Biol Trace Elem Res (2014) 158:342–352
DOI 10.1007/s12011-014-9944-4

Effects of Acute Dietary Iron Overload in Pigs (*Sus scrofa*) with Induced Type 2 Diabetes Mellitus

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Received: 19 January 2014 / Accepted: 12 March 2014 / Published online: 4 April 2014

Abstract Epidemiological studies have reported an association between high iron (Fe) levels and elevated risk of developing type 2 diabetes mellitus (T2D). It is believed that the formation of Fe-catalyzed hydroxyl radicals may contribute to the development of diabetes. Our goal was to determine the effect of a diet with a high Fe content on type 2 diabetic pigs. Four groups of piglets were studied: (1) control group, basal diet; (2) Fe group, basal diet with 3,000 ppm ferrous sulfate; (3) diabetic group (streptozotocin-induced type 2 diabetes) with basal diet; (4) diabetic/Fe group, diabetic animals/3,000 ppm ferrous sulfate. For 2 months, biochemical and hematological parameters were evaluated. Tissue samples of liver and duodenum were obtained to determine mRNA relative abundance of DMT1, ferroportin (Fpn), ferritin (Fn), hepcidin (Hpc), and transferrin receptor by qRT-PCR. Fe group presented increased levels of hematological (erythrocytes, hematocrit, and hemoglobin) and iron parameters. Diabetic/Fe group showed similar behavior as Fe group but in lesser extent. The relative abundance of different genes in the four study groups yielded a different expression pattern. DMT1 showed a lower expression in the two iron groups compared with control and diabetic animals, and Hpc showed an increased on its expression in Fe and diabetic/Fe groups. Diabetic/Fe group presents greater expression of Fn and Fpn. These results suggest that there is an interaction between Fe nutrition, inflammation, and oxidative stress in the diabetes development.

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Keywords Iron · Diabetes · DMT1 transporter · Hepcidin · Iron overload

Introduction

Iron overload has been associated with different clinical conditions such as neoplasms, cardiomyopathy, atherosclerosis, and some chronic diseases such as cirrhosis and type 2 diabetes mellitus (T2D) [1]. The first evidence that systemic Fe overload may contribute to abnormal glucose metabolism derived from the observation that the frequency of diabetes is increased in patients with hereditary hemochromatosis (HH) [2, 3], genetic disorder characterized by mutations in the gene coding for the HFE protein, which leads to a high absorption of dietary iron, and finally to a large accumulation of the metal in different parenchymal cells of organs such as the liver, pancreas, and heart [4, 5]. It has been established that between 53 and 80 % of people with HH finally develop T2D [6]. Furthermore, it has been suggested a pathological role of this mineral because it has been demonstrated that when loads of Fe are reduced, either by blood donations, phlebotomy, or Fe chelating therapies, there is an increase in insulin sensitivity and a decreased risk of developing T2D [7, 8].

Moreover, different epidemiological studies have reported an association between high levels of Fe storage and risk of developing T2D [9, 10] and have demonstrated an association with high consumption of Fe in the diet [11, 12]. However, what are the potential mechanisms linking iron with the development and/or consequences of T2D is an issue still under discussion. One of the mechanisms described for the association between iron and diabetes development risk has relation with oxidative stress. Reactive oxygen species (ROS) can induce direct tissue damage, but also they may play a direct role in hyperglycemia [13]. On the other hand, increased iron levels in specific tissues may increase T2D risk

through other mechanisms; for instance, the liver may induce insulin resistance by impeding its capacity for insulin extraction, thereby resulting in impaired suppression of hepatic glucose production [14]. Also, iron may impair insulin action and interfere with glucose uptake in adipocytes [15]. Further, increased muscle iron stores may enhance free fatty acid oxidation and therefore could interfere with glucose disposal [16]. Thus, both increased glucose production and decreased glucose utilization may occur with increasing levels of body iron [17]. Excess of body iron may also cause iron deposition in pancreatic β cells resulting in impaired insulin secretion [18].

So far, pigs have been widely used to study regulation pathways of nutrients. Pigs are able to accumulate high Fe concentrations in the body [19], making this animal an interesting model of Fe overload, comparable to humans. While several proteins and metal transporters that play a critical role in the homeostasis of trace minerals have been identified, only some of these molecules have been studied in domestic animals such as pigs [20, 21].

The aim of this study was to determine the effect of dietary treatment with high Fe content on Fe nutrition parameters and on the expression of transporters and proteins related to Fe metabolism in the small intestine and liver of pigs (*Sus scrofa*) with induced type-2 diabetes mellitus.

Materials and Methods

Animals and Diets We studied 24 crossbred male pigs (TEMPO×TOPIG 20), averaging 50 days of age and 15± 1.3 kg body weight. Piglets were kept in the Faculty of Veterinary and Animal Science, University of Chile, in roofed pens, cement floors, with nipple drinkers, electric heating lamps, and natural ventilation. All protocols and treatments were approved by the bioethics committee from Institute of Nutrition and Food Technology (INTA). Two diets were used for the study, the only difference being the iron content between the two diets. The basal diet corresponded to the same consumed by these animals at the origin commercial swine herd, made for pigs between 45 and 75 days of age (Table 1). We added 12 g of ferrous sulfate/kg of food to reach 3,000 ppm Fe. Each group was fed for 2 months with the different diets: (1) control group (C), normal pigs fed with basal diet; (2) Fe group (F), basal diet supplemented with 3,000 ppm Fe; (3) diabetic group (D), type 2 diabetic pigs induced with streptozotocin (STZ) (135 mg/kg iv), fed with basal diet; and (4) diabetic/Fe group (DF), STZ-induced diabetic pigs fed with basal diet/3,000 ppm Fe.

Food intake was controlled: in the first 20 days of the study, each animal received 700 g/day, the following 20 days 1 kg/day, and the last 20 days 1.5 kg/day divided into two meals/day throughout the trial. The nutritional quality of diets was checked periodically by proximate and mineral composition analysis (base fresh matter=humidity, 10 %; dry matter, 90 %; total protein, 18.7 %; crude fiber, 4.2 %; crude fat, 6.9 %; nitrogen-free extract, 54.5 %; ash, 5.8 %; calcium, 0.4 %; phosphorus, 0.5 %; energy digestible, 3,425.7 kcal/kg).

Table 1 Diet composition

Ingredient	g/kg
Corn	512
Soybean	100
Mix 80/20 (soybean/corn)	200
Olein	15
Sorghum	50
Bigolac[a]	20
Fish meal (Salmon)	32
Wheat bran	50
Carbonate	4
Phosbic[b]	3
Nucleo lechon[c]	3
Salkil	2
Lysine	3
Threonine	0.2
Natuphos premix[d]	0.5
Salt	3
Duflosan[e]	2

[a] Whey, soy concentrate, casein, and lactose, with a minimum content of dairy products of 60 %

[b] Bicalcium phosphate. Nutritional supplements: Ca 26 % and P 18 %

[c] Vitamins and minerals (per kg of premix): A (9,900 UI), D (1,650 UI), E (77 UI), K (4.4 mg), choline (330 mg), niacin (44 mg), riboflavin (9.9 mg), B_{12} (44 mcg), folic acid (770 mcg), biotin (154 mcg), thiamin (3.3 mg), pyridoxine (4.4 mg), Ca (33 mg), Zn (130 g), Fe (175 g), Mn (45 g), Cu (15 g), I (0.55 g), and Se (0.30 g)

[d] Enzyme supplement (phytase)

[e] Florfenicol 2 %

[a,b,c,d,e] Veterquímica®

Diabetes Induction For T2D induction, pigs in the diabetic and diabetic/Fe groups received a dose of 135 mg/kg of STZ (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) (day 0). Briefly, pigs were anesthetized intramuscularly with ketamine 10 % (0.75 ml/15 kg Ketamil®, Troy Laboratories, Australia) and azaperone (0.2 ml Stresnil®, Janssen Pharmaceutica, Belgium). The anesthesia was maintained on the basis of intravenous ketamine. Then, they were injected with 135 mg/kg of STZ (1 g STZ/10 ml of 0.9 % NaCl) intravenously during 30 min [22] To control diabetes induction, we measured glucose levels during the first week post-induction, before the first meal and after 2 h post-intake, using an OneTouch® UltraMini glucometer (Johnson & Johnson Medical, USA). Then, glucose levels were assessed weekly until the study ended. In addition, we monitored ketones and

glucose in urine using the Keto-Diastix strips (Bayer®, Leverkusen, Germany).

Blood and Tissue Samples At days 0, 30, and 60, blood samples were taken up for biochemical and hematological analysis. These procedures were performed under anesthesia as above. Blood samples (30 ml) were obtained after overnight fasting. For biochemical indicators, serum was obtained through centrifugation of the complete blood at 3,000 rpm× 3 min. Immediately, the serum was stored at −20 °C for subsequent analyzes: glycemia (Dialab, Austria), insulin (radioimmunoassay, Siemens, LA, USA), lipid profile (Dialab, Austria), CRP (Orion Diagnostica, Espoo, Finland), insulin (Kit Coat-A-Count, TKIN 1103, Siemens®, Malvern, Pennsylvania, USA), iron (Fe), and total iron binding capacity (TIBC) by colorimetric method. Total blood were used for hematological studies: red and white cells count, MCV, hematocrit, hemoglobin (Cell Dyn 3200 counter; Abbott Laboratories, Abbott Park, IL), and Zn protoporphyrin (ZnPP; ZP Hematofluorometer model 206D, AVIV Biomedical Inc., Lakewood, NJ).

Prior to sacrifice (day 60), we extracted from each pig blood samples. Animals sacrifice was performed by exsanguination, so that the blood in the organs did not alter the results related to iron status. The pigs were anesthetized as above and then we collect samples of the duodenum, liver, pancreas, kidney, spleen, brain, and heart. The samples were used to measure the total Fe content by AAS with graphite furnace (Simaa 6100, PerkinElmer, Waltham, Massachusetts, USA) after acidic digestion. Tissue samples were stored at −80 °C in RNAlater^{TM} solution (Life Technology, Ambion®, Austin, Texas, USA) to determine the relative expression of gene related to Fe metabolism: DMT1 transporter; transferrin receptor (TfR), ferroportin (Fpn), hepcidin (Hpc), and ferritin (Fn) in the duodenum and liver by qRT-PCR (Brilliant ® II SYBR® Green QPCR Master Mix, Stratagene, Agilent Technologies, Santa Clara, California, USA).

Reverse Transcription and Real-Time PCR Total RNA was extracted from the liver and duodenum using EZNA total RNA kit (Omega Bio-Tek, Norcross, Georgia, USA) and then treated with RNase-Free DNase Set (RNase-free DNase I, 20 Kunitz/μl, Omega Bio-Tek, Norcross, Georgia, USA) according to product protocol. Total RNA (1.5 μg) was reverse transcribed using an Affinity Script QPCR cDNA Synthesis Kit (Stratagene, Agilent Technologies, Santa Clara, California, USA). Real-time PCR was performed using Brilliant II SYBR Green QPCR Master Mix (Stratagene, Agilent Technologies, Santa Clara, California, USA) in a Max Pro System 3000. PCR products were confirmed using a 2 % agarose gel electrophoresis. The number of mRNA copies of target and housekeeping genes was calculated according to the Pfaffl method [23]. The primers used were as follows (5′ and 3′; respectively): **DMT1** (EU647217.1): TGGTCCTCATCGTCTGTTCCATCA and AAGGACAT GCCCAGTGCAATCA; **Fn L chain** (AF 288821.1): GGACGTGCAGAAACCATCTCAA and AGTGGTTCTC CAGGAAGTCACA; **TfR** (AF416763.1): AGCTGCAAAG TCCAGTGTAGGAAC and TCAGTGGCACCAATAGCT CCAA; **Hpc (**AF516143.1): TTCTCCCATCCCAGACAA GACA and CACATCCCACAGATTGCTTTGC; **Fpn** (DFCI Sus Scrofa Gene Index TC374597): AGTTGGGATG CCAGGACATTGA and AAATGGACTCCAGGGCAAGT CA. As a housekeeping gene, we used the following: **SDHA** (DQ402993.1): TTGCGCCCTGCAGACCATTTAT and AGAGACCTTCCCGGTCTTGACAT; **RPL4** (DQ845176.1): GGGACGTTTCTGCATTTGGACTGA and GGCTCTTTGGATCTCTGGGCTTTT; **HPRT1 (**DQ 136030.1): GCAAACCTTGCTTTCCTTGGTC and AGGG CATAGCCTACCACAAACT. Agarose gel electrophoresis was performed to test amplicon specificity. Final results were reported according to Pfaffl [23].

Statistical Analysis The analysis included descriptive statistics, one-way ANOVA and two-way ANOVA, Kruskal–Wallis test, and Tukey, Dunnett's, Dunn's and Bonferroni's as a posttest. Differences were considered significant when $p<0.05$.

Results

Type 2 diabetes mellitus induction was effective with a dose of 135 mg/kg of STZ. The criteria used to define type 2 diabetes were as follows: fasting hyperglycemia (>150 mg/dl) and/or postprandial (>200 mg/dl), fasting normo-insulinemia (insulin levels comparable to baseline or control group), absence of ketones in urine and positive energy balance without exogenous insulin treatment. Fasting and postprandial glucose levels increased in diabetic and diabetic/Fe groups at days 2 and 1, respectively (Fig. 1). Regarding serum insulin measurements at times 0, 10, 20, 30, 40, and 60 days of trial, no significant differences between control group and diabetic/Fe were observed in serum insulin, while diabetic group presented a significant decrease compared to control group only at days 30 and 40. Fe group in turn had higher insulin levels at day 60 (8.2±3.8), a difference that was significant to control (4.0±1.9), diabetic (2.1±0.4), and diabetic/Fe group (2.5± 0.4) (two-way ANOVA: treatment: $p<0.0001$, time: $p=0.0708$, interaction: $p<0.0001$). In urine samples of both diabetic and diabetic/Fe groups, we did not find presence of ketones. The concentration of glucose in urine was variable in these groups, ranging from 0 to 2,000 mg/dl, depending on the day and time of sampling. Both determinations were completely negative in control and Fe animals. The animals

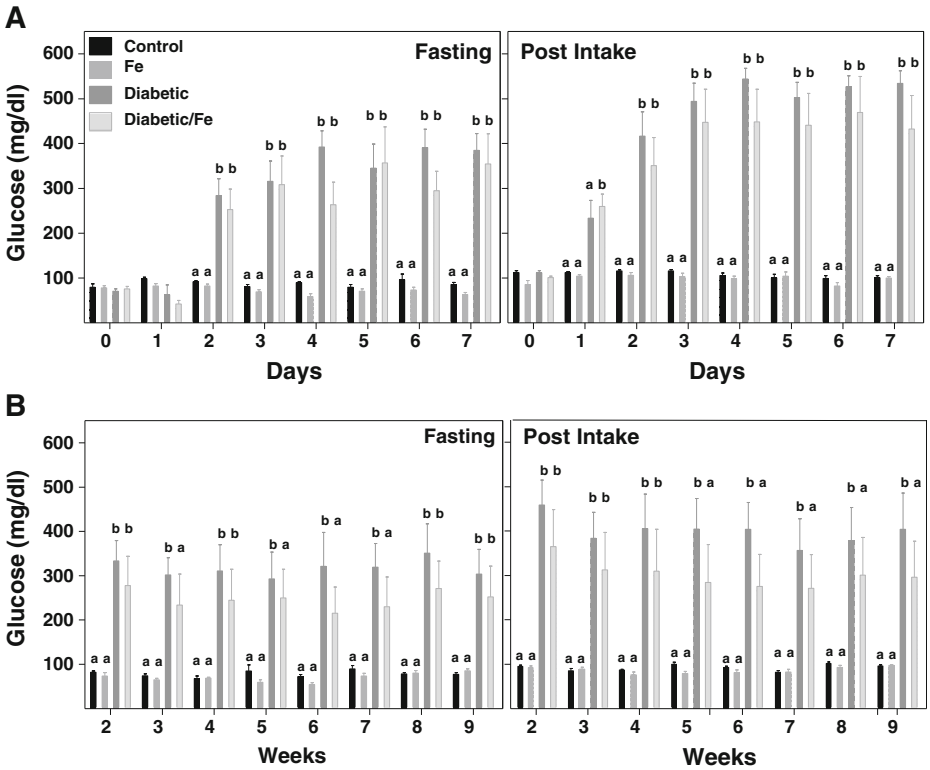

Fig. 1 Fasting glycemia and after 2 h post-intake during the first week (**a**) and during 8 weeks (**b**). Two-way ANOVA: $p<0.0001$ for interaction, treatment, and time and Dunnett's posttest: *different letters* mean significant difference from control group. Mean ± SEM. $N=6$

were weighed every 10 days, and we observed that diabetic and diabetic/Fe groups showed less weight gain compared to control and Fe groups (two-way ANOVA: treatment and time: $p<0.0001$, interaction: p=NS), however, these animals continued to grow normally throughout the study without requiring external insulin administration, an indication of a positive energy balance. These results along with high blood sugar levels in diabetic and diabetic/Fe groups during the study confirm the induction of T2D (Fig. 1).

Hematological and Blood Biochemical Characteristics At baseline (time 0), no differences were found in any of the hematological parameters studied between the groups. However, after 2 months of treatment, we observed differences in the number of erythrocytes, hematocrit, and hemoglobin (Tables 2 and 3), being the Fe group who had greater levels of these three parameters. When comparing baseline values with those obtained on day 60, we observed that the number of erythrocytes varied only in the Fe group (two-way ANOVA, treatment: $p = 0.0038$; time: $p < 0.0001$; interaction: p = NS). While hematocrit varied in the four studied groups (two-way ANOVA, treatment and time: $p<0.0001$; interaction: p=NS) and hemoglobin differences were observed in control, Fe, and diabetic groups (two-way ANOVA, treatment and time: $p<0.0001$; interaction: $p=0.0475$). For CRP, despite the fact that the (two-way Anova, treatment: $p=0.1095$; time: $p=0.4159$; interaction: $p=0.1759$), Bonferroni's posttest showed a difference between diabetic/Fe and control and diabetic groups ($p<0.05$).

At baseline, serum Fe, total capacity to bind iron (TIBC), and percent transferrin saturation (Tf Sat) did not differ between the groups; however, at day 60, we observed a significant increase in serum Fe in Fe group compared to control and diabetic groups, but not with diabetic/Fe group (two-way Anova, treatment: $p = 0.0452$; time: $p = 0.0171$; interaction: $p = 0.0046$) (Tables 2 and 3). For TIBC, at day 60, the Fe and diabetic/Fe groups showed a decreased capacity in TIBC, while the control and diabetic groups experienced a slight increase (two-way Anova, treatment: $p = 0.0002$; time: $p < 0.0001$; interaction: p = NS) (Tables 2 and 3). The main changes in the Tf Sat (%) were observed at day 60 (Tables 2), where the Fe and diabetic/Fe had the greater percentage of saturation compared with control and diabetic groups (two-way Anova, treatment: $p = 0.0067$; time: $p = 0.0265$; interaction: $p = 0.0140$) (Table 3).

By the other hand, biochemical parameters showed no significant differences between groups at baseline (Table 4). At day 30 of the study, we observed significant differences in fasting plasma glucose levels (two way ANOVA, treatment and time: $p<0.0001$; interaction $p=0.0016$) and in triglycerides (two-way ANOVA, treatment: $p=0.0612$; time: $p=0.9577$; interaction: $p=0.2100$) (Table 4 and 5). At day 60 of the study, we observed significant differences only in fasting plasma glucose levels, mainly between diabetic and diabetic/Fe

Table 2 Hematological and iron nutrition parameters at days 0, 30, and 60

	Control (n = 6)	Fe (n = 6)	Diabetic (n = 6)	Diabetic/Fe (n = 6)
Day 0				
Red cells×10^6×mm^3	6.3±0.3	6.7±0.6	6.6±0.7	6.3±0.5
MCV (fl)	54.3±2.5	54.8±2.1	53.2±1.6	55.8±1.5
Hematocrit (%)	34.2±1.6	36.5±2.4	34.0±2.5	35.9±2.9
Hemoglobin (gr/dl)	10.8±0.8	11.8±0.6	10.9±0.9	11.6±1.1
White cells×mm^3	12,300±2,963	11,300±4,376	11,890±2,520	10,610±4,090
ZnPP (μg/dl RC)	119±23.0	106±34.8	126±15.5	110±13.2
Fe (μg/dl)	125±31.3	130±14.1	133±7.2	144±43.0
TIBC (μg/dl)	565±18.3	521±77.1	587±61.7	544±59.6
Tf Sat (%)	24.7±8.2	24.5±3.0	26.6±4.3	33.0±4.8
Day 30				
Red cells×10^6×mm^3	7.4±0.9	8.7±1.5	7.7±1.1	8.3±0.8
MCV (fl)	53.0±3.4ab	54.0±2.6ab	52.0±2.4a	56.0±1.5b
Hematocrit (%)	38.0±2.8a	43.6±4.5b	36.0±2.9a	42.4±3.2b
Hemoglobin (gr/dl)	12.7±1.0 ac	14.5±1.6b	11.5±0.9a	14.1±0.9bc
White cells×mm^3	10,700±2,228	11,280±4,279	11,430±3,048	10,290±2,596
ZnPP (μg/dl RC)	106±22.9	86.2±21.3	90.9±18.6	86.6±17.0
Fe (μg/dl)	163±48.3	140±30.0	156±51.4	175±25.5
TIBC (μg/dl)	470±24.2	439±40.2	488±41.0	427±30.1
Tf Sat (%)	34.7±10.0	31.9±7.0	31.7±9.0	41.1±6.6
Day 60				
Red cells×10^6×mm^3	7.4±0.4a	8.7±0.9b	7.4±0.5a	7.0±1.0a
MCV (fl)	54.5±3.4	53.8±3.0	53.3±2.3	55.8±2.0
Hematocrit (%)	39.6±1.7a	44.6±1.7b	39.1±2.1a	40.1±2.5a
Hemoglobin (gr/dl)	12.9±1.2a	15.0±0.6b	12.6±0.8a	13.0±1.0a
White cells×mm^3	10,300±3,790	12,170±3,705	10,690±1,631	9,827±2,558
ZnPP (μg/dl RC)	107±33.3	90.5±18.9	99.0±8.3	96.7±12.4
Fe (μg/dl)	126±26.7a	219±66.3b	127±32.6a	177±30.0ab
TIBC (μg/dl)	519±41.8ab	465±68.5bc	541±29.8a	449±36.9c
Tf Sat (%)	26.6±6.0 ac	48.3±21.0b	23.5±5.3a	39.5±7.2bc

Data are expressed as mean ± SD. Two-way ANOVA; posttest Bonferroni, different letters indicates significant difference, $p<0.05$

MCV mean corpuscular volume, *ZnPP* zinc-free erythrocyte protoporphyrin, *TIBC* total iron binding capacity, *Tf Sat* transferrin saturation

groups vs. control and Fe groups (two-way ANOVA, Treatment and Time <0.0001 and interaction p=0.0016)

Table 3 Results of the two-way ANOVA analysis of hematological and iron nutrition parameters

	Interaction	Time	Treatment
Red cells	p = 0.2821	p < 0.0001	p = 0.0038
MVC	p = 0.9497	p = 0.3564	p = 0.0047
Hematocrit	p = 0.671	p < 0.0001	p < 0.0001
Hemoglobin	p = 0.0475	p < 0.0001	p < 0.0001
White cells	p = 0.9643	p = 0.6907	p = 0.6321
ZnPP	p = 0.9126	p = 0.0014	p = 0.0899
Fe	p = 0.0046	p = 0.0171	p = 0.0452
TIBC	p = 0.8641	p < 0.0001	p = 0.0002
Tf Sat	p = 0.0140	p = 0.0265	p = 0.0067

(Tables 4 and 5). The diabetic and diabetic/Fe groups showed differences between baseline glycemia and those obtained at day 60 (two-way ANOVA, Treatment and Time <0.0001 and interaction p=0.0016). The HDL cholesterol did not differ between groups at day 60; however in the diabetic and diabetic/Fe groups, there was an increase with time (two-way ANOVA, time: $p<0.0001$; treatment and interaction: p=NS). LDL cholesterol levels were not different between groups at day 60; however, there was a significant decrease between days 0 and 30 of treatment in the control and diabetic groups (two-way ANOVA, time: $p<0.0001$; treatment and interaction: p=NS) (Tables 4 and 5).

Iron Content in Organs The Fe group presented the highest concentrations of this mineral in the liver, spleen, kidney, heart, and pancreas; but mainly in the liver (41.8±9.0 mg/100 g) and spleen (30.1±5.2 mg/100 g) (one-way ANOVA, $p<0.0001$)

Table 4 Biochemistry parameters at days 0, 30, and 60

	Control (n = 6)	Fe (n = 6)	Diabetic (n = 6)	Diabetic/Fe (n = 6)
Day 0				
Glycemia (mg/dl)	114±15.7	104±13.4	106±11.4	107±14.2
Cholesterol (mg/dl)	90.8±11.6	86.0±8.3	75.4±11.7	89.4±14.7
HDL Chol. (mg/dl)	22.2±4.7	21.1±3.4	19.3±4.6	23.2±4.0
LDL Chol. (mg/dl)	62.7±10.4	58.5±5.4	47.7±10.5	58.7±12.6
Triglycerides (mg/dl)[a]	28.9	30.4	40.5	35.0
	(22.4–37.2)	(21.5–43.1)	(29.9–54.9)	(23.6–51.9)
CRP (mg/l)	9.0±2.7	8.7±2.9	9.2±1.8	8.0±2.2
Day 30				
Glycemia (mg/dl)	121±17.4a	101±13.4a	282±94.3b	248±113b
Cholesterol (mg/dl)	79.0±8.4	82.2±17.2	77.2±9.7	84.6±12.0
HDL Chol. (mg/dl)	29.3±6.5	31.6±6.7	29.3±2.7	32.4±6.8
LDL Chol. (mg/dl)	41.1±6.0	42.6±14.1	41.3±11.1	39.6±10.2
Triglycerides (mg/dl)[a]	28.3	25.6	32.5	61.5
	(17.7–45.0)a	(16.3–40.2)a	(21.3–49.6)ab	(46.9–80.5)b
CRP (mg/l)	8.9±3.2	10.4±2.1	8.3±2.7	11.0±3.5
Day 60				
Glycemia (mg/dl)	104±11.7a	110±13.5a	253±84.6b	232±118b
Cholesterol (mg/dl)	79.3±8.8	82.3±11.2	84.2±9.7	92.6±19.6
HDL Chol. (mg/dl)	26.8±3.8	27.4±4.7	27.3±4.3	31.8±8.8
LDL Chol. (mg/dl)	46.3±3.9	47.2±5.7	50.2±10.8	50.2±20.9
Triglycerides (mg/dl)[a]	31.0	38.1	30.9	38.2
	(20.3–47.3)	(25.1–57.8)	(16.5–57.6)	(16.8–87.1)
CRP (mg/l)	8.3±0.9a	9.3±0.8ab	8.0±1.8a	11.7±2.0b

Data are expressed as mean ± SD

CRP C-reactive protein

[a] Triglycerides are expressed as geometric mean (range). Two-way ANOVA; posttest Bonferroni, different letters indicates significant difference, $p<0.05$

(Table 6). On the other hand, the diabetic group had the lower concentration of Fe in liver compared to the others groups (11.6 ±5.2 mg/100 g), while in the other organs did not observe differences in relation to the control group (Table 6). The brain was the only organ without significant changes between treatments (one-way ANOVA, p=NS). In diabetic/Fe group, we observed that generally it reaches the levels of the Fe group.

Relative Abundance of Different Genes Related to Metabolism of Fe In the duodenum, the relative abundance of DMT1 mRNA showed a lower expression in the diabetic/Fe and Fe groups compared with control and diabetic groups (Kruskal–Wallis, $p<0.0001$) (Fig. 2a). Ferroportin showed increased expression in the diabetic/Fe group compared with the control group ($p=0.0327$) (Fig. 2b). Ferritin in the duodenum showed no significant differences (Fig. 2c), whereas Hcp expression showed a decrease in expression in the Fe and diabetic/Fe groups ($p<0.001$) (Fig. 2d). In the liver, Hcp expression increased in diabetic/Fe and Fe groups ($p<0.0001$) (Fig. 3a). The TfR showed a lower abundance in Fe and diabetic/Fe groups compared to control group ($p<0.001$) (Fig. 3b).

Table 5 Results of the two-way ANOVA analysis of biochemistry parameters

	Interaction	Time	Treatment
Glycemia	$p = 0.0016$	$p < 0.0001$	$p < 0.0001$
Total cholesterol	$p = 0.5614$	$p = 0.3790$	$p = 0.1277$
HDL cholesterol	$p = 0.9453$	$p < 0.0001$	$p = 0.1824$
LDL cholesterol	$p = 0.4725$	$p < 0.0001$	$p = 0.7513$
Triglycerides	$p = 0.2100$	$p = 0.9577$	$p = 0.0612$
CRP	$p = 0.1759$	$p = 0.4159$	$p = 0.1095$

Table 6 Total Fe content (mg/100 g wet tissue) in organs

	Control (n = 6)	Fe (n = 6)	Diabetic (n = 6)	Diabetic/Fe (n = 6)	p value[a]
Liver	19.3±5.0a	41.8±9.0c	11.6±5.2a	31.4±10.0b	<0.001
Spleen	14.6±2.2a	30.1±5.1b	16.8±3.6a	29.1±10.3b	<0.001
Kidney	5.0±1.5a	7.1±1.6b	4.5±1.1a	7.3±1.0b	<0.001
Heart	3.5±0.7a	4.2±0.7b	3.0±0.5a	3.0±0.1a	<0.001
Pancreas	1.2±0.2a	2.0±0.3b	1.3±0.4a	1.7±0.2b	<0.001
Brain	0.9±0.2	0.9±0.3	0.9±0.2	0.7±0.1	0.188

Data is expressed as mean ± SD

[a] One-way ANOVA; different letters indicates significant difference

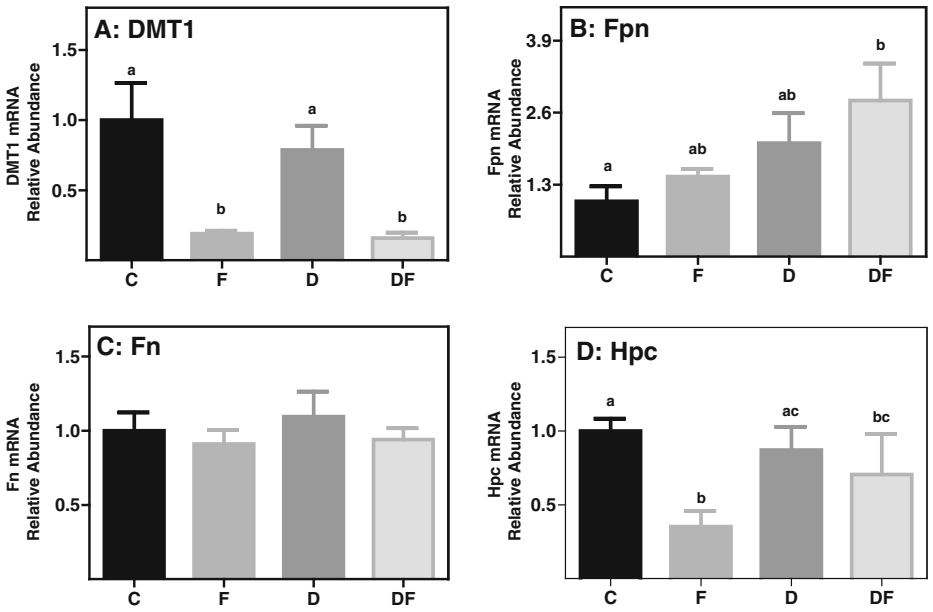

Fig. 2 mRNA relative abundance of genes related to Fe metabolism in duodenum. Mean ± SEM. *N*=6; Kruskal–Wallis test, Dunn's posttest **a** DMT1 ($p<0.0001$); **b** Fpn ($p=0.033$); **c** Fn (p=NS); **d** Hcp ($p<0.001$). Different letters indicates significant difference

Ferritin in the liver was significantly increased only in the diabetic/Fe group ($p<0.05$) (Fig. 3c) and DMT1 expression showed a pattern similar to that observed in the duodenum ($p<0.001$) (Fig. 3d).

Discussion

The pig (*S. scrofa*) as an animal model has generated high expectations not only nutritionally but also in different areas of science because of its similarities to human beings especially in relation to their physiology, organ development, and progression of disease. Also, to understand the underlying mechanisms of chronic human diseases, being used as a model of pharmacologically induced diabetes mellitus by administering drugs such as streptozotocin, alloxan, and nicotinamide, which depending dosage and rate of administration may induce similar syndromes to type 1 or type 2 diabetes mellitus or impaired glucose tolerance [22–24]. In this study, a dose of 135 mg/kg of STZ was effective in inducing type 2 diabetes achieving fasting hyperglycemia (>150 mg/dl) and/or postprandial (>200 mg/dl), fasting normo-insulinemia, no ketones in urine, and positive energy balance without exogenous insulin treatment.

In normal pigs, a diet with high content of Fe administered for 60 days resulted in significant changes on hematological and nutrition parameters and Fe metabolism. Increased iron

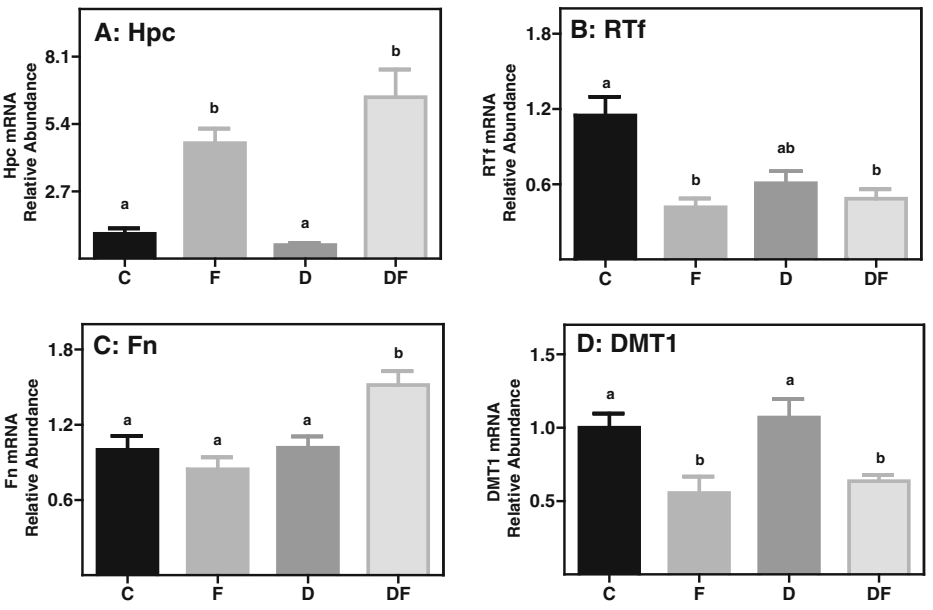

Fig. 3 mRNA relative abundance of genes related to Fe metabolism in the liver. Mean ± SEM. *N*=6; Kruskal–Wallis test, Dunn's posttest **a** Hcp ($p<0.0001$); **b** TfR ($p<0.001$); **c** Fn ($p<0.001$); **d** DMT1 ($p<0.001$). Different letters indicates significant difference

stores in the liver and other organs suggest that the storage capacity of Fe is relatively high. However, these changes occur to a lesser extent in diabetic pigs fed with the same diet (diabetic/Fe group); some of these parameters did not differ significantly from the control group, for example, in the hematological characteristics of these animals. However, in nutritional parameters of Fe, such as serum Fe, TIBC, Tf Sat, and Fe content in organs, we observed a greater Fe status compared to control and diabetic groups.

It is important to note that the observed changes in hematologic parameters, as well as the content of Fe in organs may indicate that indeed the animals fed with diet with high Fe content were loaded with this metal, which could lead to damage associated with oxidative stress due to the increased availability of Fe to react with other molecules, generating free radical production. Given this evidence, it would be essential to evaluate different markers of oxidative stress, either at blood level or more specifically at level of tissues.

Although several proteins and metal transporters that play a critical role in the trace minerals homeostasis have been identified, only few of these molecules have been studied in domestic animals such as pigs [19–21], and in relation to diabetic pigs, there is no previous history.

The results for the transporter DMT1 are consistent with those described in the literature. DMT1 is the primary transporter of Fe at enterocyte level and also it is present in the liver, the main organ for Fe deposit [25]. This transporter is expressed in relation to the availability of Fe, greater amount of Fe available DMT1 decreases its expression mainly in the duodenum, in order to protect the cell from an overload of this mineral [26–28].

This observation is consistent with that observed in pigs of groups fed diets high in Fe, which showed a significant decrease in the expression of DMT1 mRNA in both liver and duodenum. Moreover, the entry of Fe into the cells of the body is a process primarily mediated by the TfR, since a significant portion of the circulating Fe is mobilized attached to the transferrin. Like DMT1, TfR expression is mediated by intracellular Fe concentrations, decreasing its expression when it is iron overload, while in front mineral deficiencies increases its expression [29]. This is consistent with what was observed in Fe and diabetic/Fe groups, which reduced receptor expression in relation to the control group. It was expected to find a similar expression in the diabetic group compared to control group because Fe status is comparable in both groups; however, a slight decrease in the expression of TfR in the diabetic group compared with the control group (p=NS) was found. This reduced expression could explain the lower levels of total Fe found in the liver of these animals. On the other hand, it shows that in diabetic animals there exist a degree of alteration in the metabolism of Fe.

For ferritin, no significant difference in expression in the duodenum was found, which is consistent with that observed by Hansen et al. [20], who fed pigs with diets high in Fe (700 mg Fe/kg dry matter in the form of $FeSO_4$). In this case, Fn expression was not affected by dietary Fe concentration and the age of the animals. In rats, no significant differences were observed in the relative abundance of Fn in the liver. In this study, there were four groups (control rats, rats with high Fe diet, diabetic rats, and diabetic rats with high Fe diet), making it comparable with our work [30]. Hentze et al. [31] demonstrated that human Fn biosynthesis is dependent on Fe, but this regulation is not accompanied by any change in the levels of total or cytoplasmic Fn mRNA. Moreover, in different brain areas, it has been seen that the mRNA levels of Fn and protein levels do not correlate with each other, indicating that there would be posttranscriptional regulation mediated by Fe status through system IRP-IRE [32].

Regarding expression of ferritin, a significant increase in its expression in the liver was found only in the diabetic/Fe group. Fn is recognized as an acute phase protein and a marker of acute and chronic inflammation, and nonspecifically rises against a wide range of inflammatory conditions [33]. Fn increased induced by inflammation would be at translational level and no changes would be observed relative to Fn mRNA levels [34–36]. However, it has also been postulated to be an increase in mRNA expression of Fn [37]. Furthermore, Pang et al. [38] demonstrated that the mRNA of both Fn subunits was induced in endothelial cells and macrophages of early atherosclerotic lesions of human aorta and in smooth muscle cells at later injury. Furthermore, a significant increase in both Fn mRNA levels in cultures of THP-1 cells (human monocytes) treated for 24 h with TNF was observed. Heller et al. [39] examined peripheral blood lymphocytes from patients with rheumatoid arthritis and inflammatory bowel disease for identifying gene expression infiltrating inflamed tissues from the circulating blood. Among its findings, some of the genes found upregulated in rheumatoid arthritis were the L Apoferritin unit and Mn-SOD, indicating that Fn is the main Fe storage protein, which responds to intracellular oxidative stress and reactive substances to oxygen generated during inflammation. Therefore, the key to increased Fn expression observed in the diabetic/Fe group would be the sum of several factors, such as excess Fe, inflammation, and probably oxidative stress. The fact that Fn is expressed in response to oxidative stress could indicate an important role of Fn in decreasing the pool of labile Fe to minimize the possible formation of ROS via Fenton reaction [40].

Hpc mRNA expression, principal Fe metabolism regulating hormone, was measured in the liver and intestine. In the liver, Hpc expression was higher in both groups exposed to high concentrations of Fe in the diet vs. control and diabetic groups. Hpc is regulated by Fe concentrations and by erythropoietic requirements for Fe; therefore, when it exceed the supply of Fe, this hormone is secreted by the liver to control the entry of Fe into the bloodstream from erythrocytes. Furthermore, it has

been described that they have a direct effect on the expression of DMT1 in the apical membrane of the intestinal cells, inhibiting their transcription [41]. However, Hpc expression not only is controlled by Fe, since it is known that inflammation and hypoxia are all factors that promote expression. During inflammatory processes, Hpc is stimulated by IL-6, generating anemia of inflammatory response, during which a redistribution of Fe reserves mainly accumulate in macrophages is generated [42, 43]. This phenomenon could explain in some way the lower levels of Fe in serum, as well as lower levels in different nutritional parameters of Fe found in the diabetic/Fe group compared to the Fe group. So the higher Hpc expression, the elevated CRP levels and the increased Fn expression in the diabetic/Fe group could be due to the inflammatory process that would be developing these animals.

For diabetic patients, several studies show increased expression of Hcp or higher circulating levels of this hormone. Jian et al. [44] showed that type 2 diabetic patients had Hpc levels significantly higher than control subjects, as well as serum ferritin and TfR. To examine whether the increase in Hpc was partly due to inflammation, they measured hsCRP and IL-6, which were significantly higher in diabetic patients, so they conclude that higher levels of Hcp in type 2 diabetic patients is due to both high ferritin levels due to an accumulation of Fe and increased amount of inflammatory proteins. When Hpc was added to macrophages, an increase in proinflammatory cytokines production is observed, thereby contribute to the vicious circle that perpetuates the inflammation state.

With respect to the relative abundance of Hcp in the duodenum in the four study groups, no published information was found. Most papers describe the Hpc mRNA expression in the liver because hepatocytes are the major source of circulating Hcp; however, other cell types such as macrophages, adipocytes, kidney cells, epithelial cells of the airways, and β pancreatic cells expressed Hpc mRNA but in smaller amounts. The relevance of extra hepatic Hcp production is still not very clear, but could have an important role in the local regulation of flows of Fe [45–48]. This study showed that there is Hpc mRNA expression in pig duodenum and could see that there is a different pattern of expression among the four groups, showing a decrease in expression in Fe group compared to control group. The importance of expression of Hpc in duodenum is an issue that requires further study, since it probably has some function in regulating enterocyte Fe level. Also, there is differential expression between groups and we can confirm that the process of diabetes does have an effect on the metabolism of Fe also at this level.

Ferroportin, main exporter of Fe, showed no significant difference in expression between groups control and Fe. This is consistent with that observed in other works where young pigs were fed with diet with high levels of Fe [20, 21]. It has been demonstrated that regulation of Fpn by Hcp would be at posttranscriptional level. Hpc treatment of duodenum and spleen of mice showed that changes in protein expression of Fpn are produced, but mRNA levels were not altered [49]. Regarding the expression of Fpn in diabetic animals, no previous works were found. The increased expression of Fpn in the diabetic/Fe group found in this study could be explained in part by Khan et al. [50], where hemin-treated rats showed an increased Fpn mRNA expression, HO activity and Fe content in hepatocytes and Kupffer cells, and an increased inmuno-staining of 8-OHdG marker of oxidative DNA damage in liver tissue, suggesting that increased expression of Fpn would represent an adaptive mechanism to remove Fe from the cell.

In summary, pigs are a good model to study different conditions including pathologies or nutrient deficiencies or excess. The characteristics of iron metabolism in pigs are similar to the humans, and using a induced type 2 diabetes model with normal and high levels of iron in diet, showed similar behavior as type-2 diabetes in human, i.e., high levels of fasting and postprandial glucose, fasting normoinsulinemia, with a positive energy balance, high levels CRP and iron storage, and oxidative stress.

Acknowledgments We thank Angelica Letelier and Amaya Oyarzún for technical assistance, and Maureen Middleton for helping with care and management of animals. This project was funded in part by (1) Comisión Nacional de Investigación Científica y Tecnológica (CONICYT)—Scholarship for Doctoral Thesis 2010; (2) Programa de Investigación Domeyko en Salud, Universidad de Chile—Scholarship Doctoral Thesis 2010; and (3) FONDECYT Grant 1110080 to MA

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