INNOVATIVE ORAL SUCROSOMIAL FERRIC PYROPHOSPHATE-BASED SUPPLEMENTATION RESCUES SUCKLING PIGLETS FROM IRON DEFICIENCY ANEMIA SIMILARLY TO COMMONLY USED PARENTERAL THERAPY WITH IRON DEXTRAN*

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Abstract

Iron deficiency is the most common mammalian nutritional deficiency during the neonatal period. However, among mammalian species neonatal iron deficiency anemia (IDA), the most severe consequence of iron scarcity, occurs regularly in pigs. Although intramuscular supplementation of piglets with high amounts of iron dextran (FeDex) is largely considered an appropriate preventive therapy for IDA prophylaxis, an increasing evidence shows that it negatively affects pig physiology. The aim of our study was to evaluate the efficacy of non-invasive supplementation of piglets with sucrosomial ferric pyrophosphate (SFP), a highly bioavailable dietary iron supplement in preventing IDA, in humans and mice. Results of our study show that SFP given to piglets *per os* in the amount of 6 mg Fe daily efficiently counteracts IDA at a rate comparable with the traditional FeDex-based supplementation (100 mgFe/kg b.w.; i.m. injection). This was indicated by physiological values of red blood cell indices and plasma iron parameters measured in 28-day-old piglets. Moreover, SFP-supplemented piglets showed significantly lower ($P \leq 0.05$) plasma level of 8-isoprostane, a biomarker for oxidative stress compared to FeDex-treated animals, implying lesser toxicity of this order of iron replenishment. Finally, supplementation with SFP does not increase considerably the blood plasma hepcidin, a peptide that acts to inhibit iron absorption from the diet. SFP emerges as a promising nutritional iron supplement, with a high potential to be adopted in the postnatal period.

Key words: sucrosomial iron, iron deficiency anemia, piglets

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Iron deficiency anemia (IDA) is the most prevalent micronutrient deficiency disorder in suckling piglets of high performance pig (*Sus scrofa domestica*) breeds (Svoboda and Drabek, 2005; Kim et al., 2018; Szudzik et al., 2018). Selective breeding of domestic pigs has been used to control desirable reproduction and growth traits of newborn piglets for centuries (Rubin et al., 2012). It seems, therefore, that both high litter size and daily gain in body weight (connected with the increase in blood volume and number of red blood cells) delineate in piglets an unattainable physiological standard for iron demands and thus are among major etiological factors in pig IDA (Svoboda and Drabek, 2005; Rincker et al., 2005; Kim et al., 2018; Szudzik et al., 2018). Early supplementation of iron to piglets (on days 3–6 postpartum) by the intramuscular injection of large amounts (100 Fe mg/kg b.w or 200 mg Fe per piglet on day 3 after birth) of iron dextran (FeDex) is the most frequent way to prevent the development of IDA in piglets (Egeli and Framstad, 1999). Although this treatment has an undeniable potential to sustain iron balance and provide sufficient amount of this microelement for erythropoietic needs, it may induce harmful reactive oxygen species. It may also incidentally induce sudden cardiovascular collapse and respiratory failure, caused by fatal anaphylaxis leading to asphyxia and hypotension (Ueberschär, 1966). Alternatively, oral iron supplementation in piglets with iron salts and chelates seems to be relatively inefficient (Szabo and Bilkei, 2002; Egeli and Framstad, 1998; Svoboda and Pištková, 2018) except when applied in very high amounts (Maes et al. 2011), mainly because inorganic iron displays low bioavailability and molecular mechanisms of iron absorption in newborn animals are immature (Lipiński et al., 2010).

Sucrosomial® iron is an innovative sucrosomial ferric pyrophosphate (SFP) supplement containing phospholipids and sucrose esters of fatty acids assuring its high bioavailability and tolerability (Pisani et al., 2015; Gómez-Ramírez et al., 2018). The efficacy of SFP in correcting iron deficiency attested by the restoration of physiological hemoglobin levels has been proven in human and animal studies (Giordano et al., 2015; Parisi et al., 2017). Here, we show that SFP given orally to suckling piglets is a suitable feed additive for the reinforcement of iron status in piglets in the critical period from birth to weaning. Compared to parental FeDex, oral supplementation with SFP is not only as effective but also resulted in lower levels of the oxidative stress biomarker 8-isoprostane. To our knowledge, this study provides first demonstration of successful preventive/therapeutic use of SFP in mammalian neonates. This suggests that iron contained in SFP can be efficiently transported across the intestinal barrier and become bioavailable despite immaturity of molecular mechanisms of iron absorption due to its specific chemical formula.

Material and methods

Ethic statement

The experimental procedures used in this study were in compliance with the EU guidelines for the care and handling of research animals (EU Directive 2010/63/EU for animal experiments). The Second Local Ethical Committee on Animal Testing at

the Warsaw University of Life Sciences in Warsaw granted a formal waiver of the ethical approval because the only procedure involved in the study was euthanasia and intramuscular injection of FeDex to piglets is a routine veterinary procedure. Moreover, administration of a dietary supplement such as sucrosomial ferric pyrophosphate is not considered a research procedure.

Piglets, experimental design and biological sample collection

Experiment was conducted at the Pig Hybridization Centre in Pawłowice belonging to the National Research Institute of Animal Production (Balice, Poland). A total of 18 Polish Landrace piglets (males) from 6 different litters housed in standard conditions (70% humidity and a temperature of 22°C in cages with straw bedding) were used. During the experiment sows were allowed to nurse their piglets and piglets had no access to the sows' feed. The Prestarter Wigor 1 Plus feed (containing 238 mg Fe per 1 kg as estimated by flame air-acetylene AAS using PerkinElmer Life Sciences 1100B, MA, USA instrument at 248.3 nm) was offered to piglets from day 5 to day 28 after birth. The composition and nutritive value of Prestarter Wigor 1 Plus is shown in Supplementary Table 1. Piglets from 6 different litters were randomly allotted to 2 experimental groups on the basis of balanced body weight and equalized red blood cell indices at day 3 after birth. Each litter provided piglets for two experimental groups. Other piglets from the litter not selected for experiment did not participate in them but remained with their mothers. First group of piglets (n=9; FeDex) received parenteral iron supplementation (Ferran®100, Vet-Agro, Lublin, Poland, Fe³⁺100 mg/ml; 100 Fe mg/kg BW) on the third day of life, by intramuscular injections of FeDex in the neck. This routinely applied iron supplement is a complex of ferric ions with low molecular weight dextran. The second group of piglets (n=9; SFP) received oral iron supplementation (Sucrosomial® iron; (PharmaNutra, Pisa, Italy, Ingredients: Ferro Sucrosomiale® (iron pyrophosphate – 6 mg of Fe/g of powder, pregelatinised rice starch, saccharic esters of fatty acids, sunflower lecithin supported on glucose syrup, milk proteins, tricalcium phosphate) in a daily dose of 6 mg/kg BW from day 5 to 28 of life. SFP powder was suspended in 2 ml of milk replacer (application with a sterile syringe). Sucrosomial ferric pyrophosphate (SFP) represents an innovative oral iron-containing carrier in which ferric pyrophosphate is protected by a phospholipid bilayer membrane mainly from sunflower lecithin and sucrose matrix (Gómez-Ramírez et al., 2018). Feed intake was calculated as the intake per litter divided by the number of piglets in the litter. Weighed Prestarter was applied to play-feeder® troughs (Middelkoop et al., 2019). When the piglet lost interest in fodder, the remaining Prestarter was weighed. Average total Fe intake from creep feed/piglet (mg) has been calculated. Blood was drawn on day 28 after birth in both experimental groups by venipuncture of the jugular vein (*Vena jugularis externa*) into tubes coated with heparin as an anticoagulant. The blood samples were centrifuged (1200 x g, 10 min, 4°C) to separate the plasma. Plasma samples were immediately aliquoted and stored at -80°C. All experimental piglets were euthanized by the intracardiac injection of 0.5 mL/kg b.w. of Morbital (133.3 mg/mL of sodium pentobarbital + 26.7 mg/mL of pentobarbital, Biowet, Puławy, Poland). Sow's milk was collected at day 21 of lactation. Tissue samples were collected, rinsed with PBS

(phosphate buffered saline) and then stored at -80°C until they were used for biochemical analyses.

Supplementary Table 1. The composition and nutritive value of the creep feeding diet -Wigor 1 Plus

Ingredients (g/kg)	
Ground barley	400.0
Ground wheat	200.0
Ground maize	100.0
HP300 ^a	140.0
Skimmed milk powder	70.0
Pszenmix ^b	35.3
Bergafat HTL ^c	20.0
Calcium formate	12.0
Monocalcium phosphate	8.0
Premix ^d	5.0
Probiotic ^e	0.4
NaCl	3.0
L-Lysine	4.2
DL-Methionine	0.5
L-Threonine	1.3
L-Tryptophan	0.3
Chemicals composition (g/kg)	
ME (MJ/kg)	15.0
Dry matter	887.0
Crude protein	200.0
Crude fat	70.0
NDF	118.4
ADF	42.2
Ash	45.0
Phosphorus	6.0
Calcium	7.5
Iron (mg/kg)	238.0
Lysine	13.5
Methionine	4.9
Threonine	8.2
Tryptophan	2.5

^aSoy protein concentrate.

^bProduct formed during the production of isoglucose; crude protein 17.4%, Ether extract 3.85%, Crude fibre 7.48%, Crude ash 5.26%.

^cPalm fat combined with lecithin.

^dPremix provided the following per kg of diets: Na: 0.28 g, Ca: 2.15 g, P: 0.7 g, Mg: 0.1 g, Lysine: 0.2 g, Fe: 37 mg, Mn: 30 mg, I: 0.75 mg, Zn: 15 mg, Cu: 2.55 mg, Co: 0.1 mg, Se: 0.1 mg, Methionine: 50 mg, Threonine: 90 mg, Tryptophan: 15 mg, Histidine: 17 mg, Vitamin A: 10000 IU, Vitamin D3: 1000 IU, Vitamin E: 20 mg, Vitamin K3: 1.5 mg, Vitamin B1: 0.3 mg, Vitamin B2: 0.8 mg, Vitamin B6: 1.0 mg, Vitamin B12: 10 µg, Niacin: 5 mg, Folic acid: 0.5 mg, Biotin: 20 µg, Nicotinic acid: 6.5 mg, Pantothenic acid: 8 mg, Choline: 25 mg.

^eBonvital, lactic acid bacteria – Enterococcus faecium (DSM 7134, 4b1841), 1×10^{10} CFU/g (SCHAUMANN Agri International GmbH).

Table 1. Comparison of red blood cell indices of piglets supplemented with FeDex^a and SFP^b

Parameter	Groups		SEM	P
	FeDex	SFP		
RBC ^c (x 10 ¹² /L)	5.89	6.05	0.207	0.714
HGB ^d (g/dL)	8.90	8.80	0.381	0.517
HCT ^e (fraction)	0.20	0.26.1	1.827	0.535
MCV ^f (fL)	48.85	41.08	1.971	0.245
RDW ^g (%)	37.28	36.30	2.384	0.832
MCH ^h (pg)	14.40	13.54	0.531	0.451

Values are expressed as the mean $\pm$ SEM (nine male piglets per treatment). ^aIron dextran. ^bSucrosomial ferric pyrophosphate. Blood cell indices were determined for piglets from each group on day 28 after birth. ^cRed blood cell count ($\times 106/\mu\text{L}$). ^dHemoglobin concentration (g/dL). ^eHematocrit value (%). ^fMean corpuscular volume (fL). ^gRed cell distribution width (%). ^hMean cell hemoglobin (pg).

Table 2. Growth performance and sow's milk iron content

Parameter	Groups		SEM	P
	FeDex ^a	SFP ^b		
Initial BW (kg)	1.89	2.02	0.056	0.971
Final BW (kg)	7.16	7.14	0.210	0.957
Creep feed intake (g/day/piglet)	32.66	33.92	1.270	0.584
ADG ^c (g)	194.97	189.46	7.780	0.947
F:G ratio ^d (kg/kg)	0.16	0.17	0.009	0.949
Sow's milk Fe ($\mu\text{g/g}$)	0.33	0.34	0.090	0.990

Values are expressed as the mean $\pm$ SEM (nine male piglets per treatment). ^aIron dextran. ^bSucrosomial ferric pyrophosphate. ^cAverage daily gain in piglets during the period between days 1 and 28 after birth (g). ^dCreep feed to gain ratio.

Measurement of red blood cell indices and plasma iron level

Hematological indices were determined using an automated ADVIA 2010 analyzer (Siemens, Munich, Germany). The plasma iron concentration was determined by colorimetric measurement of an iron-chromazurol complex according to the manufacturer's protocol (Alpha Diagnostic, Poland).

Measurement of 8-isoprostane level

Levels of 8-isoprostane, an oxidative stress marker were measured in plasma, spleen and liver according to the manufacturer's protocol (8 isoprostane ELISA Kit (ab175819), Cambridge Biomedical Campus, UK) (Rajendran et al., 2019; Starczyński et al., 2017).

Measurement of iron content and distribution in tissues

The non-heme iron contents of liver, spleen, kidney, heart, bone marrow and brain (100 mg), as well as milk samples (200 μL) were determined by acid digestion

of the samples at 100°C for 10 minutes, followed by colorimetric measurement of the absorbance of the iron-ferrozine complex at 560 nm (Beckman DU®-68 Spectrophotometer, CA, USA) as described previously (Torrance and Bothwell, 1980).

The heme content of formic acid-solubilized tissues was determined spectrophotometrically at 398 nm (Beckman DU®-68 Spectrophotometer, CA, USA) using hemin standards prepared in formic acid and a molar absorption coefficient of $1.5 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (Motterlini et al., 1995). Non-heme hepatic, splenic and bone marrow iron deposits were analyzed using the Accustain Iron Deposition Kit (Sigma Aldrich, St. Louis, MO, USA). Briefly, liver and spleen samples excised immediately after sacrifice of piglets were fixed in Bouin's solution for 24 h, then stored in 70% ethanol. After embedding in paraffin, the samples were cut into 7- μm sections using a microtome (Reichert-Jung, 2030; Nussloch, Germany). The sections were placed on a slide, deparaffinized and incubated with Perls' Prussian Blue solution for 30 minutes. Slides were counterstained with pararosaniline solution for 2 minutes and examined with a standard compound light microscope (Olympus, type CH2, Tokyo, Japan). The same procedure of staining with Prussian Blue was performed using bone marrow smears prepared from piglets.

Blood plasma hepcidin-25 quantification

Hepcidin-25 measurements were performed as described previously for porcine plasma samples (Starzyński et al., 2013; Staroń et al., 2015) by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF MS) using a stable hepcidin-25+40 isotope as internal standard for quantification. Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionisation TOF MS platform (Bruker Daltonics, Billerica, MA, USA). Hepcidin-25 concentrations were expressed as nmol/L (nM). The lower limit of quantification of this method was 0.5 nM. The concentration of pig hepcidin-25 was calculated by comparing its mass peak height with that of the internal standard (Laarakkers et al., 2013).

Statistical analysis

Data normality tests including the Shapiro-Wilk were performed. Results were statistically analyzed with Student's t-test for normal distributed data or Mann-Whitney test for non normal distributed data using GraphPad Prism version 8.0.1 for Windows (GraphPad Software, USA). $P \leq 0.05$, was considered significant. For the statistical analysis, each individual animal ($n = 18$) was considered as the experimental unit. Data are presented as mean values $\pm$ SEM. All measurements were made in two technical replicates.

Results

Red blood cell indices in piglets supplemented with FeDex and SFP

Oral supplementation of piglets with SFP (daily dose of 6 mg Fe) from day 5 after birth up to weaning resulted in maintenance of values of red blood cell indices

of 28-day-old animals at physiological levels (Table 1). Importantly, hemoglobin concentration in these piglets (8.8 ± 1.64 g/dL) was above the threshold value for anemia in pigs, i.e. 8 g/dL (Egeli and Framstad, 1998). In piglets receiving SFP, values of all hematological parameters were similar (showed no statistically significant differences) to those evaluated in animals given parenteral injection with FeDex, i.e. supplementation considered as highly efficient in preventing the occurrence of IDA and commonly used in pig rearing (Egeli and Framstad, 1999).

Importantly for pig farming and future fattening, feed intake was measured in both groups of piglets and showed no statistically significant differences (Table 2). All growth performance parameters (Table 2) including daily body weight gain (from the 1st to the 28th day after birth), initial and final body weight and tissue weight (Table 2) of 28-day-old piglets receiving SFP *per os* were similar to those of animals given FeDex injections. Iron content in sow's milk were similar (Table 2) and very low compared with creep feed offered to piglets during experiment (Supplementary Table 1).

Blood plasma iron levels and tissue iron content and distribution in piglets supplemented with FeDex and SFP

To compare iron status of piglets from both experimental groups, we performed a comprehensive analysis of iron content in their tissues including the measurement of iron concentration in blood plasma, non-heme and heme iron content in liver, spleen, kidney, heart and brain (Table 4). Considering that liver, spleen and bone marrow are key organs for maintaining systemic iron balance, in addition, we analyzed the distribution of non-heme iron deposits in these tissues by staining with Perls' Prussian Blue (Figure 1). We found that plasma iron levels in piglets supplemented with SFP were almost 2-fold higher compared with FeDex-treated animals ($P < 0.05$) (Table 4), which attests to high nutritional bioavailability of SFP-derived iron and denotes adequate iron provision for piglets erythropoietic demand. In contrast to FeDex-derived iron, iron absorbed from oral supplement is not preferentially stored in the liver as evaluated by biochemical analysis of non-heme (Table 4) and histological analysis of iron deposit (Figure 1). Interestingly, supplementation of piglets with SFP resulted in significantly lower non-heme iron loading of the bone marrow compared to piglets receiving FeDex ($P < 0.05$) (Figure 1). However, using Prussian Blue staining of bone marrow smears we failed to detect any iron deposits in samples from both SFP and FeDex supplemented piglets (Figure 1). In other tissues analyzed for iron content such as spleen, heart, brain, and kidney we detected no significant differences in non-heme iron level between SFP- and FeDex-supplemented animals (Table 4). Regarding tissue heme iron, its levels, regardless of the tissue, did not differ between piglets from two experimental groups (Table 3).

Blood plasma and hepatic 8-isoprostane levels in piglets supplemented with FeDex and SFP

Application of supplemental iron to suckling piglets raises questions about the toxic effects of exposure to high iron levels at an early age. Considering that excess iron induces reactive oxygen species, we determined in 28-day-old piglets from both

experimental groups blood plasma and hepatic levels of 8-isoprostane, a biomarker providing a reliable measure of oxidative stress (Tsikas and Suchy, 2016). We found that in plasma of SFP-supplemented piglets, 8-isoprostane level was only half that measured in FeDex-treated animals (Table 4). In the liver of piglets receiving *per os* SFP we also observed decreased 8-isoprostane concentration, although this decline was not statistically significant. We did not observe a decrease in 8-isoprostane level in the spleen (Table 4).

Table 3. Comparison of different tissues weight in piglets supplemented with FeDex^a and SFP^b

Tissues (g/100 g of body weight)	Groups		SEM	P
	FeDex	SFP		
Liver	2.72	2.86	0.180	0.579
Kidney	0.30	0.38	0.025	0.106
Spleen	0.27	0.27	0.012	0.972

Tissues weight are calculated on 100 g of body weight. Values are expressed as the mean $\pm$ SEM (nine male piglets per treatment). ^aIron dextran. ^bSucrosomial ferric pyrophosphate.

Table 4. Comparison of the tissular non-heme and heme iron content and 8-isoprostane levels in piglets supplemented with FeDex^a and SFP^b

Parameter	Tissue	Groups		SEM	P
		FeDex	SFP		
Non heme iron level ^c (mg Fe/kg of wet tissue)	liver	116.55	29.50	14.110	0.001
	spleen	55.66	76.44	7.951	0.205
	kidney	21.00	21.60	4.160	0.868
	heart	35.44	46.88	4.330	0.545
	brain	9.60	8.80	3.010	0.879
	bone marrow	9.20	4.00	1.110	0.008
Heme iron level ^d (mg Fe/kg of wet tissue)	liver	8.66	10.00	0.612	0.300
	spleen	30.33	31.66	1.856	0.609
	kidney	8.33	11.33	1.160	0.214
	heart	8.66	11.16	1.077	0.060
	brain	3.66	3.33	0.420	0.580
8-isoprostane level ^e (pg/ml)	plasma	63.33	39.33	6.600	0.052
	liver	765.66	613.83	58.153	0.090
	spleen	28.67	30.19	1.976	0.723
Plasma iron level (μ mol/L)		7.0857	9.3563	1.4128	0.043

Values are expressed as the means $\pm$ SEM for tissue samples obtained from pigs of each group (nine male piglets per treatment).

^aIron dextran. ^bSucrosomial ferric pyrophosphate. ^cMeasured in following tissues: plasma, liver, spleen, kidney, heart, brain, bone marrow (mg Fe/kg of wet tissue). ^dMeasured in following tissues: liver, spleen, kidney, heart, brain (mg Fe/kg of wet tissue). ^eOxidative stress biomarker level was measured in plasma, liver and spleen (pg/ml).

Figure 1. Comparison of iron deposits in spleen, bone marrow and liver in piglets supplemented with FeDex^a and SFP^b. Histological examination of iron loading in liver, spleen and bone marrow. Non-heme iron deposits were detected by staining with Perl's Prussian Blue (blue stain) exclusively in Kupffer cells of piglets supplemented with FeDex (indicated by arrow heads). ^aIron dextran. ^bSucrosomial ferric pyrophosphate

Concentration of hepcidin-25 in the blood plasma of piglets supplemented with FeDex and SFP

Analysis of hepcidin level is helpful for guiding safe iron supplementation in piglets. Here, we found that the concentration of hepcidin-25 in heparin-treated plasma

of piglets supplemented orally with SFP and parenterally with FeDex was not detectable in any of the samples (below the lower limit of detection, i.e. 0.5 nM).

Discussion

In most mammals reserves of iron accumulated in the liver during gestation are primary source of this microelement in early postnatal life. However, in piglets hepatic iron stores are relatively low and are rapidly exhausted during few days after birth (Peters and Mahan, 2008; Lipiński et al., 2010). Our previous data indicated that between day 1 and 3 postpartum in the Polish Landrace piglets iron content in the liver is drastically decreased to a barely detectable level (Lipiński et al., 2010). For this reason, exogenous iron fortification is crucial to prevent and treat iron deficiency in piglets. Intramuscular bolus injection of high amounts of iron in the form of FeDex to piglets, has emerged as an efficient technique to counteracting iron deficiency in pig breeding (Egeli and Framstad, 1999; Svoboda and Drabek, 2005; Szudzik et al., 2018). Interestingly, dietary supplements are usually much less effective (Egeli and Framstad, 1998; Szabo and Blikei, 2002; Svoboda and Pištková, 2018), unless iron is given in excessive (grams) quantities (Maes et al., 2011).

Iron absorption is a tightly regulated process, in which several iron transporters, oxidoreductases, and regulatory proteins act in concert to transfer adequate amount of dietary iron successively across the apical and basolateral membrane of duodenal enterocytes (Gulec et al., 2014). Poor responsiveness of suckling piglets during the neonatal period to orally administered iron salts and chelates is caused by impaired molecular mechanisms of iron absorption, i.e. very low expression of the main iron transporters in the duodenum (Lipiński et al., 2010). It seems that immaturity of molecular absorption machinery is a common developmental insufficiency in mammalian neonates as demonstrated in mice, rats and humans (reviewed in Lipiński et al., 2013). Nevertheless, the oral route of iron administration to piglets remains potentially an attractive procedure as it is noninvasive and enables accurate, gradual iron dosing during consecutive days of supplementation.

Liposomes (small vesicles enclosed by a lipid bilayer membrane) are now well recognized and widely used delivery carriers for drugs that can reduce systemic toxicity and increase drug delivery to the target sites in the body (Alavi et al., 2017). Importantly for this study, iron compounds encapsulated in liposomes have been successfully used to relieve the iron deficiency in various animal models (Zimmermann, 2004). Here, we made an attempt to verify efficacy of SFP, liposomal iron preparation to rescue piglets from neonatal IDA. A strong indication for using SFP in the treatment of neonatal IDA in pig model was that its liposomal formulation allows transfer of iron (III) pyrophosphate encapsulated in liposomes across the duodenal barrier independently of established molecular pathways of iron absorption (Rodriguez et al., 2019; Pisani et al., 2015; Mazgaj et al., 2020). Furthermore, sucrose ester has the dual function of *protecting* the iron from *stomach* acid while interacting with the absorptive enterocytes. Preventive/curative effect of piglets supplementa-

tion with SFP, was evaluated in comparison with the parenteral treatment of piglets with FeDex, a procedure with proven efficacy which is widely used in pig rearing (Egeli et al., 1998). In both groups of animals all analyzed RBC indices were similar and perfectly within the physiological range established for this age group of pigs (Egeli and Framstad, 1998). RBC count was insignificantly higher in piglets given SFP. Importantly, physiological RBC status of piglets receiving SFP was accompanied by the normal growth performance as their body weight gain during the first 4 weeks of life met the growth standards for the Polish Landrace breed and was higher than in anemic piglets. Our results prove that the amount and bioavailability of iron provided with SFP is adequate for meeting erythropoietic needs of suckling piglets.

It is worth noting that the appearance of first IDA symptoms is usually preceded by the deterioration of systemic iron status. It often happens that while RBC indices remain within standards, hepatic iron stores decline. Therefore, we evaluated body iron content of piglets supplemented with SFP and found that these animals are sufficiently but not excessively replenished with this microelement, compared with FeDex-treated animals. Firstly, we found elevated iron level in blood plasma, which is a source of iron for erythropoiesis as it is known that diferric transferrin in the blood plasma is the major molecule providing iron for erythroid heme synthesis (Papanikolaou and Pantopoulos, 2017). Secondly, higher (but not significant) iron content detected in the spleen may reflect its contribution to erythropoietic activity. Accordingly, spleen is one of the extramarrow soft tissues that produce red blood cells (Orphanidou-Vlachou et al., 2014). Lower hepatic iron content in piglets supplemented with SFP compared with those given FeDex may be due to the fact that after intramuscular injection of FeDex to animals, it enters the reticuloendothelial cells in the liver through lymphatic circulation and thus contributes to the excessive iron accumulation in this tissue (Geisser et al., 1992). Indeed, in our studies (Lipiński et al., 2010; Staroń et al., 2015; Starzyński et al., 2013; present study) we constantly observed increased deposits of non-heme iron in hepatic macrophages (Browicz-Kupffer cells) after supplementation of piglets with FeDex. Saving reticuloendothelial macrophages from iron overload may be an advantage of SFP-based supplementation as high iron level in hepatic macrophages may contribute to the induction of hepcidin expression (Rausa et al., 2015), a peptide that inhibits absorption of inorganic iron through canonical molecular mechanisms (Lipiński et al., 2010; Staroń et al., 2015). Hepcidin is an iron-regulatory hormone synthesized mainly by hepatocytes and secreted into the circulation as a 25 aa peptide. It acts as a negative regulator of iron absorption from the duodenum and iron release from macrophages recycling old red blood cells and thus adjusts iron supplies to body as per iron requirements (Muckenthaler et al., 2017). Animals supplemented with SFP showed no evidence of increased blood plasma hepcidin concentration, which was below the lower limit of detection, i.e. 0.5 nM. The expression of hepcidin is induced transcriptionally mainly in response to iron. There is experimental evidence that both hepatic iron loading (Pigeon et al., 2001) and high saturation of plasma transferrin with iron (Lin et al., 2007) stimulate hepcidin synthesis. We hypothesize that in piglets supplemented with SFP neither transferrin saturation nor hepatic iron level reached a threshold that could have induced hepcidin. Interestingly, we also found a very low

blood plasma hepcidin level (below 0.5 nM) in FeDex-treated animals. By contrast, hepcidin-25 concentration was strongly elevated in the plasma of 28-day-old piglets subjected to the procedure of 2-fold FeDex injection on days 3 and 21 after birth used in our previous studies (Starzyński et al., 2013; Staroń et al., 2015).

According to the double-edge sword nature of iron, this biometal, apart from being an essential micronutrient, is potentially toxic due to its redox properties leading to the generation of highly toxic reactive oxygen species. In this context, evaluation of any iron supplementation procedure must involve an assessment of its toxic effects. Administration of iron to piglets in the form of FeDex carries a risk of over-supplementation. During this procedure, piglets usually receive in a bolus injection an amount of iron, which exceeds 4–5 times the total content of this element in their body. Consequently, only a part of supplemental iron may be used for physiological purposes. The remaining part may be potentially toxic. We have previously demonstrated that supplementation of piglets with FeDex induces oxidative damage to DNA in the liver (Lipiński et al., 2010). In this study, despite using lower dose of FeDex, piglets receiving this treatment showed the plasma concentration of 8-isoprostane, a popular lipid peroxidation marker (Tsikas and Suchy, 2016) at level almost twice as high as animals supplemented with SFP. As 8-isoprostane found in blood plasma originates from lipid membranes in any tissue of the piglet's body, its plasma levels furnishes a systematic and integrated evaluation of oxidative stress in the whole organism. Our results indicate considerably reduced levels of oxidative stress in piglets fed with SFP compared to animals injected with FeDex. It is noteworthy that the total amount of supplemental iron given per piglet from each experimental group is higher in FeDex group than in SFP group (~200 *versus* 138 mg). Although we cannot calculate the exact amount of iron absorbed by piglets from SFP, our observation shows that the content of iron in feces of piglets from both group is similar (FeDex 0.645 vs. SFP 0.747 mg/kg feces). This suggests that the major part of iron applied orally with SFP is absorbed by piglets. Moreover, our time consuming procedure of individual SFP application to animals may be justified by the fact that we wanted to strictly control the amount of supplemental iron. Such control is indispensable when testing for the first time a new iron supplement. Previously, we have tested the efficacy of heme iron in prophylaxis of IDA in piglets by adding powdered hemoglobin directly to the feed. We hypothesize that conducting a large-scale experiment using SFP will reveal the utility of this supplement to prevent IDA in pigs rearing/farming.

Conclusion

We conclude that oral supplementation of suckling piglets with sucrosomial ferric pyrophosphate during a period of 23 consecutive days, resulted in similar therapeutic effect and caused decreased toxicity of supplemental iron compared to the procedure of iron dextran injection on day 3 after birth. In this context, supplementation with sucrosomial ferric pyrophosphate appears a promising procedure that can be applied for the correction of iron deficiency anemia in suckling piglets. However, the details about molecular mechanisms of sucrosomial ferric pyrophosphate absorption in piglets after *per os* application remain to be elucidated. Appropriate studies in this direction are currently being carried out in our laboratory.

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