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Feed exposure to FB1 can aggravate pneumonic damages in pigs provoked by *P. multocida*

Melinda Kovács [a,b], Roland Pósa [a], Tamás Tuboly [c], Tamás Donkó [a], Imre Repa [a], János Tossenberger [a], Judit Szabó-Fodor [b], Stoycho Stoev [d,*,1], Tibor Magyar [e]

[a] Faculty of Animal Science, Kaposvár University, Guba Sándor u. 40, H-7400 Kaposvár, Hungary
[b] MTA-KE Mycotoxins in the food chain Research Group, Guba Sándor u. 40, H-7400 Kaposvár, Hungary
[c] Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University, Hungária krt 23-25, H-1143 Budapest, Hungary
[d] Dept of General and clinical pathology, Faculty of Veterinary Medicine, Trakia University, Stara Zagora 6000, Bulgaria
[e] Institute for Veterinary Medical Research, Centre for Agricultural Research, Hungarian Academy of Sciences, Hungária krt. 21, H-1143, Budapest, Hungary

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ABSTRACT

The possible interaction between *Pasteurella multocida* and the mycotoxin fumonisin B1 (FB1), recognised as one of the most often food/feed contaminant, was studied with the aim to evaluate whether and how FB1 can influence and/or complicate the development and severity of various pathological damages provoked by *Pasteurella multocida* in some internal organs of pigs. Heavier lung pathology was seen in pigs experimentally infected with *Pasteurella multocida*, when the same were exposed to 20 ppm dietary levels of fumonisin B_1 (FB_1) as was assessed by gross pathology, pathomorphological examinations, clinical biochemistry and some immunological investigations. The most typical damages in FB_1 treated pigs were the strong oedema in the lung and the slight oedema in the other internal organs and mild degenerative changes in the kidneys, whereas the typical pathomorphological findings in pigs infected with *Pasteurella multocida* was broncho-interstitial pneumonia. FB1 was found to aggravate pneumonic changes provoked by *P. multocida* in the cranial lobes of the lung and to complicate pneumonic damages with interstitial oedema in the lung. No macroscopic damages were observed in the pigs infected only with *Pasteurella multocida.* It can be concluded that the feed intake of FB1 in pigs may complicate or exacerbate the course of P. multocida serotype A infection.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by certain fungi that grow in target agricultural products susceptible to mold infestation. The most mycotoxins have strong immunosuppressive effects and often compromise animals and human health provoking development of secondary bacterial infections (Stoev et al., 2000) or deteriorating some existing diseases (Pósa et al., 2011, 2013). It is well known, that Fumonisin B_1 (FB_1) is one of the most frequent contaminant of the feeds for pigs in all over the world (Dutton, 2009). In Hungary, FB_1 was found in relatively high percentage of maize used as animal feed (Fazekas et al., 1998).

FB_1 was found to be the cause of a number of outbreaks of equine leukoencephalomalacia (Conkova et al., 2003) and porcine pulmonary oedema killing many horses and pigs in the U.S. during 1989 and 1990 (Marasas, 2001). These lesions appeared to be a consequence of impairment of vascular permeability provoked by disruption of sphingolipid metabolism by FB_1 (Ramasamy et al., 1995). FB_1 is also suspected to be responsible for the pulmonary fibrosis in pigs, which develops in the case of chronic exposure and follows oedematous changes in the lung (Zomborszky-Kovács et al., 2002).

Some studies clearly confirmed that immunosuppression may be the first expressed toxic effect of some mycotoxins, such as ochratoxin A (Stoev et al., 2000). It was also found that FB_1 given at diet levels of 10 ppm, has immunosuppressive effect on humoral immune response in pigs (Stoev et al., 2012) and therefore can provoke some secondary bacterial infections, which arise in immunocompromised animals. In this regard, Oswald and collaborators observed a significant increase of intestinal colonization of pathogenic *Escherichia coli* in FB_1-treated pigs (Oswald et al., 2003). On the other hand, a heavy progression of *Pasteurella multocida* (Halloy et al., 2005) or porcine reproductive and respiratory syndrome (PRRS) virus infections in swine (Ramos et al., 2010) were seen when the same animals were compromised by feed exposure to FB_1. There are also some recent reports about aggravations of pathological infections in pigs, when fed on FB1 contaminated diet (Pósa et al.,

Abbreviations: BAL, Bronchoalveolar lavage; FB_1, fumonisin B1; PA, phagocytic activity; PBS, phosphate buffered saline; SA, sphinganine; SO, sphingosine; TNF, tumor necrosis factor.

* Corresponding author at: Department of General and Clinical Pathology, Faculty of Veterinary Medicine, Trakia University, Students campus, 6000 Stara Zagora, Bulgaria.
E-mail address: stoev@uni-sz.bg (S. Stoev).
[1] Currently in Faculty of Science, University of Johannesburg, South Africa.

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2011, 2013). Nevertheless, the data available about the possible interaction between FB_1 and some frequent infectious pathogens in pigs can be evaluated as insuficient and scarce. As FB1 is a frequent contaminant of pigs' diet, and *P. multocida* is the most frequent secondary pathogen which can generate a respiratory disorder in predisposed or immunocompromised pigs (Halloy et al., 2005), such studies could have significant scientific and/or practical importance. In this regard, the data on the predisposing effect of FB_1 on the appearance and/or progression of the lesions provoked by toxigenic strain of *P. multocida,* serotype A and the interaction between the toxin and the bacterium are scarce (Halloy et al., 2005), independently of the circumstance that both agents (toxic and infectious) have the same target organ localization (lung), and the same can be encountered simultaneously in the pig production farms.

It is also implied the problem of recognising the pathological damages provoked by FB_1 and/or the respective infectious agents, especially in the case when such damages are very similar or localized in the same internal organ such as lung. In our former experiment we found that feeding a diet containing 10 ppm FB_1, in combination with *Bordetella bronchiseptica* and toxigenic *Pasteurella multocida* serotype D infection, increased the incidence and the extension of pulmonary lesions in pigs as well as aggravated the severity of the same lesions (Pósa et al., 2011). This experiment is the next step of our framework programme designed to reveal what can be the real adverse effects of FB_1 on various contagious or non-contagious diseases having similar pathology and whether the same mycotoxin is able to facilitate or complicate the typical pathological finding of these diseases. In the current experiment, we also studied the possible interaction of *Pasteurella multocida* with the mycotoxin FB_1, recognised respectively as one of the most often food/feed contaminant, with the aim to evaluate whether and how FB_1 can influence and/or complicate the development and/or severity of various pathological changes provoked by *Pasteurella multocida* in some internal organs of pigs.

2. Materials and methods

2.1. Experimental design

The piglets used in this study were obtained from a Seghers hybrid herd (Hungaro-Seghers Ltd., Mohács) in which the incidence of respiratory diseases was low and were free from five major infectious diseases (Brucellosis, Leptospirosis, Pasteurellosis, Aujeszky and porcine reproductive and respiratory syndrome – PRRS). The sows ($n = 10$) delivering the piglets were also serologically free from *Pasteurella multocida* and serologically negative for *M. hyopneumoniae*. Twenty eight 3-days old female piglets of 2.25 ± 0.3 kg average body weight, obtained from the same sows and received colostrum to get maternal (colostral) immunity were divided in four groups (7 piglets in each) using the principle of equality, and housed in two separate rooms in elevated-level battery cages of identical size as follow: (1) the two non-infected groups (Group A — controls and Group B — FB_1 exposed) were kept in one of the rooms in two separated cages, and (2) the infected with *Pasteurella multocida* groups (Group C and Group D — FB_1 exposed) were housed in the other room in a similar way. Both rooms were adjusted to identical air temperature (27 °C) using thermostat-regulated central heating, while the required air exchange was ensured by the use of exhaust fans. The battery cages, the drinkers and the rooms were cleaned twice a day, and the piglet-rearing installations were dismantled and washed off every second day. The respective hygienic and/or protective measures as wearing of protective clothes and foot & hand disinfection with the aqueous solution of Virkon S (KRKA d. d./Antec International Ltd., Novo Mesto, Croatia) were strictly respected at the time of entering or exiting the rooms.

2.2. Feeding and water supply

Up to day 16 from the birth all experimental piglets were fed on milk replacer consisting of skimmed milk powder, vegetable fats and whey powder, containing 23% crude protein, 23% crude fat and 1.6% lysine (Salvana Ferkel Ammen Milch, Salvana Tiernahrung GmbH, Klein-Offenseth Sparrieshoop, Germany) from an automatic feeder (Sloten B.V., Deventer, The Netherlands).

From day 7, a dry creep feed of coarse meal form, containing 16 MJ/kg energy, 18.5% crude protein, 9% crude fat and 1.65% lysine (Salvana Pre-meal, Salvana Tiernahrung GmbH, Klein-Offenseth Sparrieshoop, Germany) was also given *ad libitum* to the piglets, and then, from day 16 until the end of the experiment (day 40) only the latter was available to them.

Fresh drinking water was available *ad libitum* from nipple drinkers. At the beginning of the experiment (between day 0 and 7), drinking water was also provided by the free water surface of the plastics drinking bowls.

2.3. Mycotoxin exposure

The necessary quantity of FB_1 was produced by the fungus *Fusarium verticillioides* as described previously (Fodor et al., 2006) as the final fungal culture typically contained 3–4 mg/g FB_1, and small quantities of less toxic compounds FB_2 and FB_3 (0.3–0.6 mg/g). Starting from day 14, a defined quantity of the same fungal culture was thoroughly homogenised into the piglets' ration of groups B and D to give the required concentration of 20 ppm (mg/kg feed) FB_1 in the diet and these groups were exposed to the same feed levels of FB_1 until the end of the experiment (day 40), i.e. over a period of 26 days.

FB1 and FB2 concentration in the fungal culture and in the diet was checked using LC-MS system (LC-MS 2020 Single Quadrupole Mass Spectrometer, LC-20AD pumps with DGU-20A degasser, SIL-20ACHT autosampler, CTO-20-AC Column Owen and CBM-20A Interface, SHIMADZU, Kyoto, Japan), and the diet did not contain other mycotoxins (such as T-2, zearalenone, deoxynivalenol, ochratoxin A, aflatoxins, etc.) in detectable quantities. Concentration of T-2 and total aflatoxin was measured by ELISA kits, AgraQuant® T-2 Toxin Assay and AgraQuant® Total Aflatoxin Assay (RomerLabs, Singapur), respectively, following the instructions of the producer. The basic diet was also free from detectable quantities of the mycotoxins assayed.

2.4. Experimental infection

On day 14, the pigs of groups C and D were infected with toxigenic strain of *Pasteurella multocida* serotype A (strain DE3011, 4.4×10^8 CFU/mL). The bacterial suspensions were prepared as described previously (Magyar et al., 2002). A volume of 1.0 mL was inoculated through an endotracheal tube in all cases.

Microbiological investigations revealed no presence of other respiratory pathogens in the inoculum as well as in the lungs of the control and infected pigs at the end of the study except the main pathogen *Pasteurella multocida* found only in experimentally infected pigs.

2.5. Immunisation with ovalbumin

All piglets were injected intraperitoneally with 100 μg ovalbumin/animal on days 19 and 27 in order to provoke immune response. 100 μg ovalbumin was solved in 400 μL phosphate buffered saline (PBS, Sigma-Aldrich, Hungary) and thereafter 400 μL incomplete Freund adjuvant (Sigma-Aldrich, Hungary) was added.

2.6. Blood clinico-biochemical and immunological investigations

Blood samples were taken from the *v. cava cranialis* on days 14 (the beginning of the experiment), 27 and 39 after the respective narcosis and premedication as described below.

Serum clinical/biochemical parameters were determined in a professional veterinary laboratory (Vet-med Laboratory, Budapest, Hungary), using Roche Hitachi 912 Chemistry Analyzer (Hitachi, Tokyo, Japan)

with commercial diagnostic kits (Diagnosticum, Budapest, Hungary), respectively. The concentration of total protein (TP), albumin (ALB), glucose (G), total cholesterol (CHOL), urea, and the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) were measured as appropriate.

At the end of the experiment the blood samples taken during exsanguination (see later) were analysed for certain immunological parameters.

2.7. Phagocyte number and phagocytic activity

The determination of number of phagocytic cells and phagocytic activity (PA) was done according to Simpson et al. (1979) as described in Material and Methods with some modifications. Peripheral white blood cells (WBC) were isolated by density gradient centrifugation (400 ×g for 15 min) using Ficoll-Paque (Pharmacia) according to standard protocols. The number of viable PBLs was determined by trypan blue exclusion in a haemocytometer. The cells were diluted in DMEM supplemented with penicillin and streptomycin (Sigma-Aldrich, Hungary) antibiotics and 10% fetal bovine serum. Cells were plated at 1×10^5 cells/well density into 6 well plates. The cultures were incubated for 4 days at 37 °C under 5% CO_2 tension.

PA was determined with Congo-red stained yeast cells by incubating overnight at 37 °C. Phagocytic ability was expressed as percentage of phagocytic cells quantified from 100 cells observed under a microscope.

2.8. Ovalbumin Specific IgG and tumor necrosis factor alpha (TNF) from the blood

Specific circulating antibody titers were measured using microtiter plates (Sigma-Aldrich, Hungary) coated with 100 μL of ovalbumin solution (10 μg/mL ovalbumin in PBS) per each well, incubated overnight at 4 °C in a humidified environment. Excess protein was removed by washing with 3 × 150 μL PBS-Tween 20 (Sigma-Aldrich, Hungary). Plasma samples were twofold serially diluted in the washing solution, starting with 1:50, in a separate plate. Aliquots of 100 μL of each dilution (1:50 and 1:100) were transferred to the microtiter plate (two wells for each dilution) and incubated for 1 h at room temperature. Wells were washed again 3 times with PBS-Tween and anti-swine IgG-HRPO conjugate (Sigma-Aldrich, 1:25,000) was added to each well, followed by incubation at RT for 1 h. The wash step was then repeated and the substrate (3,3′,5,5′-Tetramethylbenzidine, Sigma-Aldrich) added. Optical density was measured in an ELISA reader at 450 nm.

For tumor necrosis factor alpha (TNF-α) quantification total RNA was purified from blood samples using the InnuPREP Blood RNA Kit (Analytikjena) as recommended by the manufacturer. Reverse transcription of the RNA templates was performed with the M-MuLV RevertAid™ Reverse Transcriptase using random hexamer primers (100 pmol/sample) (Fermentas, Lithuania). Polymerase chain reaction was done according to the method described by Jung et al. (2007), serial dilutions of cloned cDNA were used for generating a standard curve of concentration. Melting curve analysis to confirm target-specific amplification was performed following PCR.

2.9. IgA from the lung lavage fluid taken at necropsy

Total amount of IgA antibodies present in bronchoalveolar lavage was measured using a direct sandwich IgA ELISA kit (Abcam) following the manufacturer's recommendations. Samples were diluted 1:1000 in sample diluent, standard curve was constructed by measuring the OD of twofold dilutions of the standard IgA provided with the kit.

2.10. Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage (BAL) was performed aseptically at necropsy (on day 40) with 50 ml PBS. Lavage fluid was gently dispensed into and aspirated from the right cranial, middle, and caudal lung lobes.

2.11. Gross pathology and pathomorphological examination

At the end of the experiment (on day 40) the pigs were narkotized with sodium-pentobarbital (Euthanyl, Bimeda, MTC Animal Health) and thereafter exsanguinated and subsequently examined for macroscopical and pathomorphological changes in various internal organs. Materials for histological examination were taken from the lung (areas with and without macroscopic changes), trachea, kidneys, liver, hearth, brain, cerebellum, spleen, mesenterial lymph nodes, small and large intestine and then fixed in 10% neutral buffered formalin. Fixed tissues were embedded in paraffin wax, sectioned at 6 μm and stained with hematoxylin-eosin. Periodic acid — Schiff stain was also used for proving of lipoproteid, glycoproteid or mucoproteid substances in various tissues and cell components. Some materials were stained according to Weigert iron hematoxylin for proving the presence or absence of fibrin in lymphatic cysts or oedematous areas.

2.12. Body weight and health status

The clinical signs (including nasal discharge, sneezing, panting, hoarseness, coughing and dyspnoea) as well as body temperature were recorded daily during the experiment. The piglets were weighed on 3, 14, 27 and 40 days of age. Feed consumption of the groups was registered daily.

2.13. Sphingolipid profile test

At the end of the experiment (on day 40) the free sphinganine to sphingosine ratio, the most sensitive biomarker of fumonisin toxicosis (Riley et al., 1993), was determined in the blood serum by a method described by Kametler et al. (2006).

2.14. Statistical analysis

Statistical analysis of the data obtained was carried out by the SPSS for Windows statistical software package using the version 11.5 (SPSS, 2002). Effect of treatment was analyzed by the One-Way Analysis of Variance. The significance of between group differences was tested by the LSD post hoc test.

2.15. Animal welfare permission and ethical standards

The experimental design as well as the housing, maintaining and slaughtering of pigs was performed in accordance with European Guidelines and Ethic Principles for the care and use of animals for research purpose and the Hungarian Welfare Regulations. The study protocol, i.e., experimental infection, blood sampling applied in this study were authorised by the Food Chain Safety and Animal Health Directorate of the Somogy County Agricultural Office, under the permission number SOI/31/241–3/2013.

3. Results

3.1. Growth rate, clinical signs

There were no significant differences in the body weight of the pigs between various experimental groups as measured on days 14, 27 and 39 of the experiment.

No clinical signs were observed in the control and FB_1 treated groups throughout the experiment. In *P. multocida* infected groups (C and D),

starting from day 3 after the pathogen *inoculation*, part of the infected animals in the group exposed simultaneously to FB_1 and *P. multocida* exhibited a temporary increase in the body temperature and slight coughing in addition to occasional sneezing and a slight serous nasal discharge.

3.2. Biochemical and immunological investigations

On day 39, the free sphinganine (SA) to sphingosine (SO) ratio (SA/SO), known to be a biomarker of FB_1 toxin, was significantly elevated ($P < 0.05$) in the blood of groups B and D (2.41 ± 0.49 and 2.7 ± 0.45, respectively) exposed to FB1 as compared to the groups A and C fed on toxin free diet (0.68 ± 0.11 and 0.72 ± 0.44, respectively), indicating that there was an effect exerted by the toxin at a cellular level.

Among blood clinical chemical parameters ALT and AST revealed mild hepatic injury in toxin treated animals, as enzyme activities were significantly higher on the 3rd sampling dates in these animals compared to controls or infected (but not FB_1 treated) animals (Table 1).

Phagocyte count and activity, as well as specific IgG and the cytokine TNF did not respond on any of the treatments. Only locally secreted IgA concentration in the lung was altered in FB_1 treated groups, resulted in less antibody production (Table 2).

3.3. Gross pathology

The blood vessels in the mesenterium in some pigs of the control and experimental groups were slightly hyperaemic. Mesenterial lymph nodes of the same pigs were slightly enlarged and small foci of local peritonitis were seen in most of the pigs (experimental and control).

3.3.1. Control pigs

Generally, there were not any persistent macroscopical changes in the internal organs evaluated in the pigs from the control group, excluding a small swelling in the thymus of one pig from this group looking-like an abscess.

3.3.2. Fumonisin B_1 treated pigs

Macroscopic findings during the necropsy revealed that kidneys of pigs from this group were slightly pale than normal with rarely seen focal hyperaemic areas. The interstitial tissue of lung in all examined pigs was well visible and thickened, which suggested about interstitial oedema in the lung (Fig. 1). Slight hyperaemia was seen in the brain envelopes. Gall bladder was slightly enlarged only in one pig of this group.

Table 1
Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) activity in the blood serum of pigs exposed to FB1 and/or infected with *P. multocida* compared to control animals.

Age (days) at sampling						
	14		27		39	
AST (U/l)	Mean	SD	Mean	SD	Mean	SD
A (control)	31,9	8,9	47,6	14	$21,7^{a}$	8,1
B (FB_1)	$29,9^{A}$	4,7	$62,8^{B}$	11,2	$42,6^{bAB}$	15
C (Pm)	29,1	7,7	43,3	18	$31,8^{a}$	6,5
D (Pm + FB_1)	$31,3^{A}$	6,4	$50,6^{AB}$	17,9	$47,3^{bB}$	4,1
	14		27		39	
ALT (U/l)	Mean	SD	Mean	SD	Mean	SD
A (control)	$37,3^{A}$	6,8	$51,4^{AB}$	8,7	$63,8^{aB}$	5,6
B (FB_1)	$44,7^{A}$	5,8	$56,7^{A}$	10,5	$75,7^{bB}$	5,3
C (Pm)	$37,9^{A}$	5,4	$57,3^{B}$	9	$60,6^{aB}$	4,3
D (Pm + FB_1)	$40,1^{A}$	3,7	60^{AB}	15,4	$75,7b^{B}$	7,8

Different indices indicate significant difference between (^{a,b}) treatments or (^{A,B}) sampling dates.
FB_1: fumonisin B1, Pm: *Pasteurella multocida*, C: control.

3.3.3. Pasteurella multocida infected pigs

Macroscopic findings during the necropsy revealed that kidneys of pigs were slightly pale. Spleen of one pig was only enlarged. Jejunum of pigs showed signs of local inflammation (jejunitis). The gall bladder was slightly enlarged in part of the pigs of this group. In the lung, only small bluish to reddish pneumonic areas were sometimes observed in the cranial lobes.

3.3.4. Fumonisin B_1 treated and Pasteurella multocida infected pigs

Macroscopic findings during the necropsy revealed that kidneys of pigs from this group were slightly pale than normal with rarely seen focal hyperaemic areas. The interstitial tissue of lung in all examined pigs was well visible and thickened, which suggested about interstitial oedema or pneumonia in the lung. Small bluish to reddish pneumonic areas were observed sometimes in the cranial lobes. Slight hyperaemia and purulent exudate was observed in the nasal cavity around the conch. Slight hyperaemia was seen in the brain envelopes. Jejunum of pigs showed signs of local inflammation (jejunitis). Gall bladder was slightly enlarged in most of the pigs of this group. Spleen of 3 pigs was enlarged.

3.4. Pathomorphological findings

3.4.1. Control pigs

Generally, there were not any persistent pathomorphological changes in various internal organs of the pigs from control group, excluding slight degenerative changes in the liver and some degenerative and/or necrotic changes in addition to leucocyte infiltration in the thymus of one pig, which could be bound to macroscopic visible abscess in the same organ.

3.4.2. Fumonisin B_1 treated pigs

Pathomorphological investigation revealed that the most typical pathomorphologiacal damage in the pigs of this group was seen in the permeability of vessels, which was responsible for perivascular and especially pericapillary oedema in various internal organs and especially in the lung.

In the lung, the most typical pathomorphological changes were the oedema and accumulation of serous or sero-fibrinous exudate in the interlobular or perivascular tissue of all examined pigs (Fig. 2). Increased quantity of the mucus on the mucosal surface and a slight oedema in the mucosa or submucasa were seen also in the trachea (Table 3).

In the brain, only slight hyperaemia and perivascular or pericellular oedema and slight vacuolization or colliquative (lytic) changes in the cortex of the brain as well as lytic changes in some of the neurons and glia cells were seen. Slight hyperaemia and oedema were also seen in the brain envelopes and/or ventricles.

In the cerebellum, the only recognized damages were the slight edema and degenerative changes (mainly lysis) in the region of the Purkinje's cells as well as the slight lytic or colliquative changes in the corpus medullare or laminae medullares of white matter.

In the kidneys, the most persistent pathomorphological changes were the slight or moderate vacuolar/granular degeneration in the epithelium of proximal tubules, the hyperaemia of vessels and peritubular capillaries, the slight activation of capillary endothelium, the perivascular or pericapillary edema and the slight enlargement of lymphatic spaces.

In the liver, the irregular staining (eosinophilia or cloudy swelling) or slight degenerative changes in some hepatocytes and the slight hyperaemia of vessels and the slight activation of capillary endothelium were observed.

In the spleen, there was only a slight hyperaemia and sometimes a slight enlargement or oedema in the trabeculae.

In the mesenterial lymph nodes, pathomorphological changes corresponding to lymphadenitis simplex such as enlargement and

Table 2
Immunological parameters (mean ± SD) of pigs exposed to FB1 and/or infected with *P. multocida* compared to control animals at the end of the experiment (at day 39 of their age).

Group	Phagocyte count/ml[1]	Phagocyte activity (%)[1]	TNF[2] (Ct value)	IgG[2] (mg/ml)	IgA[3] (mg/ml)
A (control)	366 ± 15	81 ± 3	20.0 ± 1.6	6.7 ± 0.8	5.4 ± 0.3[a]
B (FB_1)	362 ± 26	80 ± 3	19.7 ± 1.9	5.4 ± 0.9	4.4 ± 0.4[b]
C (Pm)	375 ± 13	80 ± 3	20.4 ± 1.2	5.7 ± 0.9	4.9 ± 0.3[a]
D (Pm + FB_1)	383 ± 41	79 ± 3	19.4 ± 2.0	5.7 ± 0.7	4.4 ± 0.3[b]

Different indices ([a,b]) indicate a significant difference between treatments.
FB_1: fumonisin B1, Pm: *Pasteurella multocida*, C: controls.
[1] Measured in whole blood.
[2] Blood plasma.
[3] Lung lavage fluid — it is only taken at day 40.

hyperplasia of lymph follicles and lymphoid tissue were rarely seen in some of the pigs.

In the small- and large intestines, only slight degenerative changes in superficial epithelium or slight increasing of the glandular secretion and the quantity of Goblet cells, were seen.

In the myocardium, the only observed changes were irregular staining or slight lytic changes in some myofibrils.

3.4.3. Pasteurella multocida infected pigs

Pathomorphological investigation revealed that the most common pathomorphologiacal damage in the pigs of this group was seen in the lung. The thickening of the alveolar septa due to a slight to moderate mononuclear inflammatory infiltration containing mainly macrophages and a small quantity of lymphocytes and/or leucocytes was seen in some regions of the lung. The same mononuclear cell infiltration was also seen in interlobular and perivascular or peribronchiolar interstitial tissue. An exudate containing the same mononuclear cells and leucocytes was rarely present in the alveolar lumens and sometimes in the bronchioles only in two pigs of this group. A slight proliferation and/or hyperplasia of alveolar/bronchiolar epithelium were also observed (Fig. 3). A slight to moderate congestion of capillaries and larger vessels was seen. The same lesions were located in the cranial and slightly in the caudal lobes. Increased quantity of the mucus was seen also in the trachea.

In the brain, there were not any visible pathological changes. A slight perivascular and/or pericellular oedema and a slight vacuolization or colliquative (lytic) changes in the cortex under the brain envelopes was observed only in one of the pigs examined. A slight hyperaimia of vessels in the brain envelopes was rarely seen.

In the cerebellum, there were not any visible pathological damages. Only a slight hyperaemia of some vessels was seen.

In the kidneys, there were not any significant pathological damages.

In the liver, the irregular staining (eosinophilia or cloudy swelling) or slight degenerative changes, mainly granular degeneration in some hepatocytes were only observed.

In the spleen, there were not any visible pathological changes, excluding the slight predomination of red pulp over the white pulp in some of the pigs.

In the mesenterial lymph nodes, pathomorphological changes corresponding to lymphadenitis simplex were rarely seen in some of the pigs.

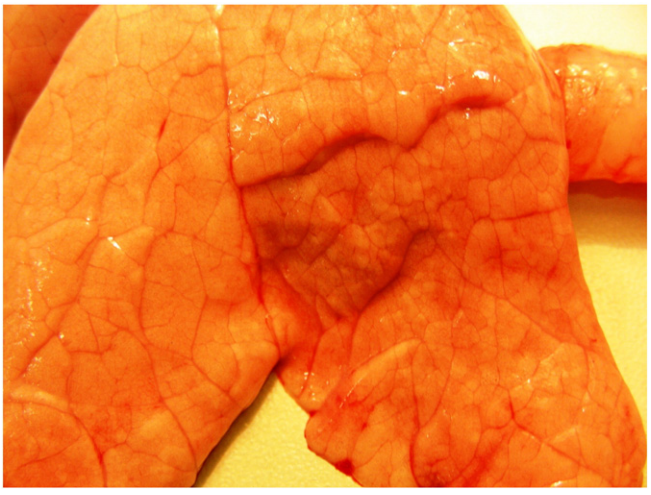
In the small- and large intestines, slight degenerative changes were seen in superficial epithelium in addition to a slight increasing of the glandular secretion, incl. The increasing of the quantity of Goblet cells and a local histiocyte proliferation.

In the myocardium, no pathological changes were seen.

3.4.4. Fumonisin B_1 treated and Pasteurella multocida infected pigs

Pathomorphological investigation confirmed that the most common pathomorphologiacal damage in the pigs of this group was seen again in the permeability of vessels and the subsequent perivascular and especially pericapillary oedema in various internal organs and especially in the lung.

The most typical pulmonary damages were the thickening of the alveolar septa due to a slight to moderate mononuclear inflammatory infiltration containing mainly macrophages and a small quantity of lymphocytes and/or leucocytes. Slight or moderate oedema and accumulation of serous or sero-fibrinous exudate in the interlobular or perivascular tissue were seen. The same inflammatory mononuclear cell infiltration was also seen in interlobular and perivascular or peribronchiolar interstitial tissue. An exudate containing the same mononuclear cells, desquamated epithelial cells and a lot of leucocytes was often present in the alveolar lumens and sometimes in the



Fig, 1. Thickened interstitial tissue suggesting for interstitial oedema in the lung. Lung of pig given FB1.

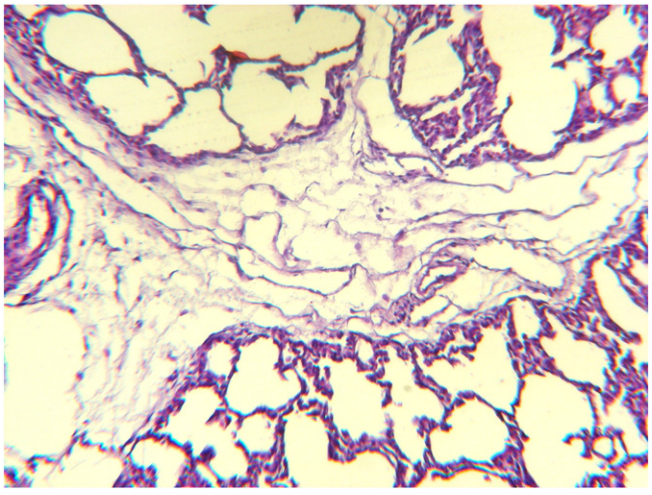


Fig. 2. Oedema and accumulation of serous or sero-fibrinous fluid in the interlobular tissue. Lung of pig given FB1. X/E. ×200.

Table 3
Pathomorphological lesions in some internal organs of the pigs exposed to FB1 and/or infected with *P. multocida* at the end of the experiment (at day 40 of their age).

Pathomorphological lesions in the lung	FB_1	Pm	FB_1 + Pm
Oedema and accumulation of serous or serofibrinous exudate in interlobular and/or perivascular tissue	++++	−	+++++
Thickening of interalveolar septa due to inflammatory cell infiltration of macrophages, lymphocytes and leucocytes	−	++	+++
Proliferation and/or hyperplasia of alveolar/bronchiolar epithelium	−	++	++
Perivascular/peribronchiolar/interlobular mononuclear cell infiltration of macrophages, lymphocytes and leucocytes	−	++	+++
Accumulation of exudate with a lot of leucocytes, macrophages and/or desquamated epithelial cells in the alveolar lumina and in the bronchi or bronchioles	−	+	+++
Increased quantity of mucus on the mucosal surface and/or oedema in the tracheal mucosa or submucosa	++	+	+++
Hyperaemia of capillaries or larger vessels	−	++	+++
Pathomorphological lesions in the brain			
Perivascular and/or pericellular oedema	+++	+	+++
Oedema in the meninges or ventricles	++	−	++
Hyperaemia in the brain or meninges	++	+	++
Lytic changes and/or vacuolization in the cortex	++	+	++
Lytic changes in the neurons and glia cells	++	−	++
Pathomorphological lesions in the cerebellum			
Oedema and lysis in the region of the Purkinje's cells	++	−	++
Lytic changes in the white matter	++	−	++
Hyperaemia of vessels	−	+	+
Pathomorphological lesions in the kidneys			
Vacuolar or granular degeneration in the tubular epithelium	++	−	+++
Hyperaemia of vessels and peritubular capillaries	++	−	++
Activation of capillary endothelium	+	−	+
Perivascular oedema and enlargement of lymphatic spaces	++	−	++
Oedema around the ureters and hyaline casts in tubules	−	−	+
Pathomorphological lesions in the liver			
Cloudy swelling and granular degeneration in hepatocytes	+	+	+
Hyperaemia and activation of capillary endothelium	+	−	+
Pathomorphological lesions in the spleen			
Oedema and/or hyperaemia in the trabeculae	+	−	+
Predomination of red pulp over the white pulp	−	+	+
Pathomorphological lesions in the intesines/mesent. lns			
Enlargement of lymph follicles in mesenterial lymph nodes	+	+	+
Increase in glandular secretion and degenerative changes	+	+	+
Pathomorphological lesions in the myocardiums			
Irregular staining or lytic changes in some myofibrils	+	−	+

+ − slight damage or damage seen occasionally in few pigs.
++ − slight to moderate damage seen in less than half of the pigs.
+++ − moderate damage seen in all pigs or strong damage seen in less than half of the pigs.
++++ − strong damage seen in more than half of the pigs, but not all of them.
+++++ − strong damage seen in all pigs.
FB_1: fumonisin B1, Pm: *Pasteurella multocida.*
Notes: No damages were found in control animals (Group A) except slight hyperaemia of vessels, which was presumably due to the narcosis.

bronchioles in five pigs of this group (Fig. 4). A slight proliferation and/or hyperplasia of alveolar/bronchiolar epithelium were also observed. A slight to moderate congestion of capillaries and larger vessels was observed. The same lesions were located in the cranial and slightly in the caudal lobes. An increased quantity of the mucus and a slight oedema in the mucosa or submucosa were seen in the trachea.

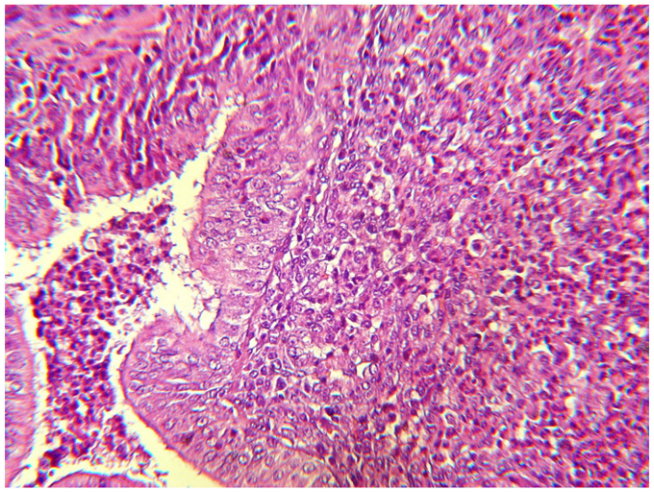


Fig. 3. Thickening of the alveolar septa due to a slight to moderate mononuclear inflammatory infiltration of macrophages and lymphocytes and/or leucocytes in addition to proliferation of alveolar and/or bronchiolar epithelium. An exudate containing the same cells and leucocytes in alveolar/bronchiolar lumens. Lung of pig infected with P. multocida. X/E. ×260.

In the brain, only slight hyperaemia and perivascular or pericellular oedema and slight vacuolization or colliquative (lytic) changes in the cortex of the brain as well as lytic changes in some of the neurons and glia cells were seen. A slight focal proliferation of the glia cells was also observed. Slight hyperaemia and oedema were seen in the brain envelopes and/or ventricles (Fig. 5).

In the cerebellum, there were only slight edema and degenerative changes (mainly lysis) in the region of the Purkinje's cells as well as slight lytic or colliquative changes in the corpus medullare or laminae medullares of white matter. A slight hyperaemia of some vessels was also seen.

In the kidneys, the most common pathomorphological changes were the slight or moderate vacuolar/granular or hyaline degeneration in the epithelium of proximal tubules, the hyperaemia of vessels and/or peritubular capillaries, the slight activation of capillary endothelium, the perivascular or pericapillary edema and the slight enlargement of lymphatic spaces. Hyaline casts were seen in some tubules. Slight oedematous changes were seen around the ureters of some pigs.

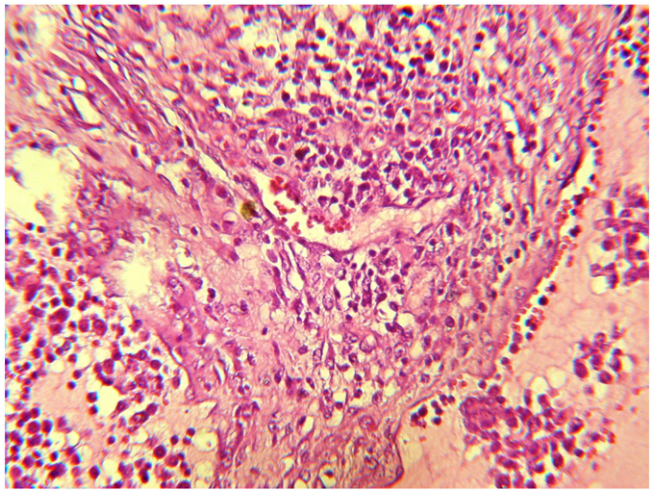


Fig. 4. An exudate containing mononuclear cells, desquamated epithelial cells and a lot of leucocytes in the alveolar lumens and bronchioles. Accumulation of serous or serofibrinous exudate in the interlobular or perivascular tissue. Lung of pig given FB1 and infected with P. multocida. X/E. ×260.

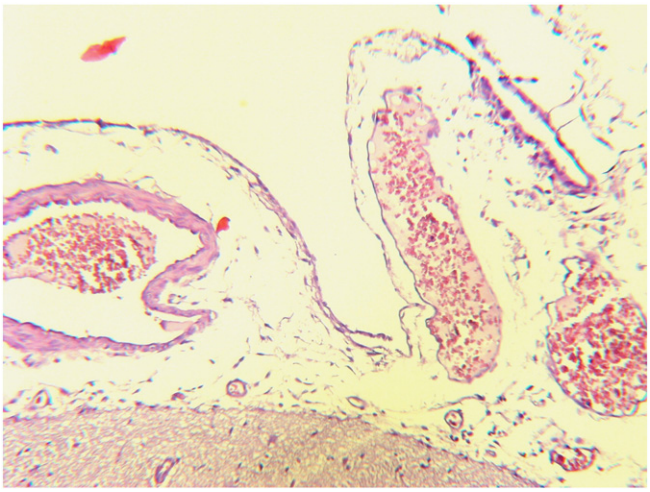


Fig. 5. Slight hyperaemia and oedema in the brain envelopes. Brain of pig given FB1 and infected with *Pasteurella multocida*. X/E. ×200.

In the liver, there were slight degenerative changes, mainly granular degeneration, in some hepatocytes and/or irregular staining (eosinophilia or cloudy swelling). A slight hyperaemia of vessels and slight activation of capillary endothelium were also observed.

In the spleen, there was only a slight hyperaemia and a slight enlargement or oedema in the trabeculae. A slight predomination of immature lymphocytes in the lymph follicles and rarefied white pulp were observed. A slight predomination of the red pulp over the white pulp in some of the pigs was also seen.

In the mesenterial lymph nodes enlargement and hyperplasia of lymph follicles and lymphoid tissue were rarely seen in some of the pigs.

In the small- and large intestines, slight degenerative changes in superficial epithelium or slight increasing of the glandular secretion such as increasing of the quantity of Goblet cells and a local histiocyte/lymphocyte proliferation in mucosal propria, were rarely seen (Fig. 6).

In the myocardium, there was only irregular staining or slight lytic changes in some myofibrils.

4. Discussion

FB_1 was found to have a suppressing effect on phagocytic activity of pulmonary macrophages and to prevent the removal of particulate matter and bacteria from the circulation (Smith et al., 1996). This circumstance is considered to be a possible reason for the increased susceptibility to *P. multocida* infection in swine (Halloy et al., 2005) as seen in the present study. It was also supposed, that the increased susceptibility of porcine pulmonary capillary endothelium to FB_1 (Gumprecht et al., 2001) may also contribute to the increased susceptibility to infectious and/or inflammatory diseases (Halloy et al., 2005), which was supported in the present study. Some other studies have also confirmed the pro-inflammatory effects of FB_1 and contributed significantly to the explanation of these effects via some investigations on the cytokine profile of FB_1 exposed pigs (Taranu et al., 2005).

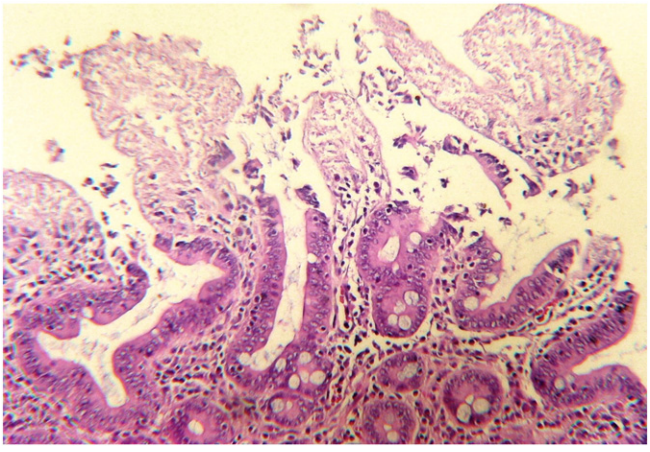


Fig. 6. Degenerative changes in superficial epithelium and increased quantity of Goblet cells. Jejunum of pig given FB1 and infected with *Pasteurella multocida*. X/E. ×200.

The FB_1-induced increase in the permeability of vessels could also contribute to the dissemination of various infections in the lung of pigs, resulting in further distribution and aggravation of pneumonic damages out of its typical localisation as was seen in this study. This process could be facilitated by the immunosuppressive effect of FB_1 on humoral immune response in pigs (Stoev et al., 2012). In this regard, a study of Ramos et al. (2010) demonstrated that lung damages are much complicated in swine inoculated with PRRS virus, when the same are exposed additionally to FB_1 in the feed diet. The authors explained this deterioration of the disease by immunosuppressive effect of FB_1, supposed to be due to the accumulation of free sphingoid bases and a subsequent inhibition of lymphocytes proliferation (Taranu et al., 2005).

In the literature, there are some controversial evidences about the adverse effect of FB_1 on pigs. Some authors report that even high feed levels of FB_1 such as 40 ppm don't affect feed intake and body weight gain. Pigs exposed to such high contamination levels of FB_1 develop usually a strong pulmonary oedema which, however, is not always accompanied with visible clinical signs (Tóth et al., 2000). Such clinical signs can develop only after exposure to very high doses of FB_1 such as 100–300 ppm (Harrison et al., 1990; Haschek et al., 1992). However, other studies revealed that dietary exposure of pigs to significantly lower feed levels of FB_1 such as 1–10 ppm, can provoke a partial decrease in body weight gain in the first 4 weeks of life, which can be compensated in a later stage (Rotter et al., 1996). In this regard, our studies support the observations of the authors who observed no visible clinical signs and significant differences in the body weight of the pigs exposed to high levels FB_1, but it should be taken into account that our levels of FB_1 were significantly lower than the levels used by these authors.

Some previous studies of Zomborszky-Kovács et al. (2002) revealed that the pulmonary oedema usually appears in the early stage of fumonisin exposure, whereas in the later stages (>6–8 weeks of exposure to low doses 1–10 ppm of FB_1) a pulmonary fibrosis is observed, often without any clinical signs (Zomborszky-Kovács et al., 2002). The clinical manifestation may only appear after much higher doses of FB_1 (100–300 ppm or above 15 mg/kg b.w.) as reported by Harrison et al. (1990) and Haschek et al. (1992) and this finding was confirmed in the present study, where no clinical signs were observed in FB_1 treated pigs.

The slight enlargement of mesenterial lymph nodes and the slight hyperaemia in mesenterial vessels observed in some of the control pigs can be attributed to some individual features and/or to the small foci of local peritonitis seen in most of the pigs (experimental and control), due to intraperitoneal injections with ovalbumin.

The most obvious changes in FB_1 exposed pigs can be considered as a result of the increased permeability of vessels, which is responsible for perivascular and especially pericapillary oedema in the lung, brain, cerebellum and partially in kidneys seen in the present study. However, these oedematous changes were much stronger in the lung than in the other organs. It is also reported that FB_1 can decrease myocardial contractility in pigs. Such decrease could be due to sphingosine inhibiting the L-type calcium channels in the myocardium (Haschek et al., 2009). This heart damage could additionally contribute to the oedematous changes in the lung. The lung oedema of pigs exposed to FB_1 is similar to that described in our previous studies (Zomborszky-Kovács et al., 2000; Pósa et al., 2011, 2013; Stoev et al.,

2012) or that found by some other authors, e.g. interlobular oedema of non-inflammatory origin found in the peribronchial, peribronchiolar and perivascular areas of the lungs (Fazekas et al., 1998; Haschek et al., 1992).

FB_1 has been reported to be a nephrotoxic for kidney (Bucci et al., 1998; Voss et al., 2001; Howard et al., 2001) and to provoke *in vitro* and *in vivo* degenerative and/or apoptotic damages in tubular epithelium of kidneys in rats or pigs (Dragan et al., 2001, Stoev et al., 2012), which was confirmed by the present study. FB_1 is also reported to impair protein synthesis in cells, which could lead to further degenerative changes in kidneys or liver, the target organs responsible for elimination or detoxification of mycotoxins (Abado-Becognee et al., 1998).

The lung is also the target organ of the *P. multocida*-induced damage, but the pathomorphological changes corresponded to the morphologic pattern of a broncho-interstitial pneumonia, with development of prominent interalveolar, peribronchiolar, peribronchial and perivascular mononuclear infiltration. These changes were quite different as compared to the lung changes provoked by FB_1.

Histopathological damages in *P. multocida* infected pigs were similar, but less expressed than those described by Halloy et al. (2005), who observed inflammatory mononuclear proliferation in the interstitium of lung. Mononuclear and neutrophils infiltration in the interstitium and alveolar or bronchial lumina in the present experiment were mainly observed in infected animals compromised by FB_1, which is in agreement with pathomorphological findings reported by Halloy et al. (2005).

A complicated course of *P.multocida* serotype A infection is also observed when the same pathogen is interacted with other respiratory infectious agents (Amass et al., 1994; Ciprian et al., 1988; Pósa et al., 2011) instead FB_1.

It can be concluded, that pathomorphological changes in various internal organs in the pigs of the group infected with *P. multocida* and treated simultaneously with FB_1 should be considered not only as a simple combination of the pathomorphological changes characteristic for both: *P. multocida* infection and FB_1 intoxication, but also as a complication and deterioration of the same changes. One of the target changes in the pigs of this group is due to the increased permeability of vessels leading to a strong perivascular and especially pericapillary oedema in the lung and subsequently in decreasing order in the brain, cerebellum and kidneys, which can be considered as a consequence of FB_1 action. The other target damage was seen also in the lung, where a focal broncho-interstitial pneumonia, with peribronchial, peribronchiolar and perivascular inflammatory cell infiltration and/or intra-alveolar/intrabronchiolar leucocyte infiltration were also seen, which was mainly due to the complicated *P. multocida* infection.

We can conclude that FB_1 exposure of pigs may complicate or exacerbate the course of *P. multocida* serotype A infection as was demonstrated in the present study. Some additional experiments would be necessary in order to clarify more precisely the target mechanism and/or the extent of this exacerbation.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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