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Original article

Blood antioxidant enzymes (SOD, GPX), biochemical and haematological parameters in pigs naturally infected with porcine reproductive and respiratory syndrome virus

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) has become one of the most economically important diseases for the swine industry worldwide. The objective of the study was to determine selected blood antioxidant enzymes (glutathione peroxidase (GPX), superoxide dismutase (SOD)), biochemical and haematological parameters in PRRS positive and negative pigs of three different categories, mainly to test oxidative stress hypothesis in pigs naturally infected with PRRS virus. Ninety PRRS positive and 90 PRRS negative pigs were included in the study. The presence of PRRS was confirmed by serological detection of antibodies against PRRS virus (PRRSV) and detection of PRRS viral RNA by RT-PCR. Pigs were further divided into three groups of 30: piglets just before weaning (weaners), fatteners and finishers. Blood samples for determining selected blood parameters were collected from the vena cava cranialis. Significantly ($P < 0.05$) higher activities of SOD in weaners and fatteners and of GPX in weaners were determined in PRRS positive pigs than in corresponding groups of PRRS negative pigs. In contrast, significantly ($P < 0.05$) lower GPX activity was observed in finishers of PRRS positive pigs than in the corresponding group of PRRS negative pigs. Concentrations of serum total protein in PRRS positive weaners and fatteners were significantly ($P < 0.05$) higher than those found in PRRS negative pigs. Leukopenia was observed in all three groups of PRRS positive pigs. It has been demonstrated, for the first time, that oxidative stress might be increased in PRRSV naturally infected pigs, especially in weaners.

Key words: pig, porcine reproductive and respiratory syndrome, blood antioxidant enzymes (SOD, GPX), biochemistry, haematology

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of the porcine reproductive and respiratory syndrome (PRRS), which is a devastating multisystem infection of pigs. PRRS has become one of the most economically important diseases for the swine industry worldwide (Rossow 1998, Stadejek et al. 2002, Neumann et al. 2005). The total production cost for PRRS in the Slovenian swine industry (30,000 breeding sows) is estimated at two million Euros per year.

PRRS is characterized primarily by severe reproductive failure and a high rate of late abortion and early farrowing in sows, and respiratory tract disease and mortality in pigs of all ages, but especially in suckling and nursery-age pigs (Wensvoort et al. 1993, Pejsek et al. 1997, Rossow 1998). The primary host cell for PRRSV is the macrophage (Rossow 1998). Replication of PRRS virus in macrophages in lung and lymphoid tissues and, to a lesser extent, in other tissues, induces lesions and clinical disease by a variety of mechanisms. These include apoptosis of infected cells, apoptotic death of proximate non-infected cells, induction of inflammatory cytokines, induction of polyclonal B cell activation, and reduction in the bacterial phagocytosis and killing by macrophages that result in increased susceptibility to septicaemia (Lemke et al. 2004, Butler et al. 2007, Zimmerman et al. 2012). The cause of PRRSV-induced indirect apoptosis is unknown, but is probably due to substances released from, or secreted by, infected macrophages – apoptotic cytokines, reactive oxygen species (ROS) or nitric oxide (Chiou et al. 2000, Choi et al. 2002, Labarque et al. 2003). In primary bovine turbinate cells infected with bovine viral diarrhoea virus (BVDV) oxidative stress was suggested to be a crucial event in the sequence leading to apoptotic cell death (Schweizer and Peterhans 1999). Secretion of pro-inflammatory cytokines from PRRSV-infected macrophages probably results in effects that are both positive (recruitment of leukocytes, initiation of immune response, and reduction in viral replication) and negative (increased vascular permeability resulting in pulmonary oedema and bronchial constriction) (Zimmerman et al. 2012).

Oxidative stress has been implicated in the pathogenesis of many diseases and inflammatory conditions. It occurs when redox homeostasis within the cell is altered due either to an overproduction of ROS and/or to deficiency of the counteracting antioxidant system (Valko et al. 2007). The pathogenesis of several viral diseases in animals, among them PRRS, has been linked to oxidative stress (Schwartz 1996, Beck et al. 2000, Chiou et al. 2000, Karadeniz et al. 2008,

Panda et al. 2009, Kataria and Kataria 2012). Virus-induced activation of phagocytes is associated with oxidative stress, not only because ROS are released but also because activated phagocytes release pro-oxidant cytokines. The ROS released play the positive modulatory role in immune activation, eradication of viral infection and immune-induced cellular injury (Beck et al. 2000). It has been reported that mononuclear phagocytic cells infected by PRRSV not only produce copious amounts of inflammatory cytokines (Thanawongnuwech et al. 1997, Van Reeth and Nauwynck 2000, Xiao et al. 2010) but also generate ROS (Chiou et al. 2000). Oxidative stress and an overabundance of inflammatory cytokines are known to cause tissue damage in a number of diseases and also in PRRS (Xiao et al. 2010).

There are few data in the veterinary literature on haematological parameters in pigs infected experimentally with different strains of the PRRSV (Christianson et al. 1993, Nielsen and Bøtner 1997, Halbur et al. 2002), but no data on blood antioxidant enzymes and biochemical parameters in PRRSV naturally infected pigs.

Blood intracellular antioxidant enzymes and biochemical parameters have not been studied in pigs naturally infected with PRRSV. The major aim of the study was to test the hypothesis that oxidative stress is increased in PRRSV naturally infected pigs. The objective of the present study was therefore to determine selected blood antioxidant enzymes, glutathione peroxidase (GPX) and superoxide dismutase (SOD), and also biochemical and haematological parameters in PRRS positive and negative pigs of three different age categories.

Materials and Methods

The study involved two one-site (farrow to finish), large pig farms in Slovenia. Both farms were free of Aujeszky's disease and Classical swine fever. Farm 1 had 2300 breeding sows and 25 boars. The presence of PRRS on farm 1 was confirmed by detecting antibodies against PRRSV (ELISA) and detection of PRRSV by RT-PCR. The clinical signs of respiratory and reproductive disorders of PRRS were noticed in infected pigs (abortions, irregular returns to oestrus or non-pregnant sows, lack of libido and reduction in semen quality in boars, and respiratory disorders, reduction in average daily gain and elevated mortality in weaners and growers). Farm 2 had 5000 breeding sows and 35 boars. The farm was free of PRRS, as was confirmed serologically (ELISA) and by RT-PCR. Ninety pigs from the PRRS positive farm and 90 pigs from the PRRS negative farm were included in the

study. At each farm pigs were further divided into three groups: thirty 28 days old piglets, just before weaning (weaners), thirty 120 days old fatteners between 60-70 kg and thirty 160 days old finishers around 100 kg. The pigs were reared according to the Council directive for minimum standards for the protection of pigs (2008/120/EC). All procedures complied with the relevant Slovenian legislation (Animal Protection Act, Official Gazette of the Republic of Slovenia, No. 43/2007).

Blood samples for all blood analyses were collected from the vena cava cranialis. Blood samples for the determination of biochemical profiles, serological and molecular detection of PRRSV were collected into serum separator tubes (Vacuette, Greiner Bio-one, Kremsmunster, Austria). The tubes were stood for 2 hours to clot prior to centrifugation at 1300 x g at 4°C for 10 minutes. Serum samples were stored at -20°C until analyzed. Blood samples for the determination of complete blood count (CBC) were collected into tubes with K₃EDTA anticoagulant (Vacuette, Greiner Bio-One, Kremsmunster, Austria). Blood samples for determining antioxidant enzyme parameters, activity of GPX in whole blood and SOD in washed red blood cells, were collected into tubes containing anticoagulant lithium heparin (Vacuette, Greiner Bio-One, Kremsmunster, Austria). Aliquots of heparinised whole blood were prepared and immediately frozen at -80°C until analysis. Haemolyzed red blood cells were prepared immediately after blood collection, following the manufacturer's (Ransod kit, Randox, Crumlin, UK) instructions, and stored at -80°C until analysis. Haemoglobin concentration in the red blood cell haemolyzates was determined spectrophotometrically by the cyano-methaemoglobin method using an automated biochemistry analyzer RX Daytona (Randox, Crumlin, UK).

180 individual pig samples were tested by ELISA (Ingezim PRRS DR, Ingenasa test kit, Ingenasa, Madrid, Spain) for the presence of antibodies against PRRSV, following the manufacturer's instructions.

Samples from individual pigs were tested in pools (five samples/pool) by reverse transcription and polymerase chain reaction (RT-PCR), using primers for Type I and Type II PRRSV detection (Donadeu et al. 1999).

Biochemical profiles, which involved determination of urea, creatinine (Crea), albumins (Alb), serum total protein (TP), cholesterol (Chol) and triglycerides (Trig), were determined using an automated biochemistry analyzer RX-Daytona (Randox, Crumlin, UK).

CBC was determined, within 6 hours of collection, with an automated haematological analyzer Techni-

con H*1 (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany). CBC included white blood cell count (WBC), red blood cell count (RBC), haemoglobin concentration (Hgb), haematocrit (HCT) and platelet count (PLT).

GPX and SOD activities were determined spectrophotometrically with an automated biochemistry analyzer RX-Daytona (Randox, Crumlin, UK) using the commercial Ransel and Ransod kits (Randox Laboratories, Crumlin, UK), respectively. The Ransel kit is based on the method of Paglia and Valentine (1967) and the Ransod kit on the method of McCord and Fridovich (1969). Activity of GPX and SOD was expressed as units per gram of haemoglobin (U/g Hgb).

Data were analyzed using the SPSS computer program (SPSS 17.0 for Windows, Chicago, Illinois, USA). Results are expressed as means \pm standard deviation. Independent t-test was used to test for differences in all selected blood parameters between PRRS positive and negative pigs of all three categories. The minimum level of significance was defined at $P < 0.05$.

Results

The presence of antibodies against PRRSV and the presence of PRRS viral RNA at farm 1 was confirmed in pigs in all three age categories. Serological analyses revealed no antibodies against PRRSV and no PRRS viral RNA on farm 2 in all pigs tested.

GPX and SOD activities in three groups of PRRS positive and negative pigs are shown in Table 1. The values of GPX in pigs of all categories from both farms were in general agreement with published values (Štukelj et al. 2010). With the exception of SOD activity in PRRS positive weaners, the values of SOD in other groups of pigs from both farms were in general agreement with published values (Štukelj et al. 2010). SOD activities were significantly ($P < 0.05$) higher in PRRS positive weaners and fatteners than in corresponding groups of negative pigs. GPX activities were significantly ($P < 0.05$) higher in PRRS positive weaners and significantly lower in PRRS positive finishers than in the corresponding groups of PRRS negative pigs.

All biochemical parameters (Table 2) were within their reference ranges (Kaneko et al. 2008) in all three groups of PRRS positive and negative pigs with the exception of hypoproteinemia found in PRRS negative weaners. There were significant ($P < 0.05$) differences in biochemical parameters between PRRS positive and negative pigs in each group. In PRRS positive weaners, urea, TP and cholesterol were significantly ($P < 0.05$) higher than those found in PRRS

Table 1. Antioxidant enzyme parameters (means \pm SD) in three groups of PRRS positive and negative pigs.

	PRRS	PRRS	P value	PRRS	PRRS	P value
	negative pigs	positive pigs		negative pigs	positive pigs	
	SOD (U/g Hgb)	SOD (U/g Hgb)		GPX (U/g Hgb)	GPX (U/g Hgb)	
Weaners	1522.5 \pm 142.8	1708.9 \pm 103.8	0.000	154.9 \pm 29.6	173.5 \pm 21.1	0.037
Fatteners	1428.3 \pm 144.2	1585.9 \pm 172.4	0.001	233.9 \pm 56.1	221.8 \pm 35.8	0.394
Finishers	1345.9 \pm 164.9	1381.6 \pm 195.1	0.479	229.5 \pm 38.9	204.9 \pm 31.2	0.016

The P value indicates the significance of differences ($P < 0.05$) in SOD and GPX activities between PRRS positive and negative pigs. SOD – superoxide dismutase; GPX – glutathione peroxidase; U/g Hgb – units per gram of haemoglobin.

Table 2. Biochemical parameters (means \pm SD) in three groups of PRRS positive and negative pigs.

	PRRS positive pigs	PRRS negative pigs	P value	Reference range (Kaneko et al. 2008)
Urea (mmol/L)				2.33-6.66
weaners	4.72 \pm 1.99	2.96 \pm 0.76	0.001	
fatteners	3.44 \pm 0.87	4.66 \pm 0.96	0.000	
finishers	4.74 \pm 1.03	4.99 \pm 1.07	0.355	
Creatinine (μ mol/L)				80-221
weaners	126.77 \pm 21.18	124.46 \pm 10.99	0.664	
fatteners	134.7 \pm 14.65	120.38 \pm 11.64	0.000	
finishers	149.8 \pm 11.97	153.63 \pm 17.19	0.321	
TP (g/L)				62-82
weaners	67.51 \pm 10.26	51.84 \pm 5.84	0.000	
fatteners	77.70 \pm 7.19	72.62 \pm 6.90	0.009	
finishers	77.79 \pm 3.68	78.49 \pm 5.92	0.589	
Alb (g/L)				30-40
weaners	34.31 \pm 4.49	33.22 \pm 3.38	0.298	
fatteners	36.86 \pm 3.56	33.06 \pm 2.96	0.000	
finishers	40.74 \pm 4.76	39.87 \pm 4.63	0.470	
Chol (mmol/L)				1.94-3.10
weaners	2.55 \pm 0.85	2.15 \pm 0.57	0.039	
fatteners	2.55 \pm 0.37	2.89 \pm 0.32	0.000	
finishers	2.65 \pm 0.32	3.01 \pm 0.35	0.000	
Trig (mmol/L)				(data not available)
weaners	0.81 \pm 0.24	0.73 \pm 0.16	0.147	
fatteners	0.74 \pm 0.19	0.62 \pm 0.12	0.010	
finishers	0.56 \pm 0.10	0.46 \pm 0.10	0.008	

The P value indicates the significance of differences ($P < 0.05$) in biochemical parameters between PRRS positive and negative pigs. TP – serum total protein; Alb – albumins; Chol – cholesterol; Trig – triglycerides.

negative weaners. In PRRS positive fatteners, urea and cholesterol were significantly ($P < 0.05$) lower and creatinine, TP, albumin and triglycerides significantly ($P < 0.05$) higher than those determined in corresponding group of PRRS negative pigs. In PRRS positive finishers, cholesterol was significantly ($P < 0.05$) lower and triglycerides higher than those observed in the corresponding group of PRRS negative pigs.

Haematological parameters, RBC, Hgb and HCT, (Table 3) were within their reference ranges (Thorn 2000) in all three groups of PRRS positive and nega-

tive pigs. On the other hand, WBC was significantly ($P < 0.05$) lower in all three groups of PRRS positive pigs than that found in PRRS negative pigs (Table 3).

Leukopenia was found in all three groups of PRRS positive pigs, and thrombocytopenia in weaners and finishers. In PRRS negative pigs WBC remained in the normal range in all three groups; thrombocytopenia was detected only in finishers. RBC and PLT were significantly ($P < 0.05$) lower in PRRS positive than in PRRS negative weaners, but no such differences were detected in fatteners and finishers. On the other hand, HCT was significantly ($P < 0.05$) lower

Table 3. Haematological parameters (means \pm SD) in three groups of PRRS positive and negative pigs.

	PRRS positive pigs	PRRS negative pigs	P value	Reference range (Thorn et al. 2000)
WBC ($10^9/L$)				18.9-26.9
weaners	14.1 \pm 5.2	23.0 \pm 6.1	0.000	
fatteners	19.6 \pm 4.5	28.5 \pm 11.1	0.000	
finishers	16.9 \pm 5.1	22.12 \pm 6.7	0.002	
RBC ($10^{12}/L$)				5.0-8.0
weaners	6.18 \pm 0.66	6.68 \pm 0.61	0.020	
fatteners	6.51 \pm 0.37	6.45 \pm 0.52	0.757	
finishers	6.86 \pm 0.78	6.64 \pm 0.51	0.258	
Hgb (g/L)				100-160
weaners	117.69 \pm 11.82	115.7 \pm 11.36	0.605	
fatteners	113.13 \pm 5.76	114.4 \pm 9.61	0.630	
finishers	126.26 \pm 11.49	129.17 \pm 8.34	0.311	
HCT (L/L)				0.32-0.50
weaners	0.350 \pm 0.035	0.349 \pm 0.030	0.975	
fatteners	0.326 \pm 0.023	0.356 \pm 0.032	0.001	
finishers	0.361 \pm 0.033	0.389 \pm 0.027	0.002	
PLT ($10^9/L$)				325-715
weaners	257.15 \pm 108.85	478.07 \pm 203.58	0.001	
fatteners	393.50 \pm 135.02	461.73 \pm 160.27	0.154	
finishers	266.95 \pm 110.65	284.43 \pm 111.61	0.594	

The P value indicates the significance of differences ($P < 0.05$) in haematological parameters between PRRS positive and negative pigs. WBC – white blood cell count; RBC – red blood cell count; Hgb – haemoglobin; HCT – haematocrit; PLT – platelet count.

in PRRS positive fatteners and finishers than that determined in the corresponding groups of PRRS negative pigs.

Discussion

The present study is the first report on selected blood antioxidant enzymes and biochemical and haematological parameters in three age categories of naturally infected PRRS positive and negative pigs. Significantly increased activity of SOD and GPX has been demonstrated, which suggests that oxidative stress might be increased in PRRSV naturally infected pigs, especially in weaners. Several factors, such as animal age and bacterial co-infection, can influence virus replication and clinical signs. Younger pigs (4-8 weeks of age) infected with PRRSV have been shown to exhibit a longer viraemia and higher excretion and replication rates in macrophages when compared to older (16-24 weeks of age) animals (Van der Linden et al. 2003).

Oxidative stress has been implicated as a pathogenic factor in a number of viral infections, such as in gastroenteritis in dogs with canine parvovirus (CPV) infection (Panda et al. 2009) and in dogs naturally infected with canine distemper virus (CDV) (Karadeniz et al. 2008), in pigs naturally infected with classical swine fever virus (CSFV) (Kataria

and Kataria 2012) and some others. ROS resulting from viral effects on phagocytic and/or host cells might be accompanied by increased cellular antioxidant defences (Schwartz 1996). In dogs infected with CPV significantly increased activity of antioxidant enzymes, SOD and catalase were determined (Panda et al. 2009) in blood samples, suggesting enhanced synthesis of antioxidant enzymes as in-built compensatory mechanism. Additionally, in CSFV infected pigs activities of blood antioxidant enzymes, catalase, SOD, glutathione reductase and xanthine oxidase, increased significantly in affected pigs as compared to those in healthy ones (Kataria and Kataria 2012). It has been reported that mononuclear phagocytic cells infected by PRRSV generate ROS (Chiou et al. 2000). Moreover, PRRSV infection has been shown to induce oxidative stress in MARC-145 cells and alveolar macrophages by generating ROS, and antioxidants inhibited NF- κ B DNA binding activity in PRRSV infected cells, suggesting ROS as a mechanism by which NF- κ B, a critical regulator of innate and adaptive immune function as well as cell proliferation and survival, is activated by PRRSV infection (Lee and Kleiboeker 2005). Oxidative stress and an overabundance of inflammatory cytokines are known to cause tissue damage in a number of diseases including PRRS (Xiao et al. 2010). In order to reduce the consequences of oxidative stress in nursery pigs infected with PRRSV, the effects of a vitamin E rich

diet on growth performance, circulating cytokines, and several haematological traits were studied. However, increasing the antioxidant defences by feeding high levels of vitamin E did not ameliorate the effects of PRRSV – decreased growth, leukopenia, and increased levels of serum IL-1 β and IFN- γ (Toepfer-Berg et al. 2004).

Activities of blood antioxidant enzymes, SOD and GPX, were determined in the present study. These intracellular antioxidant enzymes constitute the primary antioxidant defence system against ROS and are thus sensitive markers of oxidative stress. Both increased and decreased levels have been reported in different diseases as a consequence of enhanced ROS production either by up-regulation of enzyme activity or utilization of the antioxidant enzymes to counter the ROS (Mates 2000, Valko et al. 2007). In the present study significantly increased activities of SOD in PRRS positive weaners and fatteners, and GPX in weaners were observed in contrast to corresponding groups of PRRS negative pigs, which could be ascribed to enhanced synthesis of antioxidant enzymes as an in-built compensatory mechanism in these groups of infected pigs. Similarly, increased activity of blood antioxidant enzymes has been reported in moderately severe cases of gastroenteritis in dogs naturally infected with CPV (Panda et al. 2009) and in pigs naturally infected with CSFV (Kataria and Kataria 2012). In contrast to weaners, in PRRS positive finishers GPX activity was significantly lower than that found in the corresponding group of PRRS negative pigs. The decreased activity of GPX could result from long-term utilization of this enzyme to counter the ROS during PRRS infection. The results on antioxidant enzyme parameters suggest that increased oxidative stress occur in PRRSV infected pigs, especially in weaners.

All measured biochemical parameters were within their reference ranges (Kaneko et al. 2008) in all three groups of PRRSV positive and negative pigs, with the exception of the hypoproteinemia found in PRRS negative weaners. The latter could be due to differences in weaners included in the study. There were significant differences in biochemical parameters, for each group, between PRRS positive and negative pigs. Concentrations of TP in PRRS positive weaners and fatteners were significantly higher than those found in PRRS negative pigs. The main reason is most probably the polyclonal activation of B cells which has been reported to cause hypergammaglobulinemia (Lemke et al. 2004, Butler et al. 2007, Sun et al. 2012). The significant differences in other biochemical parameters, between PRRS positive and negative pigs, could be due to variations between the pigs studied.

Significant differences in haematological parameters were observed between PRRS positive and negative pigs. WBC was significantly lower in all three groups of PRRS positive pigs than that found in PRRS negative pigs. Similarly, Nielsen and Brtner (1997) found significantly decreased WBC in weaners experimentally infected with PRRSV and Christianson et al. (1993) in mid-gestation sows. In contrast, Halbur et al. (2002) reported no significant changes in WBC in four-week-old pigs experimentally infected with four different strains of PRRS from three to 21 days after inoculation and the values of WBC remained within the reference range. In the present study, leukopenia, a consequence of viral infection, was observed in all three groups of PRRS positive pigs, while the values of WBC remained within the reference range in the corresponding groups of PRRS negative pigs. Leukopenia was also described in PRRSV-infected sows (Christianson et al. 1993).

Non-regenerative anaemia was observed in pigs infected experimentally with four different PRRSV isolates. The most highly pneumovirulent strains induced more severe anaemia than the least virulent isolate (Halbur et al. 2002). In contrast, anaemia was not detected in PRRS positive pigs in the present study. However, there were some significant differences in RBC and HCT between PRRS positive and negative pigs. RBC values were significantly lower in PRRS positive than in PRRS negative weaners, suggesting that PRRSV might have an effect on red blood cell precursors, possibly as a direct result of virus replication, although the presence of PRRSV in the bone marrow has not been demonstrated consistently by virus isolation. As in the study of Halbur et al. (2002), significantly lower platelet counts, but within the normal range, were observed in PRRS positive than in PRRS negative weaners. Thrombocytopenia was detected in PRRS positive weaners and finishers and in PRRS negative finishers, which could also be ascribed to PLT aggregation in these groups due to problems arising during collection of blood samples.

It has been demonstrated for the first time that oxidative stress might be increased in PRRSV naturally infected pigs, especially in weaners. It may also be concluded that leukopenia, but not anaemia and thrombocytopenia were detected in all three age categories of PRRSV naturally infected pigs. In order to confirm the results of the present study that was conducted in the field conditions, an experimental study in smaller number of pigs under strictly controlled conditions is warranted. Additionally, the results point to the need for further studies on antioxidant supplementation and subsequent determination of different oxidative stress markers in PRRSV naturally infected pigs.

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