A study on the changes in hepcidin in an animal model of anemia of chronic inflammation: mechanistic insights related to iron supplementation and hepcidin regulation

Hye-Bin Kim

Department of Medicine

The Graduate School, Yonsei University

A study on the changes in hepcidin in an animal model of anemia of chronic inflammation: mechanistic insights related to iron supplementation and hepcidin regulation

Directed by Professor Young-Lan Kwak

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Hye-Bin Kim

June 2021

This certifies that the Doctoral Dissertation
of Hye-Bin Kim is approved.

Thesis Supervisor : Young-Lan Kwak

Thesis Committee Member #1 : Jae-Kwang Shim

Thesis Committee Member #2 : Sung Yong Park

Thesis Committee Member #3 : Sak Lee

Thesis Committee Member #4 : Chi Young Shim

The Graduate School
Yonsei University

June 2021

ACKNOWLEDGEMENTS

First, I would like to express my sincerest gratitude to my supervisor and invaluable mentor, Professor Young-Lan Kwak. She has inspired and encouraged me throughout my research journey, and this dissertation would not have been possible without her insights and guidance.

I would also like to thank Professor Jae-Kwang Shim. His enduring encouragement and practical advice have been inestimable sources of support during this venture.

I would like to extend my gratitude to my thesis committee members, Professor Sung Yong Park, Sak Lee, and Chi Young Shim for their valuable advice and comments. I also wish to thank Ji Hae Jun for being a great teacher and a model researcher during my doctoral program.

I would like to take this opportunity to express the deepest sense of appreciation for the support granted by my colleagues at the Department of Anesthesia and Pain Medicine.

Finally, a word of gratitude to my loving family and friends, for their affection and encouragement.

Hye-Bin Kim

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

A study on the changes in hepcidin in an animal model of anemia of chronic inflammation: mechanistic insights related to iron supplementation and hepcidin regulation

Hye-Bin Kim

Department of Medicine
The Graduate School, Yonsei University

Directed by Professor Young-Lan Kwak.

Anemia of chronic inflammation (ACI), also known as anemia of chronic disease, is the second most frequent form of anemia in hospitalized patients and a harbinger of adverse health outcomes. Hepcidin, the master regulator of iron homeostasis, is expressed mainly in the liver and induces the degradation of ferroportin, the sole iron exporter. Chronic inflammatory conditions lead to an increase in the levels of hepcidin, resulting in a sequestration of iron inside cells in the form of ferritin, and this hinders iron utilization for erythropoiesis despite sufficient iron stores in the body. However, anemia is often clinically treated with iron supplementation, regardless of the underlying cause, with varying results in patients with ACI. Changes in hepcidin levels and activity in ACI patients have not been fully elucidated, and understanding them may provide new insights for planning treatment strategies. Thus, the primary aim of this study was to establish an animal model of ACI that resembled clinical ACI and use it to investigate changes in hepcidin and upstream regulatory pathways

in the liver after intravenous (IV) iron supplementation. Additionally, we examined whether oxidative stress and concomitant changes in the major erythropoiesis-stimulating pathways involving erythropoietin (EPO) and erythroferrone (ERFE), was induced by iron supplementation. We also aimed to identify the role of signaling pathways upstream of hepcidin in HepG2 cells treated with iron using short interfering RNAs.

ACI was induced in rats by intraperitoneal administration of Complete Freund's Adjuvant (CFA, 0.2 mg in oil, 3 times/2 weeks). Male Sprague Dawley rats (n = 30) were randomly assigned to 3 groups: control-saline (n = 10), CFA-saline (n = 10), and CFA-IV iron (n = 10). Rats showing a decline in hemoglobin (Hb) levels >2 g/dL from baseline at 2 weeks after CFA administration were designated as ACI rats. ACI rats received iron (20 mg/kg of iron isomaltoside, CFA-IV iron) or vehicle (CFA-saline) intravenously. Three days after which changes in hematologic parameters, inflammatory cytokine levels, iron profiles, expression of proteins involved in signaling pathways upstream of hepcidin transcription, and iron metabolism gene expression were evaluated in the liver.

ACI rats with anemia showed a concomitant increase in the expression of inflammatory mediators (i.e., interleukin [IL]-1β, IL-6, tumor necrosis factor-α [TNF-α]) and reactive oxygen species (ROS). Three days after IV iron administration, Hb levels in ACI rats did not fully recover to the corresponding baseline values. Hepcidin levels in the liver of ACI rats were upregulated with a concomitant increase in ferritin levels in the liver compared to those in CFA-saline rats. Iron supplementation also induced an increase in ROS-generating NADPH oxidase (NOX)-2, NOX-4, and superoxide dismutase in the liver compared to those in CFA-saline rats. In ACI rats, activity of the IL-6/signal transducer and activator of transcription-3 (IL-6/STAT-3) pathway and the bone morphogenetic protein/Sma mothers against decapentaplegic (BMP/SMAD) pathway were increased in the liver compared to those in vehicle treated rats. IV iron supplementation further increased SMAD1/5 phosphorylation and STAT-3

phosphorylation in CFA-IV iron rats compared to those in CFA-saline rats, while there was no difference in IL-6 expression between both groups. Iron caused phosphorylation of STAT-3 and SMAD1/5 in HepG2 cells, and knockdown of *STAT-3* and *SMAD1/5* using short interfering RNAs in HepG2 cells was found to be associated with diminished iron-induced upregulation in hepcidin transcription compared to that in the corresponding control cells. IV iron supplementation was also associated with decrease in EPO mRNA expression in the kidney and serum ERFE concentration in CFA-IV iron rats compared to those in control-saline rats.

In conclusion, in this rat model of ACI, IV iron administration did not effectively ameliorate anemia within 3 days despite an increase in ferritin levels in the liver, which might be related to an additional increase in hepcidin levels that was already upregulated under chronic inflammatory conditions. Both STAT-3 and SMAD1/5 phosphorylation were found to be associated with hepcidin upregulation after IV iron treatment, and this is potentially linked to iron-induced oxidative stress. Concurrent reductions in renal EPO expression and serum ERFE activity after IV iron supplementation imply a complex interplay between erythroid regulatory molecules that hinders effective erythropoiesis despite sufficient iron supplementation under ACI conditions. These results provide primary evidence regarding the importance of hepcidin regulation and EPO availability in increasing the efficacy of IV iron supplementation for the treatment of anemia under chronic inflammatory conditions.

Key words: anemia of chronic inflammation, erythropoietin, hepcidin, intravenous iron supplementation, oxidative stress

A study on the changes in hepcidin in an animal model of anemia of chronic inflammation: mechanistic insights related to iron supplementation and hepcidin regulation

Hye-Bin Kim

Department of Medicine
The Graduate School, Yonsei University

Directed by Professor Young-Lan Kwak.

I. INTRODUCTION

Anemia of chronic inflammation (ACI), also referred to as anemia of chronic disease, is the second most common anemia after iron deficiency anemia (IDA) in hospitalized patients, and is associated with increased morbidity.[1,2] Inadequate utilization of iron despite sufficient body iron stores leads to the development of ACI, whereas IDA is caused by an absolute iron deficiency.[3] Although, iron is an essential micronutrient for cellular processes, such as DNA synthesis, ATP production, and oxygen transport, an overload of iron can be toxic to cells and result in the production of reactive oxygen species (ROS).[4,5] Excess iron is sequestered within ferritin, which is capable of storing a large number of iron atoms.[6]

Hepcidin, a key regulator of iron homeostasis, is mainly produced by hepatocytes and is abundantly expressed in the heart. Hepcidin regulates iron metabolism through ferroportin (FPN), which is the sole known iron exporter.[7,8] Hepcidin binds to FPN and induces FPN internalization and lysosomal degradation, causing iron sequestration in macrophages, enterocytes, and hepatocytes. The expression of these two proteins is tightly regulated. Hepcidin is upregulated by an increase in iron stores in the body and inflammatory

conditions, whereas it is downregulated by erythropoiesis.[7,8] Iron-mediated regulation of hepcidin acts mainly through the bone morphogenetic protein/Sma mothers against decapentaplegic (BMP/SMAD) signaling pathway. Inflammation and ROS mainly trigger the signal transducer and activator of transcription-3 (STAT-3) pathway which also influences the expression of hepcidin.[7] Erythroferrone (ERFE), secreted by erythropoietin (EPO)-stimulated erythroblasts during erythropoiesis, causes a suppression in hepcidin expression.[9]

In ACI, increased hepcidin expression induces a decrease in FPN expression and iron sequestration in iron storage cells, resulting in functional iron deficiency and anemia.[10] Thus, the hepcidin/FPN axis is an ideal target in the treatment of ACI; however, no currently available drugs can directly regulate this axis. Iron supplementation and blood transfusion are the only clinical treatment regimens for anemia, regardless of the underlying mechanisms.

In current clinical practice, intravenous (IV) administration of iron is preferred over oral administration of iron because of its superior efficacy in terms of bioavailability and simple administration protocol.[11,12] Additionally, the absorption of dietary iron through duodenal enterocytes decreases as hepcidin expression increases, and therefore, IV administration is recommended for iron supplementation. Iron isomaltoside 1000 is an iron complex that is used in IV administration and has robust and reliable pharmacokinetic properties, and thus, it is well-tolerated by patients even at high doses.[13] Recent studies demonstrated that a single administration of high-dose IV iron can replenish iron stores of the body, which was not always followed by meaningful increases in hemoglobin (Hb) depending on the treatment protocols, especially during a short period time.[14-17] Of note, there is a paucity of data on changes in hepcidin levels in relation to iron supplementation under ACI conditions in both experimental and clinical studies, and this information is essential as these changes may seriously counteract the therapeutic efficacy of IV iron supplementation in boosting erythropoiesis.

Thus, the primary aim of this study was to establish a relevant animal model of ACI that resembled its clinical presentation and to use the model to investigate short-term changes in hepcidin expression and its regulatory pathways in the liver after IV iron supplementation. The secondary aim of this study was to evaluate potential oxidative stress and concomitant changes in EPO expression in the kidney and ERFE concentration in the serum after IV iron supplementation. We also aimed to identify the role of signaling pathways upstream to hepcidin in HepG2 cells loaded with iron using short interfering RNAs (*si*RNAs).

II. MATERIALS AND METHODS

1. Animal preparation

All animal procedures were approved by the Committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine and were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (USA). Male Sprague Dawley rats (130–150 g) were housed in a temperature-controlled environment at 23–25 °C with a 12/12 h light/dark cycle and were given *ad libitum* access to food and water.

2. Study groups and experimental models

Animals were randomly assigned to 3 groups: control-saline (n = 10), Complete Freund's Adjuvant (CFA, InvivoGen, USA)-saline (n = 10), and CFA-IV iron (Monofer®, Pharmacosmos A/S, Holbaek, Denmark; n = 10).

For establishment of the ACI model, rats were intraperitoneally administered 0.2 mL of CFA containing 1 mg/mL of heat-killed *Mycobacterium tuberculosis* mixed with phosphate buffer saline (in the ratio of 1:1, thrice across 2 weeks), while control rats were administered the same volume of saline .[18] Rats that exhibited a decline >2 g/dL in hemoglobin (Hb) from baseline after 2 weeks were designated as ACI rats.[19] ACI rats intravenously received 20 mg/kg of iron, which is the highest clinical dose currently used, or an equivalent volume of saline. Serum and tissues were harvested from euthanized rats 3 days after IV iron or saline administration.

3. Hematologic studies

Blood samples were subjected to complete blood counts obtained using a Mindray BC-5000 Vet hematology analyzer (Shenzhen Mindray Bio-Medical Electronics, China).

4. Measurement of iron parameters

Serum iron level, total iron-binding capacity (TIBC), and transferrin saturation (TSAT) were assessed at Seoul Clinical Laboratories (Seoul, Korea).

5. Immunohistochemistry (IHC)

Liver tissue samples were subjected to IHC analysis. Samples were washed in physiological saline, fixed in 10% buffered formalin, and embedded in paraffin. Embedded sections were then stained with rabbit anti-NADPH oxidase (NOX)-2 (1:100, Novus) and anti-NOX-4 (1:500, Abcam), followed by staining with the chromogen 3,3-diaminobenzidine (Abcam, USA). Slides were viewed with an Olympus IX73P2F microscope (Olympus America, NY) equipped with an Olympus DP71 digital camera (20×).

6. Cell culture

HepG2 cells (human liver cancer cell line) were purchased from American Type Culture Collection (USA) and were maintained in Eagle's minimal essential medium (EMEM) + 2 mM glutamine supplemented with 10 % fetal bovine serum (FBS), 100 unit/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. Culture media and supplements were purchased from Gibco (Carlsbad, CA, USA).

7. Cell viability assay

HepG2 cells (5×10^3) were seeded into 96-well culture plates and incubated overnight. The cells were then incubated for 0–2 days in EMEM + 2 mM glutamine supplemented with 10% FBS with or without iron, at the concentrations indicated. Finally, cell viability was evaluated using the Cell Counting Kit (CCK)-8 assay (Dojindo, Japan), according to the manufacturer's protocol. Experiments were performed in triplicate.

8. Immunoblot analysis

Tissue specimens were lysed in radioimmunoprecipitation assay buffer supplemented with 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, a protease inhibitor mixture and phosphatase inhibitor cocktail-2 and cocktail-3 (Sigma-Aldrich, USA). Protein concentrations were measured using the bicinchoninic acid assay, and equal amounts of protein from each sample were subjected to immunoblot assay. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with anti-hepcidin, anti-ferritin (R&D), anti-superoxide dismutase (SOD) (Santa Cruz, USA), anti-NOX-2, anti-NOX-4, anti-Phospho-STAT-3, anti-STAT-3, anti-Phospho-SMAD1/5, anti-SMAD1, anti-SMAD5, and anti- glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Beverly, MA). Each experiment was performed in triplicate.

9. Enzyme-linked immunosorbent assay (ELISA)

Levels of hepcidin and erythroferrone (ERFE, Mybiosource, USA), interleukin (IL)-1β (R&D, USA), IL-6 (Biolegend, USA), and tumor necrosis factor-α (TNF-α, BD Life Science, USA) in the serum samples were determined using ELISA commercial kits according to the manufacturer's instructions.

10. Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol reagent (Thermo Fisher, USA). Complementary DNA was synthesized from 1 μg of total RNA using *AccuPower* RT premix kits (BIONEER, Korea). RT-PCR analysis was performed using TB Green™ Premix Ex Taq II kit (TAKARA, Japan) and AB7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Each sample was analyzed in quadruplicate, and target genes were normalized to the reference housekeeping gene, *GAPDH*. Fold

differences were then calculated for each group using C_T values normalized to those of the control groups. The primer sequences used for RT-PCR are listed in Table 1.

11. Gene knockdown by small interfering RNA (siRNA)

*si*RNAs for human *STAT-3*, *SMAD1*, and *SMAD5* genes and a non-targeting *si*RNA (control *si*RNA) were purchased from Santa Cruz (CA, USA). *STAT-3 si*RNA was a mixture of 3 *si*RNAs, providing advantages in terms of both potency and specificity. Transfection of *si*RNA into HepG2 cells was performed according to the manufacturer's instructions. Subsequently, the cells were incubated for an additional 24 h with or without iron. Whole cell lysates were prepared and subjected to immunoblotting.

12. Statistical analysis

Data are expressed as mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA), Student's *t-test* followed by Tukey correction, or repeated measures ANOVA. Statistical significance was set at $P < 0.05$.

Table 1. Primer sequences for real-time polymerase chain reaction (RT-PCR)

Gene name	Accession number	Primers	
Hepcidin	NM_053469	F	5'-ACAGACGAGACAGACTACGG-3'
		R	5'-GAGGCATATGGGGAAGTTGG-3'
Ferroportin (FPN)	NM_133315	F	5'-CTTCACTTGGCTACGTCGAA-3'
		R	5'-GAACACGGAGATCACACACA-3'
Homeostatic iron regulator (HFE)	XM_032884765	F	5'-AGATGCCAAGGATGTCAACC-3'
		R	5'-TCTTGTCTCTTCTCCAGGGG-3'
Hemojuvelin (HJV)	NM_001012080	F	5'-TGCAGCCTTTGAAGATGGTT-3'
		R	5'-TGTTCCAATGTAGGCAGCTC -3'

Bone morphogenetic protein-6 (BMP-6)	NM_013107.1	F	5'-GCTCCAGTGCTTCAGACTAC-3'
		R	5'-GATGATCCAGTCCTGCCATC-3'
Erythropoietin (EPO)	NM_017001	F	5'-CTATTTACGGGGTGCTGGAC-3'
		R	5'-ATGAGTTTGGCTGTCTCTGC-3'
GAPDH	NM_017008	F	5'-AACGACCCCTTCATTGACCT-3'
		R	5'-TGACCAGCTTCCCATTCTCA-3'

GAPDH; glyceraldehyde-3-phosphate dehydrogenase.

III. RESULTS

1. Establishment of a rat model of ACI

CFA-injected rats (CFA-treated group) gained an average of 88 g, while rats in the control group gained an average of 102 g over the course of the 2-week experimental period. The rate of weight gain in normal rats was higher than that in the CFA-treated rats (Fig. 1A).

At 2 weeks after the start of the CFA treatment, Hb levels decreased (Fig. 1B), while serum levels of inflammatory cytokines (i.e., IL-1β, IL-6, and TNF-α) significantly increased in the CFA-treated group compared with those in the control group (Fig. 1C, 1D, and 1E), indicating the successful establishment of ACI in the rats.

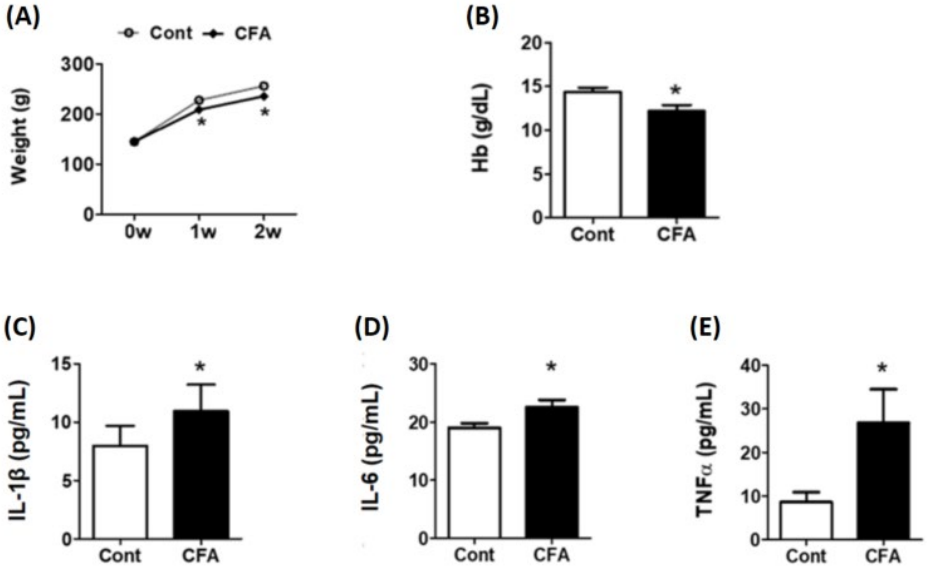

Figure 1. Changes in weight, hemoglobin (Hb) and serum inflammatory cytokines 2 weeks after administration of Complete Freund's Adjuvant (CFA). Cont; control group, CFA; CFA-treated group, IL-1β; interleukin-1β, IL-6;

interleukin-6, TNF-α; tumor necrosis factor-α. *P < 0.05 compared with the control group.

2. Leukocytes with differential counts and red cell indices in ACI rats

At 2 weeks after the start of the CFA treatment, white blood cell and neutrophil counts increased in the CFA-treated rats compared to those in control rats. Additionally, mean corpuscular volume decreased, while red cell distribution width increased in the CFA-treated group compared to those in the control group (Table 2).

Table 2. Leukocytes counts and red cell indices at 2 weeks after Complete Freund's Adjuvant (CFA) treatment

	Control	**CFA-treated group**
White blood cells ($\times 10^3/\mu L$)	5.6 ± 0.3	9.9 ± 1.2 *
Neutrophil ($\times 10^3/\mu L$)	1.17 ± 0.1	3.80 ± 0.7 *
Lymphocyte ($\times 10^3/\mu L$)	4.0 ± 0.2	5.2 ± 0.8
MCV (fL)	68.3 ± 0.4	66.9 ± 0.7 *
MCH (pg)	22.7 ± 0.2	22.2 ± 0.3
MCHC (g/dL)	33.2 ± 0.3	33.2 ± 0.2
RDW (%)	12.4 ± 0.1	15.9 ± 0.4 *

MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width. *P < 0.05, compared with the control group.

3. Changes in Hb, iron profiles, IL-6, hepcidin, and erythroferrone (ERFE) in blood 3 days after iron treatment

Hb and serum iron levels were decreased in both of the CFA-saline and CFA-IV iron groups when compared to those in the control group (Fig. 2A and 2 B). TSAT was lower in the CFA-saline group than that in the control group (Fig. 2C), while the TIBC did not differ between the treatment and control groups (Fig. 2D). IL-6 levels were increased in the CFA-saline and CFA-IV iron groups compared to that in the control group (Fig. 2E). Serum hepcidin levels were higher in the CFA-IV iron group than that in the control group (Fig. 2F). ERFE was decreased in the CFA-saline and CFA-IV iron groups compared with that in the control group (Fig. 2G).

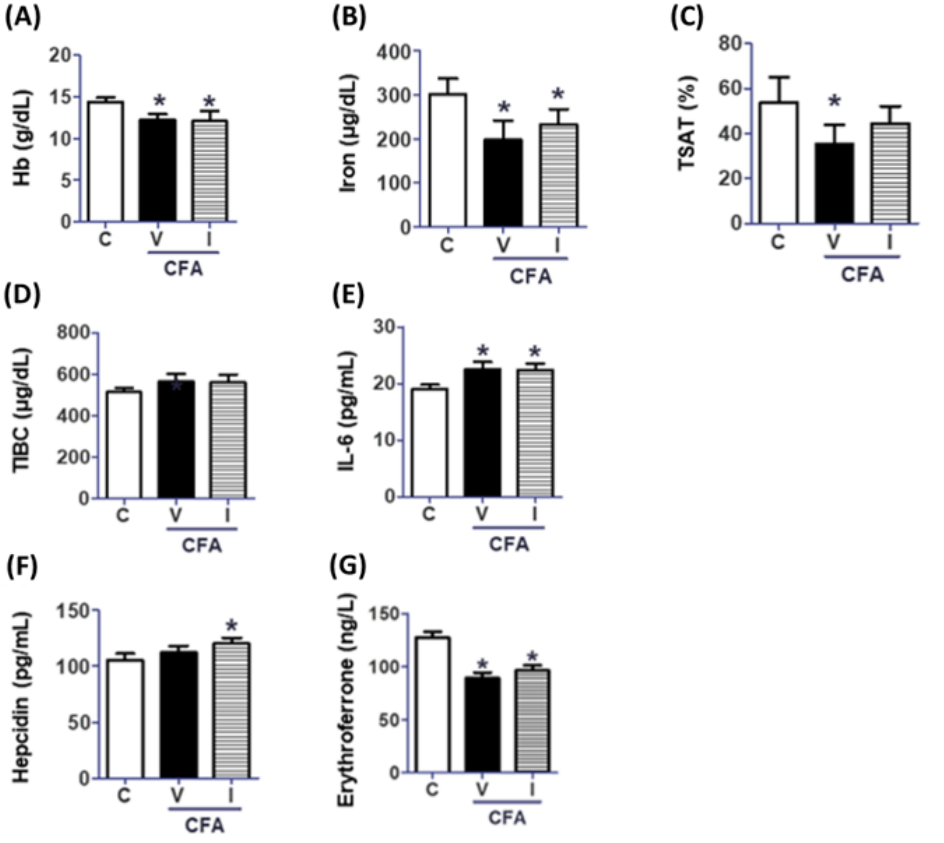

Figure 2. Effects of Complete Freund's Adjuvant (CFA) and intravenous iron treatment on the serum levels of hemoglobin (Hb), iron profiles, interleukin-6 (IL-6), hepcidin, and erythroferrone.

C; control-saline group, V; CFA-saline group, I; CFA-IV iron group, TSAT; transferrin saturation, TIBC; total iron binding capacity. $^{*}P < 0.05$ compared with the control group.

4. Changes in ROS-generating NAPDH oxidase-2 (NOX-2), NOX-4, and superoxide dismutase (SOD) levels in the rat liver

CFA induced significant increases in the protein levels of hepatic NOX-2, NOX-4, and SOD compared to their levels in the control group. IV iron supplementation resulted in further increases in these protein levels compared to those in the CFA-saline group (Fig. 3).

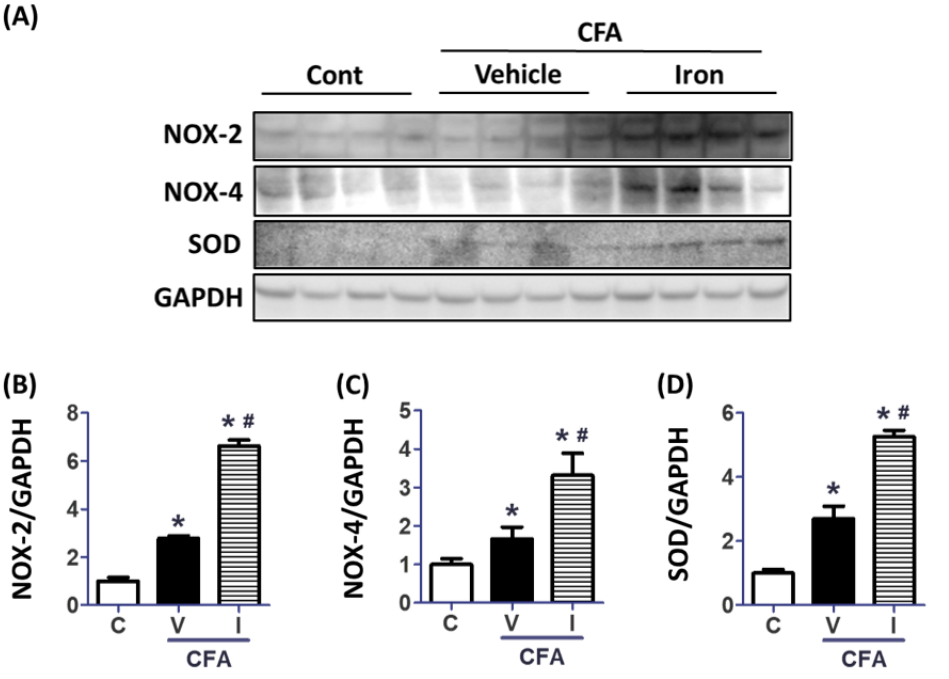

Figure 3. Effect of Complete Freund's Adjuvant (CFA) and intravenous iron treatment on the protein levels of NAPDH oxidase-2 (NOX-2), NOX-4, and superoxide dismutase (SOD) in the rat liver. The relative protein levels were quantified by densitometry (lower panel).

C; control-saline group, V; CFA-saline group, I; CFA-IV iron group, GAPDH; glyceraldehyde-3-phosphate dehydrogenase. $^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the CFA-saline group.

In order to analyze whether NOX-2 and NOX-4 expression in hepatocytes were altered with IV iron supplementation, liver specimens were subjected to IHC analyses of NOX-2 and NOX-4. The numbers of NOX-2 and NOX-4 positive hepatocytes were higher in the CFA-saline group than those in the control group. Moreover, stronger immunopositivity for NOX-2 and NOX-4 was observed in the CFA-IV iron group compared to that in the control and CFA-saline groups (Fig. 4).

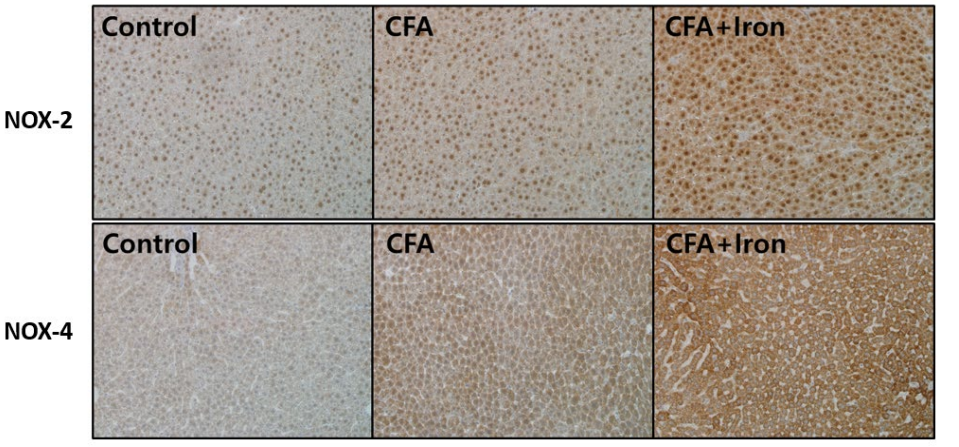

Figure 4. Immunohistochemistry of NAPDH oxidase-2 (NOX-2) and NOX-4 in the rat liver after Complete Freund's Adjuvant (CFA) and intravenous iron treatment.
Control; control-saline group, CFA; CFA-saline group, CFA+Iron; CFA-IV iron group.

5. Changes in hepcidin and related parameters in the rat liver

CFA induced significant increases in the protein levels of hepcidin and ferritin in the liver compared with those in the control group, and they were significantly higher in the CFA- IV iron group compared with those in the control and CFA-saline groups (Fig. 5).

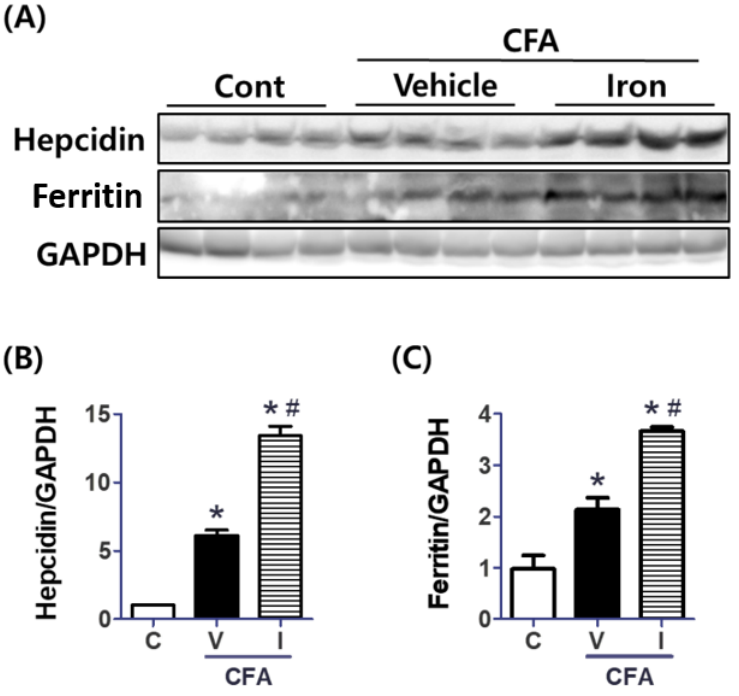

Figure 5. Effect of Complete Freund's Adjuvant (CFA) and intravenous iron treatment on the protein levels of hepcidin and ferritin in the rat liver. The relative protein levels were quantified by densitometric analysis (lower panel).

C; control-saline group, V; CFA-saline group, I; CFA-IV iron group, GAPDH; glyceraldehyde-3-phosphate dehydrogenase. $^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the CFA-saline group.

The mRNA expression levels of hepatic FPN did not change with CFA treatment and iron supplementation (Fig. 6).

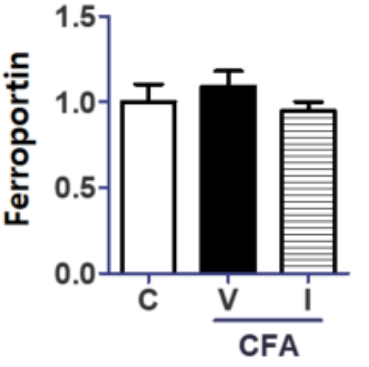

Figure 6. Effect of Complete Freund's Adjuvant (CFA) and intravenous iron treatment on the mRNA expression of ferroportin (FPN) in the rat liver. The mRNA level of FPN was analyzed by real-time polymerase chain reaction. Relative transcript levels are C_T values normalized to that of the control group. C; control-saline group, V; CFA-saline group, I; CFA-IV iron group.

BMP-6, the primary BMP associated with regulation of hepcidin transcription via the BMP/SMAD pathway, was increased in the CFA-IV iron group compared to that in the control and CFA-saline groups. (Fig. 7A). mRNA expression of homeostatic iron regulator (HFE), one of the mediators of iron-mediated signaling pathways in hepatocytes, was increased in the CFA-IV iron group compared to that in the CFA-saline group (Fig. 7B). mRNA expression of Hemojuvelin, a co-receptor of BMP receptors, did not differ between the groups (Fig. 7C).

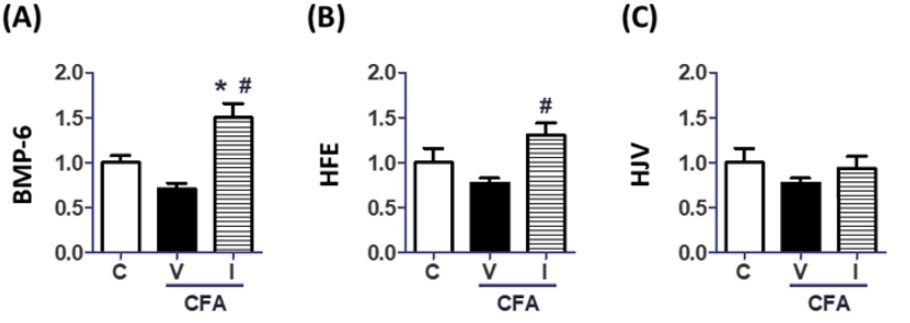

Figure 7. Effect of Complete Freund's Adjuvant (CFA) and intravenous iron treatment on the mRNA expression of bone morphogenetic protein-6 (BMP-6), homeostatic iron regulator (HFE), and hemojuvelin (HJV) in the rat liver. Real-time polymerase chain reaction was performed. Relative transcript levels are C_T values normalized to those of the control group.

C; control-saline group, V; CFA-saline group, I; CFA-IV iron group. $^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the CFA-saline group.

Phosphorylation of STAT-3 was increased in the CFA-saline group compared with that in the control group, and that in the CFA-IV iron group was higher than that in the CFA-saline group (Fig. 8A and 8 B). Additionally, phosphorylated levels of SMAD1/5 were increased in the CFA-saline group compared with that in the control group, and that in the CFA-IV iron group was higher than that in the CFA-saline group (Fig. 8A, 8C, and 8D).

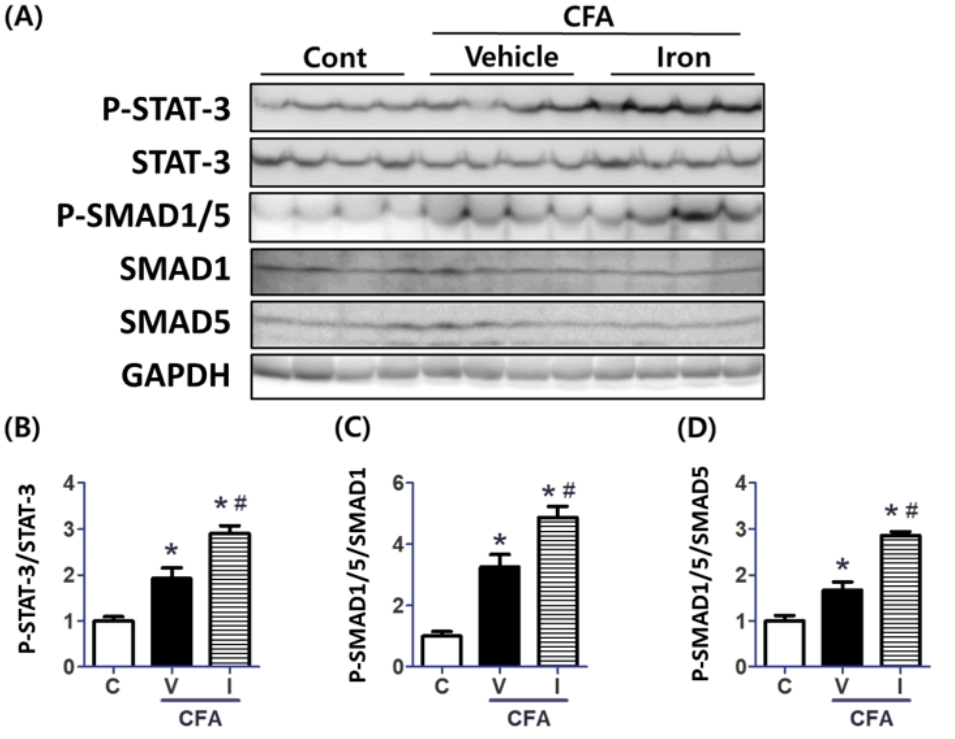

Figure 8. Effect of Complete Freund's Adjuvant (CFA) and intravenous iron treatment on the hepcidin regulation pathways in the rat liver. The relative protein levels were quantified by densitometry (lower panel).

C, control-saline group, V; CFA-saline group, I; CFA-IV iron group, STAT-3; signal transducer and activator of transcription-3, P-STAT-3; phosphorylated STAT-3; SMAD; sma mothers against decapentaplegic, P-SMAD;

phosphorylated SMAD, GAPDH; glyceraldehyde-3-phosphate dehydrogenase. *P < 0.05 compared with the control group; #P < 0.05 compared with the CFA-saline group.

6. Changes in EPO mRNA expression in the rat kidney

The mRNA expression levels of EPO in the rat kidney were significantly lower in the CFA-IV iron group than that in the control and CFA-saline group (Fig. 9).

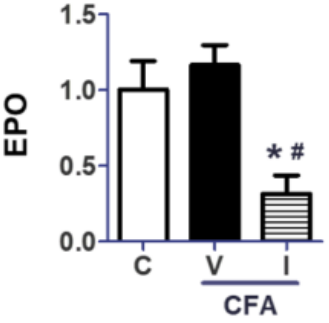

Figure 9. Effect of Complete Freund's Adjuvant (CFA) and intravenous iron treatment on the mRNA expression of erythropoietin (EPO) in the rat kidney. Real-time polymerase chain reaction was performed. Relative transcript levels are C_T values normalized to that of the control group.
C; control-saline group, V; CFA-saline group, I; CFA-IV iron group. *P < 0.05 compared with the control group; #P < 0.05 compared with the CFA-saline group.

7. Effects of iron treatment on hepcidin, ferritin, P-STAT-3 and P-SMAD1/5 expressions in HepG2 cells

Cell viability was measured using the CCK-8 assay after the treatment of HepG2 cells with various concentrations of iron for varying time durations. Cell viability of cells treated with 5–10 mg/mL of iron was significantly lower than that of control cells (Fig. 10).

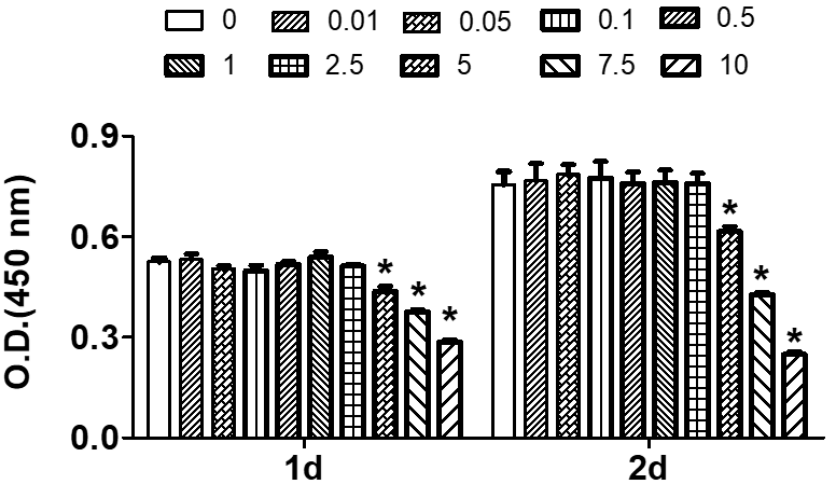

Figure 10. Cell viability of HepG2 cells treated with varying concentrations of iron. Cells were incubated for 1–2 days in the presence of iron at the concentrations indicated, after which the number of viable cells was analyzed by the Cell Counting Kit-8 assay.

O.D.; optical density. $^{*}P < 0.05$ compared with the control group.

The protein levels of phosphorylated STAT-3 and phosphorylated SMAD1/5 increased 24 h after iron administration in a dose-dependent manner in HepG2 cells compared to those in control cells. The protein levels of hepcidin and ferritin were subsequently increased by iron treatment in HepG2 cells compared to those in control cells (Fig. 11).

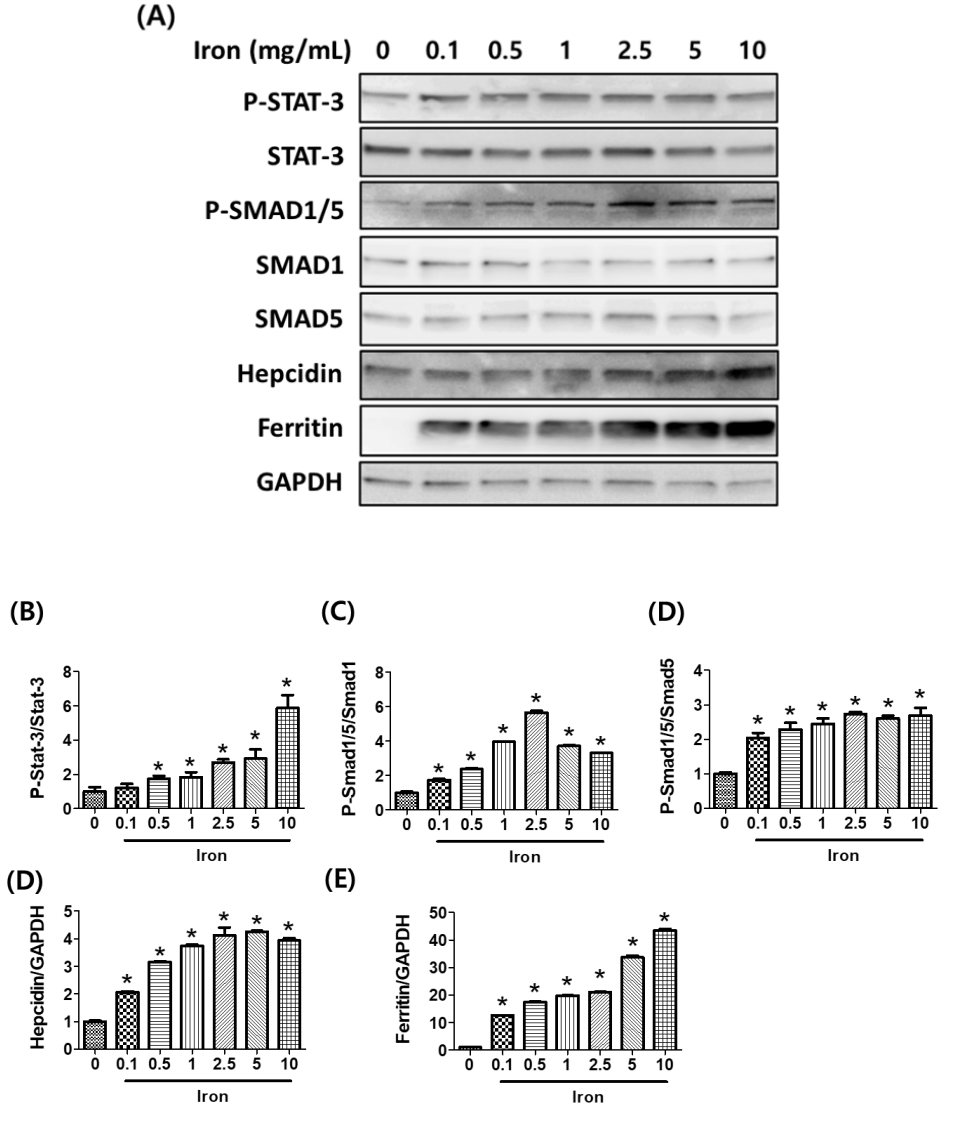

Figure 11. The effect of iron on the phosphorylation of STAT-3 and SMAD1/5 in HepG2 cells. Iron-induced STAT-3 and SMAD1/5 phosphorylation in HepG2 cells were examined by immunoblot analysis in a dose-dependent manner. The relative protein levels were quantified by densitometry (lower panel).
STAT-3; signal transducer and activator of transcription-3, P-STAT-3; phosphorylated STAT-3, SMAD; sma mothers against decapentaplegic, P-SMAD; phosphorylated SMAD, GAPDH; glyceraldehyde-3-phosphate dehydrogenase.
$^{*}P < 0.05$ compared with the control group.

8. Iron-induced hepcidin upregulation involves STAT-3 and SMAD1/5 activation in HepG2 cells

To further define the function of STAT-3 and SMAD1/5 in iron-mediated regulation of hepcidin, we performed *si*RNA-mediated knockdown of endogenous *STAT-3 and SMAD1/5*. The efficacy of *si*RNA-mediated knockdown was confirmed by immunoblotting (Fig. 12A and 12D). Iron treatment led to an increase in hepcidin levels in HepG2 cells transfected with *si*Control, and knockdown of *STAT-3 and SMAD1/5* diminished this iron-induced upregulation of hepcidin. Hepcidin expression in HepG2 cells treated with *si*STAT3, was still significantly greater with iron treatment than that in the control group, whereas that in cells treated with *si*SMAD1/5 was comparable between the groups. These results suggest that iron-mediated upregulation of hepcidin involves activation of STAT-3 and SMAD1/5 (Fig. 12).

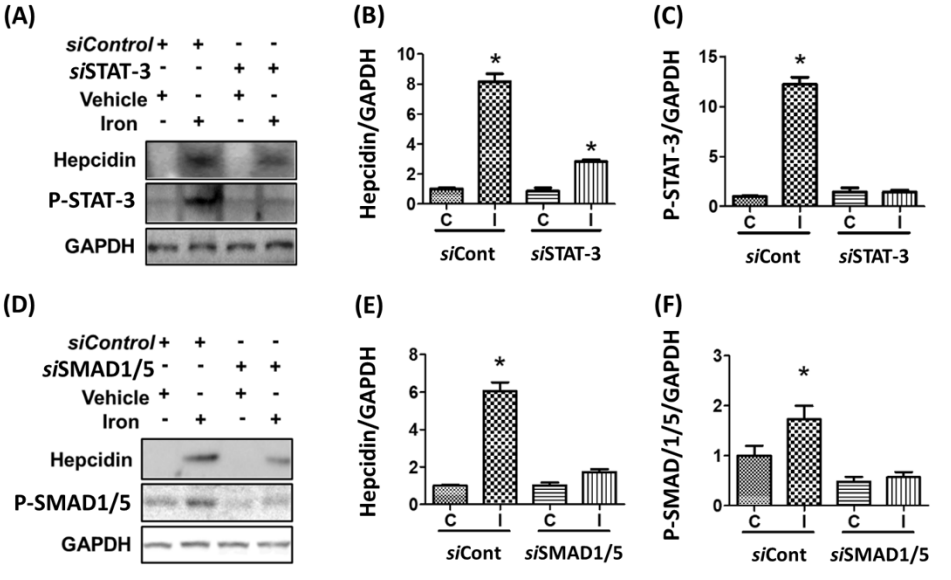

Figure 12. Western blot analysis of HepG2 cells transfected with control small interfering RNA (*si*RNA) or *si*RNA against *STAT-3* and *SMAD1/5* regarding

hepcidin expression according to iron treatment. The relative protein levels were quantified by densitometry (middle, right panel).

*si*Control; control *si*RNA, *si*STAT-3; *si*RNA against STAT-3, *si*SMAD-1/5; *si*RNA against SMAD1/5, STAT-3; signal transducer and activator of transcription-3, SMAD; sma mothers against decapentaplegic, GAPDH; glyceraldehyde-3-phosphate dehydrogenase, C; control group, I; iron-treated group. $^{*}P < 0.05$ compared with the control group.

IV. DISCUSSION

In this study, we successfully established a rat model of ACI, which resembled the clinical presentations of ACI, involving iron deficiency and systemic inflammation, and observed that systemic and hepatic levels of hepcidin were significantly increased by IV iron supplementation in rats with ACI. At 3 days after iron supplementation, we found that hepcidin activity was upregulated along with increase in the levels of both phosphorylated SMAD1/5 and STAT-3, which was also be confirmed by the lack of hepcidin expression to iron-treated HepG2 cells knocked down for the endogenous *STAT-3* and *SMAD1/5*. Of note, we did not observe changes in IL-6 levels with iron supplementation in rats with ACI. Additionally, concurrent hepatic upregulation of NOX-2, NOX-4, and SOD protein levels following iron supplementation is likely to implicate a potential link between iron-induced oxidative stress and the activation of STAT-3. ACI in rats was also associated with normal levels of renal EPO expression, which decreased after iron supplementation. Serum ERFE levels were reduced in ACI rats, and did not change with iron supplementation.

ACI in rats and the short-term effect of iron supplementation

In this study, we established a rat model of ACI, which was confirmed by decreased Hb, serum levels of iron, and TSAT and an increase in leukocytes and inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, compared to those in control rats.[20] Of note, elevated hepatic levels of hepcidin after ACI induction are in line with previous animal studies involving pseudo-infectious conditions induced by immunogenic particles.[18] In the previous models of ACI, hepcidin expression increased with a peak at 6 h after administration of the substance (e.g., CFA), remained slightly increased through the following 7 days, and significantly decreased after 14–28 days.[21,22] These changes in hepcidin levels may be associated with stress erythropoiesis that produces large amounts of new

erythrocytes when steady-state erythropoiesis is impaired by inflammatory states.[22,23] In this study, rats were euthanized for analyses 3 days after IV iron treatment, which was 1 week after the last CFA administration. Thus, the increase in hepcidin levels in our study conforms to the time course of changes in hepcidin levels upon iron supplementation reported in previous studies.

Since the short-term treatment effect of high-dose IV iron supplementation during the perioperative period has been a concern in clinical practice, we examined Hb and signaling pathways associated with hepcidin regulation at 3 days after IV iron supplementation in the current study.[15,24] As observed in several clinical studies, Hb did not increase 3 days after IV iron supplementation in CFA-treated rats compared to that in CFA-saline rats in this study. In humans, erythropoiesis is known to increase 5–7 days after IV iron replenishment. Considering that the lifespan of erythrocytes in humans is 2–3 times that of rats, a duration of 2–4 days to activate erythropoiesis was expected in response to increased iron load in rats,[25] and we decided to assess the results 3 days after IV iron supplementation in ACI rats. Clinically despite an increase in erythropoiesis in a short period, Hb increases 2–3 weeks after IV iron supplementation in patients,[26,27] and this is in line with the results of this study showing a lack of Hb recovery 3 days after iron supplementation. A substantial increase in hepcidin levels in response to IV iron could have potentially sequestered iron as ferritin in the storage cells (e.g., hepatocytes), impeding iron utilization for erythropoiesis and hindering Hb increase. Notably, in addition to elevated hepcidin levels, we saw that mRNA expression of EPO in the kidney decreased whereas serum ERFE levels were not further reduced after iron supplementation in ACI rats, compared with saline-treated ACI rats. From these results, we can assume that the regulation of both hepcidin and EPO is required to induce effective erythropoiesis after IV iron supplementation in a clinically relevant short period in ACI patients.

Iron supplementation and hepcidin activity in ACI rats: role of oxidative stress

Both the BMP/SMAD and IL-6/STAT3 pathways play pivotal roles in the regulation of hepcidin expression. Interestingly, in addition to the well-known direct activation of BMP/SMAD pathways by iron, STAT-3 activity was significantly increased with iron supplementation in the CFA-IV iron group compared to the CFA-saline group, while IL-6 activities were similar between the groups. Additionally, in HepG2 cells transfected with *si*STAT-3, hepcidin upregulation by iron administration was significantly attenuated compared with that in the control cells. IV iron has been reported to cause oxidative stress, and STAT-3 is known to be involved in not only inflammation but also in ROS-mediated hepcidin regulation, mainly involving IL-6 and NOX-4.[28,29] NOX-4 is a member of a family of enzymes consisting of 7 isoforms responsible for producing various ROS. Of these family members, 2 isoforms, NOX-2 and NOX-4 have been previously demonstrated to be powerful inducers of hepcidin expression in the liver via the STAT-3 signaling pathway in a previous study.[30] Simultaneous increase in NOX-2, NOX-4, and STAT-3 activities after iron supplementation in this study are consistent with the results of previous studies. These findings imply a potential relationship between ROS-generating NOX-2, NOX-4, and SOD, with an additional increase in hepcidin expression after iron supplementation.

Iron supplementation and erythropoiesis regulation in ACI rats

Increased hepcidin levels in chronic inflammatory conditions lead to hypoferremia and iron-restricted erythropoiesis. Homeostatic iron regulator (HFE) acts as an iron sensor and upstream regulator of hepcidin transcription.[31] The pathophysiological relevance of the cross talk among hepcidin, iron, and erythropoiesis has not been elucidated in ACI models, especially in association with IV iron supplementation. In this study, renal EPO mRNA expression did not

change in ACI rats, which is considered a characteristic of the disease.[32] However, serum ERFE produced by EPO-stimulated erythroblasts during erythropoiesis was decreased in ACI rats compared to that in control rats, possibly due to a blunted response to EPO under chronic inflammatory conditions.[32] It is noteworthy that IV iron supplementation in ACI rats significantly decreased EPO mRNA expression without further decrease in serum ERFE levels compared to that in saline-treated CFA rats. EPO gene transcription is controlled by hypoxia-inducible transcription factor 2α (HIF2α). Oxygen and iron are well-known factors that determine the activity of HIF2α through HIF-prolyl hydroxylase (PHD)-induced degradation.[33-35] In this context, IV administration of iron could have caused an increase in PHD activity, leading to subsequent decreases in HIF2α and EPO mRNA expression. Since we did not evaluate the corresponding signaling pathways, further studies are needed. Considering that erythropoiesis-stimulating agent inhibits hepcidin expression through ERFE, these changes might also have contributed to enhanced hepcidin activity.

Limitations

This study has the following limitations. First, although the ACI rat model used in this study seems to be clinically relevant, we focused on the short-term effect of IV iron supplementation. Anticipating that its effects on hepcidin and erythropoiesis would be dynamic, significant hematopoiesis may have appeared after this period, and further studies are warranted to investigate the response of hepcidin and its regulatory pathways to IV iron supplementation across a longer period. Second, although we observed concomitant changes in STAT-3 activity and NOX-2 and NOX-4 expression in the liver, we did not confirm whether simultaneous treatment with antioxidant and IV iron could prevent the activation of STAT-3 and subsequent elevation of hepcidin levels through NOX inhibition in the ACI rat model.

V. CONCLUSION

In conclusion, hepatic hepcidin protein activity was upregulated with IV iron supplementation in rats under chronic inflammatory conditions. IV iron supplementation resulted in an increase in liver ferritin; however, anemia was not mitigated at 3 days after iron treatment, and this may be associated with hepcidin upregulation as well as reduced renal EPO mRNA expression and serum ERFE levels. Both SMAD1/5 and STAT-3 were identified to be as relevant pathways of hepcidin upregulation in relation to IV iron supplementation in ACI rats. In addition, iron-induced increase in oxidative stress, indicated by an increase in NOX-2 and NOX-4 activity, seems to be a potential mechanism of STAT-3 activation in the absence of IL-6 increase. The current study provides primary evidence regarding the need for hepcidin and EPO regulation to increase the efficacy of IV iron supplementation for the treatment of anemia under chronic inflammatory conditions.

REFERENCES

1. Agarwal N, Prchal JT: Anemia of Chronic Disease (Anemia of Inflammation). Acta Haematologica 2009; 122:103-8
2. Weiss G, Goodnough LT: Anemia of chronic disease. N Engl J Med 2005; 352:1011-23
3. Poggiali E, Migone De Amicis M, Motta I: Anemia of chronic disease: A unique defect of iron recycling for many different chronic diseases. European Journal of Internal Medicine 2014; 25:12-7
4. Papanikolaou G, Pantopoulos K: Iron metabolism and toxicity. Toxicol Appl Pharmacol 2005; 202:199-211
5. Papanikolaou G, Pantopoulos K: Systemic iron homeostasis and erythropoiesis. IUBMB Life 2017; 69:399-413
6. Chiou B, Connor JR: Emerging and dynamic biomedical uses of ferritin. Pharmaceuticals 2018; 11:124
7. Hawula ZJ, Wallace DF, Subramaniam VN, Rishi G: Therapeutic advances in regulating the hepcidin/ferroportin axis. Pharmaceuticals 2019; 12:170
8. Vela D: Balance of cardiac and systemic hepcidin and its role in heart physiology and pathology. Laboratory Investigation 2018; 98:315-26
9. Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T: Identification of erythroferrone as an erythroid regulator of iron metabolism. Nat Genet 2014; 46:678-84
10. Pagani A, Nai A, Silvestri L, Camaschella C: Hepcidin and anemia: a tight relationship. Frontiers in physiology 2019; 10:1294
11. Girelli D, Ugolini S, Busti F, Marchi G, Castagna A: Modern iron replacement therapy: clinical and pathophysiological insights. International journal of hematology 2018; 107:16-30
12. Schaefer B, Meindl E, Wagner S, Tilg H, Zoller H: Intravenous iron supplementation therapy. Molecular Aspects of Medicine 2020:100862

13. Kalra PA, Bhandari S: Efficacy and safety of iron isomaltoside (Monofer®) in the management of patients with iron deficiency anemia. International journal of nephrology and renovascular disease 2016; 9:53
14. Johansson P, Rasmussen A, Thomsen L: Intravenous iron isomaltoside 1000 (Monofer®) reduces postoperative anaemia in preoperatively non-anaemic patients undergoing elective or subacute coronary artery bypass graft, valve replacement or a combination thereof: a randomized double-blind placebo-controlled clinical trial (the PROTECT trial). Vox Sanguinis 2015; 109:257-66
15. Spahn DR, Schoenrath F, Spahn GH, Seifert B, Stein P, Theusinger OM, Kaserer A, Hegemann I, Hofmann A, Maisano F: Effect of ultra-short-term treatment of patients with iron deficiency or anaemia undergoing cardiac surgery: a prospective randomised trial. The Lancet 2019; 393:2201-12
16. Johansson PI, Rasmussen AS, Thomsen LL: Intravenous iron isomaltoside 1000 (Monofer®) reduces postoperative anaemia in preoperatively non-anaemic patients undergoing elective or subacute coronary artery bypass graft, valve replacement or a combination thereof: a randomized double-blind placebo-controlled clinical trial (the PROTECT trial). Vox Sanguinis 2015; 109:257-66
17. Moon T, Smith A, Pak T, Park BH, Beutler SS, Brown T, Kaye AD, Urman RD: Preoperative Anemia Treatment with Intravenous Iron Therapy in Patients Undergoing Abdominal Surgery: A Systematic Review. Adv Ther 2021; 38:1447-69
18. Rivera S, Ganz T: Animal models of anemia of inflammation. Semin Hematol 2009; 46:351-7
19. Theurl I, Schroll A, Sonnweber T, Nairz M, Theurl M, Willenbacher W, Eller K, Wolf D, Seifert M, Sun CC, Babitt JL, Hong CC, Menhall T, Gearing P, Lin HY, Weiss G: Pharmacologic inhibition of hepcidin

expression reverses anemia of chronic inflammation in rats. Blood 2011; 118:4977-84

20. Ganz T: Anemia of Inflammation. N Engl J Med 2019; 381:1148-57
21. Kim A, Fung E, Parikh SG, Valore EV, Gabayan V, Nemeth E, Ganz T: A mouse model of anemia of inflammation: complex pathogenesis with partial dependence on hepcidin. Blood 2014; 123:1129-36
22. Lofruthe N, Gallitz I, Traeger L, Bäumer N, Schulze I, Kuhlmann T, Müller-Tidow C, Steinbicker AU: Intravenous Iron Carboxymaltose as a Potential Therapeutic in Anemia of Inflammation. PLoS One 2016; 11:e0158599
23. Paulson RF, Ruan B, Hao S, Chen Y: Stress Erythropoiesis is a Key Inflammatory Response. Cells 2020; 9
24. Muñoz M, Gómez-Ramírez S, Cuenca J, García-Erce JA, Iglesias-Aparicio D, Haman-Alcober S, Ariza D, Naveira E: Very-short-term perioperative intravenous iron administration and postoperative outcome in major orthopedic surgery: a pooled analysis of observational data from 2547 patients. Transfusion 2014; 54:289-99
25. Van Putten LM: The life span of red cells in the rat and the mouse as determined by labeling with DFP32 in vivo. Blood 1958; 13:789-94
26. Mueller MM, Van Remoortel H, Meybohm P, Aranko K, Aubron C, Burger R, Carson JL, Cichutek K, De Buck E, Devine D, Fergusson D, Folléa G, French C, Frey KP, Gammon R, Levy JH, Murphy MF, Ozier Y, Pavenski K, So-Osman C, Tiberghien P, Volmink J, Waters JH, Wood EM, Seifried E: Patient Blood Management: Recommendations From the 2018 Frankfurt Consensus Conference. Jama 2019; 321:983-97
27. Muñoz M, Acheson AG, Auerbach M, Besser M, Habler O, Kehlet H, Liumbruno GM, Lasocki S, Meybohm P, Rao Baikady R, Richards T, Shander A, So-Osman C, Spahn DR, Klein AA: International consensus statement on the peri-operative management of anaemia and iron

deficiency. Anaesthesia 2017; 72:233-47

28. Li J, Lan T, Zhang C, Zeng C, Hou J, Yang Z, Zhang M, Liu J, Liu B: Reciprocal activation between IL-6/STAT3 and NOX4/Akt signalings promotes proliferation and survival of non-small cell lung cancer cells. Oncotarget 2015; 6:1031
29. Pan M-S, Wang H, Ansari KH, Li X-P, Sun W, Fan Y-Z: Gallbladder cancer-associated fibroblasts promote vasculogenic mimicry formation and tumor growth in gallbladder cancer via upregulating the expression of NOX4, a poor prognosis factor, through IL-6-JAK-STAT3 signal pathway. Journal of Experimental & Clinical Cancer Research 2020; 39:1-21
30. Silva I, Peccerella T, Mueller S, Rausch V: IL-1 beta-mediated macrophage-hepatocyte crosstalk upregulates hepcidin under physiological low oxygen levels. Redox Biol 2019; 24:101209
31. Zhao N, Zhang AS, Enns CA: Iron regulation by hepcidin. J Clin Invest 2013; 123:2337-43
32. Roy CN: Anemia of Inflammation. Hematology 2010; 2010:276-80
33. Suzuki N, Matsuo-Tezuka Y, Sasaki Y, Sato K, Miyauchi K, Kato K, Saito S, Shimonaka Y, Hirata M, Yamamoto M: Iron attenuates erythropoietin production by decreasing hypoxia-inducible transcription factor 2α concentrations in renal interstitial fibroblasts. Kidney Int 2018; 94:900-11
34. Ganz T: Erythropoietin and iron-a conflicted alliance? Kidney Int 2018; 94:851-3
35. Jelkmann W: Regulation of erythropoietin production. J Physiol 2011; 589:1251-8

ABSTRACT (IN KOREAN)

만성 염증성 빈혈에서 hepcidin 변화에 관한 연구 : 철분제 투약 후 hepcidin 조절 기전

<지도교수 곽영란>

연세대학교 대학원 의학과

김 혜 빈

만성 염증성 빈혈(anemia of chronic inflammation, ACI)은 환자의 부정적 예후와 연관된 것으로 알려져 있다. Hepcidin은 체내의 철 항상성을 조절하는 주요 조절 인자로, 주로 간에서 생성된다. 만성 염증 상태에서는 hepcidin이 증가하여 체내의 철이 활용 불가능한 ferritin의 형태로 세포 내에 저장되는데, 이로 인해 체내에 충분한 철 저장량이 있음에도 불구하고 철의 이용에 제한이 발생하여 적혈구 생성이 어려워지고, 빈혈이 발생하게 된다. 임상에서는 모든 종류의 빈혈을 철분 보충으로 치료하곤 하는데, ACI의 근본적인 원인을 고려하였을 때 이것은 적절하지 못한 치료법일 가능성이 있다. 따라서, 본 연구에서는 임상과 유사한 ACI 쥐 모델을 확립하고, 정맥 내 철분을 투여한 후 단기간에 걸쳐 간에서 hepcidin 및 그 조절 경로의 변화를 알아보는 것을 주요 연구 목표로 하였다. 또한 철분 투여로 인해 발생 가능한 산화 스트레스를 평가하고, 신장에서의

erythropoietin (EPO) 발현 및 erythroferrone (ERFE) 변화를 함께 살펴보고자 하였다. 동시에, knockdown HepG2 세포에 철을 처리하는 실험을 통해 hepcidin 조절 경로를 확인하고자 하였다.

ACI는 Complete Freund's Adjuvant (CFA)를 2주간 3회 복강 내 투여하여 유발하였다. 수컷 Sprague-Dawley 쥐 (n = 30)는 다음의 3개 그룹으로 나누어 배정되었다: 대조-식염수군 (n = 10), CFA-식염수군 (n = 10), CFA-철분군 (n = 10). CFA 투약 시작 2주 뒤, 혈색소 수치가 기저치에 비해 >2 g/dL 감소한 경우 ACI가 유발된 것으로 보았다. ACI 쥐는 정맥으로 철분 (iron isomaltoside, 20mg/kg) 또는 동량의 식염수를 투여 받았다. 투약 3일 후, 혈액학적 변수, 염증 표지자, iron profile과 함께 간에서의 hepcidin 및 그 전사 조절 경로를 확인하였다.

ACI 쥐에서는 빈혈과 함께 염증 표지자와 활성 산소의 증가가 확인되었다. 정맥 철분 투여 3일 뒤, 혈색소 수치에는 변화가 없었으나, 간 및 혈청에서 hepcidin 농도가 증가하였고, 간에서의 ferritin 증가도 확인되었다. 철분 투약은 간에서 ROS-generating NADPH oxidase-2 (NOX-2), NOX-4 및 superoxide dismutase (SOD)의 증가를 유발하였다. ACI 쥐의 간에서 interleukin-6/signal transducer and activator of transcription-3 (IL-6/STAT-3) 경로와 bone morphogenetic protein/Sma mothers against decapentaplegic (BMP/SMAD) 경로 활성화가 확인되었으며, 정맥 철분 투여는 IL-6의 증가 없이 ACI 조건 하에서 활성화 되어 있던 SMAD1/5와 STAT-3를 더 활성화시켰다. 철을 처리했을 때 HepG2 세포에서도 SMAD1/5와 STAT-3가 모두 활성화 되었으며,

소간섭 RNAs (short interfering RNAs, *si*RNAs)를 이용한 *STAT-3*와 *SMAD1/5*의 knockdown을 시행했을 때 철 처리로 인한 hepcidin 전사 증가 정도가 억제됨을 확인하였다. 정맥 철분 투여는 신장에서 EPO mRNA 발현량을 감소시켰으며, 이는 혈청에서의 ERFE 농도 감소와 동반되었다.

결론적으로, ACI 쥐 모델에서 정맥 철분을 투여하고 3일 뒤 혈색소 수치가 호전되지 않았는데, 이는 만성 염증 상태에서 증가해 있던 hepcidin이 철분 투약으로 인해 더 증가한 것과 연관되어 있을 수 있다. 실제로 철분 투약 후 간에서 hepcidin 및 ferritin의 유의한 증가가 확인되었다. 철분 투약 후 hepcidin의 발현 증가는 SMAD1/5 외에 STAT-3 관련 경로의 활성화와도 관련이 있음을 관찰할 수 있었는데, 철분 투여로 산화 스트레스 지표들이 증가한 것과 연관성이 있을 것으로 생각된다. 철분 투약 후 신장 EPO 발현 및 혈청 ERFE 농도가 동시에 감소하는 것은 ACI 조건 하에서 충분한 철분 보충에도 불구하고 효과적인 조혈 작용을 방해하는 적혈구 조절 인자들의 복잡한 상호작용이 발생함을 보여준다. 이러한 결과들을 고려할 때 만성 염증 상태에서 정맥 철분제 투약으로 빈혈을 효율적으로 치료하기 위해서는 hepcidin과 EPO 조절을 함께 고려하는 것이 도움이 될 것으로 생각한다.

핵심되는 말: 만성 염증성 빈혈, erythropoietin, hepcidin, 정맥용 철분제 치료, 산화 스트레스