Original Article

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

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ABSTRACT

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 that clinicians can give proper treatment and management to pregnant women with inflammation. Therefore, this study aims to prove changes in hepcidin and serum iron levels in pregnant mice with inflammation. This study involved sixteen second week pregnant mice which were 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, while 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. Our results show that pregnant mice with inflammation show that 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). 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 regulation of hepcidin in the fetus.

Keywords: Inflammation, Hamp1, Hepcidin, Pregnancy, Serum iron

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Introduction

Anemia is a condition in which 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), the most common cause of anemia globally is iron deficiency [1]. Pregnant women are individuals who are vulnerable to iron deficiency and iron deficiency anemia because they experience an increased need for iron, estimated iron requirement during pregnancy of 1000 to 1200 mg [2-4]. 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 [5, 6]. By regulating plasma iron hemostasis and systemic iron, hepcidin and ferroportin profoundly influence erythropoiesis [7].

During pregnancy there is an suppression of hepcidin 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 [8, 9]. The mechanism of suppression is due to the increased E2 during pregnancy interacting with the ER (estrogen receptor) especially ERα in the cytoplasm so as to form a complex that can bind half of the ERE (estrogen responsive element) site on the hepcidin gene promoter and inhibit hepcidin formation [10-12].

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

Materials and Methods

Animal

Sixteen ±12 week-old female female mice strain ddy (39±6g) in the second week pregnancy were purchased from the Farma Veterinary Center (Surabaya, Indonesia). The day of 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, Merck, Singapore) (1 µg/g body weight in PBS) intraperitoneally as the treatment group.

Sample collection

After four hours of treatment, the mice were sacrificed and blood was collected intracardiacly using a syringe and transferred into a collection tube. The blood was allowed to clot for at least 1 hour, then centrifuged at 3000 rpm for 20 minutes to obtain serum. All sera were stored at -80°C until used for analysis.

Furthermore, the mice were dissected and the uterus was checked to make sure the mice were pregnant by finding a fetus. After that, the liver was taken and washed using cold PBS until it was clean from blood. Subsequently, the liver was put into a container containing PBS and stored at -80°C until used for analysis.

Enzyme-linked immunosorbent assay

After four hours of treatment, the mice were sacrificed and Serum was measured using an enzyme-linked immunosorbent assay (ELISA) to determine hepcidin levels (Cat. No. E1467Mo, Bioassay Technology Laboratory, Shanghai Korain Biotech Co., Ltd., Shanghai, China). Measurements were carried out according to the manufacturer's instructions.

Iron serum measurement

For iron serum measurement, 50 µL of serum was pipetted and added to 450 µL of PBS. Then, serum iron was measured using a colorimetric assay (Elabscience Biotechnology Inc., Houston, TX, USA). Measurements were carried out according to the manufacturer's instructions. The serum iron concentration obtained was multiplied by 10 times.

RNA isolation

RNA isolation was performed using the Total RNA Mini Kit (Cat. No. RT 100, Geneaid Biotech, Xizhi District, New Taipei City, Taiwan). Liver organs were weighed as much as 25 mg then placed in a 1.5 ml microcentrifuge tube. Liquid nitrogen was added and then the liver was crushed using a micropestle. RNA isolation was carried out according to the manufacturer's instructions. RNA stored at -20℃ or used immediately.

cDNA synthesis

cDNA was synthesized using iScript™ cDNA Synthesis (Cat. No. 1708891, Biorad Laboratories Inc, Hercules, CA, US). The reagent components consisted of 5x iScript™ Reaction mix 4µl, iScript™ Reverse Transcriptase 1µl, Nuclease free water and RNA template 1µg. The reagent mix was incubated using a thermal cycler using the cycle program: Priming 5 minutes at 25°C, Reverse transcription 20 minutes at 46°C, 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

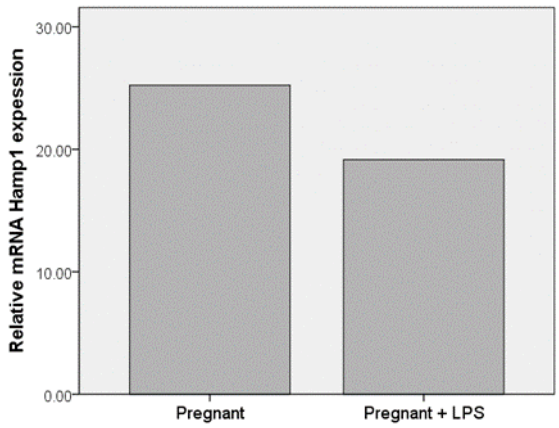
mRNA expression was analyzed using the SensiFast SYBR™ No-ROX kit (Cat. No. BIO-98005, Bioline, Memphis, Tennessee, USA). β-actin was used as the positive control amplification using the following primers: F: 5'-ACC ATG TAC CCA GGC ATT GC -3' and R: 5'-CAC ACA GAG TAC TTG CGC TC-3', while target gene 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, denaturation at 95°C for 5 seconds, annealing at 55°C for 30 seconds. Amplification was carried out for 40 cycles.

Statictical analysis

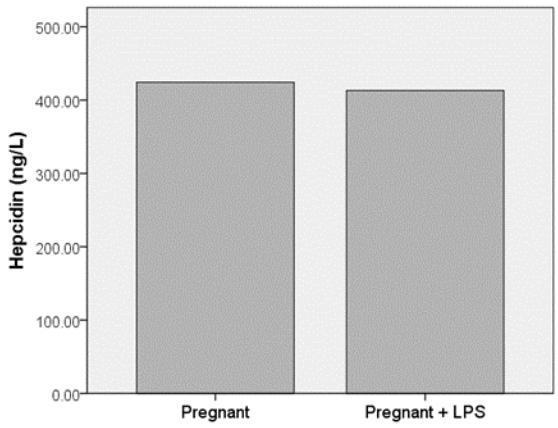
Data are shown as mean and standard deviation (SD). The normally distributed samples were analyzed statistical by t-test to determine differences between groups. All statistical tests used IBM SPSS statistics for Windows version 21.0 (IBM Corp., Armonk, NY, USA). A p-value of less than 0.05 was considered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

Results and Discussion

We observed hepcidin expression by measuring Hamp1 mRNA in hepatocytes and hepcidin protein in 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). Thus, hepcidin levels in pregnant mice was not affected by LPS **(Figure 1)**.



a)



b)

Figure 1. Comparison of hepcidin expression in non-LPS-induced pregnant mice and LPS-induced mice. a) Hamp1 mRNA expression in hepatocytes. b) Hepcidin level in blood.

We also observed the response of serum iron, our observations showed that LPS-induced pregnant mice (229.07 ± 80.63 µmol/L) had a significantly lower serum iron levels compared to control group (338.65 ± 115.11 µmol /L), with p-value 0.035 **(Figure 2)**.

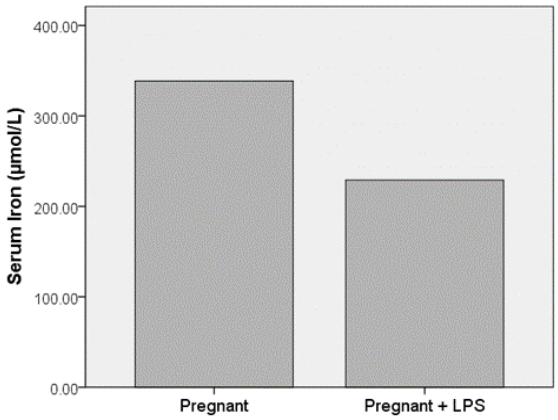


Figure 2. Induction of LPS in pregnant mice reduces serum iron levels.

In this study, we showed that LPS induction in pregnant mice gave no significant increase in Hamp1 mRNA expression in the liver. To analyze at the protein level, we measured hepcidin level in the blood. Our results also showed no significant increase in blood hepcidin levels. LPS-induced pregnant mice appear to maintain hepcidin levels at the same level to maintain iron homeostasis during gestation.

Several studies have shown that LPS is a protein that can induce hepcidin levels in hepatocytes [17, 18]. LPS-induced hepcidin is also expressed in brain and heart cells [19, 20], however, the liver is the main source of hepcidin in vitro [17]. Increased expression in hepatocytes is followed by increased hepcidin levels in blood and reaches a peak at 6 hours [21, 22]. Intriguingly, our present study showed different results with the previous studies.

It is not known what causes hepcidin to be retained in LPS-induced pregnant mice. LPS is a macromolecule that strongly mediates the inflammatory response, resulting in the production of proteins, especially interlequin-6 (IL-6) as strong inducers of hepcidin expression [23, 24]. Based on the available information, we speculate that estradiol (E2) may be the key in hepcidin regulation, since during pregnancy there is an increase in E2 production [25, 26]. 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) [27]. 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 [11, 28].

Interestingly, in our study, LPS-induced pregnant mice showed a decrease in serum iron, even though the hepcidin level was maintained. Generally, decreased serum iron is accompanied by increased hepcidin [24]. It should be understood that hepcidin is also produced by the fetus and can regulate fetal iron content by controlling placental iron export [10, 29]. 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 [30].

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 [10, 29, 31]. The study conducted by Sangkhae, *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 have any impact on fetal iron. As a result, the hepcidin of pregnant women and the hepcidin of the fetus do not mix with each other. Besides of 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 transfer of iron from mother to fetus [29].

A study by Kammerer *et al*. (2020) reported the autocrine role of hepcidin in regulating hepatic iron stores. 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 be probably due to the increased transfer of serum iron to the fetus [31].

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.

Conclusion

Our study shows that LPS-induced inflammation in pregnancy did not affect changes in Hamp1 mRNA expression and hepcidin level. Howecer, 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.

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Conflict of interest: None

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