigma-Aldrich), followed by secondary Texas Red®-labelled goat anti-mouse antibodies (ThermoFisher Scientific). Nuclei were detected by staining with Hoechst 33342 (ThermoFisher Scientific). Slides were mounted with glycerol-DABCO and analysed using a Leica (TCS SPE) confocal microscope. The total number of plaques was counted on 50 cryosections and plaque latitude was measured using the Leica confocal software package.

2.6.2. EREC

Antibodies were incubated directly in the transwells for 1 h at 37 °C. Cells were first incubated with a 1:1000 dilution of a polyclonal rabbit anti-immediate early protein (IEP) antibody, kindly provided by Dr. D. O'Callaghan, Louisiana State University, USA. The diluent used was PBS containing 10% negative goat serum. This was followed by incubation with a goat anti-rabbit IgG FITC®-conjugated antibody (ThermoFisher Scientific). Nuclei were counterstained with Hoechst 33342 for 10 min at 37 °C. Transwell membranes were excised from the culture inserts and mounted on glass slides using glycerol-DABCO. Slides were examined using a Leica confocal microscope. The total number of plaques was counted on 5 random fields of approximately 3×10^4 cells per insert. Plaque latitude was measured on 10 individual plaques using the Leica confocal software package.

2.7. Virus titration

Twenty-four hours after inoculation, explant supernatant was collected and stored at −80 °C until titration. EHV1 titrations were conducted on RK13 cells, which were incubated at 37 °C for 7 days. Titers were expressed as $TCID_{50}$.

2.8. Statistical analyses

Significant differences ($P < 0.05$) between different treatments were identified by one-way analysis of variances (ANOVA) followed by Tukey's post-hoc test. If homoscedasticity of the variables was not met as assessed by Levene's test, the data were log-transformed prior to ANOVA. Normality of the residuals was verified by the use of the Shapiro-Wilk test. If the variables remained heteroscedastic or normality was not met after log-transformation, a Kruskall-Wallis' test, followed by a Mann-Whitney's post-hoc test were performed. All analyses were conducted in IBM SPSS Statistics for Windows, version 25.0 (IBM Corp, Armonck, NY, USA).

3. Results

3.1. Deoxynivalenol (DON), but not alfatoxin B1 (AFB1), fumonisin B1 (FB1) and diesel exhaust particles (DEP) disrupt respiratory epithelial intercellular junctions

Mycotoxins and diesel exhaust particles (DEP) have already been shown to alter the integrity of intestinal and lung epithelial cells, respectively (Bracarense et al., 2012; Gao et al., 2017; Gerez et al., 2015; Lehmann et al., 2009; Van De Walle et al., 2010). Here, we examined for the first time their effect on the horse's respiratory epithelium using *ex vivo* respiratory mucosal explants and *in vitro* primary equine respiratory epithelial cells (EREC). The examined concentrations were based on data from the above-mentioned studies and most likely correspond to clinically relevant concentrations. For instance, diesel exhaust particle concentrations in the ambient air of urban regions go up to 25 µg/m^3 (Steiner et al., 2016). In addition, a global mycotoxin survey found deoxynivalenol (DON) and fumonisin B1 (FB1) doses of up to 2–20 mg/kg in European roughage and cereal feeds (Biomin, 2016). Taking into account that the horse's nose is often in direct contact with these feeds, it seems reasonable to assume that inhaled mycotoxin concentrations range from several nanograms to even micrograms per mL.

3.1.1. Respiratory mucosal explants

As shown in Fig. 1A left panel, the percentage of intercellular spaces in the respiratory epithelium of equine mucosal explants was significantly ($P < 0.05$) higher after treatment with 50 µM DON (10 ± 2%), but not after treatment with 2 nM AFB1 (3 ± 1%), 10 µM FB1 (2 ± 1%) or 1 µg/mL DEP (2 ± 1%), compared to after treatment with control diluent (2 ± 1%). Increasing the concentration of AFB1, FB1 or DEP by ten-fold did not alter the intercellular spaces, but did decrease cell viability, as assessed with TUNEL staining. Cell viability in the respiratory mucosal explants did not significantly drop after treatment with 50 µM DON or control diluent. Increasing the concentration of DON by 2-fold resulted in a significant drop in cell viability. Twenty-four hours after the treatment, samples were analysed to determine whether the respiratory epithelium was able to repair its intercellular junctions. Indeed, the percentage of intercellular space in the respiratory epithelium of the explants pretreated with DON decreased from 10 ± 2% to 3 ± 1% (Fig. 1A, right panel). Fig. 1B shows representative haematoxylin-eosin-stained images of explants fixed at 3 h post-treatment (upper panel) or 24 h after the 3 h treatment (lower panel).

3.1.2. EREC

EREC attained a steady transepithelial electrical resistance (TEER) of ~500–700 Ω·cm^{-2} after 5–7 days of incubation at the air-liquid interface in a transwell cell culture system. The TEER significantly dropped to baseline levels after treatment with DON, but not after

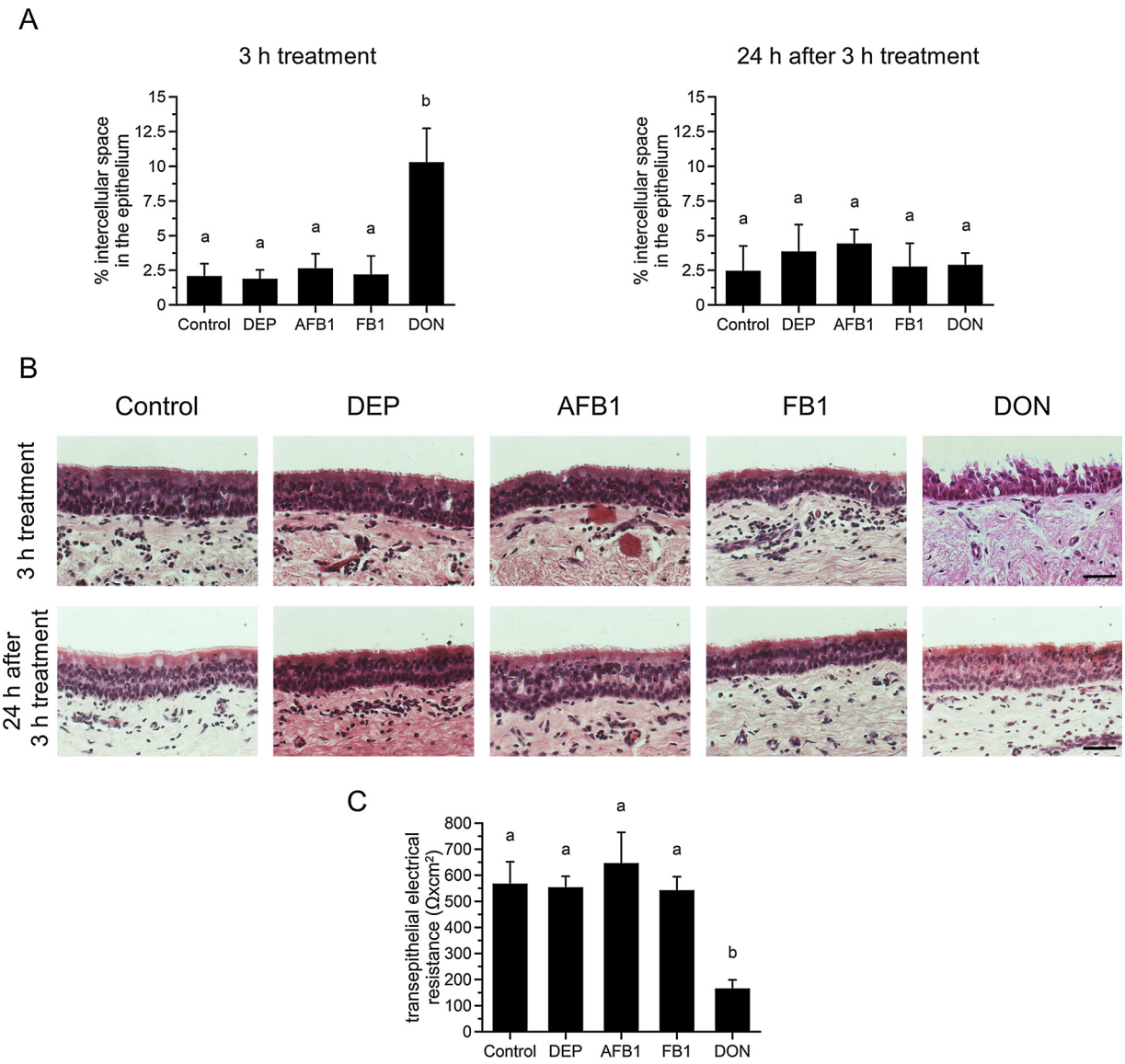

Fig. 1. The effect of diesel exhaust particles (DEP) and mycotoxins (aflatoxin B1 [AFB1], fumonisin B1 [FB1] and deoxynivalenol [DON]) on respiratory epithelial integrity. (A) The percentage of intercellular space in the epithelium of equine respiratory mucosal explants after 3 h treatment (left) and 24 h after the 3 h treatment (right). Experiments were performed on respiratory mucosal explants of 3 individual horses and data are represented as means + SD. Different letters indicate significant ($P < 0.05$) differences between different treatments. (B) Representative haematoxylin-eosin-stained images of respiratory mucosal explants 3 h after treatment (upper panel) and 24 h after the 3 h treatment (lower panel). The scale bar measures 50 µm. (C) The transepithelial electrical resistance of EREC 3 h after treatment. Experiments were performed on the EREC of 3 individual horses and data are represented as means + SD. Different letters indicate significant ($P < 0.05$) differences between different treatments.

treatment with AFB1, FB1, DEP or control diluent (Fig. 1C). DON (50 µM) did not alter cell viability, as determined by EMA-staining.

3.2. DON predisposes respiratory epithelial cells for subsequent EHV1 infection

We previously showed that disruption of intercellular junctions predisposes respiratory epithelial cells for EHV1 infection (Van Cleemput et al., 2017). Since this phenomenon was observed for both neurovirulent and non-neurovirulent EHV1 strains, we chose to include only one important EHV1 field strain (03P37) in the present study. DON affected epithelial integrity in both respiratory mucosal explants and EREC. Therefore, we analysed the effect of DON on subsequent EHV1 infection. FB1 was included as an internal control, as this mycotoxin did not affect the intercellular junctions of explants or EREC.

3.2.1. Respiratory mucosal explants

Number of plaques - As shown in Fig. 2A left panel, the number of plaques per 50 cryosections significantly ($P < 0.05$) increased from 5 ± 5 in control diluent-pretreated explants to 32 ± 20 in DON-pretreated explants. The number of plaques following FB1 pretreatment (5 ± 2) did not significantly differ from the number of plaques in control diluent-pretreated explants.

Plaque latitude - The plaque latitude gives an indication about the ease of viral spread in the explant epithelium and is shown in Fig. 2A, middle panel. The average latitude of EHV1 plaques increased significantly from 101 ± 14 µm in control diluent-pretreated explants to 180 ± 73 µm after DON pretreatment. The average EHV1 plaque latitude in FB1-pretreated explants (103 ± 41 µm) was similar to control diluent-pretreated explants.

Virus titer - Increased viral replication in the epithelium most likely

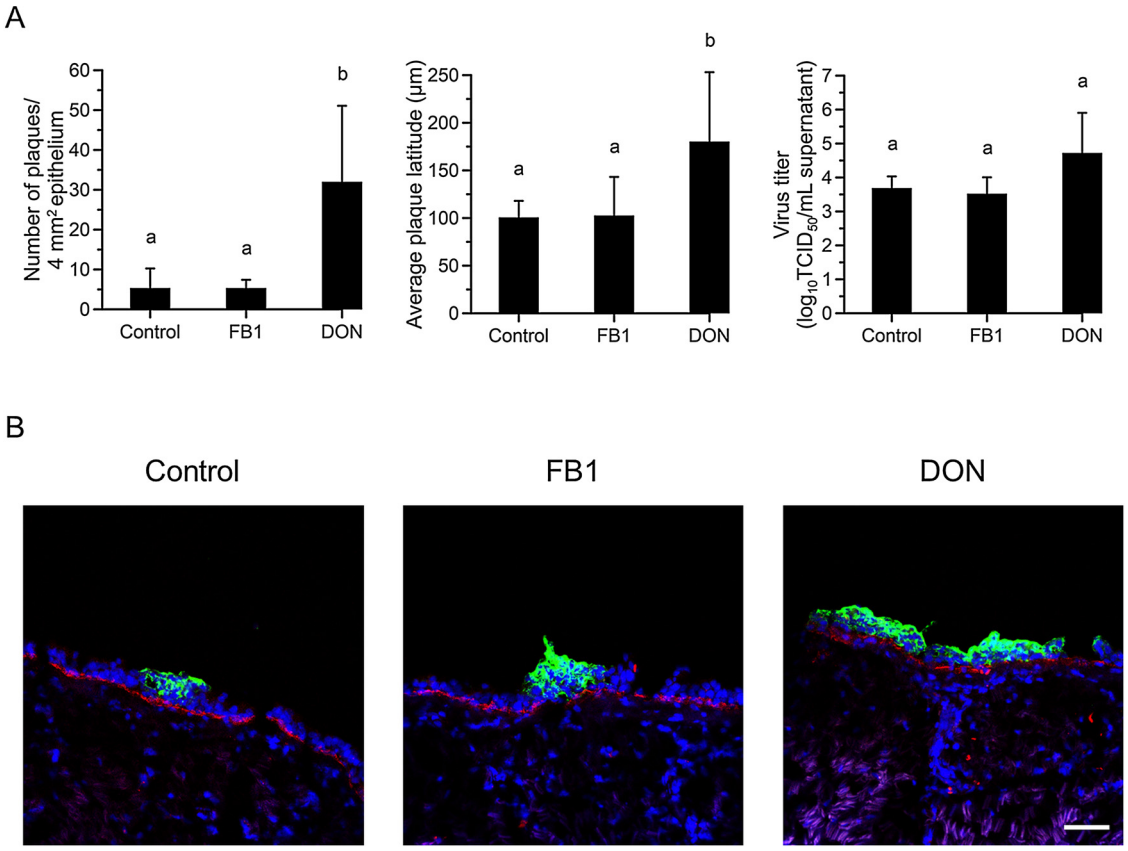

Fig. 2. EHV1 infection of respiratory mucosal explants after pretreatment with PBS (control) or mycotoxins (fumonisin B1 [FB1] and deoxynivalenol [DON]). Explants were frozen 24 hpi and cryosections were stained for late viral antigens. (A) The total number of plaques was counted on 50 consecutive cryosections (left panel), the average plaque latitude was calculated based on a maximum of 10 individual plaques (middle panel) and the extracellular virus titer was determined by titration on RK13 cells (right panel). Experiments were performed on 3 individual horses and data are represented as means + SD. Different letters indicate significant ($P < 0.05$) differences in pretreatments. (B) Representative confocal images of EHV1 plaques (green) in respiratory mucosal explants. The basement membrane is shown in red. Cell nuclei were counterstained with Hoechst 33342 (blue). The scale bar represents 50 µm..

results in an increased production of extracellular infectious virus particles. Indeed, virus titrations (Fig. 2A, right panel) showed that the supernatant of DON-pretreated explants contained on average a 1 $\log_{10}$ higher virus titer (4.74 ± 1.18) than control- or FB1-pretreated explants (3.69 ± 0.34 and 3.52 ± 0.48). However, this increase was not significant ($P = 0.196$). Representative confocal images of EHV1 plaques in the respiratory mucosal explants are shown in Fig. 2B.

3.2.2. EREC

Number of plaques - On 3×10^4 EREC, we counted an average of 4 ± 2 EHV1 plaques following control diluent pretreatment of the cells (Fig. 3A, left panel). Following disruption of intercellular junctions with DON, the number of EHV1 plaques in 3×10^4 EREC significantly ($P < 0.05$) increased to 16 ± 3. Pretreatment of EREC with FB1 did not significantly increase the number of EHV1 plaques (3 ± 3 on 3×10^4 EREC), compared to control diluent pretreatment.

Plaque latitude - No significant difference in EHV1 plaque latitude was found between different pretreatments (Fig. 3A, right panel). Representative confocal images of EHV1 plaques in EREC are shown in Fig. 3B.

4. Discussion

With every breath, a number of potential hazards enter the horse's respiratory tract. In today's equestrian world, these hazards range from air pollutants to dusts and moulds. For example, horses kept in (sub) urban regions are constantly exposed to air pollution caused by traffic and industrial activities. Although Lehmann et al. (2009) reported that diesel exhaust particles (DEP) affected integrity of a human bronchial epithelial cell (16HBE) monolayer, we did not observe this in primary isolated equine respiratory epithelial cells (EREC). The loss of specific differentiation markers during the immortalization process of 16HBE cells might have caused this discrepancy. Indeed, the formation of a tight and realistic intercellular network within a respiratory epithelial cell monolayer is one of the hallmarks of differentiation (Knust and Bossinger, 2002). Furthermore, the authors showed that by co-culturing 16HBE cells with dendritic cells, the damaging effect of DEP on epithelial integrity was no longer observed. The authors proposed that by phagocytosing a major amount of deposited DEP, dendritic cells lowered the DEP burden on the epithelial cells. Similarly, dendritic cells might have phagocytosed DEP in our *ex vivo* respiratory mucosal explants. In our primary EREC culture system, residual dendritic cells could presumably also have removed part of the DEP.

Moulds and their toxic secondary metabolites, *i.e.* mycotoxins, are ubiquitous contaminants in equine feeds such as green forages, hay, silages and grains (Liesener et al., 2010; Ogunade et al., 2018). While eating, especially from up-hanging hay racks, the horse's nose is in close contact with food and thus potentially breaths in a considerable amount of these toxic substances. The respiratory epithelium is the first barrier to encounter incoming hazards and could therefore be exposed to high doses of mycotoxins. Until now, the specific impact of these mycotoxins on the equine respiratory epithelium has not been studied. Previous studies examined the role of mycotoxins on epithelial integrity focussing on intestinal cells (Bracarense et al., 2012; Gao et al., 2017; Gerez et al., 2015; Van De Walle et al., 2010). Here, we used an

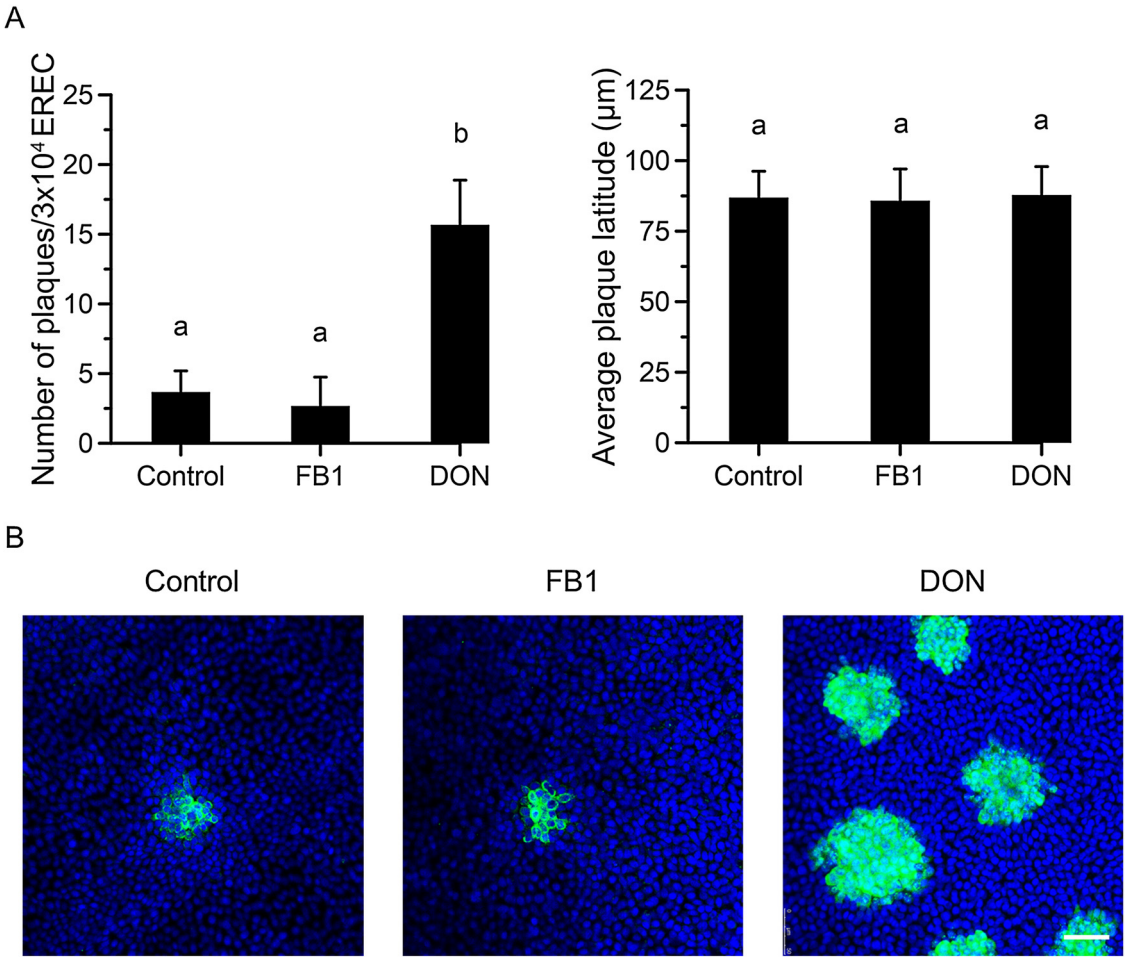

Fig. 3. EHV1 infection of equine respiratory epithelial cells (EREC) after pretreatment with PBS (control) or mycotoxins (fumonisin B1 [FB1] and deoxynivalenol [DON]). Cells were fixed 10 hpi and stained for immediate early protein (IEP). (A) The total number of plaques was counted in five different fields of approximately 3×10^4 cells for each condition (left). Average plaque latitudes were measured on 10 individual plaques (right). Experiments were performed in triplicate on primary EREC of 3 different horses. Data are represented as means + SD and the different letters indicate significant ($P < 0.05$) differences between pretreatments. (B) Representative confocal images of EHV1 IEP-positive plaques (green) in EREC monolayers. Nuclei were detected with Hoechst 33342 (blue). The scale bar represents 50 µm.

established *ex vivo* equine respiratory mucosal explant model and *in vitro* primary equine respiratory epithelial cells, grown on transwells, to study the effect of mycotoxins.

Deoxynivalenol (DON), but not fumonisin B1 (FB1) or aflatoxin B1 (AFB1) damaged epithelial integrity in both mucosal explants and EREC. Several other groups already reported that exposure to DON decreases the transepithelial electrical resistance (TEER) of intestinal epithelial cell monolayers (Kasuga et al., 1998; Pinton et al., 2009; Van De Walle et al., 2010). This decrease in TEER was caused by a selective downregulation of claudin-4 and E-cadherin, but not of occludin or zonula occludens (ZO)1 protein expression upon DON exposure (Bracarense et al., 2012; Pinton et al., 2009; Van De Walle et al., 2010). It has already been described that tricothecenes (*e.g.* DON) inhibit protein neosynthesis by blocking the peptidyl transferase subunit of actively translating ribosomes (Feinberg et al., 1989). Consequently, DON exposure of intestinal epithelial cells led to a significant decrease in the level of alkaline phosphatase and several nutrient transporters (Kasuga et al., 1998; Maresca et al., 2002; Turner et al., 2008). Therefore, most authors concluded that the DON-induced down-regulation of claudin-4 and E-cadherin was caused by a block in protein neosynthesis. It is not surprising that the expression of occludin was not affected upon exposure to DON, as this tight junction protein is recycled from and to the cell surface and thus, its formation depends less on protein neosynthesis (Morimoto et al., 2005). In addition, blocking the ribosomal subunit by DON also induced the onset of an inflammatory cascade in both intestinal cells, as well as in immune cells. (Moon and Pestka, 2002; Moon et al., 2007; Ouyang et al., 1996; Sergent et al., 2006; Van De Walle et al., 2008; Zhou et al., 2003). By impacting on protein synthesis and increasing inflammation, a thin line exists between a cytotoxic or non-cytotoxic outcome upon exposure to DON. Indeed, in our experiment, DON did not affect cell viability at the carefully chosen concentration and duration of exposure. However, increasing the concentrating of DON by 2-fold or the duration time by 6-fold rapidly resulted in a significant loss of cell viability. Likewise, very low concentrations of AFB1 and FB1 were required in order to avoid the onset of apoptosis. These results complement those of others, reporting AFB1- and FB1-induced cytotoxicity at low concentrations (Du et al., 2017; Wan et al., 2013; Zhang et al., 2015).

In contrast to previous studies examining the effects of chronic dietary mycotoxin exposure, we studied merely the acute effect on respiratory epithelial cells. As the nasal cavities filter out incoming debris, mycotoxin concentrations are likely to reach peak values in the surface-lining epithelium (Harkema et al., 2006). However, the mucociliary escalator and sneezing/coughing reflex rapidly remove incoming hazards upon inhalation, limiting the duration of exposure (Cohn and Reinero, 2007). Nonetheless, this acute exposure was sufficient to destroy epithelial integrity in both our respiratory mucosal explants and EREC.

Here, we demonstrated that the respiratory epithelium is more susceptible to EHV1 infection after complete disruption of the epithelial barrier by the mycotoxin DON. We previously demonstrated that EHV1 targets a receptor located at the basolateral surface of epithelial cells (Van Cleemput et al., 2017). Consequently, this receptor becomes exposed upon disruption of epithelial integrity and subsequent loss of epithelial polarity. These data strongly suggest that feeding deoxynivalenol-contaminated roughage or grain predisposes horses for a primary EHV1 infection. Therefore, mould and mycotoxin levels in equine feeds and surroundings should be better monitored. In this context, a global mycotoxin survey found DON doses of up to 2–20 mg/kg in European roughage and cereal feeds (Biomin, 2016). It seems reasonable to propose that these food-derived mycotoxins are inhaled and deposited on the horse's respiratory epithelium during feeding. For instance, roughage is often put in up-hanging hay racks or nets, forcing horses to feed with an upright head position. In this way, horses inevitably inhale the accompanying dust, moulds and mycotoxins. Future studies should measure the exact amount of mycotoxins in equine feeds. Next, the exact percentage of these food-derived mycotoxins that ends up in the horse's respiratory tract should be determined. Based on these results, new scientifically approved threshold levels of such mycotoxins in equine feeds could be implemented.

Finally, besides EHV1, multiple other airborne pathogens might benefit from this defective barrier. For instance, adenoviruses and reoviruses likewise preferentially infect the basolateral surface of respiratory epithelial cells (Excoffon et al., 2008; Zabner et al., 1997).

5. Conclusions

Our study identified for the first time deoxynivalenol, a mycotoxin ubiquitously present in all animal feeds, as a major airborne threat to the horse, as it significantly affected respiratory epithelial integrity and morphology. In contrast, two other mycotoxins (fumonisin B1 and aflatoxin B1) did not affect respiratory epithelial integrity, although they were cytotoxic at very low concentrations. Similarly, diesel exhaust particles had no effect on equine respiratory epithelial integrity. Furthermore, DON pretreatment predisposed the horse's respiratory epithelium for EHV1 infection. Thus, our data strongly suggest that feeding mycotoxin-contaminated roughage or grains impacts on the equine respiratory tract and thereby favours a primary EHV1 infection. In this context, the horse's nose is typically in close contact with food while eating, especially in up-hanging hack racks, and thus potentially breaths in a considerable amount of these toxic substances. Based on our results, we want to emphasize that horse owners should feed their horses hay and grain from ground level. In addition, future studies should determine threshold levels of mycotoxins in horse feeds and surroundings.

Together, these novel insights can help us better understand the complex impact of ambient respirable hazards on the horse's respiratory tract and the onset of EHV1 infection.

Conflicts of interest

The authors declare no conflict of interest.

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