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Pre-infection of pigs with *Mycoplasma hyopneumoniae* induces oxidative stress that influences outcomes of a subsequent infection with a swine influenza virus of H1N1 subtype

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ABSTRACT

The severity of swine influenza is highly variable and can be exacerbated by many factors, such as a pre-infection of pigs with *Mycoplasma hyopneumoniae* (Mhp). The aim of this study was to investigate the oxidative stress induced by Mhp and the impact of this stress on the evolution of an infection with the European avian-like swine H1N1 influenza virus. Two experimental trials (E1 and E2), which differed only by the feed delivered to the animals, were conducted on SPF pigs. In each trial, one group of nine 6-week-old pigs was inoculated intra-tracheally with Mhp and H1N1 at 21 days intervals and a mock-infected group (8 pigs) was included. Clinical signs were observed, blood samples were collected throughout the study and pathogens were detected in nasal swabs and lung tissues. Results indicated that Mhp infection induced an oxidative stress in E1 and E2, but its level was more important in E2 than in E1 three weeks post-Mhp inoculation, before H1N1 infection. In both trials, a strong inflammatory response and a response to the oxidative stress previously induced by Mhp appeared after H1N1 infection. However, the severity of influenza disease was significantly more marked in E2 as compared to E1, as revealed by prolonged hyperthermia, stronger reduction in mean daily weight gain and earlier viral shedding. These results suggested that severity of flu syndrome and reduction in animal performance may vary depending on the level of oxidative stress at the moment of the influenza infection, and that host responses could be influenced by the feed.

1. Introduction

Influenza in swine is caused by type A influenza viruses that belong to the *Orthomyxoviridae* family. Three subtypes of swine influenza viruses (SIV), H1N1, H1N2 and H3N2, are simultaneously circulating among pigs worldwide, whereas lineages may vary within each subtype depending on the region, i.e. North America, Europe and Asia (Kuntz-Simon and Madec, 2009). These viruses have become enzootic in areas densely populated with pigs and may occasionally be transmitted to humans. Swine influenza is an acute respiratory disease that is characterized by hyperthermia, loss of appetite, lethargy, respiratory problems and coughing. This infection is highly contagious and affects 50% of French livestock. A flu outbreak has a substantial economic impact for an infected farm, particularly due to the decline in animal performance but also due to the cost of

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medication, especially in case of complications. The severity of clinical symptoms is highly variable. It can be influenced by many factors including the virus strain, the age and the immune status of the infected pig, the climatic condition, the housing type, the presence of other respiratory pathogens, or other still unknown parameters (Deblanc et al., 2012; Kuntz-Simon et al., 2010). A better understanding of these factors could help in developing strategies for disease control, aiming to improve animal health status and welfare, as well as to reduce medication and zoonotic risk.

It is recognized that some viral infections induce an oxidative stress in the infected host. This is particularly the case during infection with RNA viruses, including influenza A viruses (Hennet et al., 1992; Peterhans, 1997; Schwarz, 1996). Oxidative stress is commonly defined as an imbalance between the production of reactive oxygen species (ROS) and the antioxidant system of an organism, in favor of ROS (Sies, 1991). It results in a stimulation of enzymes involved in its regulation (glutathione peroxidase, superoxide dismutase), oxidation of antioxidant components (glutathione, thiol proteins, vitamins E, C, A), and accumulation of oxidation products in tissues, with an increase in the amount of hydroperoxides in the blood. ROS can damage many biological molecules such as DNA, lipids and proteins. In case of flu, ROS are produced in excess during the inflammatory response following the entry of the virus in the body (Buffinton et al., 1992). Thus, oxidative stress induced by the infection itself or already established in host at the time of infection, could participate in the virus pathogenicity. Various studies in mice showed that compounds with antioxidant properties reduced the severity of the disease, especially with a reduction in the severity of lung injury (Cai et al., 2003; Ghezzi, 2011; Ghezzi and Ungheri, 2004; Kumar et al., 2003). The role of oxidative stress in pulmonary damage caused by influenza virus is well characterized in the mouse model but no study has been conducted in pigs to date. Yet, many situations are associated with oxidative stress in pigs, such as weaning (Robert et al., 2009), high density housing (Marco-Ramell et al., 2011) or infectious diseases responsible for pneumonia, enteritis and sepsis (Lykkesfeldt and Svendsen, 2007). We have therefore undertaken to study the impact of oxidative stress on response to influenza infection in pigs. This study has been conducted in pigs successively infected, 3 weeks apart, with *Mycoplasma hyopneumoniae* (Mhp), a respiratory pathogen widespread in French farms, and a European avian-like swine H1N1 virus. Both pathogens are involved in the porcine respiratory disease complex and were demonstrated to be significantly associated in induction of pneumonia in swine herds (Fablet et al., 2012). Under experimental conditions, pre-infection of pigs with *M. hyopneumoniae* enhanced the outcomes of infection with the H1N1 virus, especially lung injury, providing a suitable animal model to study host factors that may modulate the Influenza A virus pathogenicity (Deblanc et al., 2012). In the present study, we investigated the oxidative stress induced by *M. hyopneumoniae* and the effect of oxidative stress on the outcomes of infection with H1N1 virus.

2. Materials and methods

2.1. Pathogens

M. hyopneumoniae (Mhp, strain 116) was isolated from an outbreak of enzootic pneumonia in France and cultivated in Friis broth medium (FBM) at 37 °C (Marois et al., 2007). Three 10-fold serial dilutions of the Mhp stock were prepared in FBM and incubated at 37 °C. After 5–10 days, color changes of medium were observed and the Mhp stock titer was calculated and expressed as color-changing units per milliliter (CCU/ml).

The SIV strain A/Sw/Cotes d'Armor/0231/06 (H1N1) was isolated from a nasal swab taken from a pig with acute respiratory disease in a French herd. It was isolated onto Madin Darby Canine Kidney (MDCK) cells and further propagated in the allantoic cavity of 9-day-old embryonated chicken eggs at 36 °C for 3 days for inoculum production, following standard procedure (OIE, 2008). Allantoic fluid was tested for haemagglutinating activity with 0.5% chicken erythrocytes. Virus titer was determined by inoculating 5 embryonated chicken eggs with 150 μl of 10-fold serial dilutions of the virus stock. After 9 days of incubation at 36 °C, the embryonic lethal dose (ELD_{50}/ml) was calculated by the method of Reed and Muench.

2.2. Animals, experimental protocol and sample collection

Two similar experimental trials (E1 and E2) were performed in the experimental pig herd of the French Agency for Food, Environmental and Occupational Health and Safety (Anses) at Ploufragan. Experiments were performed in accordance with the animal welfare experimentation recommendations granted by the *Directions Départementales de la Protection des Populations des Côtes d'Armor* (Anses registration number B-22-745-1), under the responsibility of G. Simon (authorization number 22-26). E1 and E2 experimental protocols were identical, except in the feed delivered to the animals, as E1 and E2 feed had similar energy and protein intake but differed in some micro-nutrients. E1 and E2 were performed at the same time but in two separate units of the experimental pig herd. Thirty-four specific pathogen-free (SPF) 6-week-old pigs (Large White) were randomly allocated into these trials. The animals were known to be free from SIV and Mhp at the beginning of the study. Very strict biosecurity measures were implemented in order to avoid undesirable contamination of the pigs: air filtration system and airlocks for each unit, unit specific clothes and compulsory showering before and after visiting the pigs (BSL3). In each trial, nine pigs (MH1N1-E1 and MH1N1-E2 groups) were inoculated intra-tracheally on two consecutive days with 5 ml of 10^8 CCU/ml of Mhp 116 (day 0 and day 1) and 3 weeks later (day 21) with 10^5 EID_{50} in a total volume of 5 ml of SIV H1N1. Simultaneously, eight pigs served as control and received intra-tracheally 5 ml of Friis broth medium at days 0 and 1 and 5 ml of allantoic fluid at day 21 (C-E1 and C-E2 groups) (Table 1). Clinical signs including cough (number of coughs for 15 min), respiratory rate and rectal temperature were evaluated daily, throughout the study. Pigs were weighted weekly for four weeks. Blood

Table 1
Experimental design.

Experimental trial	Groups	No. of pigs	Intra-tracheal inoculations		Day of necropsy
			Day 0 + Day 1	Day 21	
E1	MH1N1-E1	9	*Mycoplasma hyopneumoniae*	H1N1	Day 28
E1	C-E1	8	Friis broth medium	Allantoic fluid	Day 31
E2	MH1N1-E2	9	*Mycoplasma hyopneumoniae*	H1N1	Day 29
E2	C-E2	8	Friis broth medium	Allantoic fluid	Day 32

samples were collected on lithium heparin and sodium heparin 4 days before Mhp-inoculation then at days 18, 23, 25 and 28. Nasal swabs were taken at days 23, 25 and 28, i.e. 2, 4 and 7 days post-infection (DPI) with SIV. Animals were euthanised by intravenous booster of sodium pentobarbital at the end of the experiment. Pigs from MH1N1-E1 and MH1N1-E2 groups were necropsied at day 28 and day 29, respectively, and pigs from C-E1 and C-E2 groups were necropsied at day 31 and day 32, respectively. Post mortem examinations were carried out on each animal and macroscopic pulmonary lesions were scored visually as previously described (Madec and Kobisch, 1982). The maximum total score possible for each lung was 28. A sample of each left (apical, cardiac and diaphragmatic) lobe of the lungs was collected for detection of SIV and Mhp and stored at −70 °C until use. A sample of the left diaphragmatic lobe was also fixed in 10% neutral buffered formalin for histopathological examination, as previously described (Deblanc et al., 2012).

2.3. Measurements of oxidative stress markers in blood

Blood samples were placed in dark immediately after the collection, then centrifugated at 4000 × *g*, 4 °C, for 10 min and supernatants were stored at −70 °C until use. Blood samples were collected on sodium heparin for measurement of trace elements and on lithium heparin for measurement of other markers. Thus, typical profiles of oxidative stress markers have been realized, including measurement of zinc, selenium and copper (trace elements), measurement of vitamins A, C, E and γ-tocopherol, measurement of total glutathione, reduced glutathione (GSH), oxidized glutathione (GSSG), thiol proteins and glutathione peroxidase (GPx), as well as measurement of malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS) and reactive oxygen metabolites (ROM), some components that result from oxidative damage (Cornelli et al., 2001; de la Villehuchet et al., 2009; Miller et al., 1993). Briefly, zinc and copper were analyzed by flame atomic absorption spectrophotometry and selenium was measured by electrothermal vaporization atomic absorption spectrophotometry. Vitamins A and E were evaluated by high performance liquid chromatography (HPLC), with detection after extraction by hexane, evaporation to dryness and redissolving. For the vitamin C quantification, 5% metaphosphoric acid was added to the blood samples before centrifugation. Stabilized plasmas were then analyzed by HPLC with fluorometric detection after total oxidation in dehydroascorbate by ascorbate oxidase and the derivatization by ortho-phenylene diamine. For the GSSG and GSH measurements, 6% metaphoshoric acid was added to the blood samples before centrifugation and storage. Quantifications of the GSSG and the GSH were realized by measurement of the thio-bis 2-nitrobenzoic acid by spectrophotemetry at 412 nm. This acid was formed after reaction between the 5,5'-dithio-bis 2-nitrobenzoic acid which is a reducing agent of disulfide bonds. The GPx activity was measured by enzymatic assay, in the presence of glutathione, glutathione reductase and NADPH, and by monitoring the change in optical density over time at 340 nm. MDA plasmatic levels were evaluated by HPLC with detection at 532 nm after derivatization by thiobarbituric acid. TBARS were quantified by fluorometry after reaction with thiobarbituric acid. The total thiol proteins were evaluated by colorimetry at 450 nm after reaction with 5,5'-dithio-bis 2-nitrobenzoic acid.

ROMs were quantified by a dROM-test that measured the blood concentration of hydroperoxides. Hydroperoxides are generated by the oxidation of proteins, amino acids, lipids and nucleotides. They reacted with a chromogen substrate to develop a colored derivative (Cornelli et al., 2001). The color intensity was then quantified at 505 nm. Results were expressed in Carratelli Units (U. Carr.).

2.4. Determination of acute phase protein concentration

Blood samples were collected on lithium heparin for the determination of haptoglobin level. Plasmas were separated by centrifugation and stored at −70 °C until use.

Haptoglobin was measured using the colorimetric test Phase Haptoglobin Assay (AbCys, Paris, France), following manufacturer's instructions. The absorbance was read at 620 nm using an ELISA plate reader (Multiskan, Thermo Fisher Scientific, Saint Herblain, France).

2.5. Pathogens detection

After collection, nasal swabs were suspended in 2 ml of Eagle's minimum essential medium (EMEM, LONZA, Levallois-Perret, France) containing penicillin and streptomycin (Sigma, Saint-Quentin Fallavier, France) and vigorously vortexed. Supernatants were aliquoted and stored at −70 °C.

For the detection of SIV, nasal swab supernatants taken at 23, 25 and 28 DPI and lung samples obtained at necropsy were analyzed by a matrix (M) gene real-time RT-PCR protocol adapted from Spackman et al. (2002) with some modifications. Briefly, RNA was extracted from 200 μl of a nasal swab specimen or from 30 mg of an organ sample with the NucleoSpin 8 RNA kit (Macherey-Nagel, Hoerdt,

France). 2.5 μl of RNA template was tested using the Qiagen One step RT-PCR kit (Qiagen, Courtaboeuf, France) in a total volume of 25 μl. The RT-PCR was run in a Chromo4 Real-Time Detector (Bio-Rad, Marnes-la-Coquette, France) and the thermal profile used was 50 °C for 30 min followed by 95 °C for 15 min and 45 cycles of 94 °C for 5 s and 60 °C for 60 s.

For Mhp detection, DNA was extracted from 25 mg of lung sample with DNeasy Blood and Tissue kit (Qiagen, Courtaboeuf, France) for detection of Mhp by quantitative PCR, as previously described (Marois et al., 2010).

2.6. Statistical analysis

Influences of the feed, of the infection and of both the feed plus the infection on the biochemical parameters were studied by means of analysis of covariance (ANCOVA) using SPSS 19 software for Windows. The covariate was the oxidative stress marker at the previous time of sampling. Thus, means of oxidative stress markers were corrected by those evaluated at the previous sampling time. The feed, the infection and the interaction between food and infection were defined as fixed factors in the ANCOVA. Other data (rectal temperature, daily weight gain, haptoglobin concentration and Mhp DNA quantification) were compared by the Mann–Whitney test using Systat 9 software. Differences were considered significant when $P \leq 0.05$.

3. Results

3.1. Clinical disease

Following Mhp infection, coughing started at 7 DPI in the MH1N1-E1 group and at 10 DPI in the MH1N1-E2 group (data not shown). During the third week post-infection, the frequency of coughing reached similar averages of coughs per pig per 15 min in both Mhp infected groups, i.e. 0.55 and 1.02 in the MH1N1-E1 group and the MH1N1-E2 group, respectively. Subsequent SIV inoculation at 21 DPI did not induce an increase in the frequency of coughing, which was sustained at 0.64 coughs per pig per 15 min between the 21st and the 25th day following Mhp inoculation in both infected groups. No cough was observed in C groups.

All pigs sustained normal rectal temperature after Mhp inoculation and during the first three weeks of the experimental assay (data not shown) but pyrexia (temperature > 40 °C) was observed in both coinfected groups from day 1 post-infection with H1N1 (Fig. 1). Mean temperatures in infected groups were significantly different from those of control groups during 3 days following H1N1 inoculation. At 4 DPI, MH1N1-E2 group was still different from C-E2 group, whereas no more significant difference was observed between MH1N1-E1 and C-E1 groups, what led to a significant difference between both coinfected groups.

In addition to hyperthermia, increases in respiratory rates were observed after H1N1 inoculation. Indeed, respiratory rates reached 66–86 and 72–88 breaths per minute in MH1N1-E1 and MH1N1-E2 groups, respectively, while there were only 32–44 breaths per minute in C-E1 and C-E2 groups.

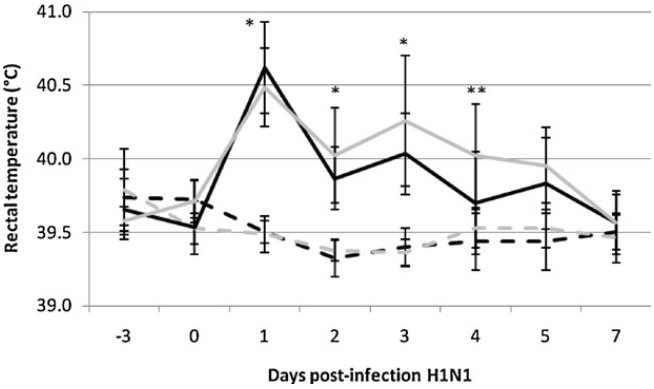

Fig. 1. Mean of rectal temperature (°C) of pigs after H1N1 inoculation. Mean ± standard deviation of rectal temperature of C-E1 group (- -), C-E2 group (▬ ▬), MH1N1-E1 group (—) and MH1N1-E2 group (▬) after H1N1 inoculation. *MH1N1-E1 and MH1N1-E2 are significantly different from C-E1 and C-E2, respectively ($P \leq 0.05$). **MH1N1-E2 is significantly different from C-E2 and MH1N1-E1 ($P \leq 0.05$).

Pigs from both infected groups also showed inappetence after H1N1 inoculation. Over the 4 first days post-infection with SIV, they ate a mean of 725 g/pig/day in the MH1N1-E1 group and 511 g/pig/day in the MH1N1-E2 group, whereas pigs from C-E1 and C-E2 groups ate in an average of 1746 and 1743 g/pig/day respectively. In infected groups, the feed consumption decrease induced a very strong reduction of the mean weight gain (MWG) during the first week following H1N1 inoculation (Fig. 2). As a consequence, significant differences in MWG were observed between MH1N1 groups and corresponding C groups, when calculated over the periods D21–D25 and D21–D28. It has to be noticed that the MWG reduction observed in coinfected groups as compared to the control groups, was significantly stronger for the MH1N1-E2 group than for the MH1N1-E1 group. Indeed, a negative MWG was observed in MH1N1-E2 group over the D21–D25 period, but such a weight loss was not observed in the MH1N1-E1 group. This difference in the severity of MWG

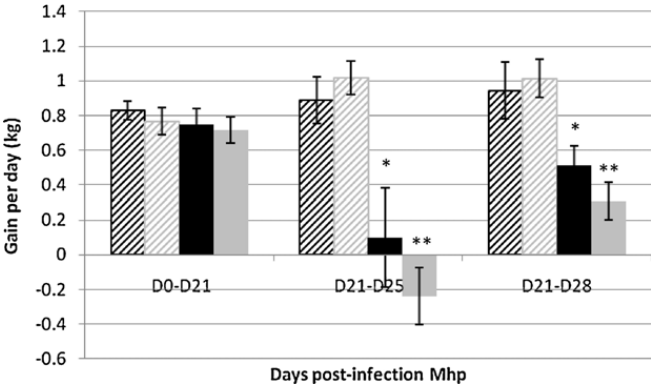

Fig. 2. Mean daily weight gain (kg) of pigs after Mhp and H1N1 inoculations. Mean daily weight gain between 0 and 21 DPI Mhp (before H1N1 infection), between 21 and 25 DPI Mhp (between 0 and 4 DPI H1N1) and between 21 and 28 DPI Mhp (between 0 and 7 DPI H1N1) of C-E1 group (▨), C-E2 group (■), MH1N1-E1 group (■) and MH1N1-E2 group (▬). *MH1N1-E1 is significantly different from C-E1 and MH1N1-E2 groups ($P \leq 0.05$). **MH1N1-E2 is significantly different from C-E2 and MH1N1-E1 groups ($P \leq 0.05$).

reduction between both infected groups was still significant over the period D21–D28.

At necropsy, lung lesions typical of pneumonia (purple–red and firm lesions) were observed in all pigs from MH1N1 groups (data not shown). These lesions were extensive and reached the diaphragmatic lung lobes. No difference in the severity of the macroscopic lesions was observed between coinfected groups, as calculated mean scores were 13.1/28 and 13.7/28 in MH1N1-E1 and MH1N1-E2 groups, respectively. Also, histological analyses did not show any significant difference in mean microscopic lesion scores between both infected groups (data not shown). All pigs from MH1N1-E1 and MH1N1-E2 groups developed bronchiolitis, broncho-interstitial and interstitial pneumonia.

No macroscopic or microscopic lesions were observed in both control groups.

3.2. Pathogens detection

SIV RNA detection performed on nasal swabs collected at 2, 4 and 7 DPI H1N1 showed that SIV was shed from 9/9 pigs in the MH1N1-E2 group at 2 DPI, whereas in the MH1N1-E1 group only 4/9 pigs shed virus at that time (Table 2). At 7 DPI, viral genome was still detected in nasal secretions of 8/9 pigs from both coinfected groups. At necropsy, H1N1 genome was detected in apical lobes of 9/9 pigs in the MH1N1-E2 group, against only 7/9 pigs in the MH1N1-E1 group. More diaphragmatic lobes were also detected positive in MH1N1-E2 group than in MH1N1-E1 group (8/9 against 6/9 pigs, respectively).

Mhp genome was detected in all Mhp-inoculated animals. However, whereas DNA quantities varied a lot from an animal to another whatever the lung lobe, no significant difference was observed between both infected groups.

No viral RNA, nor Mhp genome, was detected in samples from both control groups (data not shown).

3.3. Blood data

The analysis of plasma parameters at day 18 revealed that Mhp inoculation induced a significant increase in ROM levels, a reduction in total glutathione concentration and a reduction of the ratio GSH/GSSG in both MH1N1 groups (Table 3). However, it has to be noted that the ROM concentration appeared two times higher in MH1N1-E2 group than in MH1N1-E1 group. Similarly, a reduction in copper's concentration was also observed at day 18 in both inoculated groups, but the severity of this reduction was higher in MH1N1-E2 group than in MH1N1-E1 group. Thus, differences in oxidative stress levels tended to be due to the combined action of Mhp-inoculation and feed ($P = 0.086$).

After H1N1 infection, an increase in the concentration of haptoglobin was observed in both infected groups at 2 DPI H1N1 (Fig. 3). Also, increases in amounts of ROM, glutathione peroxidase and selenium, as well as decreases in concentrations of vitamin A, γ-tocopherol and zinc were observed (Table 4).

Globally, the co-infection with Mhp and H1N1 induced, in both infected groups, MH1N1-E1 and MH1N1-E2, an

Table 2
Pathogens detection in nasal swabs and lung tissues of inoculated pigs.

Group	Pig	H1N1 RNA detection[a]						Mhp DNA quantification[b] (pg DNA/25 mg organ)		
		Nasal swabs			Left lung tissue			Left lung tissue		
		2 DPI	4 DPI	7 DPI	Apical lobe	Cardiac lobe	Diaphragmatic lobe	Apical lobe	Cardiac lobe	Diaphragmatic lobe
MH1N1-E1	1	+	+	+	+	+	-	42.0	330.6	0.4
	2	-	+	+	+	+	-	57.1	65.0	4.1
	3	+	+	+	+	+	+	52.0	10.3	35.4
	4	–	+	–	+	+	+	30.9	73.1	32.5
	5	–	+	+	–	+	+	0.5	64.0	0.2
	6	–	+	+	–	+	–	6.9	448.8	0.3
	7	+	+	+	+	+	+	77.2	58.8	53.2
	8	+	+	+	+	+	+	0.7	0.2	0.0
	9	–	+	+	+	+	+	12.4	0.6	36.4
	Mean (SD)							31.1 (27.7)	116.8 (160.1)	18.1 (21.1)
MH1N1-E2	10	+	+	+	+	+	+	109.9	164.9	74.2
	11	+	+	+	+	+	+	23.4	85.4	2.5
	12	+	+	+	+	+	+	1.5	0.7	126.7
	13	+	+	–	+	+	+	0.0	0.8	0.2
	14	+	+	+	+	+	–	0.0	903.0	0.3
	15	+	+	+	+	+	+	88.6	621.1	2.3
	16	+	+	+	+	+	+	161.5	8.7	69.4
	17	+	+	+	+	+	+	13.0	254.2	691.9
	18	+	+	+	+	+	+	119.7	174.4	60.6
	Mean (SD)							57.5 (62.5)	245.9 (313.6)	114.2 (221.2)

SD, standard deviation.

[a] Results of the real-time RT-PCR targeting the Influenza A matrix (M) gene, in nasal swabs collected at 2, 4 and 7 DPI H1N1 and in lung tissues collected at necropsy (+ and –: presence or absence of SIV genome).

[b] Results of the quantification of Mhp in lungs at necropsy.

Table 3
Effects of the Mhp inoculation on plasma parameters of pigs of C and MH1N1 groups in E1 and E2. Data are expressed as estimated means ± standard deviation at D18, means at D-4 is used as covariate. ROM: reactive oxygen metabolites; TBARS: thiobarbituric acid reactive substances; MDA: malondialdehyde; GSH: reduced glutathione; GSSG: oxidized glutathione. nd: not determined.

Variable	Unit	E 1		E 2		*P*-Value		
		Control	MH1N1	Control	MH1N1	Feed	Inoculation	Feed × inoculation
ROM	U. Carr	562 ± 202	787 ± 190	470 ± 214	1400 ± 201	0.19	0.014	0.086
TBARS	µmol/l	2.58 ± 0.09	2.56 ± 0.09	2.55 ± 0.09	2.55 ± 0.08	0.79	0.9	0.88
MDA	µmol/l	1.47 ± 0.06	1.45 ± 0.06	1.4 ± 0.06	1.46 ± 0.06	0.59	0.718	0.49
Thiol protein/total protein	µmol/g	2.32 ± 0.10	2.46 ± 0.09	2.12 ± 0.1	2.44 ± 0.1	0.23	0.06	0.34
GSH/GSSG		44.3 ± 5.7	13 ± 5.2	32 ± 5.7	13.8 ± 5.2	0.31	0.0001	0.24
Glutathione peroxidase	U/l	1782 ± 78.6	1847 ± 74.6	1684 ± 81.4	1700 ± 74.2	0.13	0.61	0.75
Total glutathione	µmol/l	842.9 ± 53.7	551.5 ± 47.4	812.4 ± 52.2	550.1 ± 52.9	0.75	0.0001	0.77
Vitamin C	µmol/l	nd	38.4 ± 3.3	34.7 ± 3.2	36.6 ± 3.1	nd	nd	nd
Vitamin A	µmol/l	1.2 ± 0.08	1.12 ± 0.08	1.04 ± 0.08	1.23 ± 0.08	0.44	0.82	0.12
Vitamin E	µmol/l	2.58 ± 0.65	1.97 ± 0.57	1.43 ± 0.61	2.3 ± 0.59	0.53	0.83	0.22
Selenium	µmol/l	1.39 ± 0.05	1.36 ± 0.05	1.44 ± 0.05	1.38 ± 0.06	0.53	0.36	0.8
Zinc	µmol/l	22.7 ± 0.82	20.03 ± 0.78	20.3 ± 0.82	20.13 ± 0.77	0.21	0.077	0.12
Copper	µmol/l	29.49 ± 0.72	25.58 ± 0.67	26.76 ± 0.72	26.4 ± 0.69	0.16	0.012	0.013

increase in the plasmatic concentration of ROM, a decrease in the level of thiol proteins and total glutathione, and a stimulation of the glutathione peroxidase (Table 5). Concentrations of vitamin A, γ-tocopherol and zinc also decreased and concentrations of vitamin C and selenium increased.

4. Discussion

In this study, we first showed that Mhp infection induced an oxidative stress in pigs, as revealed by an increase in ROM concentration three weeks post-infection. The predominant phagocytic cells infiltrating the lung of a Mhp-affected pig are macrophages (Sarradell et al., 2003). Once recruited on the site of an infection, macrophages use superoxides (hydrogen peroxide, nitric oxide and superoxide anions) to kill the pathogen during the respiratory burst (Avron and Gallily, 1995; Babior, 1984). However, Mhp seems to be able to evade this host immune system (Schafer et al., 2007) as the important production of oxidative radicals by macrophages may contribute to cell damages and thereby to the clinical pathology, as previously seen in mycoplasma infections in other animal species (Avron and Gallily, 1995; Hermeyer et al., 2011).

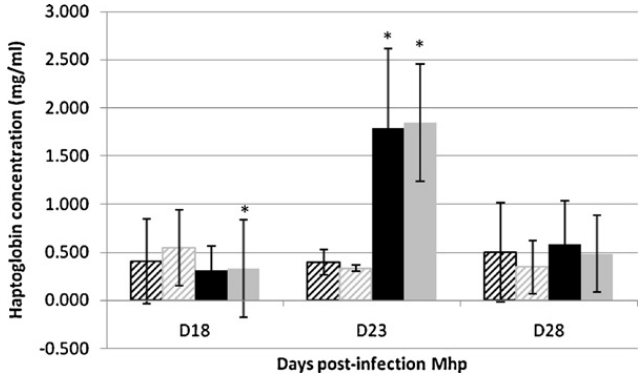

Fig. 3. Concentration of haptoglobin (mg/ml) in plasmas of pigs at 18, 23 and 28 DPI Mhp, i.e. 4 days before H1N1-inoculation, 2 and 7 DPI H1N1. Mean ± standard deviation of haptoglobin levels of C-E1 group (▨), C-E2 group (■), MH1N1-E1 group (■) and MH1N1-E2 group (■). *MH1N1 group is significantly different from C group ($P \leq 0.05$).

In this study, we also showed that the level of ROM after mycoplasma infection was two times higher in the MH1N1-E2 group, as compared to the MH1N1-E1 group and this difference tends to be significant ($P = 0.086$). As the feed formulation was the only parameter that changed between E1 and E2 experiments, one can hypothesize that this difference in oxidative stress was related to that parameter. It has also to be noted that animals from the MH1N1-E2 group were slightly more coughing than those from MH1N1-E1 group during the third week post-infection. Further investigations should be necessary to confirm or not a correlation between the oxidative stress level, the feed and the severity of clinical signs induced by Mhp infection.

It also appeared that the coinfection with Mhp and H1N1 induced an oxidative stress, as well as a response to this stress. Indeed, the increase in glutathione peroxidase and the decrease in the ratio thiol proteins/total proteins indicated a stimulation of the antioxidant system in order to eliminate ROS. Another response to the coinfection was an increase in the availability of the selenium, which is a major antioxidant trace element and a co-factor of glutathione peroxidase. Concentrations of other trace elements as copper and zinc did not increase. The plasmatic level of copper was unchanged and the level of zinc decreased after H1N1 infection. Similar data on selenium, copper and zinc plasmatic levels were obtained in previous studies, when pigs were subjected to oxidative stress after weaning (Robert et al., 2009) or after lipopolysaccharide (LPS) inoculation (Robert et al., personnel communication). Such modulations were also observed during inflammation in guinea pigs (Akcil et al., 2003).

MDA and TBARS are usually used as biomarkers of lipid oxidation in studies related to oxidative stress in humans

Table 4
Effects of the H1N1 inoculation on plasma parameters of Mhp-infected pigs in E1 and E2. Data are expressed as estimated means ± standard deviation at D23, means at D18 are used as covariate. ROM: reactive oxygen metabolites, TBARS: thiobarbituric acid reactive substances. MDA: malondialdehyde; GSH: reduced glutathione; GSSG: oxidized glutathione.

Variable	Unit	E1		E2		*P*-value		
		Control	MH1N1	Control	MH1N1	Feed	Inoculation	Feed × inoculation
ROM	U. Carr	1199 ± 191	1744 ± 179	1144 ± 192	1521 ± 194	0.47	0.025	0.66
TBARS	µmol/l	2.19 ± 0.07	2.38 ± 0.07	2.42 ± 0.07	2.46 ± 0.07	0.034	0.13	0.29
MDA	µmol/l	1.50 ± 0.06	1.34 ± 0.06	1.51 ± 0.07	1.39 ± 0.06	0.66	0.03	0.71
Thiol protein/total protein	µmol/g	2.34 ± 0.09	1.75 ± 0.09	2.22 ± 0.1	1.68 ± 0.09	0.29	0.0001	0.79
GSH/GSSG		33.4 ± 4.3	32.8 ± 3.7	38.7 ± 3.7	35.7 ± 3.6	0.26	0.7	0.74
Glutathione peroxidase	U/l	1871 ± 53	2128 ± 50	1810 ± 53	2254 ± 51	0.536	0.0001	0.084
Total glutathione	µmol/l	649 ± 29.2	573 ± 27.5	699 ± 28.4	593 ± 26.5	0.19	0.01	0.57
Vitamin C	µmol/l	35.9 ± 4.58[a]	37.82 ± 2.17	42 ± 2.29	47.28 ± 2.2	0.024	0.32	0.7
Vitamin A	µmol/l	1.14 ± 0.09	0.84 ± 0.09	1.32 ± 0.09	0.70 ± 0.09	0.84	0.0001	0.11
Vitamin E	µmol/l	2.30 ± 0.49	2.55 ± 0.46	3.3 ± 0.49	3.12 ± 0.46	0.12	0.9	0.16
Selenium	µmol/l	1.35 ± 0.04	1.65 ± 0.04	1.42 ± 0.04	1.63 ± 0.04	0.47	0.0001	0.26
Zinc	µmol/l	19.88 ± 0.67	14.58 ± 0.56	22.13 ± 0.6	14.3 ± 0.57	0.126	0.0001	0.043
Copper	µmol/l	26.86 ± 0.74	28.73 ± 0.66	28.85 ± 0.7	29.35 ± 0.65	0.065	0.089	0.356
γ-Tocopherol	µmol/l	0.29 ± 0.05	0.04 ± 0.04	0.038 ± 0.04	0 ± 0.04	0.07	0.0001	0.28

[a] Mean calculated with values of only 2 pigs.

Table 5
Effects of the coinfection Mhp + H1N1 on plasma parameters of pigs of C and MH1N1 groups in E1 and E2. Data are expressed as estimated means ± standard deviation at D23, means at D-4 are used as covariate. ROM: reactive oxygen metabolites; TBARS: thiobarbituric acid reactive substances; MDA: malondialdehyde; GSH: reduced glutathione; GSSG: oxidized glutathione.

Variable	Unit	E1		E2		*P*-Value		
		Control	MH1N1	Control	MH1N1	Feed	Inoculation	Feed × inoculation
ROM	U. Carr	1159 ± 196	1728 ± 184	1103 ± 207	1609 ± 194	0.64	0.018	0.87
TBARS	µmol/l	2.2 ± 0.07	2.37 ± 0.07	2.43 ± 0.07	2.46 ± 0.69	0.035	0.16	0.31
MDA	µmol/l	1.51 ± 0.07	1.35 ± 0.06	1.5 ± 0.07	1.38 ± 0.06	0.85	0.046	0.78
Thiol protein/total protein	µmol/g	2.36 ± 0.1	1.76 ± 0.09	2.19 ± 0.1	1.67 ± 0.1	0.17	0.0001	0.66
GSH/GSSG		36.6 ± 3.5	32.1 ± 3.2	37.5 ± 3.5	34.5 ± 3.2	0.64	0.26	0.83
Glutathione peroxidase	U/l	1893 ± 80.5	2204 ± 76.4	1749 ± 83.4	2212 ± 76	0.4	0.0001	0.34
Total glutathione	µmol/l	687 ± 32.9	540 ± 29	730 ± 32	565 ± 32	0.27	0.0001	0.77
Vitamin C	µmol/l	35.2 ± 2.1	39.2 ± 2.18	41.1 ± 2.1	45.5 ± 2.09	0.05	0.013	0.93
Vitamin A	µmol/l	1.17 ± 0.09	0.81 ± 0.09	1.29 ± 0.09	0.72 ± 0.09	0.83	0.0001	0.24
Vitamin E	µmol/l	2.08 ± 0.51	2.51 ± 0.45	3.4 ± 0.49	3.22 ± 0.47	0.056	0.79	0.52
Selenium	µmol/l	1.35 ± 0.05	1.59 ± 0.05	1.49 ± 0.05	1.63 ± 0.04	0.11	0.0001	0.26
Zinc	µmol/l	20.69 ± 0.7	14.33 ± 0.67	21.9 ± 0.7	14.0 ± 0.66	0.55	0.0001	0.25
Copper	µmol/l	28.7 ± 0.7	27.6 ± 0.66	29.2 ± 0.71	28.6 ± 0.68	0.29	0.28	0.69
γ-Tocopherol	µmol/l	0.3 ± 0.05	0.04 ± 0.04	0.14 ± 0.05	0 ± 0.44	0.039	0.0001	0.19

(Mayne, 2003). However, MDA and TBARS levels were not modified throughout the course of our experiments, confirming that they are not suitable to evaluate the oxidative stress status in pigs, as previously suggested from observations made at weaning (Robert et al., 2009) and after LPS inoculation (Robert et al., personnel communication). Thus, the ROM level appeared to be the most relevant indicator for estimating ROS damages and assessing oxidative stress in pigs.

In addition to the oxidative stress, the increase in haptoglobin concentration at 2 DPI H1N1 indicated that the SIV infection induced a strong inflammatory response, of equal importance in both MH1N1 groups. This is in agreement with previous studies on the acute phase protein response after influenza infection in pigs (Barbe et al., 2011; Pomorska-Mol et al., 2012). The albumin, which is a negative acute phase protein (Alava et al., 1997), was also measured but there was no difference between control groups and infected groups (data not shown). This suggests that albumin measurement is not appropriate for detection of inflammation after influenza infection, as recently shown for Pig-MAP (Pomorska-Mol et al., 2012).

Interestingly, the H1N1 virus appeared more pathogenic in E2 than in E1: hyperthermia was prolonged until 4 DPI H1N1 and the weight loss over the week after infection was more important. In addition, H1N1 virus was detected at 2 DPI in nasal secretions of all animals

from the MH1N1-E2 group as compared to less than a half in the MH1N1-E1 group, indicating that virus shedding was earlier in the MH1N1-E2 group. Moreover, although there was no difference in lung lesions between both infected groups, virus spread in lungs was enhanced in the MH1N1-E2 group as compared to the MH1N1-E1 group. Altogether, these data showed that H1N1 multiplication was facilitated in animals from MH1N1-E2 group and first suggested that outcomes of H1N1 infection in Mhp-infected pig could be directly linked to the level of oxidative stress that is observed at the moment of the influenza virus infection. This statement can be related to a previous study in humans that showed that symptomatic hosts established multiple pattern recognition receptors-mediated antiviral and inflammatory responses that may relate to virus-induced oxidative stress, whereas asymptomatic hosts regulated these responses and exhibited elevated expression of genes that function in antioxidant responses and cell-mediated responses (Huang et al., 2011). Therefore, it can be hypothesized that the severity of the influenza disease in Mhp preinfected pig would be minimized by reducing oxidative stress of animals, for example through antioxidant intakes, as already shown in mouse (Cai et al., 2003; Ghezzi, 2011; Ghezzi and Ungheri, 2004; Kumar et al., 2003).

In conclusion, our findings indicated that coinfection with Mhp and H1N1 induced an inflammation, an oxidative stress and a response to this stress. They also suggested that the severity of the flu syndrome and the reduction in animal performance may vary depending on the level of oxidative stress at the moment of the influenza infection, also explaining how Mhp infection leads to the enhancement of H1N1 infection outcomes. Finally, this study suggests that the provision of feed supplemented with antioxidants could be a strategy to better prepare animals to overcome influenza infection. Further tests would be necessary to investigate such a novel prophylaxis approach as an useful alternative to medication in farms, especially in case of co-infections with several pathogens.

Conflict of interest

None of the authors of this paper has any financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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