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Effect of Deferiprone on Hepatic Expression of *Hamp*, *Ftl*, and *Tfr1* Genes in an Iron-Overloaded Rat (*Rattus norvegicus*) Model

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Abstract: Iron overload is linked to progressive impairment of organ function, with the liver being the primary site of deposition due to the lack of a physiological route for iron elimination. The maintenance of systemic iron balance depends on key regulatory proteins, including hepcidin (*Hamp* gene), ferritin light chain (*Ftl* gene), and transferrin receptor 1 (*Tfr1* gene). This study tested the hypothesis that Deferiprone (DFP), an oral iron chelator, modulates the hepatic expression of *Hamp*, *Ftl*, and *Tfr1* genes in an iron-overloaded rat model. Eighteen male Wistar rats (150-200 g) were randomly assigned into three groups: Normal (N), Negative Control (NC; induced with Iron Dextran), and Treatment (T; Iron Dextran + DFP). Iron overload was induced via intravenous injection of Iron Dextran (120 mg/kg BW) over 15 days at 3-day intervals, while DFP was administered orally (100 mg/kg BW) in three divided doses for 28 consecutive days. Gene expression was assessed using RT-PCR, and relative quantification was performed using the Livak method. The iron-overloaded rats showed marked upregulation of *Hamp* and *Ftl* and downregulation of *Tfr1*. Administration of DFP significantly reversed these alterations, decreasing *Hamp* and *Ftl* levels while restoring *Tfr1* expression to levels comparable to normal controls. These results highlight the potential role of DFP in modulating hepatic iron-regulatory genes under iron overload conditions.

Introduction

Iron is a vital micronutrient involved in numerous physiological processes, including redox reactions, oxygen transport, DNA synthesis, and various enzymatic activities (1). Despite a relatively small daily requirement of approximately 20–30 mg, the human body contains 3–6 grams of iron, predominantly stored in the ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms (2). However, excess iron can be toxic, catalyzing the formation of reactive oxygen species (ROS) and triggering oxidative stress and cellular damage. Iron overload frequently occurs in individuals with thalassemia or other transfusion-dependent conditions, as each transfusion unit contributes an additional 200–250 mg of iron to the body (3, 4). Notably, the liver serves as a central hub for iron metabolism and homeostasis.

Among various organs, the liver plays a key role in regulating iron metabolism and maintaining systemic iron balance (5). Its primary functions include storing excess iron and controlling the amount of iron released into circulation (6). This process is mainly governed by the production and release of hepcidin, a peptide hormone that modulates iron entry into the bloodstream (7). Hepcidin stimulates the degradation of ferroportin, an iron exporter, thereby regulating iron release from cells (8). Furthermore, the liver participates in the absorption of dietary iron via Divalent Metal Transporter 1 (*Dmt1*) and Transferrin Receptor 1 (*Tfr1*). A key function of *Tfr1* in maintaining iron homeostasis is its interaction with the hemochromatosis protein Hfe, which is critical for regulating hepcidin production (9).

Several hepatic genes are critical in iron metabolism, including *Tfr1*, Hepcidin Antimicrobial Peptide (*Hamp*), and Ferritin Light Chain (*Ftl*). The *Hamp* gene encodes hepcidin, the principal hormone governing iron homeostasis. Hepcidin expression increases in response to elevated iron levels or inflammatory stimuli, which is commonly associated with hyperferritinemia (10). Conversely, in healthy individuals, hepcidin production decreases in response to low iron levels and other stimuli (8). Ferritin, encoded in part by *Ftl*, is an intracellular iron-storage protein, and its serum levels are widely used as a surrogate marker for body iron status, increasing during iron overload (11). Serum ferritin levels correlate directly with total body iron stores, making it a valuable biomarker for estimating iron status (12, 13). Meanwhile, *Tfr1* facilitates cellular iron uptake and can become saturated in individuals with HFE gene mutations (14). To prevent iron accumulation and its detrimental effects, iron chelation therapy is employed as an effective intervention.

Iron chelation therapy is the primary treatment for iron overload, utilizing pharmacological agents such as Deferiprone (DFP), Deferoxamine (DFO), and Deferasirox (DFX) to bind excess circulating iron (15). Beyond reducing iron burden, these agents also suppress ROS formation, thereby mitigating oxidative damage (16). Among available chelators, DFP is particularly effective at reducing intracellular labile iron and curbing oxidative stress (17). Its mechanism involves chelating Fe^{2+} ions at the active site of iron-dependent histone lysine demethylases, inhibiting their enzymatic function (18).

This study aims to evaluate the expression of *Hamp*, *Ftl*, and *Tfr1* genes in the liver of rats under normal and iron overload conditions. Additionally, it investigates the effects of DFP administration on the expression levels of these genes in a rat model of iron overload.

Experimental Section

Materials

The materials used in this study included Deferiprone, Iron Dextran (Hemadex; Sanbe Farma, Bandung, Indonesia), RNA isolation kit (Quick-RNA™ Miniprep Plus Kit, Zymo Research), cDNA synthesis kit (FIREScript RT cDNA Synthesis Kit, Solis BioDyne), and primers (Macrogen).

In Vivo Test

Animal Preparation

This study was approved by the Animal Research Ethics Committee of Universitas Padjadjaran, Indonesia (Approval No. 75/UN6.KEP/EC/2023). A total of 18 healthy male Wistar rats (150–200 g) were housed in standard cages under controlled environmental conditions, with a 12-hour light/dark cycle. Food and water were provided *ad libitum* throughout the study (19). Animals were acclimatized for 7 days prior to experimental procedures.

Sample Administration and Liver Collection

Rats were randomly divided into three groups (n = 6 per group): Normal (N), Negative Control (NC, Iron Dextran), and Treatment (T, Iron Dextran + DFP). The sample size was calculated using Mead's Resource Equation method (E = N − B − T) to ensure both ethical animal use and sufficient statistical power. Iron dextran (120 mg/kg BW) was administered intravenously every 3 days for a total of 15 days (20). The Treatment group received DFP orally at a dose of 100 mg/kg BW/day, divided into three equal doses, for 28 consecutive days. At the end of the treatment period, rats were fasted for 16 h and anesthetized via intramuscular injection of Ketamine-Xylazine (0.2 mL). Liver tissues were collected, rinsed with physiological saline, weighed, and stored at −20°C for subsequent molecular analysis.

RNA Isolation and Extraction

Total RNA was extracted from liver tissues using the Quick-RNA™ Miniprep Plus Kit (Zymo Research), following the manufacturer's instructions. The protocol included lysis buffer preparation, tissue homogenization, and RNA purification. RNA integrity and purity were assessed using a Nanodrop spectrophotometer, with acceptable purity defined by a 260/280 absorbance ratio of 1.8–2.0.

cDNA Synthesis and RT-PCR

First-strand cDNA synthesis was performed using the FIREScript RT cDNA Synthesis Kit (Solis BioDyne) according to the manufacturer's protocol. RT-PCR conditions were optimized by testing annealing temperatures between 55–65°C. Amplification was carried out using the GENECHECKER UF-300 real-time thermal cycler. The thermal cycling protocol is detailed in **Table 1**. Primer sequences were synthesized by Macrogen, and the complete list of primer pairs used in the analysis is presented in **Table 2**.

Table 1. RT-PCR cycling steps.

Step	Temperature (°C)	Time	Cycles
Initial Activation	95	12 min	1
Denaturation	95	15 s	40
Annealing	58	20 s	
Extension	72	20 s	

Table 2. Primer sequences used for RT-PCR.

Gene	Primer Sequence 5' 3'	Tm (°C)	Ref.
Hamp	Forward: 5'-CTG CCT GTC TCC TGC TTC TCC-3'	65.9	[21]
	Reverse: 5'-AGT TGG TGT CTC GCT TCC TTC G-3'	64.2	
Ftl	Forward: 5'-GCT GGC TTC TTG ATG TCC-3'	58.5	
	Reverse: 5'-CCT CCT ACA CCT ACC TCT C-3'	59.7	
Tfr1	Forward: 5'-AGT CAT CTG GAT TGC CTT CTA TAC C-3'	60.1	
	Reverse: 5'-TAG CGG TCT GGT TCC TCA TAG C-3'	63.1	
Gapdh	Forward: 5′-CCATCAACGACCCCTTCATT-3′	58	[22]
	Reverse: 5′-CACGACATACTCAGCACCAGC-3′	58	

Data Analysis

Cycle threshold (Ct) values obtained from RT-PCR for the Normal (N), Negative Control (NC), and Treatment (T) groups were analyzed. Relative expression (RE) was calculated using the Livak method: RE = 2^−ΔΔCt (14). Statistical analyses were performed using GraphPad Prism 9 software. Data were first assessed for normality using the Shapiro–Wilk test. For normally distributed data, a one-way analysis of variance (ANOVA) followed by Dunn's multiple comparisons test was used to evaluate significant differences. All analyses were conducted at a 95% confidence level ($p < 0.05$), and results are presented graphically.

Results and Discussion

Relative Expression of mRNA *Hamp*

Quantitative RT-PCR analysis demonstrated that administration of Iron Dextran (ID) significantly elevated hepatic *Hamp* mRNA expression. Cycle threshold (Ct) values obtained from liver tissue RT-PCR were used to assess gene expression across the three experimental groups. Relative expression (RE) was calculated using the 2^−ΔΔCt method described by Livak and Schmittgen (2001), with results illustrated in **Figure 1**. This approach allows normalized comparison of gene expression among the experimental groups.

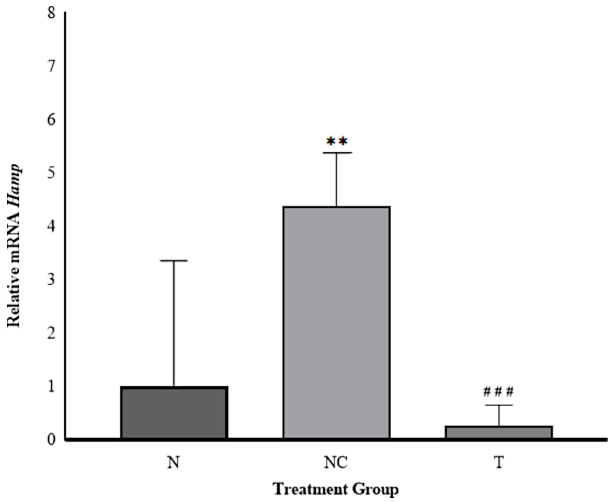

Figure 1. Relative Expression of Hamp Gene in Rat Liver. The expression of Hamp was significantly increased in the NC group (iron dextran) and decreased in the T group (iron dextran + DFP). Data are presented as mean ± SD (n = 6) and were analyzed using GraphPad Prism software. *Significant difference compared with the N group ($p < 0.05$); #Significant difference compared with the NC group ($p < 0.05$).

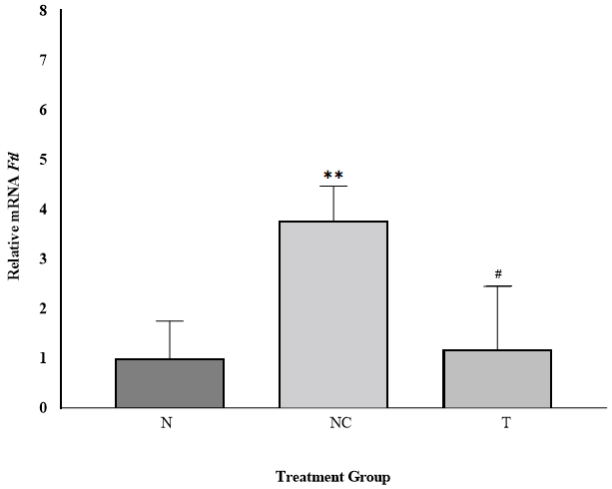

Figure 2. Relative Expression of Ftl Gene in Rat Liver. The expression of Ftl was significantly increased in the NC group (iron dextran) and decreased in the T group (iron dextran + DFP). Data are presented as mean ± SD (n = 6) and were analyzed using GraphPad Prism software. *Significant difference compared with the N group ($p < 0.05$); #Significant difference compared with the NC group ($p < 0.05$).

In the NC group, *Hamp* expression increased 4.39-fold relative to the N group, indicating substantial upregulation in response to iron overload. This aligns with the physiological upregulation of hepcidin under conditions of excessive hepatic iron deposition (21, 22). Hepcidin synthesis is modulated by multiple regulatory signals, including systemic iron levels, inflammation, and erythropoietic activity, with iron predominantly acting via the Bone Morphogenetic Protein (BMP)/Smad pathway. In this mechanism, BMPs bind to BMP receptors (BMPRs) and form complexes with the co-receptor hemojuvelin (HJV), triggering Smad phosphorylation and transcriptional activation of *Hamp* (23, 24).

Rats in the T group exhibited a marked reduction in *Hamp* expression (~0.27-fold relative to the N group), representing a 93.85% decrease compared to the NC group. This suggests that DFP administration effectively attenuated hepcidin gene transcription, consistent with previous reports demonstrating DFP's capacity to reduce hepatic iron burden and suppress *Hamp* expression (25). Under controlled iron levels, hepatic *Hamp* expression remains low, permitting normal iron absorption and systemic distribution (21). This pathway-mediated induction of hepcidin represents a key adaptive response to iron overload aimed at maintaining systemic iron balance.

Relative Expression of mRNA *Ftl*

Ftl gene expression among experimental groups is shown in **Figure 2**. The N group served as the control, normalized to a relative expression value of 1.00.

Ftl expression in the NC group increased approximately 3.77-fold relative to N, consistent with hepatic iron accumulation (26, 27, 28). In the T group, *Ftl* expression was only modestly elevated (~1.18-fold relative to N), indicating normalization following DFP treatment. Compared with the NC group, *Ftl* expression in the T group decreased by 0.31-fold, demonstrating effective downregulation toward baseline levels. This mirrors clinical observations where DFP monotherapy significantly reduces serum ferritin levels in transfusion-dependent thalassemia patients (29, 30, 31).

Relative Expression of mRNA *Tfr1*

Tfr1 expression across the experimental groups is illustrated in **Figure 3**. Expression values were normalized to the N group (set at 1.00) to enable fold-change comparisons.

In the NC group, *Tfr1* expression was 0.09-fold relative to N, reflecting diminished interaction between Iron Regulatory Proteins (IRPs) and Iron Responsive Elements (IREs) under iron overload (32, 33). This post-transcriptional regulation via the IRE-IRP system inversely correlates with cellular iron status. Following DFP administration, *Tfr1* expression increased (~0.29-fold relative to N), indicating normalization of gene expression. The relative *Ftl* expression in the T group increased 3.22-fold compared with the NC group, highlighting DFP's role in reducing iron-mediated toxicity by removing iron from transferrin and non-transferrin-bound iron (NTBI) pools (34, 35, 36).

Overall, DFP treatment effectively reversed iron-induced dysregulation of *Hamp*, *Ftl*, and *Tfr1* expression, demonstrating its efficacy in modulating hepatic iron-related gene expression under iron overload conditions (**Table 3**). These coordinated transcriptional changes further support the role of DFP in restoring hepatic iron homeostasis through normalization of iron sensing and transport mechanisms.

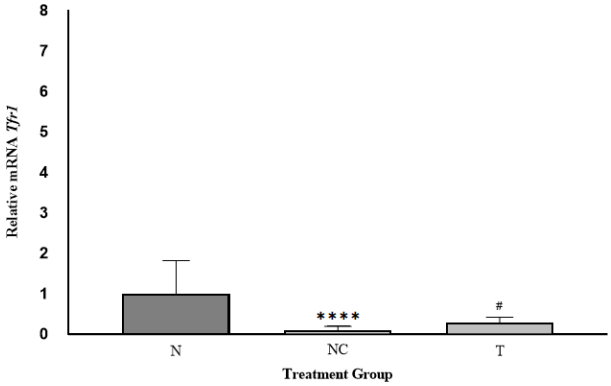

Figure 3. Relative Expression of Tfr1 Gene in Rat Liver. The expression of Tfr1 was significantly decreased in the NC group (iron dextran) and increased in the T group (iron dextran + DFP). Data are presented as mean ± SD (n = 6) and were analyzed using GraphPad Prism software. *Significant difference compared with the N group ($p < 0.05$); #Significant difference compared with the NC group ($p < 0.05$).

Table 3. Effect of Deferiprone on Hepatic Gene Expression in Iron-Overloaded Rats.

Gene	NC vs Normal	T vs NC
Hamp	↑ Upregulated	↓ Downregulated
Ftl	↑ Upregulated	↓ Downregulated
Tfr1	↓ Downregulated	↑ Upregulated

Conclusion

This study demonstrates that iron overload induces significant dysregulation of hepatic iron-regulatory genes, characterized by upregulation of *Hamp* and *Ftl* and downregulation of *Tfr1*. Treatment with Deferiprone (DFP) effectively reversed these alterations by suppressing *Hamp* and *Ftl* expression while restoring *Tfr1* levels toward those observed in the normal group. These findings indicate that DFP promotes the re-establishment of hepatic iron homeostasis at the molecular level and may be beneficial for managing iron overload–associated liver dysfunction.

Declarations

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Conflict of Interest

The authors declare no conflicting interest.

Data Availability

The unpublished data is available upon request to the corresponding author.

Ethics Statement

This research received ethical approval from the Animal Research Ethics Committee of Universitas Padjadjaran, Bandung, under approval number 75/UN6.KEP/EC/2023. All procedures involving experimental animals were conducted in strict accordance with the ethical guidelines established by Universitas Padjadjaran, based on internationally accepted principles for the humane treatment of animals.

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Additional Information

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