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Effects of iron deficiency anemia and its treatment with iron polymaltose complex in pregnant rats, their fetuses and placentas: Oxidative stress markers and pregnancy outcome

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ABSTRACT

Objective: Iron deficiency anemia (IDA) can severely impair the outcome of pregnancy. IDA has been shown to cause oxidative stress, which may be exacerbated by oral iron therapy. In this study, the effects of IDA and its treatment with iron polymaltose complex/folic acid (IPC/FA) were examined in anemic pregnant rats, their fetuses and placentas.
Study design: Hematological variables and oxidative stress markers in the liver, heart and kidney were evaluated in non-anemic, anemic and IPC/FA-treated pregnant rats and their fetuses. Markers for oxidative stress, inflammation and hypoxia were assessed in the placentas of all groups.
Results: IDA was shown to increase oxidative stress levels in all the studied organs and in placenta as well as hypoxia and inflammation in placenta. IPC/FA treatment corrected IDA measured by the hemoglobin level, serum iron level and transferrin saturation. The oxidative stress levels in all the studied organs and in placentas of the IPC/FA-treated group were comparable to those of the non-anemic group. The number of fetuses and the neonatal and placental weight were lower in the anemic group compared to the non-anemic and IPC/FA-treated groups.
Conclusions: The current study shows that IDA in pregnant rats impaired pregnancy outcome, increased the expression of hypoxia and inflammatory markers in the placenta, and increased oxidative stress in dams, fetuses and placentas. Treatment with oral IPC/FA corrected the IDA as well as reduced the levels of oxidative stress and inflammatory markers close to non-anemic control values in all the studied organs.

1. Introduction

Iron deficiency (ID) is one of the most common nutritional deficiencies in the world and the primary cause of anemia [1]. Estimated by the World Health Organization (WHO), the prevalence of anemia in pregnant women is 44% [1]. Iron deficiency anemia (IDA) during pregnancy has serious and long-lasting consequences both in the mother and the fetus [2,3]. In the mother, IDA is associated with impaired physical performance, fatigue, reduced cognitive performance, increased risk of infection and hospitalization, inhibited lactation and postpartum depression [4,5]. Anemia is associated with 40% of maternal deaths worldwide [1]. Adverse consequences for the fetus include intrauterine fetal death, low birth weight, growth retardation, hypertension, neurologic impairment and preterm birth [4,6–8]. IDA has also been shown to lead to placental hypertrophy [9] possibly depending on the severity of IDA.

Pregnancy increases the requirement for dietary iron absorption from 0.8 mg/day in the first trimester to 7.5 mg/day in the third trimester [10,11]. Thus, it is very difficult for the mother to maintain her iron stores through pregnancy without any supplementation [6,10] and, thus, the WHO has recommended iron supplementation throughout pregnancy to prevent anemia [12].

Oral iron is the first-line treatment for ID and IDA during pregnancy [4]. The most frequently used oral iron preparations are ferrous [Fe(II)] sulfate, ferrous fumarate, ferrous glycine sulfate, and ferrous gluconate. Fe(II) from these preparations is taken up in the gut not only by the physiological, active pathway but also by uncontrolled, passive diffusion directly into the blood through the paracellular route [13,14]. This may result in non-transferrin bound

Abbreviations: bw, body weight; Cu,Zn-SOD, Cu,Zn superoxide dismutase; GPx, glutathione peroxidase; GSH, glutathione; GSSG, oxidized glutathione; HIF-1α, hypoxia-inducible factor 1-alpha; ID, iron deficiency; IDA, iron deficiency anemia; IL6, interleukin-6; IPC/FA, iron polymaltose complex/folic acid; IUGR, intrauterine growth restriction; NTBI, non-transferrin bound iron; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive species; TNF-α, tumor necrosis factor-alpha; TSAT, transferrin saturation.

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iron (NTBI) [15]. NTBI triggers oxidative stress reactions [16] which have the potential to increase the risk of congenital defects, preterm delivery and low birth weight [17]. Furthermore, unabsorbed Fe(II) causes the intestinal mucosal cells of the mother to be constantly exposed to oxidative stress [17].

Because iron deficiency [18] and pregnancy [17] have both been shown to increase oxidative stress, it is important that oral iron supplementation does not cause additional oxidative stress. Iron(III)-hydroxide polymaltose complex (IPC, Maltofer®) offers a well tolerated form of oral iron with efficacy comparable to that of ferrous preparations [19]. As iron from IPC is taken up only via a regulated active mechanism the risk of oxidative stress caused by NTBI can be considered to be smaller than with ferrous salts [19]. The aim of the present non-clinical study was to examine the impact of IDA and IPC treatment on pregnancy outcome and oxidative stress markers in various organs of pregnant rats and their fetuses, and in placentas.

2. Methods

2.1. Animals and experimental procedures

All experiments were approved by the Animal Care Committee of Hospital Alemán, Buenos Aires, Argentina (Protocol No. 274-05-2008, accepted 02-09-2008), and were undertaken according to the NIH Guide for the Care and Use of Laboratory Animals. Twenty-four pregnant Sprague-Dawley rats were divided into three groups ($n = 8$): untreated non-anemic animals (control group), untreated anemic animals (anemic group) and anemic animals treated with IPC/FA [Maltofer-Fol®, lot 53001, Vifor (International) Ltd., St Gallen, Switzerland] (IPC/FA group).

To obtain the control group, 21-day old male and female rats were fed on Universal Basal Diet (TestDiet® Formula #5755, PMI International, Richmond, IN) with normal iron content (60 ppm) for eight weeks, after which male–female couples were housed together. To obtain anemic animals, 21-day old male and female rats were fed with Low-Iron Purified Diet (10–20 ppm; TestDiet®, Formula #5859, PMI International, Richmond, IN) for eight weeks. Male-female couples with Hb $\leq$ 9 g/dL [evaluated with HemoCue (HemoCue, Ängelholm, Sweden)] were housed together, and fed further with Low-Iron Purified Diet. All rats were housed in a temperature-controlled room (23 $\pm$ 2 °C) with free access to tap water. Mating was confirmed by detection of a vaginal plug, and this day was denoted day 0.

In the IPC/FA group, pregnant rats received IPC/FA at a dose of 2 mg iron/kg body weight (bw) + 7 μg FA/kg bw daily by gavage with a feeding tube. Treatment was initiated on day 0 and was adjusted weekly based on the bw of each animal.

At day 21 of gestation, dams underwent Cesarean surgery to obtain fetuses and placenta. Dams were then sacrificed by subtotal exsanguination under anesthesia (sodium thiopental 40 mg/kg bw intraperitoneal). After obtaining blood samples, dams were sacrificed by a rapid extirpation of the heart under thiopental anesthesia (sodium thiopental 40 mg/kg bw intraperitoneal). The liver, heart and kidneys from dams were perfused with ice-cold saline through the abdominal aorta until they were free of blood and then removed for oxidative stress evaluation. Placentas were weighed and either fixed in 10% (v/v) neutral buffered formalin overnight (4 °C) followed by storage in 70% (v/v) ethanol, or frozen in liquid nitrogen before being stored at −70 °C. Fetuses were rapidly dissected, weighed, and frozen in liquid nitrogen.

2.2. Biochemical procedures

Maternal Hb was determined by SYSMEX XT 1800i (Roche Diagnostic GmbH, D-68298 Mannheim, Germany). Serum iron, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels were measured with Autoanalyzer Modular P800 (Roche Diagnostic) with the correspondent reagent. Serum transferrin was determined by radial immunodiffusion (Diffu-Plate, Biocientifica, S.A., Buenos Aires, Argentina). Transferrin saturation (TSAT) was obtained by chemical methods.

2.3. Oxidative stress markers

Oxidative stress markers were analyzed as described previously [20]. Briefly, fractions of the whole placenta and the liver, heart and kidneys from mothers and fetuses were homogenized (1:3, w:v) in ice-cold 0.25 M sucrose solution. The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) was determined in the 10,000 × g supernatant. Further samples of the corresponding perfused tissues were homogenized (1:10, w:v) in 0.05 M sodium phosphate buffer (pH 7.4) for the determination of malondialdehyde to evaluate lipid peroxidation by thiobarbituric acid reactive species (TBARS). The remaining homogenate was centrifuged at 4 °C for 15 min at 9500 × g and catalase activity was measured in the supernatant. The remaining tissue samples were homogenized (1:3, w:v) in ice-cold sucrose solution (0.25 M) and centrifuged at 105,000 × g for 90 min. Cu,Zn-SOD and GPx activity were measured in the supernatant. Specific activity was expressed as U/mg protein.

2.4. Light microscopy and immunohistochemical study

Portions of placenta and liver from dams were fixed in phosphate-buffered 10% formaldehyde (pH 7.2) and embedded in paraffin. Three-micron sections of liver of dams and placentas were cut. All observations were performed with a light microscope (Nikon E400. Nikon Instrument Group, Melville, New York, USA).

In order to evaluate iron level in dam livers, Prussian blue staining was performed.

Immunolabeling of specimens was carried out by a modified avidin-biotin-peroxidase complex technique Vectastain ABC kit (Universal Elite, Vector Laboratories, CA) as described previously [20]. All samples of placenta were pre-incubated with Ultra Tech HRP (protein blocking agent PN IM2391, Immunotech SAS, Beckman Coulter Co. Marseille Cedex, France), and then with the corresponding antibody. For the negative control, the incubation was performed with non-immune serum, as recommended by the manufacturer.

Hypoxia and inflammatory markers were quantified with monoclonal antibodies against rat hypoxia-inducible factor 1-alpha (HIF-1α; Novus Biologicals, Inc., Littleton, CO), tumor necrosis factor-alpha (TNF-α; R&D Systems, Minneapolis, MN) and interleukin-6 (IL6; Santa Cruz Biotechnology, Santa Cruz, CA) at concentrations of 1:1000 (2.2 μg/ml), 1:50 (2 μg/ml) and 1:100 (2 μg/ml), respectively (phosphate-buffered saline as diluting agent).

2.5. Morphometric analysis

Histological sections were studied in each animal with an image analyzer (Image-Pro Plus version 4 for Windows, Media Cybernetics LP, Silver Spring, MD). Morphological analyses were performed at a magnification of ×400. In the placentas, inflammatory (TNF-α and IL6) and hypoxia (HIF-1α) markers were evaluated by the percentage of positive immunostaining per square millimeter, using light microscopy. Mean percentage values were calculated for each rat.

2.6. Statistical methods

Values were expressed as median and range. All statistical analyses were performed with absolute values and processed through GraphPad Prism®, version 5.0 (GraphPad Software, Inc., San Diego, CA). The assumption test to determine the Gaussian distribution was performed by the Kolmogorov and Smirnov method. For parameters with non-Gaussian distribution comparisons were performed by Kruskal-Wallis test (nonparametric ANOVA) and Dunn's multiple comparison test. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Hb, serum iron, TSAT and liver enzymes

Prior to pregnancy-values for Hb, serum iron and TSAT in the two groups fed with the low-iron diet confirmed the presence of IDA. No significant differences were observed between these two groups (Table 1). At the end of pregnancy, the IPC/FA group showed significantly higher values for Hb, serum iron and TSAT versus untreated animals although the values remained significantly lower than those in the control group (Table 1). Levels of the liver enzymes AST, ALT and ALP at the end of pregnancy were also measured, but they did not differ significantly between the control, anemic and IPC/FA groups [Results as median (range): AST (IU/L) control: 68.5 (50.9–88.5); anemic: 70.1 (55.0–86.3); IPC/FA: 74.7 (54.8–94.0). ALT (IU/L) control: 40.9 (36.9–54.0); anemic: 43.6 (31.8–57.0); IPC/FA: 51.7 (45.7–65.1) ALP (IU/L) control: 346 (274–402); anemic: 376 (278–418); IPC/FA: 406 (343–455)].

3.2. Pregnancy outcome

The number of fetuses per dam, neonatal bw and placental weight average were significantly lower in the anemic group than in the control or IPC/FA group (Table 2). In the anemic group, the mean reduction of fetuses per dam was approximately 3.5 (27%). Both the bw per neonate and the placental weight were 25% lower in the anemic group than in the control group, i.e. the anemic dams

Table 1
Hemoglobin (Hb), serum iron and transferrin saturation (TSAT) in non-anemic (control), anemic (Hb ≤ 9 g/dL) and IPC/FA-treated rats ($n = 8$/group) before and at end of pregnancy. Data are shown as median and range.

	Control	Anemic	IPC/FA
Hb (g/dL)			
Prior to pregnancy	13.9 (13.6–14.4)[a]	8.7 (7.9–8.9)	8.5 (7.8–8.9)
End of pregnancy	12.7 (11.7–13.4)[a]	8.0 (7.0–8.3)[b]	11.3 (10.6–12.2)
Serum iron (μg/dL)			
Prior to pregnancy	231.0 (199.4–270.0)[a]	96.5 (73.0–125.0)	98.0 (55.0–121.0)
End of pregnancy	201 (175.0–227.0)[a]	63.5 (35.0–76.0)[b]	131.0 (112.0–152.0)
TSAT (%)			
Prior to pregnancy	39.7 (35.0–44.4)[a]	12.6 (9.1–17.3)	11.7 (8.9–15.9)
End of pregnancy	36.9 (32.9–42.1)[a]	9.6 (6.8–12.6)[b]	28.8 (24.8–33.7)

[a] $p < 0.01$ versus all groups.
[b] $p < 0.01$ versus IPC/FA group (Kruskal–Wallis test and Dunn's Multiple Comparison test).

not only delivered fewer but also lighter offspring with lighter placentas. The number of fetuses, and neonatal and placental weight did not differ significantly between the control and IPC/FA groups.

3.3. Oxidative stress markers

In all the analyzed organs, anemic dams exhibited significantly higher levels of malondialdehyde (TBARS) and Cu,Zn-SOD activity, and significantly lower GSH:GSSG ratio, catalase and GPx activities than those of the control and IPC/FA groups (Fig. 1). There were no significant differences between the oxidative stress levels in the IPC/FA-treated dams and the control group.

All the oxidative stress markers evaluated in the fetuses (liver, heart and kidneys) presented a similar pattern as in the dams: oxidative stress was higher in the anemic animals compared to the control and IPC/FA-treated animals (Fig. 1).

Oxidative stress was also observed in the placenta (Fig. 1), although to a lower level than in the dams and the fetuses. The oxidative stress markers in the placenta of the IPC/FA group were similar to those of the control group.

3.4. Microscopy findings of the liver and placenta and immunohistochemical study of the placenta

Prussian blue staining in liver of dams revealed that control and IPC/FA group presented equivalent iron levels which were superior to the level observed in the anemic group (Fig. 2).

Most of the immunohistochemical changes were observed in the murine labyrinth, mainly in the trobhoblastic cells. The labyrinth area of placentas of the anemic dams presented a significant increase in HIF-1α in comparison to the placentas of the control animals (Fig. 3, arrows indicate cells with positive staining). IPC/FA treatment reduced the expression of HIF-1α, but the expression was still higher in the IPC/FA group than in the control group. The pro-inflammatory markers, TNF-α and IL6, were significantly higher in the placentas of anemic animals than in the control and IPC/FA groups (Fig. 3, arrows indicate cells with positive staining). Notably, the IPC/FA group presented similar levels of TNF-α and IL6 expression as the control group.

Table 2
Pregnancy outcome of non-anemic (control), anemic and IPC/FA-treated rats ($n = 8$/group) at the end of pregnancy. Data are shown as median and range.

	Control	Anemic	IPC/FA
Number of fetuses/dam	13.0 (12.0–15.0)	9.5 (8.0–12.0)[a]	12.0 (11.0–14.0)
Neonatal body weight (g)	5.1 (4.9–5.5)	3.8 (3.5–4.4)[a]	4.9 (4.8–5.2)
Placenta weight (mg)	509.5 (489.0–534.0)	387.0 (345–428.0)[a]	488.0 (460.0–501.0)

[a] $p < 0.01$ versus all groups (Kruskal-Wallis test and Dunn's Multiple Comparison test).

4. Discussion

In this study, we examined the impact of IDA and its treatment with IPC/FA in pregnant rats, their fetuses and placentas. As there exists a good correlation between human and rodent placentas, our data seems to be useful in order to translate our findings to human scenario.

IDA in pregnant women has been reported to cause decreased fetal growth [21]. Analogously, in rats, fetal growth has been shown to be dependent on the severity of maternal IDA [22,23]. In addition, maternal iron restriction prior to conception and during the first trimester was recently shown to be associated with profound changes in the developing fetus compared to iron restriction later in pregnancy [24].

Our data show that IDA has a negative effect on the number of fetuses and the weight per fetus and per placenta, which was largely overcome by IPC/FA therapy. The reduction in placental weight could be a sign that the maternal ID was very severe [22]. If this is the case, iron supplementation even during the early pregnancy may not necessarily return the iron status to the level of non-anemic controls [25]. Accordingly, our results show that, IDA was efficiently corrected by treatment with IPC/FA during pregnancy, although the rats did not reach quite the same Hb, serum iron, and TSAT values as those in the control group. Taken together, these observations confirm the importance of treating IDA in women in reproductive age to ensure adequate iron stores at the beginning of a possible pregnancy.

IDA has been associated with oxidative stress, indicated by an increased level of plasma lipid peroxidation products and decreased antioxidant defense systems both in humans and rats [18,26]. Moreover, elevated lipid peroxidation levels have also been reported in rats and humans during uncomplicated pregnancy [27,28]. Thus, the level of oxidative stress may be exacerbated by the combination of these two conditions. Oxidative stress is believed to damage the maternal-fetal unit [29] and increase the risk of preterm birth [30]. Interestingly, preeclampsia is closely associated with the level of oxidative stress during pregnancy. In agreement with previous findings [26,31,32], anemic dams in this study suffered from significantly higher levels of oxidative stress than the control animals as indicated by the oxidative stress marker levels in the liver, heart and kidneys. The Cu,Zn-SOD activity was increased and both catalase and GPx activities were decreased in the organs of anemic dams compared to controls. In pregnant women, the activity of these antioxidant enzymes in erythrocytes shows a similar trend for catalase and GPx (decrease), but a different result for that of Cu,Zn-SOD (decrease instead of increase) [33]. This apparent discrepancy may be due to different concentrations of reactive oxygen species (ROSs) and antioxidant enzymes in erythrocytes compared to liver, kidney and heart cells.

In the placentas of the anemic dams, lipid peroxidation was increased. Any stress that alters placental development or function is also likely to affect the developing fetus. Thus, it was not surprising that the levels of oxidative stress markers observed in maternal tissues between the untreated anemic and control animals were mirrored in the fetal organs. However, all oxidative stress

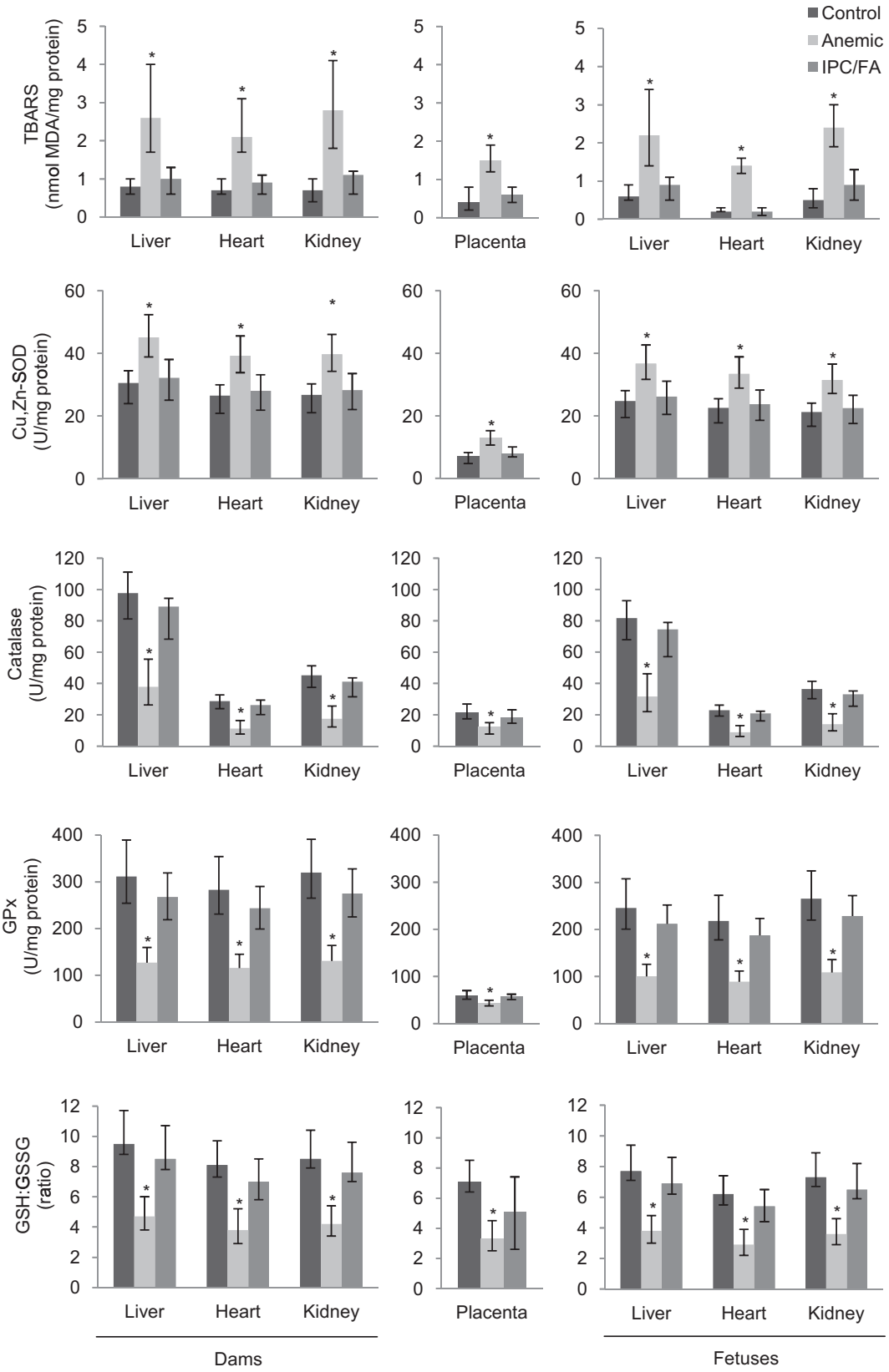

Fig. 1. Oxidative stress markers in non-anemic (control), anemic and IPC/FA-treated dams and their placentas and fetuses ($n = 8$/group) at the end of pregnancy. Values are expressed as median (range); $^{*}p < 0.01$ versus all groups (Kruskal-Wallis test and Dunn's multiple comparison test).

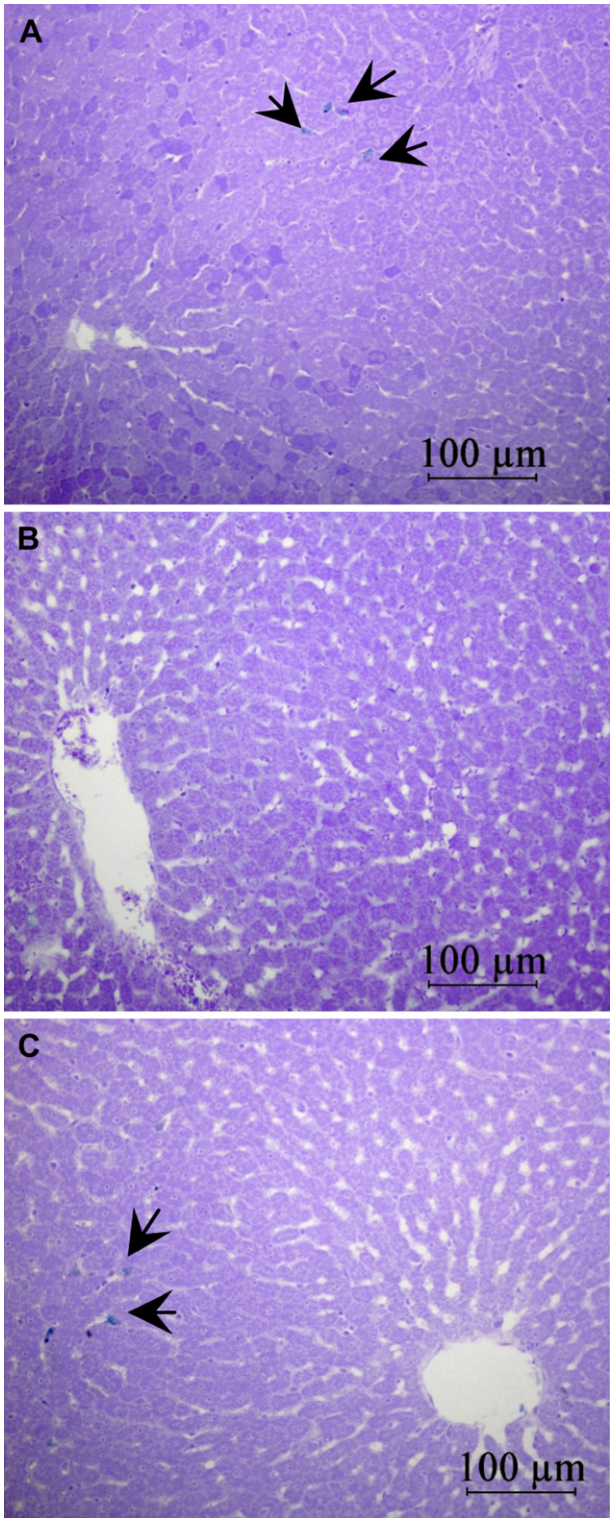

Fig. 2. Prussian blue staining of the dams' livers in all groups at the end of pregnancy. Arrows indicate positive staining in interstitial Kupffer cells in the control and IPC/FA groups.

parameters in fetuses were expressed to a lesser extent than in the dams, highlighting the protective role of the placenta against ROS.

It has been argued that presently recommended iron supplementation schemes, doses and timing during pregnancy reduce oxidative stress by correcting ID but may also produce it due to NTBI [17]. In this study, the anemic rats were treated with IPC, a stable iron(III) complex in which a polynuclear iron(III)-hydroxide core is superficially surrounded by a number of non-covalently bound polymaltose molecules. In the gut, iron from IPC is taken up only by an active, physiologically controlled pathway [13,34]. Thus, after IPC ingestion there is virtually no passive diffusion of iron into the blood that would generate NTBI [35]. In this study, the advantageous properties of IPC were confirmed by all the analyzed oxidative stress markers (in mothers, fetuses and placenta) in the IPC/FA group, which were similar to those of the control group. Thus, IPC/FA treatment corrected anemia and restored normal levels of oxidative stress markers.

A wide range of cytokines plays a role in the regulation of placental function. In rats, ID has been shown to increase the levels of TNF-α in the trophoblast giant cells of the placenta [36]. Interestingly, TNF-α is almost always elevated in infection-associated preterm labor [37]. Elevated IL6 in the amniotic fluid is associated with preterm delivery and pathogenesis of preeclampsia in women [38,39]. In this study, both TNF-α and IL6 expression was significantly elevated in the placentas of anemic animals compared with control animals. IPC/FA treatment efficiently restored normal expression levels of both pro-inflammatory markers in the placenta.

Interestingly, placental growth, differentiation and function are regulated by local endocrine and immune factors. The changing cytokine production patterns during ID have been suggested to be one of the inhibiting factors of fetal growth in rats [36], a result that we also see in this study. It may be that during IDA, oxidative stress changes the cytokine expression through activation of the nuclear factor-κB (NF-κB) [40], which is involved in the regulation of pro-inflammatory cytokines in gestational tissues, including the placenta [41].

Although a causal link has not been shown, a number of studies have suggested that anemia, possibly by causing hypoxia, induces preterm labor, pregnancy-induced hypertension, and premature rupture of the membranes [29]. HIF-1α is a key transcription factor during hypoxia. It has an important role in placental development and function. Its expression is high in the low oxygen environment of the placenta in early pregnancy and falls around nine weeks of gestation, when placental pO_2 levels are believed to increase [42]. In mice, HIF-1α overexpression leads to fetal intrauterine growth restriction (IUGR), decreased placental weights, histopathological placental abnormalities, glomerular endotheliosis, elevated liver enzyme levels, microangiopathic hemolytic anemia and thrombocytopenia [43]. In humans, HIF-1α is over expressed in the placentas of preeclamptic women and IUGR babies [43]. There is also a connection between hypoxia and the expression of several cytokines, e.g. TNF-α expression is induced by hypoxia in the placenta of mice [44]. Here, we observed significantly higher levels of HIF-1α in the placentas of untreated anemic dams than in the placentas of control animals indicating anemia-induced placental hypoxia. IPC/FA treatment reduced only partially the HIF-1α expression. It is possible that the pre-pregnancy IDA, by causing hypoxia, may have led to defective placentation [45] and possibly irreversible changes in the placenta itself. Thus, IPC/FA supplementation, although efficiently correcting maternal anemia and placenta weight, could not correct the placental hypoxia.

In conclusion, this study showed a series of negative effects of iron deficiency anemia in pregnant rats, fetuses, and placentas. Most of these effects were overcome with the IPC/FA treatment, although not all parameters returned to normal levels, a result that emphasizes the importance of ensuring adequate pre-pregnancy maternal iron levels. Ferrous salts, commonly used for oral iron therapy, have the potential to induce oxidative stress [15]. Thus, it is of interest to compare different oral iron therapies in this model. These studies are ongoing in our laboratory.

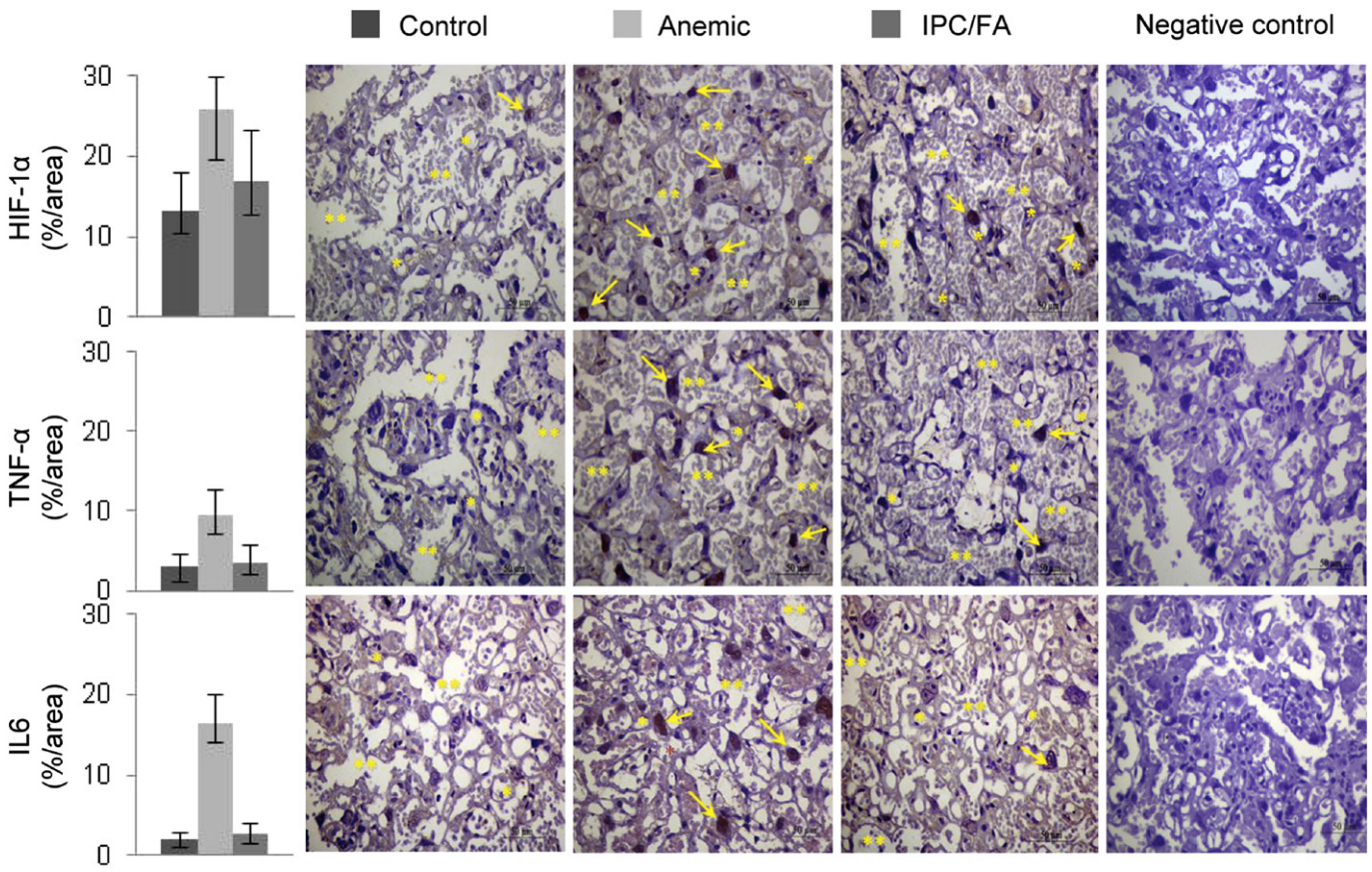

Fig. 3. Bar charts showing the percentage of positive HIF-1α, TNF-α and IL6 immunostaining area in the placentas of non-anemic (control), anemic and IPC/FA-treated rats ($n = 8$ per group). Values are expressed as median (range); $^{*}p < 0.01$ versus all groups (Kruskal–Wallis test and Dunn's multiple comparison test). Micrographs showing the immunostaining in the placenta labyrinth. Arrows indicate trophoblastic cells with positive staining. One red asterisk indicates the fetal capillary and two red asterisks the maternal blood space.

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Disclosures

All authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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