Hemochromatosis and pregnancy: iron stores in the Hfe \(-/-\) mouse are not reduced by multiple pregnancies

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1Iron Genes and Immune System (IRIS), and 2Laboratory of Animal Science, Instituto de Biologia Molecular e Celular (IMBC), and 3Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto; 4Hematologia Clínica (CHP), Hospital de Santo António, Porto, Portugal

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Neves JV, Olsson IAS, Porto G, Rodrigues PN. Hemochromatosis and pregnancy: iron stores in the Hfe \(-/-\) mouse are not reduced by multiple pregnancies. Am J Physiol Gastrointest Liver Physiol 298: G525–G529, 2010. First published January 28, 2010; doi:10.1152/ajpgi.00449.2009.—Hereditary hemochromatosis (HH), a widespread hereditary iron metabolism disorder, is characterized by an excessive absorption of dietary iron, resulting in increased body iron stores. Some studies indicate a sex difference in disease expression, with women showing a slower disease progression and a less severe clinical profile. This is usually attributed to iron loss during menstruation and pregnancy. However, this link has not been clearly demonstrated. The Hfe \(-/-\) mouse model recapitulates key aspects of HH, including an iron overload phenotype similar to that observed in human patients. In this study, we use it to test the impact of multiple pregnancies in the iron stores. One-year-old nulliparous and pluriparous (averaging 29 weaned pups per female) C57BL/6 (B6) and Hfe \(-/-\) mice were euthanized, and blood and tissues were collected. Several serological and erythroid parameters were evaluated, as well as tissue nonheme iron content and serum ferritin. Hepcidin 1, hepcidin 2, and bone morphogenetic protein 6 (BMP6) expressions in the liver were determined by real-time PCR. No significant differences were observed for many serological and erythroid parameters although differences occurred in transferrin saturation and mean corpuscular volume in Hfe \(-/-\) mice and total iron-binding capacity in B6 mice. Hepatic iron concentration was similar for nulliparous and pluriparous mice of both genotypes, but total iron per organ (liver, spleen, heart, and pancreas) was higher overall in pluriparous females than nulliparous. Hepcidin 1 and 2 and BMP6 expressions were significantly decreased in pluriparous females, when compared with nulliparous, in both genotypes. In conclusion, multiple pregnancies do not reduce body iron stores in Hfe \(-/-\) mice.

Hepcidin is now accepted as the major regulator of iron homeostasis, being downregulated in conditions of iron deficiency or increased erythropoiesis and upregulated in iron overload (23, 24). In HFE-associated hemochromatosis, hepcidin expression is greatly diminished despite the elevated iron stores (6, 14). In turn, bone morphogenetic protein 6 (BMP6), a key endogenous regulator for hepcidin (2, 19), is also known to be impaired in a mouse model of hemochromatosis (8). Other biochemical abnormalities that characterize hemochromatosis can include increased serum transferrin saturation and serum ferritin and decreased total iron binding capacity (1).

Among patients with HH, there is a male overrepresentation in terms of the number of patients diagnosed and also of the severity of iron overload (3, 4, 21, 27, 28). This apparent sex difference in disease expression is usually attributed to iron loss during menstruation and pregnancy in women (28). However, the literature is contradictory. In a cross sectional study of French and Canadian patients, Morand et al. (21) found no difference between men and women with regard to age at presentation of clinical signs. In a later study, the same group found no difference in age, number of pregnancies, or menstruation between symptomatic and nonsymptomatic women homozygous for the mutated Hfe gene (22). All these studies that evaluated the effect of sex on HH disease phenotype have used epidemiological approaches on the basis of a human population.

Using an animal model allows studies where key factors such as nutrition and reproductive experience can be controlled or experimentally manipulated. The Hfe knockout mouse model (Hfe \(-/-\)) presents an iron overload phenotype similar to that observed in human patients, recapitulating key aspects of HH. In this study, we used wild-type and Hfe \(-/-\) females to test whether multiple pregnancies would have an impact on iron stores.

MATERIALS AND METHODS

Animals. Animals generating data for the present study were part of an experiment studying mouse reproduction and maternal behavior. For the duration of the experiment, mice were fed ad libitum with a standard diet with an iron content of 312 mg/kg (8626 Teklad Mouse Breeder Diet; Teklad, Harlan, UK). The C57BL/6 (B6) and Hfe \(-/-\) (backcrossed onto B6 background) female mice were bred and housed...
at the Instituto de Biologia Molecular e Celular (IBMC) animal facilities. One-year-old nulliparous and pluriparous (on average 29 weaned pups per female 11 wk after last delivery) B6 and Hfe−/− female mice (n = 6–7) were euthanized (isoflurane anesthesia followed by cervical dislocation), and blood and tissues were collected. All animal experiments were carried out in compliance with the animal ethics guidelines at the institute and were approved by the Portuguese Veterinary Ethics Committee.

Hematological measurements. The blood samples were obtained by retro-orbital bleeding of nulliparous and pluriparous B6 and Hfe−/− mice, under anesthesia. For the erythroid parameters, blood was collected in EDTA tubes, and hemoglobin, red blood cell counts, hematocrit, and mean corpuscular volume were assessed on a Coulter-S counter (Coulter Electronics, Fullerton, CA). For iron levels, 200 μl of serum from each animal were used to determine the serum iron and total iron-binding capacity (TIBC) by the Liquid Ferrozine method (Thermo Electron, Victoria, Australia) according to the manufacturer’s instructions. Transferrin saturation was calculated as (serum iron/ TIBC) × 100.

Serum ferritin measurements. Ferritin concentrations in the serum were determined by ELISA using polyclonal antibodies (Abs) raised against mouse recombinant L-ferritin subunits and calibrated with the corresponding recombinant homopolymer. The specificity of the Abs was confirmed by immunoblotting, and the absence of cross reactivity of the Abs has been previously described (29). The microtiter plates were coated with polyclonal Abs specific for mouse L-ferritin. Serum samples or standard ferritins were diluted in 50 mM HEPES (Invitrogen, Carlsbad, CA) and 1% Igepal (Coulter Electronics, Fullerton, CA). For iron levels, 200 μl of serum from each animal were used to determine the serum iron and total iron-binding capacity (TIBC) by the Liquid Ferrozine method (Thermo Electron, Victoria, Australia) according to the manufacturer’s instructions. Transferrin saturation was calculated as (serum iron/ TIBC) × 100.

Tissue nonheme iron measurements. Liver, spleen, heart, and pancreas samples from nulliparous and pluriparous B6 and Hfe−/− mice were collected and stored at −80°C for further use. Tissue nonheme iron was measured by the bathophenanthroline method (30). Briefly, tissue samples with an average weight of 100 mg were placed in iron-free Teflon vessels (ACV-Advanced Composite Vessel; CEM, Mathews, NC) and dried in a microwave oven (MDS 2000, CEM). Subsequently, dry tissue weights were determined and samples digested in an acid mixture (30% hydrochloric acid and 10% trichloroacetic acid) for 20 h at 65°C. After digestion, a chromogen reagent (5 g of 2,2′-bipyridyl in 2 ml of 1 M HCl) was added to the samples to react with iron and obtain a colored product that was measured spectrophotometrically at 535 nm. The extinction coefficient for bathophenanthroline is 22.14 mM/cm.

Hepcidin and BMP6 expression. Total RNA from livers was isolated with the RNeasy Midi Kit for Total RNA Isolation from Animal Cells (Qiagen, Valencia, CA) with the optional On-Column DNase Digestion with RNase-free DNase (Qiagen), and 1.25 μg of total RNA were converted to cDNA by Thermoscript and an oligo (dT) 20 primer (Invitrogen), according to the manufacturer’s instructions.

Relative levels of hepcidin 1, hepcidin 2, and BMP6 mRNAs were quantified by real-time RT-PCR analysis using an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Primers used are as follows: GAPDH (housekeeping): mGAPDH forward, 5′-GCTGCCCAGAACATCATCCC-3′ and mGAPDH reverse, 5′-ATGCTGCTTCTACACCACTTTC-3′; hepcidin 1: mHepc1 forward, 5′-CCTATCTCCTCAACACAGATG-3′ and mHepc1 reverse, 5′-AACAAGATACACCTGGGAA-3′; hepcidin 2: mHepc2 forward, 5′-CCTATCTCCTCAACACAGATG-3′ and mHepc2 reverse, 5′-AAAGATACACAGAGGGTT-3′; BMP6: mBMP6 forward, 5′-TCCCACATCAACGACCAAC-3′ and mBMP6 reverse, 5′-TCCACACACAGTCTCGT-3′. A second housekeeping gene [hypoxanthine-guanine phosphoribosyl transferase (HPRT); mHPRT forward, 5′-GTAATGATCAGTCACGGGAGC-3′ and mHPRT reverse, 5′-GCCGCAAGTCTGGCAACCTTACCA-3′] was used to assure that any possible variations observed in the expression of genes of interest were not solely attributable to differences in GAPDH expression. One microliter of each cDNA sample was added to a reaction mix containing 10 μl iQ SYBR Green Supermix (Bio-Rad), 8.5 μl of H2O, and 250 nM of each primer, making a total volume of 20 μl per reaction. Each sample was prepared in triplicate. A nontemplate control was included for each set of primers. The cycling profile was the following: 94°C for 3.5 min, 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. A melting curve was generated for every PCR product to confirm the specificity of the assays, and a dilution series was prepared to check the efficiency of the reactions. The comparative Ct method (2−ΔΔCt method) based on Ct values for hepcidin 1, hepcidin 2, BMP6, and GAPDH was used to analyze the expression levels of both hepcidins and BMP6.

Statistical analysis. Statistical analysis was carried out using SPSS for Windows, version 16 (SPSS Science, Chicago, IL). Value normality was checked by performing Kolmogorof-Smirnoff test, and for Windows, version 16 (SPSS Science, Chicago, IL). Value normality was checked by performing Kolmogorof-Smirnoff test, and statistical analysis was carried out using SPSS for Windows, version 16 (SPSS Science, Chicago, IL). Value normality was checked by performing Kolmogorof-Smirnoff test, and Student’s t-test was used for estimating statistical significance and comparing nulliparous and pluriparous females within each genotype. A P value <0.01 was considered statistically significant.

RESULTS

Hematological parameters. No significant differences were observed between nulliparous and pluriparous mice in either of the genotypes regarding serum iron, red blood cells, hemoglobin, and hematocrit (Table 1). Pluriparous Hfe−/− females presented significantly lower values of transferrin saturation and mean corpuscular volume than nulliparous females of the same genotype. In B6 pluriparous mice, increased levels of

Table 1. Serological and erythroid parameters in nulliparous and pluriparous B6 and Hfe−/− female mice

<table>
<thead>
<tr>
<th></th>
<th>Nulliparous</th>
<th>Pluriparous</th>
<th>Nulliparous</th>
<th>Pluriparous</th>
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<tbody>
<tr>
<td><strong>Weaned pups per female, n</strong></td>
<td>29 ± 5</td>
<td>29 ± 4</td>
<td>208 ± 67</td>
<td>293 ± 29</td>
</tr>
<tr>
<td>Serum Iron Parameters</td>
<td></td>
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<tr>
<td>SI</td>
<td>196 ± 10</td>
<td>208 ± 67</td>
<td>347 ± 52</td>
<td>293 ± 29</td>
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<tr>
<td>TIBC</td>
<td>449 ± 22</td>
<td>547 ± 79*</td>
<td>422 ± 55</td>
<td>466 ± 27</td>
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<tr>
<td><strong>Erythroid Parameters</strong></td>
<td></td>
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<tr>
<td>RBC</td>
<td>43.7 ± 3.3</td>
<td>37.3 ± 7.9</td>
<td>82 ± 2</td>
<td>63.0 ± 6.9*</td>
</tr>
<tr>
<td>HGB</td>
<td>9.49 ± 0.46</td>
<td>9.23 ± 0.43</td>
<td>8.75 ± 0.21</td>
<td>9.26 ± 0.23</td>
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<tr>
<td>HCT</td>
<td>14.4 ± 0.6</td>
<td>13.9 ± 0.6</td>
<td>15.1 ± 0.4</td>
<td>14.8 ± 0.4</td>
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<tr>
<td>MCV</td>
<td>47.6 ± 2.3</td>
<td>45.1 ± 2.7</td>
<td>47.2 ± 0.7</td>
<td>46.8 ± 0.8</td>
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Values are represented as means ± SD (n = 6–7), and differences were considered significant at *P < 0.01. B6, C57BL/6 mice; SI, serum iron (μg/ml); TIBC, total iron-binding capacity (μg/ml); Tf Sat, transferrin saturation (%); RBC, red blood cells (×10⁶ cells/μl); HGB, hemoglobin (g/dl); HCT, hematocrit (%); MCV, mean corpuscular volume (fl).
TIBC were observed when compared with nulliparous females. The same was observed between $Hfe^{-/-}$ nulliparous and pluriparous mice although the differences were not statistically significant.

**Tissue iron.** In both B6 and $Hfe^{-/-}$ mice, iron concentration in the liver did not differ significantly between nulliparous and pluriparous females (Fig. 1). However, when measuring total iron per organ, there was an overall trend for all measured organs (liver, spleen, heart, and pancreas) from pluriparous females to contain more iron than those of nulliparous females, both in the B6 and $Hfe^{-/-}$ mice (Fig. 2, A and B). Significant differences were observed for the spleen, heart, and pancreas in B6 mice and for the spleen and heart in $Hfe^{-/-}$ mice. That trend becomes clearly evident when expressing the organ iron content as the sum of iron in the four organs. This total iron level is significantly higher in pluriparous females than in nulliparous females in both genotypes (Fig. 3).

**Serum ferritin.** Levels of L-ferritin in the serum were measured by ELISA. No significant differences were observed between nulliparous and pluriparous females from both B6 and $Hfe^{-/-}$ although there seems to be a tendency for increased levels in the pluriparous animals (Fig. 4).

**Hepcidin and BMP6 mRNA expression.** Hepatic hepcidin 1 (Fig. 5A), hepcidin 2 (Fig. 5B), and BMP6 (Fig. 5C) mRNA expressions were measured by real-time PCR in nulliparous and pluriparous B6 and $Hfe^{-/-}$ female mice. Lower expressions of hepcidin 1, hepcidin 2, and BMP6 were observed in the pluriparous females, when compared with the nulliparous, in both genotypes.

**DISCUSSION**

Reproductive iron loss is thought to underlie the observed reduction in disease expression in female HH carriers compared with men. In this study, we show experimental evidence that multiple pregnancies do not reduce body iron stores in a mouse model of hemochromatosis. In fact, most relevant clinical parameters of HH were either not significantly affected or even increased in pluriparous females compared with nulliparous females.
During normal development, the fetus has a high demand for iron, which is met from maternal sources. Iron is transferred from the maternal stores to the fetus, in amounts usually exceeding the normal maternal body iron stores. Consequently, an increase in iron absorption is required to provide the extra iron needed by the fetus and to replenish the maternal stores (20).

It has also been argued that female iron loss in reproduction reduces the expression of HH (28), in which case one would expect multiple pregnancies to result in a less severe disease phenotype. This assumption has been challenged by epidemiological data from humans (9) but has not previously been addressed experimentally. In the present experimental study of the Hfe−/− mouse model of hemochromatosis, we show for the first time that multiple pregnancies do not reduce body iron stores in mice. To the contrary, there is an increase in tissue iron stores that is not only accounted for by the increased organ size in pluriparous females but also by an increased tissue iron concentration (both in B6 and Hfe−/−). Also, with the exception of transferrin saturation, all relevant clinical parameters of HH were either not significantly affected or even increased in pluriparous females compared with nulliparous females, in both Hfe−/− and in wild-type mice. Consequently, the iron overload phenotype of Hfe−/− females remains largely unaffected by multiple pregnancies and subsequent lactations.

These results suggest that, despite abundant iron stores, female Hfe−/− mice increase their iron uptake in response to the increased demands of pregnancy and lactation. This is corroborated by the observation of a reduced expression of hepcidin 1, hepcidin 2, and BMP6 in pluriparous females compared with nulliparous females.

Such a reduction in hepcidin expression is known to abrogate iron intestinal absorption inhibition (13), and hepcidin has indeed been demonstrated to reach very low levels in pregnant rats, especially toward the end of pregnancy when the fetal iron demand is highest (20). Whereas the role of hepcidin 1 in the regulation of iron metabolism in mice has been clearly demonstrated, the hepcidin 2 gene has been proposed to play a minor role (15, 17). However, our results show that hepcidin 1 and hepcidin 2 respond in a similar fashion to the iron demands of pregnancy by a dramatic decrease in expression. This observation raises the question whether hepcidin 2 may have a more important function than previously thought, especially during pregnancy.

BMP6 has recently been demonstrated to be a key endogenous regulator of hepcidin, through the BMP/SMAD signaling pathway (2, 19). BMP6 itself seems to be regulated by iron levels, but whether it is by circulating transferrin or hepatic iron is still unknown (7). As such, a decrease in BMP6 expression is in accordance with the decrease in hepcidin expression. What is puzzling is the fact that, despite the high levels of iron in pluriparous females, BMP6 expression is nevertheless reduced.

The low hepatic hepcidin and BMP6 expressions of pluriparous females compared with nulliparous females, a mirror image of their tissue iron stores, may suggest an abrogation of iron intestinal absorption inhibition in response to pregnancy. Moreover, the fact that these results were observed both in iron overloaded in Hfe−/− and wild-type mice indicate that iron absorption during pregnancy is regulated on a level that is independent of iron stores presently available in the female body, as previously shown in experimentally iron overloaded and deficient mice (5). Reinforcing this assumption is the fact that recently, Gambling et al. (12) proposed that, at a certain point of pregnancy, maternal hepcidin levels are modulated, not by maternal iron levels, but rather by fetal iron levels.

In conclusion, multiple pregnancies do not alleviate the disease phenotype of Hfe−/− mice, an animal model of HH. In addition, the decreased hepcidin and BMP6 expression response to multiple pregnancies in both iron overloaded Hfe−/− and normal wild-type mice suggests that mechanisms regulating iron metabolism during pregnancy are independent of the iron status in hemochromatosis carriers.

Fig. 5. Hepatic hepcidin and bone morphogenetic protein 6 (BMP6) mRNA expression. Hepcidin 1 (Hepc1) (A), hepcidin 2 (Hepc2) (B), and BMP6 (C) expressions were assessed by real-time PCR in the liver of nulliparous and pluriparous B6 and Hfe−/− mice. GAPDH was used as the housekeeping gene. Values are represented as means ± SD (n = 6–7), and differences were considered significant at *P < 0.01.
However, a good deal of caution is needed when trying to extrapolate this data to humans because there are several physiological and behavioral differences between humans and mice that are not represented in the \( Hfe^{-/-} \) mouse model, the most marked being the fact that mice do not menstruate, and as such iron loss during menstruation was not mimicked in this study.

Nevertheless, these results challenge the view that pregnancy-associated iron loss explains sex differences in the expression of HH, suggesting that other possibilities should be considered to explain the differences between males and females in the regulation of iron metabolism.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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