ORIGINAL ARTICLE

The Effect of Lipopolysaccharide Exposure During Pregnancy on Hepcidin Expression in Female Mice: Involvement of Interleukin-6 and Activin B, Independent of Transforming Growth Factor-β Receptor

Gilang Nugraha[1,2,] Widjiati[3], Aryati[4], Citrawati Dyah Kencono Wungu[5], Harianto Notopuro[5], Win Darmanto[6], Agus Sulistyono[7], Hari Basuki Notobroto[8], Purwo Sri Rejeki[9]

[1] Doctoral Program of Medical Science, Faculty of Medicine, Universitas Airlangga, 60132 Surabaya, Indonesia.
[2] Department of Medical Laboratory Technology, Faculty of Health, Universitas Nahdlatul Ulama Surabaya, 60237 Surabaya, Indonesia.
[3] Department of Veterinary Anatomy, Faculty of Veterinary Medicine, Universitas Airlangga, 60115 Surabaya, Indonesia.
[4] Department of Clinical Pathology, School of Medicine, Universitas Airlangga, 60286 Surabaya, Indonesia.
[5] Department of Biochemistry, Faculty of Medicine, Universitas Airlangga, 60132 Surabaya, Indonesia.
[6] Department of Biology, Faculty of Science and Technology, Universitas Airlangga, 60115 Surabaya, Indonesia.
[7] Maternal–Fetal Medicine Division, Obstetrics & Gynecology Department, Dr. Soetomo Academic General Hospital, Universitas Airlangga, 60286 Surabaya, Indonesia.
[8] Department of Biostatics & Population, Faculty of Public Health, Universitas Airlangga, 60115 Surabaya, Indonesia.
[9] Department of Physiology, Faculty of Medicine, Universitas Airlangga, 60132 Surabaya, Indonesia.

ABSTRACT

Introduction: The expression of hepcidin in hepatocytes is induced by inflammation, primarily mediated by interleukin 6 (IL-6) and activin B. In contrast, hepcidin levels are suppressed during pregnancy via the half-site of the estrogen-responsive element (ERE) on the hepcidin gene promoter. These opposing mechanisms regulate hepcidin in pregnant women with inflammation. However, several studies report no change in hepcidin levels in the blood of pregnant women experiencing inflammation. Therefore, this study investigated the expression of hepcidin in pregnant mice with induced inflammation using LPS injection. **Materials and methods:** This study involved 26 pregnant mice. The treatment group was injected intraperitoneally with serotype O111:B4 (Sigma-Aldrich, Merck, Singapore), while the control group received Phosphate Buffered Saline (PBS). Serum levels of IL-6, activin B, estradiol, and hepcidin were measured using ELISA. The liver tissues were examined via immunohistochemistry to measure the Transforming Growth Factor-β (TGF-β) receptor. Data were analyzed using an independent t-test. **Results:** Our results demonstrated that pregnant mice with inflammation had significantly increased IL-6 (P = 0.000) and decreased activin B (P = 0.032) levels, but there were no significant differences in estradiol (P = 0.624), hepcidin (P = 0.607), and TGF-β receptor levels (P=0.662). **Conclusion:** Our study showed that inflammation during pregnancy does not impact hepcidin levels. Additionally, we observed a decrease in activin B levels in pregnant mice with inflammation. Conversely, high levels of estradiol during pregnancy may contribute to the suppression of hepcidin synthesis.
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Keywords: Activin B, Estradiol, Hepcidin, Inflammation, Interleukin-6, Pregnancy

Corresponding Author:
Prof. Dr. Aryati, dr., MS. Sp.PK(K)
Email: aryati @fk.unair.ac.id
Tel : +62 812-3057-0493

INTRODUCTION

Hepcidin is a hormone that regulates systemic iron homeostasis and is primarily produced in the liver. Its effect is to degrade ferroportin (Fpn), which decreases iron absorption in enterocytes and iron release from cells that recycle and store iron (1). Inflammation induces the expression of hepcidin mainly through the interleukin-6 (IL-6) and activin B pathways, thereby suppressing serum iron, resulting in hypoferremia, which may progress to iron deficiency anemia (2–4).

Pregnant women undergo various physiological changes, including the suppression of hepcidin production (5,6). During healthy pregnancies, maternal hepcidin concentrations typically decrease in the second and third trimesters (5). For example, the mean serum hepcidin

concentration is 19.39 ng/mL (range: 9.69–33.59 ng/mL) in the first trimester and decreases to 1.32 ng/mL (range: 0.52–6.67 ng/mL) in the third trimester (7). However, changes in hepcidin levels in pregnant women with inflammation remain ambiguous (8–10). The hepcidin concentration in pregnant women with inflammation ranges from 3.0 to 8.9 ng/mL (11,12), whereas in cases of iron deficiency anemia, it ranges from 0.7 to 1.9 ng/mL (8). This condition needs special attention due to the increased need for iron during pregnancy to support the developing fetus.

Yang et al. (2012) found that estradiol (17β-estradiol), a hormone that increases during pregnancy, can suppress hepcidin production through interaction with the estrogen receptor (ER), particularly ERα, in the cytoplasm. This interaction forms a complex that can bind to half of the estrogen-responsive element (ERE) sites on the promoter of the hepcidin gene, inhibiting hepcidin formation (13).

Interestingly, IL-6 can stimulate hepcidin production via the IL-6/STAT3 pathway. When IL-6 binds to its receptor, it activates JAK1/2 and phosphorylates STAT3, which in turn promotes the transcription of hepcidin (14). Induction of hepcidin by activin B occurs through the interaction of BMP receptors and Transforming Growth Factor-β (TGF-β) receptors, forming a phosphorylated SMAD1/5/8 complex. This complex subsequently binds to SMAD4, forming a protein complex that drives hepcidin transcription (3). Thus, there are two opposing mechanisms in the regulation of hepcidin during inflammation in pregnant women. However, several studies report no change in hepcidin levels in the blood of pregnant women experiencing inflammation (11,12,15,16). Therefore, it is crucial for clinicians to carefully address these conditions to provide suitable treatment for pregnant women with inflammation, ensuring the maintenance of iron homeostasis during pregnancy. Furthermore, there is a lack of experimental reports on hepcidin levels in pregnant women with inflammation.

The regulation of hepcidin synthesis is well-studied in inflammatory conditions (4,5), but little is known about the inflammation-mediated regulation of hepcidin during pregnancy. In this study, pregnant mice were used as models, and inflammation was induced with LPS, a bacterial cell wall component known to trigger inflammatory responses (17). This study aims to examine hepcidin expression during pregnancy in the presence of inflammation using pregnant mice models induced with LPS.

MATERIALS AND METHODS

Ethical Approval

This true experimental study was conducted in the laboratory of experimental animals. The research was approved by the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, with certificate number 2.KE.111.11.2020.

Study Period and Location

The research was conducted from March 2021 to December 2021. The maintenance and treatment of experimental animals were carried out in the Embryology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia. The sample analysis was performed at the Molecular Biology Laboratory, Biosains Institute, Universitas Brawijaya, Malang, Indonesia.

Experimental Animals

Second-week pregnant female mice with an average weight of 38.74 grams were obtained from the Farma Veterinary Center, Surabaya, Indonesia. Pregnancy was confirmed by the presence of a fetus at the time of surgery and elevated estradiol levels. The day of mating was designated as day 0 of pregnancy, and on day 14, surgery was performed, and estradiol was measured. Estradiol levels were considered elevated if the examination results were above normal values (47.22 ± 2.39 ng/L). Normal values were obtained from non-pregnant mice (18).

Mice in the second week of pregnancy were randomly divided into two equal groups. Mice were injected intraperitoneally with lipopolysaccharide (LPS) Escherichia coli serotype O111:B4 (Sigma-Aldrich, Merck, Singapore) at 1 µg/g body weight in PBS as the treatment group and PBS (Phosphate Buffered Saline) as the control group. Each group consisted of 13 pregnant female mice. The mice were sacrificed four hours after injection. This time frame was chosen based on studies by Canali S. et al. (2016) and Fillebeen et al. (2018), which showed that the immune response and increase in hepcidin in mice occurred four hours after LPS injection (2,24).

Sample Collection

Blood was collected via cardiac puncture to obtain serum and stored at -80°C until further processing using ELISA. The liver was surgically removed, washed with PBS, placed into a 10% formalin buffer container, and stored at room temperature until used for immunohistochemistry.

ELISA

The IL-6 concentration in the serum was measured using an IL-6 Mouse ELISA Kit (Cat. No. 431307, Legend MaxTM, BioLegend®, San Diego, USA). The activin B concentration was determined with an Activin B Mouse ELISA Kit (Cat. No. E1813Mo, Bioassay Technology Laboratory, Shanghai Korain Biotech Co., Ltd., Shanghai, China). The estradiol concentration of the samples was determined with a Mouse Estradiol ELISA Kit (Cat. No. E0072Mo, Bioassay Technology Laboratory, Shanghai Korain Biotech Co., Ltd., Shanghai, China), and the

hepcidin concentration was determined with a Mouse Hepcidin ELISA Kit (Cat. No. E1467Mo, Bioassay Technology Laboratory, Shanghai Korain Biotech Co., Ltd., Shanghai, China). All ELISA analyses were performed according to the manufacturer's instructions for each kit.

Immunohistochemistry

Protein expression was processed by immunohistochemistry (Cat. No. TP-015-HD, Thermo Fisher Scientific, Runcorn, UK) using the primary antibody TGFβ RII (1:250, Cat. sc-17791, Santa Cruz Biotechnology, Dallas, USA). The liver samples in 10% formalin buffer were embedded in paraffin. All specimens were sliced into 3 µm thick sections. The liver sections were deparaffinized with xylene three times for 3 minutes each, followed by rehydration with 100%, 95%, 90%, 80%, and 70% ethanol for 2 minutes each. Finally, the samples were rinsed with running water for 3 minutes. The slides were then immersed in a hydrogen peroxide block solution for 10 minutes and washed with buffer twice. Ultra V Block was added to the samples, incubated for 5 minutes, and rinsed with buffer five times. The primary antibody TGFβ RII was added for an hour at room temperature and washed with buffer four times. Biotinylated goat anti-polyvalent was added for 10 minutes at room temperature and washed with buffer four times. Streptavidin peroxidase was added for 10 minutes at room temperature and washed with buffer four times. Next, 40 µL of diaminobenzidine (DAB) plus chromogen was added to 2 mL of DAB plus substrate, stirred until homogeneous, and applied to the preparation for 15 minutes. Finally, a counterstain was performed using Mayer's hematoxylin, which was then mounted with mounting media and covered with a coverslip.

TGF-receptor expression (in brown) was observed in liver cells using a Nikon Eclipse Ci light microscope (Nikon Corporation, Tokyo, Japan) at 400x magnification, equipped with a 16-megapixel Nikon digital camera DS-Ri2 (Nikon Corporation, Tokyo, Japan) and Nikon Image Software System D (Nikon Corporation, Tokyo, Japan). The data for each sample was assessed semi-quantitatively according to the modified Remmele method (19). The results were interpreted using the immunoreactive score (IRS), as shown in Table I.

Table I: The IRS semiquantitative scale.

A	B
Score 0: No positive cells	Score 0: No color reaction
Score 1: Positive cells <10%	Score 1: Low color intensity
Score 2: Positive cells between from 11% - 50%	Score 2: Medium color intensity
Score 3: Positive cells between from 51% - 80%	Score 3: High color intensity
Score 4: Positive cells >80%	

We obtained the results by multiplying the percentage score of positive cells (A) with the color reaction intensity score (B), IRS = (A x B)
IRS: *Immuno Reactive Score*

Statistical Analysis

Data were analyzed using SPSS 21.0 software (IBM Corp., New York, USA). Results are presented as mean ± SD. Normality distribution was tested using the Kolmogorov–Smirnov test. Group differences were assessed using an independent t-test. A P-value of 0.05 was considered statistically significant.

RESULTS

LPS-Induced Inflammatory Response

Pregnancy was confirmed in the mice, which were then injected with LPS to induce inflammation. IL-6 and activin B levels in the serum were measured. LPS injection in pregnant mice resulted in a significant increase in IL-6 levels to 684.91 ± 333.87 pg/mL (P≤0.05), compared to the control group with IL-6 levels of 30.517 ± 12.633 pg/mL. Additionally, activin B levels significantly decreased to 45.66 ± 7.03 ng/L (P≤0.05) in the LPS-injected group, while the control group had activin B levels of 51.411 ± 5.75 ng/L (Table II). Thus, LPS injection in pregnant mice increased IL-6 levels but decreased activin B levels.

Table II: Mean values and standard deviation of IL-6 and activin B

	Pregnant Mice + LPS (n = 13)	Pregnant Control Mice (n = 13)	P-value
IL-6 (pg/mL)	684.91 ± 333.87	30.517 ± 12.633	0.000*
Activin B (ng/L)	45.66 ± 7.03	51.411 ± 5.75	0.032*

*Significant at α≤0.05

LPS Induction Does Not Affect Estradiol Levels

Estradiol serum levels were examined in second-week pregnant mice from each group. No significant difference in estradiol levels was observed between pregnant mice injected with LPS and the control group (P > 0.05) (Table III).

Tabel III: Mean values and standard deviation of estradiol

	Pregnant Mice + LPS (n = 13)	Pregnant Control Mice (n = 13)	P-value
Estradiol (ng/L)	71.109±20.360	76.238±31.174	0.624

*Significant at α≤0.05

Inflammation Does Not Affect Hepcidin Levels

Hepcidin levels in serum samples were measured to determine the response of hepcidin in pregnant mice injected with LPS. No significant difference in hepcidin levels was observed between pregnant mice injected with LPS and the control group (P>0.05) (Table IV).

Table IV: Mean values and standard deviation of hepcidin

	Pregnant Mice + LPS (n = 13)	Pregnant Control Mice (n = 13)	P-value
Hepcidin (ng/L)	397.981±74.761	412.156±63.414	0.607

*Significant at α≤0.05

TGF-β Receptor Expression

The expression of the TGF-β receptor in the liver was assessed semi-quantitatively using the modified Remmele method (19). No significant difference in TGF-β receptor expression was observed between pregnant mice injected with LPS and the control group ($P > 0.05$) (Table V, Figure 1).

Table V: Mean values and standard deviation of TGF-β receptor expression

	Pregnant Mice + LPS (n = 13)	Pregnant Control Mice (n = 13)	P-value
TGF-β Receptor	6.9±1.7	6.5±1.7	0.662

*Significant at α≤0.05

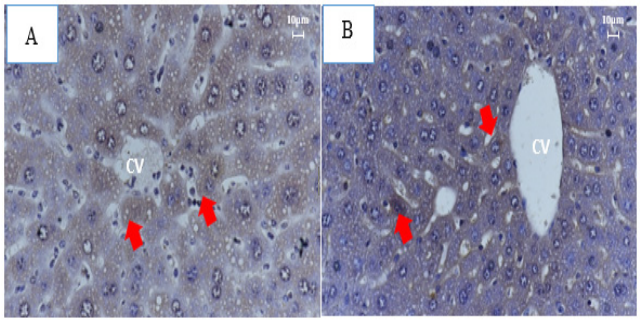

Figure 1: Comparison of the expression of TGF-β receptors in the liver.

Expression of TGF-β receptors of the treatment groups (A). Expression of TGF-β receptors of the control groups (B). Red arrows indicate the expression of TGF-β receptors on hepatocytes which are indicated by the presence of chromogen brown color. Note: cv = central vein. IHC.

DISCUSSION

IL-6 is a pro-inflammatory cytokine that can stimulate the production of hepcidin by binding to the IL-6 receptor, also known as CD126, on hepatocytes. This binding subsequently phosphorylates STAT3 via JAK1/2, promoting the dimerization and migration of the JAK/STAT complex into the nucleus, which further activates hepcidin gene transcription (1,27) . Intriguingly, this pathway requires interaction with the BMP/SMAD pathway for IL-6 to induce hepcidin. Activin B, a protein involved in activating the BMP/SMAD pathway, plays a crucial role in this process.

The BMP-SMAD pathway is the primary pathway controlling hepcidin expression in the liver. It is activated paracrinely by BMP2 and BMP6, produced by liver sinusoidal endothelial cells (28). Ligand binding activates SMAD signaling through a mechanism enhanced by hemojuvelin (HJV), leading to the phosphorylation of SMAD1/5/8, SMAD4 recruitment, and formation of a complex that translocates to the nucleus to bind the HAMP promoter, activating HAMP mRNA transcription to form hepcidin (27,29).

LPS, a major outer membrane component of Gram-negative bacteria, can induce an inflammatory response characterized by an increase in pro-inflammatory cytokines (3,20–24). In pregnant mice, LPS induction led to an increase in IL-6 levels and a decrease in activin B levels, as observed in our study (Table 2). It has been reported that the increase in IL-6 during inflammation and infection occurs due to the stimulation of cells by IL-1 or tumor necrosis factor (TNF)-α or through the stimulation of Toll-Like Receptors (25).

Uniquely, our study demonstrated decreased levels of activin B in pregnant mice injected with LPS. Previously, it was reported that LPS could induce activin B in hepatocytes, Kupffer cells, endothelial cells, and vessel lumens (3). However, this study was conducted on pregnant mice, where estradiol is expected to increase significantly during pregnancy (26).

Activin B can utilize TGF-β receptors to selectively stimulate non-canonical SMAD1/5/8 and hepcidin signaling in hepatocytes (4,27). We examined the TGF-β receptor to ensure that treatment did not affect TGF-β expression, as these receptors play a role in controlling hepcidin synthesis through non-canonical pathways (27). Our study results showed no difference in TGF-β receptor expression in pregnant mice with and without inflammation. It has been reported that inflammation and the immune response do not affect TGF-β receptor expression (30,31). Thus, the activity of hepcidin synthesis by activin B might not be affected by the TGF-β receptor. However, activin B levels decreased in pregnant mice injected with LPS, suggesting that activation of the SMAD1/5/8 signal did not occur. This condition, which may be a factor in hepcidin, did not experience changes in concentration.

During pregnancy, estradiol levels increase due to its production by the placenta (32–34). Our study showed increased estradiol levels in both inflamed and non-inflamed pregnant mice compared to a study conducted by Yan et al. (2017). Estradiol regulates many genes through various mechanisms, including direct genomic signaling, indirect genomic signaling, ER-independent, and estrogen-independent pathways (35). Yang et al. found half of the estrogen-responsive element (ERE) sites on the hepcidin gene promoter and demonstrated that treatment with estradiol inhibited hepcidin expression (13). Estradiol binds to ERα in the cytoplasm, leading to receptor dimerization and translocation to the nucleus. In the nucleus, the complex binds to chromatin in the ERE sequence, inhibiting hepcidin translation (13,35). This genomic signaling mechanism may explain why hepcidin levels in the pregnant mice with inflammation were similar to those in the control pregnant mice.

However, further studies are warranted to decipher the precise mechanism of genomic control of estrogen on the hepcidin gene promoter.

CONCLUSION

Our study suggests that inflammation during pregnancy does not significantly impact hepcidin levels but does decrease activin B. Additionally, the elevated estradiol levels observed during pregnancy may inhibit hepcidin production. Future research should delve deeper into the roles of the BMP/SMAD pathway and the JAK/STAT pathway in this context.

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