Lipopolysaccharide-induced pregnant mice had decreased serum iron while maintaining hepcidin level and Hamp1 mRNA expression

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ABSTRACT

**Introduction:** Hepcidin is a hormone that regulates systemic iron homeostasis and is mostly produced in the liver. In pregnant women with inflammation, there are two opposing mechanisms in hepcidin expression: the suppression of hepcidin synthesis by pregnancy and the induction of hepcidin by inflammation. These conditions must receive special attention so clinicians can properly treat and manage pregnant women with inflammation. Therefore, this study aims to prove changes in hepcidin and serum iron levels in pregnant mice with inflammation.

**Method:** This study involved sixteen-second-week pregnant mice divided into two groups. Pregnant mice were injected with lipopolysaccharide (LPS) *Escherichia coli* serotype O111:B4 as much as 1 µg/g body weight intraperitoneally as the treatment group. In contrast, pregnant mice were injected with phosphate buffer saline (PBS) as a control group. Serum was measured using ELISA to determine hepcidin levels and colorimetry to determine serum iron. Mice livers were measured using Real-Time PCR to determine Hamp1 mRNA expression. The data obtained were analyzed using an independent t-test.

**Result:** Our results show that pregnant mice with inflammation show there was no difference in Hamp1 mRNA expression (p-value=0.163) and hepcidin level (p-value=0.789), but there was a significant difference in serum iron level (p-value=0.035).

**Conclusion:** This study demonstrates that inflammation in pregnancy does not affect changes in Hamp1 expression and hepcidin level but reduces serum iron, which could be caused by regulating hepcidin in the fetus.

**Keywords:** hepcidin, hamp1, inflammation; pregnancy, serum iron.


INTRODUCTION

Anemia is when the body experiences a shortage of red blood cells or due to red blood cells not functioning properly. According to the World Health Organization (WHO), iron deficiency is globally the most common cause of anemia. Pregnant women are vulnerable to iron deficiency anemia because they experience an increased need for iron in the first trimester of 800 µg/day and increases up to 7500 µg/day with an estimated iron requirement during pregnancy of 1000 to 1200 mg. This condition is the body’s attempt to meet the mother’s iron needs during pregnancy and to maintain and accommodate the developing fetus.

Hepcidin is a hormone that regulates systemic iron homeostasis and is mostly produced in the liver, the effect of hepcidin is to degrade ferroportin (Fpn) so that it has the effect of reducing iron absorption in enterocytes and releasing iron in cells that recycle and store iron. Hepcidin regulation is regulated by iron status, inflammation, erythropoiesis and sex hormones. By regulating plasma iron hemostasis and systemic iron, hepcidin and ferroportin profoundly influence erythropoiesis.

During pregnancy, there is a suppression of hepicedin production so that hepcidin levels decrease in the blood, this condition is the body’s attempt to increase the absorption of dietary iron by the intestine because Fpn in enterocytes is not degraded by hepcidin. The suppression mechanism is due to the increased E2 during pregnancy interacting with the ER (estrogen receptor) especially ERα in the cytoplasm, to form a complex that can bind half of the ER (estrogen responsive element) site on the hepcidin gene promoter and inhibit hepcidin formation.

Inflammation triggers increased hepcidin expression through the Janus kinase-Signal Transducer and Activator of Transcription (JAK-
STAT) and Bone Morphogenetic Protein - Small Mothers Against Decapentaplegic (BMP-SMAD) pathways which are mediated by pro-inflammatory cytokines. Induction of hepcidin via the JAK-STAT pathway requires interaction with the BMP-SMAD pathway. The protein complex formed will undergo translocation to the nucleus for transcription of Hamp1 mRNA.13-16

The problem is that two conflicting mechanisms exist for expressing hepcidin in pregnant women who experience inflammation. Therefore this condition must receive special attention so clinicians can provide treatment and attitude to pregnant women who experience infection. Thus, iron homeostasis and adequacy for the fetus can be maintained. So it is necessary to research to get an explanation of the level of hepcidin and serum iron in pregnant women who experience inflammation.

MATERIALS AND METHODS

Animal
Sixteen pregnant female mice (39±6g) in the second-week pregnancy were purchased from the Farma Veterinary Center (Surabaya, Indonesia). The day of the breeding day was taken as day 0 of pregnancy. Mice were housed under conventional conditions and given free access to food and drink. Pregnancy in mice was confirmed by the presence or absence of a fetus at the time of surgery. This research received ethical approval from the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, with certificate number 2.KE.111.11.2020.

Mice were randomly divided equally into two groups. Control pregnant mice were injected with phosphate buffer saline (PBS). As for the treatment group, pregnant mice were injected with lipopolysaccharide (LPS) Escherichia coli serotype O111:B4 (Sigma-Aldrich, USA). The reagent components consisted of PBS. Then, serum iron was measured using a colorimetric assay (Elabscience Biotechnology Inc., Houston, TX, USA). Bio-Rad CFX96 Real-Time PCR System (Biorad Laboratories Inc, Hercules, CA, US) was used for amplification and analysis using the following cycles: Enzyme activation at 95°C for 2 minutes, followed by 40 cycles using the cycle program: Priming for 5 minutes at 25°C, Reverse transcription for 20 minutes at 46°C, and RT inactivation for 1 minute at 95°C. cDNA was analyzed quantitatively using an ND000 nanodrop (Thermo Fisher Scientific, Wilmington, DE, USA) and qualitatively using an electrophoresis gel.

Real-Time PCR
mRNA expression was analyzed using the SensiFast SYBR® No-ROX kit (Cat. No. BIO-98005, Bioline, Memphis, Tennessee, USA). β-actin was used as the positive control amplification using the following primers: F: 5’-ACC ATG TAC CCA GGC ATT GC -3’ and R: 5’-CAC ACA GAG TAC TTG CGC TC-3’, while the target gene used mouse Hamp1 primers as followings: F: 5’-AGA AAG CAG GGC AGA CAT TG-3’ and R: 5’-CCC TGT TGC TGT AGC CGT AT3’ 11. Bio-Rad CFX96 Real-Time PCR System (Biorad Laboratories Inc, Hercules, CA, US) was used for amplification and analysis using the following cycles: Enzyme activation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 5 seconds, annealing at 55°C for 30 seconds.

Statistical analysis
Data are shown as mean and standard deviation (SD). Statistical analysis was performed by t-test to determine differences between groups. All statistical tests used IBM SPSS Statistics for Windows version 21.0 (IBM Corp., Armonk, NY, USA). A p-value of less than 0.05 was considered statistically significant.

RESULTS
Hamp1 expression and hepcidin level were maintained in LPS-induced pregnant mice
We observed hepcidin expression by measuring Hamp1 mRNA in hepatocytes and hepcidin protein in the blood. Hamp1 mRNA expression in LPS-induced pregnant mice (19.16 ± 8.65) compared to control pregnant mice (24.32 ± 7.84) showed no significant difference (p-value = 0.163). Blood hepcidin levels in LPS-induced pregnant mice (413.09 ± 87.07 ng/L) compared to control pregnant mice (424.34 ± 77.73 ng/L) also showed no significant difference (p-value=0.789).
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Intriguingly, our present study showed different results from the previous studies. It is unknown what causes hepcidin to be retained in LPS-induced pregnant mice. LPS is a macromolecule that strongly mediates the inflammatory response, producing proteins, especially interleukin-6 (IL-6), as strong inducers of hepcidin expression. Based on the available information, we speculate that estradiol (E2) may be the key to hepcidin regulation since there is an increase in E2 production during pregnancy. E2 in the cytoplasm can form a complex with ER, which is then translocated into the nucleus and binds to the sequence estrogen-responsive element (ERE). The hepcidin gene promoter region was identified as having half the ERE site. Therefore, the binding of the E2 complex to the hepcidin gene promoter will inhibit transcription and translation processes.

Interestingly, in our study, LPS-induced pregnant mice showed decreased serum iron, even though the hepcidin level was maintained. Generally, decreased serum iron is accompanied by increased hepcidin. It should be understood that the fetus also produces hepcidin and can regulate fetal iron content by controlling placental iron export. We assume that serum iron is transported to the fetus in order to suppress the amount of serum iron levels in pregnant mice with inflammation and to keep up with iron in the fetus. Decreased serum iron is an immune mechanism to limit pathogenic microbes from accessing iron, further limiting their survival in the host.

The previous studies demonstrated that cord blood hepcidin was associated with cord blood iron status, but no correlation was detected between cord blood hepcidin and pregnant women. However, maternal hepcidin correlated with parameters of newborn cord blood iron status. The study by Sangkhe et al. (2020) reported that an increase in fetal hepcidin would decrease placental ferroportin (Fpn), thereby reducing the transfer of iron into the placenta. Meanwhile, decreased fetal hepcidin levels did not impact fetal iron. As a result, the hepcidin of pregnant women and the hepcidin of the fetus do not mix with each other. Besides being...
regulated by the hepcidin of the pregnant woman, the Fpn of the placenta can also be regulated by the fetus. Ultimately, the fetus has a role in controlling the iron transfer from mother to fetus.38

A study by Kammerer et al. (2020) reported the autocrine role of hepcidin in regulating hepatic iron stores.39 With this condition, autocrine impact on the liver as an iron storage organ will inhibit the release of iron in the blood, which results in a decrease in serum iron. If we look at Hamp1 and hepcidin in our study, LPS-induced pregnant mice did not show any autocrine role since Hamp1 mRNA expression and hepcidin level were not different from non-LPS-induced pregnant mice. Thus, it is suspected that decreased serum iron could probably be due to the increased transfer of serum iron to the fetus.

One of the limitations of our study is that we only examined Hamp1 expression, hepcidin and serum iron levels in the pregnant mice but not in the fetus. We also did not measure placental Fpn, which is used for iron transfer from mother to fetus. Therefore, the role of the fetus in controlling serum iron in pregnancy remains unclear.

CONCLUSIONS

Our study shows that LPS-induced inflammation in pregnancy did not affect changes in Hamp1 mRNA expression and hepcidin level. However, it decreased serum iron in pregnancy, which could possibly be caused by hepcidin regulation in the fetus. Based on the insights gained from this study, further research is needed to study the involvement of the fetus in controlling iron hemostasis in pregnant women during inflammation.

FUNDING

This research was funded by Indonesia Endowment Fund for Education (LPDP), grant number “FR19112020237466”. The researchers are also grateful for the support of the Embryology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga, East Java, Indonesia and BioScience Laboratory, Universitas Brawijaya, East Java, Indonesia.

CONFLICT OF INTERESTS

All authors declare that there are not any conflicts of interest.

ETHICAL CLEARANCE

This research received ethical approval from the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, with certificate number 2.KE.111.11.2020.

AUTHOR CONTRIBUTION

GN, conceptualization, methodology, investigation, validation, writing original draft; W, A, resources and investigation; W, A, HBN, methodology and supervision; W, A, CDKW, HN, WD, AS, HBN, PSR, methodology, writing, review, and editing; GN, W, A, supervision, conceptualization, project administration, and funding acquisition. All authors read and approved the final version of the manuscript.

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