The absence of iron deficiency effect on the humoral immune response of piglets to tetanus toxoid

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ABSTRACT: The aim of this study was to investigate an antibody response to tetanus toxoid in iron-deficient piglets. Ten piglets were not given any iron preparation until the age of 21 days. Ten piglets were given 200 mg Fe^{3+} dextran intramuscularly at the age of 3 days. All piglets were immunized with tetanus toxoid 14 days after birth. Reimmunization was performed 21 days after the first immunization. IgM and IgG titres were measured in weekly intervals after immunization. Haematological indices and iron concentration in the blood plasma of iron-deficient piglets characterized iron deficiency. The dynamic changes in IgM and IgG response to tetanus toxoid were similar in iron-deficient and iron-supplemented piglets and no significant differences in titres between the two groups were found in any period of the trial.

Keywords: antibodies; immunity; titre

Iron deficiency anaemia has been recognized as a serious problem in pig production for a long time. Piglets are born with a limited iron reserve (50 mg Fe) (Venn et al., 1947; Zimmermann, 1995). Sow's milk, however, supplies only a small part of piglets' requirements for iron (Csapo et al., 1995; Kleinbeck and McGlone, 1999). Therefore, without additional iron supplementation, suckling piglets develop anaemia in 10–14 days after birth (Framstad and Sjaastad, 1991; Zimmermann, 1995). The adverse effect of iron deficiency on the red blood cell picture and growth is well known and was described in several papers (Holter et al., 1991; Egeli et al., 1998; Svoboda and Drabek, 2002; Svoboda et al., 2004).

Iron is a vital metal for the proliferation of all cells including those of the immune system (De Sousa et al., 1988; Brock and Mulero, 2000). Contrary to this fact, relatively little is known about the impact of neonatal iron deficiency on the immune system function in pigs. Gainer and Guarnieri (1985) reported a depletion of neutrophil count in iron-deficient anaemic piglets. A low leukocyte count, resulting from a low neutrophil count, was also found in the study conducted by Egeli et al. (1998) on day 35 of age in the anaemic group compared to healthy piglets. The eosinophil counts were also lower in the anaemic group than in the non-anaemic group. Iron deficiency is also associated with the impaired ability of polymorphonuclear granulocytes to kill ingested bacteria. Neutrophils have many iron-containing compounds. Myeloperoxidase is an iron-containing enzyme that is found in primary granules and contributes to the antimicrobial activity. Cytochrome is another iron-containing compound that is found in specific granules and is required for the oxidative burst that follows phagocytosis (Murakawa et al., 1987). Dubansky et al.

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(1997) found a profound deficiency of myeloperoxidase activity in iron-deficient piglets.

In our previous study, we showed that besides anaemia iron-deficient piglets suffered from a depression of the *in vitro* lymphocyte activity as well as lymphopenia due to a decrease in IgM-positive lymphocyte counts (Svoboda et al., 2004). An impairment of lymphocyte activity during iron deficiency was also described in other animal species (Omara and Blakley, 1994; Kuvbidila et al., 2003). In contrast to the data found in other animal species (Helyar and Sherman, 1992) and human patients (Vydyborets, 2000; Bowlus, 2003), in our previous work we did not find a reduction in the count of peripheral blood T-cells of piglets (Svoboda et al., 2004).

The impact of iron-deficient anaemia on the antibody-mediated immune response has been studied rarely so far. Moreover, such an aspect is very important for the possibility to vaccinate piglets in an early stage of life. In very young piglets, maternal antibodies and innate immunity are the major defence mechanisms protecting the neonate against pathogens. With increasing age, active adaptive immunity becomes more important as effector and memory lymphocytes are generated upon stimulation with environmental antigens. The proper functioning of adaptive immunity is mainly important at the time of weaning when passive immunity transfer is ceased (Tizard, 1996; Pansaert and Van Reeth, 1997). In literature, there are only few comparative data dealing with the effect of iron deficiency on the humoral immune response in piglets. Schrama et al. (1997) found that total Ig and IgG concentrations in piglets with lower haemoglobin levels were significantly higher compared to piglets with higher haemoglobin levels.

The main goal of our study presented here was to evaluate the effect of iron deficiency in piglets on the humoral antibody response after intramuscular administration of model antigen – tetanus toxoid.

MATERIAL AND METHODS

Animals and experimental design

In total 20 piglets from two litters were equally divided into two groups; ten piglets were intramuscularly injected with 200 mg Fe^{3+} dextran (Ferridextran 10%, Spofa a.s., Prague, Czech Republic) on the 3rd day of life (hereafter supplemented piglets). Ten piglets were left untreated till the age of 21 days and served as an iron-deficient group. On day 21, the piglets were intramuscularly injected with 200 mg Fe^{3+} in the same preparation. The piglets were kept with sows in farrowing pens on a concrete floor until weaning. The piglets were weaned at the age of 28 days. Blood samples were taken from all piglets for haematological analyses on day 14, 21, and 35 of life. At the same periods, all animals were weighed.

All piglets were immunized intramuscularly with 4 mg of tetanus toxoid (TT) at the age of 14 days. Reimmunization was performed 21 days after the first administration (i.e. 35th day of life). The crude TT (Sevapharma, Prague, Czech Republic) was partially purified by centrifugation and concentrated by ultrafiltration and ammonium sulphate precipitation in 10% aluminium hydroxide (Figure 1). Blood samples were analyzed for TT-specific antibodies on the day of immunization (Day 0) and in weekly intervals after the first immunization (Days 7, 14, 21, 28, 35, 42).

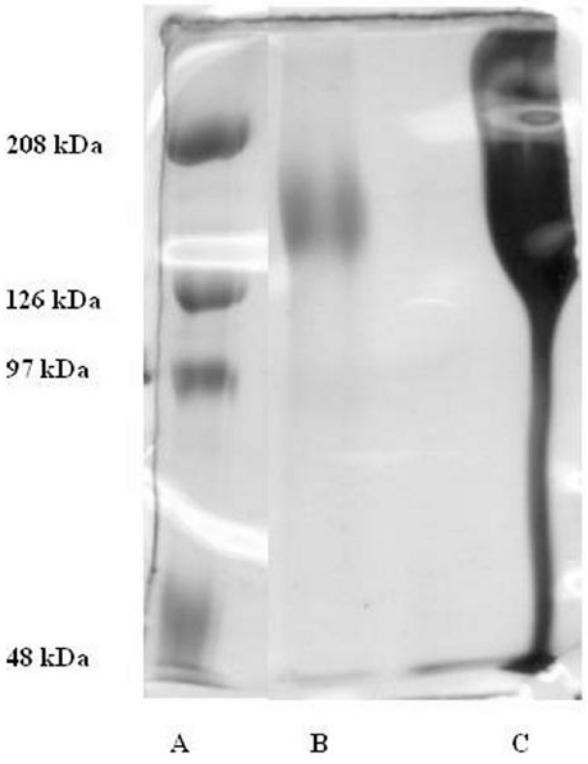

Figure 1. SDS-PAGE characterization of antigen used for vaccination (C) and ELISA detection (B). The molecular weight standard is shown as A

Haematological analyses

Blood was collected from the cranial vena cava. EDTA and heparin were used as anticoagulants for haematological examination and determination of iron concentration, respectively. Blood samples were analyzed for the following indices: haemoglobin concentration, haematocrit, erythrocyte count, mean corpuscular volume, mean corpuscular haemoglobin concentration, mean corpuscular haemoglobin and concentration of iron in blood plasma.

Haematological indices were determined by a Celtac Alfa haematological analyzer (Nihon Kohden, Japan). Iron concentration in blood plasma was determined photometrically by measuring the iron complex with ferrozin (Iron Liquid 917, Roche Diagnostic, Mannheim, Germany).

Detection of antibody response to tetanus toxoid

The detection of TT-specific antibodies was done using the home-made ELISA as described earlier (Faldyna et al., 2003) with modification for a porcine system.

The crude concentrated TT (Sevapharma Prague, Czech Republic) was purified by gel chromatography (FPLC, Pharmacia Uppsala, Sweden) using the separation medium Superosa 12 (Pharmacia Uppsala, Sweden). The fraction with a molecular mass of about 180 kDa was used as the antigen suitable for the coating of polystyrene microtitre plates. Its homogeneity and molecular mass were checked by SDS-PAGE with High Range Prestained SDS-PAGE Standard from Bio-Rad (Figure1).

Concentrations of each of the reagents used in ELISA were determined by checkerboard titrations. The antigen dissolved in carbonate-bicarbonate buffer, pH 8.6, was dispensed into the wells (0.3 μg per well) of 96-well polystyrene microtitre plates (Gama, Dalecin, Czech Republic). The plates were incubated at room temperature for 1 h and subsequently rinsed three times with PBS, pH 7.2, containing 0.05% Tween 20. Then 100 μl of diluting solution (PBS, pH 7.2, containing 0.05% Tween 20 and 0.5% casein hydrolyzate) was dispensed into each well. The wells in the first row were completed with 50 μl blood serum samples pre-diluted 1:33, and threefold dilution series were prepared transferring 50 μl of the mixture into 100 μl of diluting solution. After 60 min of incubation in a humid chamber at 37°C, the wells were rinsed three times and 100 μl sheep anti-porcine IgG and IgM horseradish peroxidase-labelled conjugate (Bethyl, Montgomery, TX) diluted 1:5 000 to 10 000 were added into the respective wells. After another one hour of incubation at room temperature, the plates were rinsed four times and 100 μl substrate + chromogen TMB mixture (Test-line, Brno, Czech Republic) was added into each well.

The reaction was stopped after 15-min incubation by adding 50 μl of 2M sulphuric acid, and absorbances were read at 450 nm using an iEMS Reader multichannel spectrometer (Labsystems, Helsinki, Finland). These measurements yielded titration curves for individual serum samples. The resulting titre was defined as the dilution at which the optical density decreased to the endpoint value ($OD^{450} = 0.1$). The samples were measured in duplicates and their mean was used as a result. Standard serum with a high antibody titre was titrated in each plate and the result was taken into account for the calculations of final titres. The serum obtained from one piglet before the first immunization was used as a negative control in each plate and its absorbance never exceeded the level 0.1 at dilution 1:33. The value of blank never exceeded 0.06.

The specificity of the reaction was checked also by Western-blot (Figure 2).

Statistical analyses

The results are presented as mean values ± SD. The significance of differences between the experimental groups was assessed by the one-tail Mann-Whitney nonparametric test. Differences at $P < 0.05$ were considered statistically significant. All calculations were performed with Prizma® (Graph Pad Software, Inc., San Diego, CA) software.

RESULTS

Haematological parameters indicating anaemia and reduced growth as a result of iron deficiency are summarized in Table 1.

The dynamics of the antigen specific IgM and IgG antibodies after repeated antigen administration are presented in Figure 3. All piglets demonstrated a systemic antibody response to TT which was shown as early as 7 days after immunization.

Table 1. Haematological parameters and body weight in iron-deficient and iron-supplemented piglets. Data are presented as mean values ± SD

Parameter	Group	14th day	21st day	35th day
Haemoglobin (g/l)	iron-deficient	54.2 ± 7.5*	43.0 ± 1.5*	102.8 ± 4.4
	iron-supplemented	104.0 ± 6.4	103.6 ± 6.3	101.7 ± 8.7
Haematocrit (l/l)	iron-deficient	0.17 ± 0.03*	0.14 ± 0.02*	0.32 ± 0.01
	iron-supplemented	0.33 ± 0.02	0.33 ± 0.02	0.31 ± 0.02
Erythrocytes (T/l)	iron-deficient	3.69 ± 0.79*	3.33 ± 0.62*	5.90 ± 0.18
	iron-supplemented	5.32 ± 0.18	5.50 ± 0.23	6.03 ± 0.51
Mean corpuscular volume (fl)	iron-deficient	46.18 ± 3.06*	42.77 ± 3.83*	53.78 ± 1.36
	iron-supplemented	62.28 ± 1.69	60.77 ± 2.13	51.51 ± 3.35
Mean corpuscular haemoglobin (pg)	iron-deficient	15.07 ± 2.33*	13.5 ± 3.33*	17.42 ± 0.73
	iron-supplemented	19.51 ± 0.65	18.8 ± 0.48	16.90 ± 1.10
Mean corpuscular haemoglobin concentration (l/l)	iron-deficient	0.324 ± 0.027	0.310 ± 0.030	0.323 ± 0.007
	iron-supplemented	0.313 ± 0.003	0.309 ± 0.006	0.328 ± 0.004
Fe (µmol/l)	iron-deficient	4.0 ± 0.7*	3.4 ± 0.8*	30.4 ± 3.4
	iron-supplemented	28.2 ± 4.1	29.2 ± 3.9	28.4 ± 3.5
Body weight (kg)	iron-deficient	3.6 ± 0.5*	4.2 ± 0.6*	6.4 ± 0.7*
	iron-supplemented	4.8 ± 0.6	6.2 ± 0.6	9.0 ± 0.8

The values with superscripts show significant differences between the groups
*$P < 0.05$

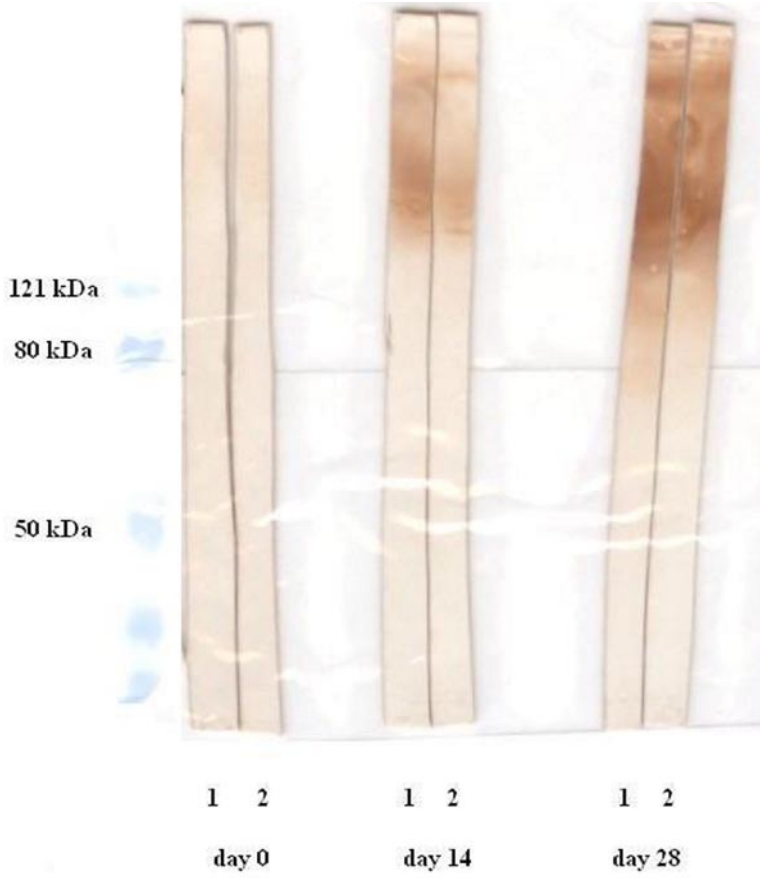

Figure 2. Western-blot characterization of a tetanus toxoid – specific antibody response of iron-supplemented (1) and iron-deficient (2) piglet. The intensity of response is compared at the time of immunization (day 0), two weeks after immunization (day 14), and one week after reimmunization (day 28). The molecular weight standard is shown on the left

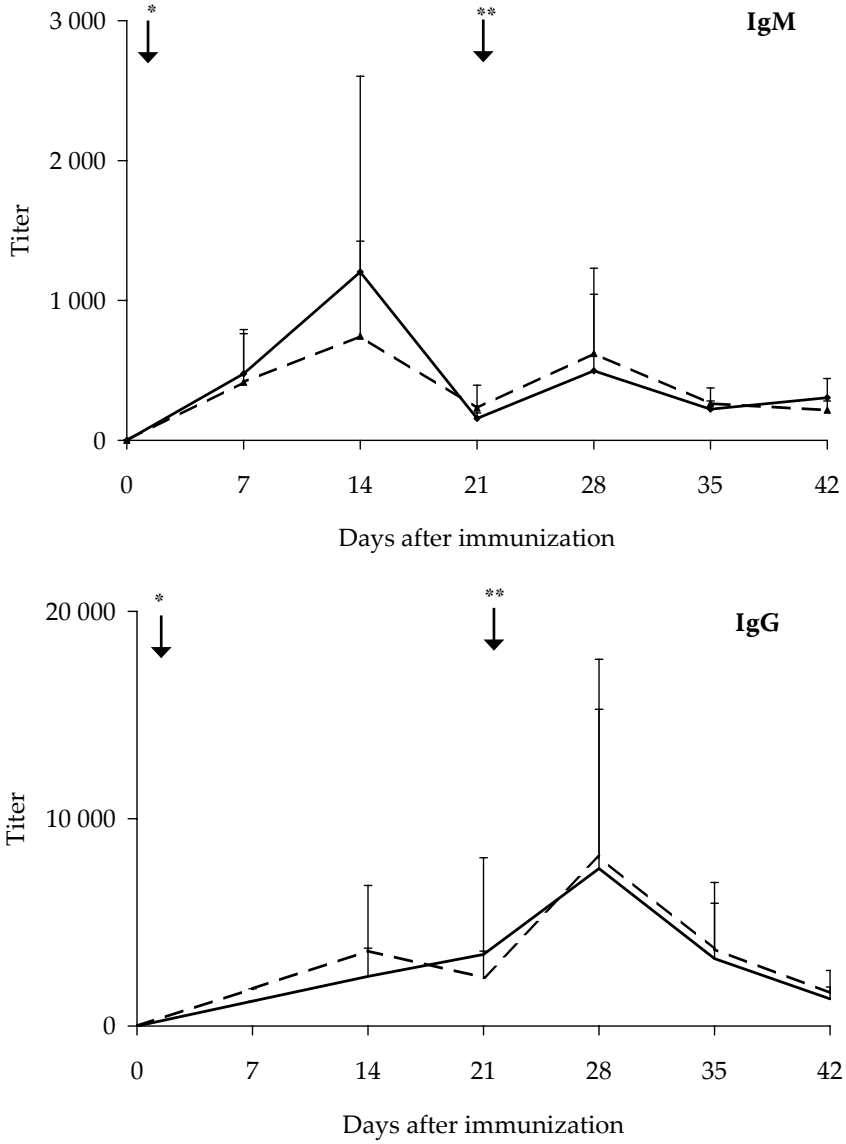

Figure 3. Dynamics of antigen-specific antibodies of IgM and IgG isotype after immunization (*) and reimmunization (**) of iron-supplemented (n = 10; dotted-line) and iron-deficient (n = 10; solid line) piglets with tetanus toxoid. Data are expressed as means and SD

IgM titres were increasing till day 14 after immunization. From day 14 to day 21 the IgM titres decreased. After reimmunization, which was performed 21 days after the first immunization, the IgM titres increased slightly and remained approximately on the same level till the end of the trial. IgG titres were increasing till day 28 after immunization. From day 28 to day 42, a decreasing tendency of IgG titres was found. The dynamics of IgM and IgG response to TT was similar in iron-deficient and iron-supplemented piglets and no significant differences in titres between the two groups were found in any period of the trial.

DISCUSSION

The differences in haematological parameters between iron-deficient piglets and iron-supplemented piglets were as expected. The anaemic limit, i.e. the point when the anaemia begins to exert a detrimental effect on the body weight or evokes clinical symptoms of anaemia, was set by most authors at a haemoglobin concentration of 80 g/l (Egeli et al., 1998). This was also confirmed in our study. The low concentration of iron in blood plasma had a detrimental effect on body weight and clinical signs of anaemia were apparent at the age of 14 and 21 days.

In contrast to those differences, the dynamic changes in IgM and IgG response to TT were similar in piglets of both the groups and no significant differences were found in titres in any period of the trial. These findings differ from the results described by Schrama et al. (1997). They found that the antibody response was either negatively related or not related to the haemoglobin status of piglets. Total Ig and IgG concentrations in piglets with lower (but not anaemic) haemoglobin levels were significantly higher compared to piglets with higher haemoglobin levels. But we must take into consideration that the experimental designs of both studies were different, which could be the cause of differences in the results.

It is difficult to exactly assess why iron deficiency did not affect the antibody response to TT in our study, but several reasons can be discussed. Since we considered the degree of iron deficiency at the age of 21 days as a menace to the future survival of piglets, we started to supplement them with iron. Therefore the piglets were found to be iron-deficient at the time of the first immunization (at the age of 14 days) and one week thereafter. At the time of reimmunization (21 days after the first immunization), all indices increased to the values comparable with those of early iron-supplemented piglets. It means that it was mainly a primary immune response that could be affected by the iron-deficient status of the organism.

The absence of iron effect on the humoral response to T-cell-dependent antigen may be due to a divergent effect of iron on various types of lymphocytes (Schrama et al., 1997). On the other hand, it should be noted here that the activity of lymphocytes was not assessed by the lymphocyte transformation test in this study. The second speculation can be that only the absolute count of B lymphocytes, but not the variability of binding sites of antigen-specific receptors of such cells is affected by iron deficiency. The third theory could be based on the fact that no mitogen used in our previous study (Svoboda et al., 2004) stimulates IgM-positive cells to significant proliferation (Dorn et al., 2002). The question if activated B-cells are not affected or if a decreased number of cells produces the same amount of antibodies might be answered in further studies with ELISPOT-assays for the detection of immunoglobulin secreting cells.

It follows from our study presented here that the antibody response to TT is not adversely influenced by the iron deficiency status of piglets in contrast to the adverse effect of iron deficiency on the cellular immune response found in our previous study (Svoboda et al., 2004).

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