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Diagnostics of iron deficiency anaemia in piglets in the early postnatal period – a review*

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Iron-deficiency anaemia in piglets causes significant losses in animal production. Although this pathology has already been extensively investigated for several decades, we are still looking for best prevention methods. Currently, molecular techniques are being used to study iron absorption in the body. Biochemical and haematological tests, however, continue to be the basis for diagnosis. The latest generation of haematology analysers make it possible to predict the function of the red cell system on the basis of reticulocyte parameters. Mean reticulocyte haemoglobin content deserves special attention. This parameter is commonly used in human medicine, and may also be applicable in veterinary studies, especially in diagnosing iron deficiency anaemia in piglets.

KEYWORDS: anaemia / piglets / iron / haemoglobin / reticulocytes

Anaemia in piglets in the neonatal period is caused by iron deficiency. Low iron content in the organism results in impairment of haem synthesis, which leads to a decrease in red blood cell parameters. Due to a decrease in red cell volume and a reduced haemoglobin content in red blood cells, iron deficiency anaemia is defined as microcytic and hypochromic anaemia. This pathology is quite common in piglets of all breeds, although it may present with different intensity, and its aetiology is

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actually unrelated with the system of piglet rearing or sow feeding. Anaemia causes low immunity of animals, low weight gains and in drastic cases even piglet death, which in turn leads to economic losses in pig production.

The objective of this article is to present a brief characteristic of anaemia in piglets in view of the latest advances concerning molecular regulation of iron metabolism and to show various possibilities of diagnosing this pathology with special regard to new reticulocyte parameters.

Iron metabolism in the body

Special redox properties of iron ions (a very broad spectrum of redox potential of the system involving two forms of iron ions: Fe^{2+} - ferrous ion and Fe^{3+} - ferric ion) are used in numerous biochemical reactions, which determine a proper course of such biological processes as oxygen transport, DNA, ATP and collagen synthesis [Pierre and Fontecave 1999]. On the other hand, uncontrolled reactions of iron ions with reactive oxygen species (ROS), such as hydrogen peroxide and superoxide anion radical, are the source of biological toxicity of iron. It involves participation of Fe ions in a Fenton reaction resulting in the production of a hydroxyl radical, one of the most reactive chemical molecules in the biological environment, which has strong oxidative properties and reacts in an uncontrolled and nonspecific way with most organic compounds [Bartosz 2003].

Due to the biological duality of iron, it is obvious that iron homoeostasis in the body must meet two basic criteria: preserving continuity of biochemical reactions dependent on iron ions and limiting participation of iron ions in reactions that generate toxic oxygen derivatives. Mammals reach the iron balance thanks to a sophisticated system of proteins, which cooperate within cellular organelles, cells and in the whole body. These include proteins involved in iron transport and storage, proteins regulating the iron redox status, as well as signalling and regulatory proteins [Harrison and Arosio 1996, Ponka and Lok 1999, Ganz 2005, Kikuchi *et al*. 2005]. Most of them were discovered at the turn of the 20th and 21st centuries. These findings contributed to a better understanding of molecular mechanisms controlling iron homoeostasis. The most significant advances in the field of iron metabolism of the past 15 years include determination of patterns of intracellular iron homoeostasis in various cell types and identification of a molecular system(s) of connections between them. Cells determining systemic iron homoeostasis include absorptive duodenal enterocytes, erythroblasts - precursor cells of mature erythrocytes found in bone marrow, liver and spleen macrophages and hepatocytes.

Absorptive duodenal enterocytes that supply iron to the whole body absorb it from food in the ionic form (inorganic iron) and in the form of haem (organic iron). These are polarized cells, which have two protein tandems (iron reductase or oxidase and a transporter) in the section of cell membrane that comes into contact with the intestinal lumen and in the section directed towards blood vessels. Ferric ions after

being reduced by duodenal cytochrome b (DcytB) located on the apical membrane of the enterocyte are then transported to its interior by a divalent metal transporter1 (DMT1) [Fleming *et al*. 1998, McKie *et al*. 2001]. From enterocytes, iron is released into the bloodstream by ferroportin with the use of hephaestin – a copper-dependent ferroxidase [Vulpe *et al*. 1999, Abboud and Haile 2000].

Erythroblasts are cells that need iron the most. Thanks to the high density of transferrin receptor 1 (TfR1) on erythroblast membranes, these cells effectively take iron bound to serum transferrin [Ponka and Lok 1999]. Iron taken up by erythroblasts is almost exclusively used for the synthesis of haem, which is then included in haemoglobin molecules. Increased haem synthesis in erythroblasts, from which mature erythrocytes develop in the process of erythropoiesis, is higher by almost an order of magnitude than in nonerythroid cells [Ponka 1999].

A process of primary importance for the maintenance of systemic iron homoeostasis in mammals is connected with the recovery of iron ions contained in haemoglobin of old red blood cells ingested in the process of phagocytosis by macrophages in order to reutilise it for haem synthesis in erythroblasts of bone barrow. Macrophages phagocytose old and damaged red cells and discharge into the bloodstream iron released as a result of haemoglobin and haem degradation. Haem degradation is an enzymatic process, in which haem oxygenase 1 is involved [Anderson *et al*. 2005]. Iron is released from macrophages into the bloodstream by ferroportin, which acts jointly with caeruloplasmin - a ferroxidase dependent on copper ions [Abboud and Haile 2000, Sharp 2004].

Discovery of hepcidin, a peptide hormone regulating systemic iron homoeostasis, and identification of hepatocytes as the main site of its synthesis have completely changed the view on the role that these cells play in iron metabolism [Pietrangelo 2011]. From cells, whose function was mainly thought to be to accumulate iron for the whole body, hepatocytes have acquired the status of cells controlling systemic iron metabolism. Hepcidin is released from hepatocytes into blood as a 25-amino-acid peptide and is bound to ferroportin, which is found on the cell membrane of both enterocytes and macrophages [Nemeth *et al*. 2004]. This interaction leads to ferroportin transfer to the cytoplasm followed by its degradation in lysosomes. Since ferroportin is the only transporter of iron from cells to the extracellular environment, its absence leads to iron accumulation in cells. In the case of enterocytes, iron accumulated in these cells located at the tip of intestinal villi is lost, because enterocytes undergo exfoliation into the intestinal lumen. On the other hand, halting iron flow from macrophages to blood due to high daily amounts of iron transported by this route may within a few days lead to hypoferremia. Iron is the main factor stimulating expression of hepcidin. This regulation is part of a feedback mechanism: high iron content in the liver and high saturation of serum transferrin with iron induce synthesis of hepcidin, which in turn inhibits iron absorption and recirculation, leading to a decreased iron level in the serum, reduced transport to hepatocytes and reduced synthesis of hepcidin [Viatte and Vaulont 2009].

It must be emphasised that most mammals have not developed physiological mechanisms to release iron from the body. It is of crucial importance in human and veterinary medicine when decisions on correcting iron deficiency are made. Special care must always be taken not to overdose iron, which if left in the body may have toxic effects.

Causes, symptoms and correction of iron deficiency in piglets

Studies concerning anaemia in piglets indicate several main causes of this pathology. These include poor iron content in the liver of newly born piglets, low iron contentration in colostrum and milk of sows and limited access of piglets to iron naturally found in soil (iron constitutes about 4-5% of the mineral composition of soil) in most rearing systems. What is also important is the fact that breeders wish to produce breeds characterised by fast weight gains and a large number of piglets born in the litter [Svoboda and Drábek 2005]. Due to changes in the breeding system, newly born piglets are increasingly often characterised by low birth weight (more frequent intrauterine growth restriction - IUGR) [Zabielski *et al*. 2002, Wu *et al*. 2006]. Fast growth requires an increased production of red blood cells, which in turn requires iron contained in systemic iron reserves. As far as pigs are concerned, iron reserve accumulated in the liver during the foetal period is not sufficient. Total Fe content in piglets is about 40 mg, whereas tissues free of fat have the 29 ppm iron concentration. Iron administration to pregnant sows does not significantly improve the status of this element in the offspring [Wei *et al.* 2005]. It seems that investigation of the molecular regulatory mechanisms of iron transport through the placenta would explain the cause of such a low iron content in pig foetuses. Finally, evaluation of a neonate for haemolysis as an element of its normal newborn physiology may be considered in the context of neonatal anaemia. The RBC life span in neonates is shorter than in adult subjects [Pearson 1967]. The reason for the reduced RBC survival observed in newborns is not known, although there are many biochemical differences between adult and neonatal RBCs [Matovcik and Mentzer 1985, Matovcikat *et al*. 1986]. Increased oxidant sensitivity of newborn red cells and the relative instability of foetal haemoglobin (still present in neonates) have been considered as possible causes for this shortened lifespan [Advani *et al*. 1992]. Also, since foetal haemoglobin does not interact with 2,3-diphosphoglycerate (2,3-DPG), it is possible that unbound 2,3-DPG may interact with the membrane and cause injury, leading to early red cell destruction.

Clinical symptoms of anaemia include pale mucous membranes, apathy, a decrease in weight gain or even weight loss, wrinkled skin and rough hair. Acute anaemia may result in respiratory and circulatory disorders or even cause animal death. [Venn *et al*. 1947, Venn and Davies 1965].

The anaemia observed in newborn animals is particularly important, because the effects of this disorder in single individuals may consequently affect the economic result for the whole process of rearing and fattening. Without additional iron supplementation,

piglets develop anaemia within 10-14 d after birth [Framstad and Sjaastad 1991]. Pig farmers rarely allow for the development of such serious health consequences of iron deficiency in piglets, preventing them by iron supplementation, mainly based on iron dextran administration [Egeli and Framstad 1999]. After intramuscular injection iron dextran reaches the lymphatic system, and then blood. There, it is taken up by macrophages (mainly hepatic), whose enzymatic activity leads to the release of iron ions. With the help of ferroportin and ceruloplasmin, iron is transferred from macrophages to blood, where it is bound to transferrin and together with this protein transported to body cells [Svoboda and Drabek 2005]. Total amount of iron administered as iron dextran varies from 200 to 400 mg Fe per piglet, which effectively corrects anaemia and prevents its development. Apart from intramuscular injections, *per os* supplementation is also used. The most frequently orally supplementation is performed by the application of iron fumarate or iron lactate preparations with bivalent iron [Kotrbáček 2001, Svoboda and Drábek, 2002 Svoboda *et al.* 2004].

Laboratory diagnostics of iron deficiency

The use of flow cytometry has significantly contributed to the development of diagnostic methods. One of the methods to apply this technology is to use haematology analysers. Combining cytometry with cytochemical, physico-chemical and biochemical methods guarantees high sensitivity and specificity of blood determinations. The advanced technology used in such a system facilitates analysis of every single blood cell [Burchardt *et al*. 2014]. Light scatter is particularly important in this context. Cells passing through the light beam cause diffraction, refraction and reflection. Then the scattered light is detected by photo detectors. Different light scatter angles provide determination of cell volume, granularity, lobularity as well as information concerning cell surface [Moritz and Becker 2010].

Red blood cell indices read by haematology analysers are crucial for establishing the presence of iron deficiency anaemia. To diagnose this condition, red blood cell indices are taken into account, including red blood cells count (RBC), haematocrit (HCT), haemoglobin level (HGB) and mean corpuscular volume (MCV). Egeli *et al*. (1998) elaborated the reference range for biochemical and haematological parameters in piglets at the 1st, 21st and 35th day of life. The animals were divided into 2 groups depending on haemoglobin concentrations measured on days 14, 21 and 28 (≤80g/l anaemic group or >80g/l normal group). Differences between groups, regarding haematological parameters, are shown in Table 1.

In some haematology instruments RBC measurement is performed using a cytochemical reaction. Firstly, RBCs are isovolumetrically sphered with sodium dodecyl sulphate, then red blood cells are fixed with glutaraldehyde. The next step of the measurement is the light scattering of homogeneous spheres, which provides cell volume and haemoglobin concentration. Then several histograms and cytograms are used to determine and display RBC parameters [Moritz and Becker 2010].

Table 1. Haematological parameters in piglets on the 1st, 21st and 35th day of life [Egeli *et al.* 1998]

Parameter	1st day of life (n=133)	21st day of life		35th day of life	
		normal piglets (n=60)	anaemic piglets (n=42)	normal piglets (n=60)	anaemic piglets (n=42)
HGB (g/l)	81	102	47***	101	66***
RBC (x10^{12}/l)	3.85	5.35	2.93***	5.79	4.41***
MCV (fl)	69.4	63.6	46.8***	56.5	44.8***
RDW (%)	18.4	20.3	35.1***	20.6	33.1***
HDW (g/l)	33.4	25.1	35.8***	22.3	29.5***
HCT (l/l)	0.26	0.34	0.14***	0.32	0.21***
MCH (pg)	21.2	19.2	16.5***	17.6	15.5***
MCHC (g/l)	306	302	361***	311	336**
WBC (x10^{9}/l)	9.07	8.40	8.47	13.58	11.25**
NEUT(x10^{9}/l)	6.56	3.08	2.68	5.56	3.73***
LYMP (x10^{9}/l)	1.77	4.57	5.02	6.78	6.51
MONO (x10^{9}/l)	0.36	0.19	0.21	0.21	0.20
EOS (x10^{9}/l)	0.08	0.14	0.09	0.20	0.12**
BASO (x10^{9}/l)	0.03	0.08	0.05	0.12	0.10
LUC (x10^{9}/l)	0.27	0.38	0.42	0.72	0.64

**P<0.01; ***P<0.001.

Haemoglobin measurements used in some analysers are based on a modification of the manual cyanmethemoglobin method. Blood samples are mixed with the haemoglobin reagent in a special chamber [Moritz and Becker 2010]. The results of the reaction include lysis of RBCs and haemoglobin release. The reaction product is measured colorimetrically. Another method used in the analysers is the cell-based haemoglobin measurement. Both these analytical methods enable determination of important HGB parameters. One of them is the corpuscular haemoglobin concentration mean (CHCM). Other parameters such as mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) or haematocrit are determined based on these RBC and haemoglobin measurements. In diagnosing anaemia, red blood cell distribution width (RDW) is also taken into account. Increased RDW (in iron deficiency anaemia) indicates the presence of both normocytes (red blood cells of normal volume) and microcytes in peripheral blood [Moritz and Becker 2010].

Biochemical indices are also helpful in assessment of iron metabolism. Veterinary studies use mainly the following parameters: iron, ferritin, transferrin concentrations in blood serum and transferrin saturation (TfS), as well as total iron binding capacity (TIBC). Measurement of serum iron concentration is usually performed by colorimetric methods based on the formation and quantitation of a coloured iron-chromogen complex. Serum ferritin level is obtained using immunologic methods. The most popular is the enzyme linked, immunosorbent assay (ELISA); however, ferritins tend to be species-specific, that is why individual assays for different animals have to be developed and calibrated. The one method which may be used to obtain the TIBC includes an addition of ferric ammonium citrate (containing excess Fe^{3+}) to the serum adjusted to a pH of 8.0

(or above) with a buffer solution. The complex (iron-transferrin) at this pH will bind iron until all binding sites are saturated. The excess Fe (unbound) is removed and the iron assay is repeated. In the other method a known amount of excess iron is added and the pH adjusted to 7.5 (or above). Under these conditions only excess free iron may be reduced to Fe^{2+} and react with the chromogen. The iron bound by transferrin remains as Fe^{3+} and it gives no colour reaction. In the supernatant the unbound iron is measured using the iron standard method [Andrews 2010].

Deficiency of Fe is accompanied by low ferritin and iron levels in blood serum, in contrast to transferrin concentration and TIBC, which are on the increase. Iron deficiency may also be indicated by an increased level of soluble transferrin receptor (sTfR) and a high level of zinc protoporphyrin in red cells [Labbe *et al*. 2004]. The value of this parameter increases with disturbed haematopoiesis, when a zinc atom is included in protoporphyrin IX, an organic component of the haem molecule, instead of iron. Although bone marrow collection by biopsy and iron content analysis are considered as a golden standard in assessing iron content in the body (identification of haemosiderin deposits in bone marrow), this procedure is very rarely performed in piglets due to the fact that it is highly invasive [Svoboda *et al*. 2008].

Since the discovery of hepcidin there has been increasing interest in developing assays to measure concentrations of this peptide in body fluids. Quantitative hepcidin methods have been developed on Mass Spectrometry (MS) and Immunochemical (IC) platforms. Currently the harmonization of hepcidin methods, facilitating the definition of universal reference values and clinical decision limits for plasma hepcidin values, is subjected to standardization procedures [MacDougall *et al*. 2010, Kroot *et al*. 2012]. However, a novel mass spectrometry assay to quantify pig plasma levels of hepcidin was developped [Starzyński *et al.* 2013]. The similar method was used to determine hepcidin-25 levels in pig urine [Staroń *et al*. 2015]. The research results may prove useful for the optimal dosing of iron treatment in piglets.

The most common diagnostics of iron-deficiency anaemia is based on the above-mentioned haematologic and biochemical tests. These methods, however, are also characterised by certain limitations. Serum iron level is subjected to considerable diurnal variations. Changes in transferrin, ferritin and sTfR levels may also be induced by factors other than iron, e.g. by inflammation [Svoboda *et al*. 2008, Weiss 2010]. Increased expression of the hepcidin gene is also observed during inflammation [Lee *et al*. 2005], which in turn may cause an erroneous interpretation of the results.

Incorrect diagnosis of iron deficiency anaemia may also be caused by the misinterpretation of haematology parameters. It often happens that changes in the red cell picture, such as reduced values of MCV or MCHC related to Fe deficiency, are revealed only a few weeks after the occurrence of pathology. A solution to this problem is currently being offered by technologically advanced haematology analysers, which apart from determining standard red blood cell indices may also detect even a small number of microcytic or hypochromic erythrocytes in peripheral blood [Weiss 2010].

Additionally, a majority of modern analysers can assess new parameters related to the reticulocyte structure. During erythropoiesis hematopoietic stem cells develop into haemoglobinised red blood cells. Each step of this process includes cell division and differentiation, which is regulated by humoral, microenvironmental, cell surface and transcription factors. In brief, the development of stem cell to erythrocytes occurs in following stages: rubriblast, prorubricyte, basophilic rubricite, polychrotophilic rubricyte, metarubricyte and reticulocyte. Nuclear expulsion from metarubricytes leads to the development of reticulocytes. The expulsion is associated with changes in intermediate filaments and microtubules that result in a rearrangement of the membrane cytoskeleton. Reticulocytes contain residual mitochondria, ribosomes, Golgi membranes and micro tubular components. Maturing reticulocytes are found primarily in the bone marrow. There they can be sheltered until better prepared for circulatory stresses. The maturation stage, at which reticulocytes normally leave marrow, varies with species. Mostly within 12-24 h released reticulocytes undergo complete maturation in the peripheral blood. During this process in the circulation, reticulocytes gain haemoglobin and decrease in volume (they lose organelles and cell surface proteins). The reticulocytes circulating in healthy organisms are found in such species as dogs, cats, rodents, rabbits and pigs. Cows, goats and sheep have circulating reticulocytes only during a regenerative response [Olver 2010].

Due to their short lifespan in the circulatory system, reticulocytes are a perfect indicator of iron deficiency. Various parameters of these cells may also be of great use in assessment of the effects of iron supplementation. According to Markowic *et a*l. [2004], the analysis of reticulocyte indices gives the most precise picture of the course of erythropoiesis and in consequence, an early indication of the subsequent circulatory red blood cell pattern.

Most haematological analysers can perform quantitative measurements of reticulocytes, as well as measurements of their maturity and the level of transformation into erythrocytes (reticulocytes at different stages of maturity have different levels of ribosomal RNA). Reticulocyte analysis is based on a cytochemical reaction. First, RBCs and platelets must be isovolumentrically sphered and the reticulocytes are differentially stained with Oxazine 750. The next step of the analysis is to obtain cell size, haemoglobin concentration and reticulocytes RNA content using low and high angle light scatter and absorption. The retic scatter cytogram is a graphical display of these results [Olver 2010].

Very useful parameters determined by modern analysers are mean corpuscular volume of reticulocytes (MCVr) and mean reticulocyte haemoglobin content (CHr). The latter parameter is a golden standard in human medicine, a diagnostic indicator of real and functional iron deficiency (helpful for patients treated with iron preparations). CHr is increasingly often used as an alternative indicator of iron deficiency in relation to serum ferritin or transferrin levels [Brugnara 2003].

Considering the fact that modern haematology analysers are also equipped with software measuring blood parameters in different species of laboratory and farm

animals, it is possible to determine reticulocyte parameters in veterinary studies. This task was undertaken by Steinberg and Olver [2005], who defined reference values for CHr and MCVr in dogs. The authors claim that a low haemoglobin content in reticulocytes, as well as a low MCVr level are good indicators of iron deficiency in these animals. Similar findings were presented by Fry and Kirk [2006]. The researchers compared reticulocyte parameters, i.e. CHr and MCVr, with standard haematologic and biochemical indices of iron deficiency in dogs. The analysis of reticulocyte parameters turned out to be more effective in assessing animal recovery from anaemia (or iron deficiency) following iron supplementation than the method based on standard indices.

Haemoglobin content in reticulocytes was one of the major indices of anaemia in piglets in studies conducted by Svoboda *et al*. [2008]. Iron supplementation used in these experiments was performed in suckling piglets of different age and with the use of different doses of iron preparations. These included intramuscular injections of 200 mg of Fe^{3+} in the form of iron dextran, and in other experimental groups it was injection of 100 mg of Fe^{3+} as the iron polymaltose complex (IPC) and 100 mg of Fe^{2+} as ferrous fumarate. Apart from CHr values, the authors also included indices of the red cell system (RBC, MCV, Hb, Ht, MCH, MCHC) and serum iron level. Moreover, the reticulocyte count (RET), percentage (RET %) and RET index were analysed. The index RET-Y provides a relative measure for the equivalent of a mean corpuscular volume of reticulocytes [Kickler *et al*. 2004]. According to Svoboda *et al*. [2008], erythropoiesis disturbed by Fe deficiency was more effectively diagnosed by the analysis of reticulocyte rather than erythrocyte indices.

Conclusion

In spite of the fact that the problem of iron deficiency anaemia in piglets was already well known several decades ago, best methods of diagnosis and prevention of this pathology are still being searched for today. Current knowledge on the molecular mechanisms of iron absorption provides new possibilities for the evaluation of results of iron administration to newly born piglets [Lipiński *et al.* 2010, Starzyński *et al.* 2013]. In studies concerning anaemia, quick and reliable diagnosis is essential. Detection of the above-mentioned disorders is typically based on haematological and biochemical parameters, although indices of reticulocyte count and structure are also worth considering. A special diagnostic importance could be attributed to the CHr parameter, which reflects directly the haemoglobin content in the reticulocyte. In human medicine, CHr has become a widely accepted and appreciated index of real and functional iron deficiencies. In veterinary studies this parameter is still rarely used, which is reflected by the limited number of publications concerning this issue. Therefore, there is a great need to continue animal studies with the use of diagnostics based on reticulocyte indices.

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