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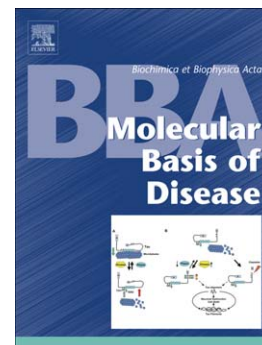
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Original Article

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(Qian et al., Expression of Fpn1, Heph and CP in rat heart)

**Expression of ferroportin1, hephaestin and ceruloplasmin in rat heart**

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## Abstract

Iron-mediated injury plays an important role in a number of heart disorders. Studies on heart iron are therefore crucial for understanding the causes of excessive heart iron. Heart cells have the ability to accumulate transferrin-bound-iron via the transferrin receptor and non-transferrin-bound-iron probably via the L-type Ca<sup>2+</sup> channel and the divalent metal transporter1. However, little is known about the mechanisms of iron export in the heart cells. Here, we investigated expression of iron exporters including ferroportin 1 (Fpn1), ceruloplasmin (CP) and hephaestin (Heph) and provided evidence for their existence in the heart. We demonstrated that iron has a significant effect on expression of Fpn1 and CP, but not Heph. Treatment of a high-iron diet induced a significant increase in Fpn1, a decrease in CP but no change in Heph mRNA and protein. The control of Fpn1 and CP protein expression by iron was parallel to that of their mRNA expression, suggesting a transcriptional regulation of Fpn1 and CP by iron. The existence of these proteins in the heart implies that they might have a role in heart iron homeostasis.

**Keywords** - Ferroportin1, Ceruloplasmin, Hephaestin, Transferrin receptor, Heart, Iron metabolism

## 1. Introduction

Iron is an essential trace element in humans. As in all cells, heart cells require iron for many aspects of their physiology. On the other hand, an excess of iron in the heart can be potentially harmful via the generation of reactive oxygen species. A vast amount of literature shows that iron-mediated injury plays an important role in the development of a number of heart disorders, including heart ischemia-reperfusion injury [1,2], acute myocardial infarction [3,4] and coronary heart diseases [5]. Iron may also be involved in the development of cardiotoxicity induced by doxorubicin [6-9] and is associated with significant cardiovascular morbidity and mortality in hemochromatosis [10,11] and beta-thalassemia [12]. Iron chelation therapy can significantly improve heart reperfusion injury, the prognosis in thalassemia patients and the endothelial function of patients with coronary artery diseases [13-16]. This implies a key role of iron in the pathogenesis of these diseases. Therefore, study on the mechanism of iron balance in the heart is crucial for the understanding of the cause of excessive iron accumulation in the heart as well as the development of new approaches for the treatment of patients with heart iron overload.

Under physiological conditions, the cell iron levels are precisely regulated. Transferrin receptor (TfR) and ferritin are two important proteins that play a primary role in the management of cell iron. By controlling the expression of these two proteins, cells can determine the amount of iron acquired and sequestered [17-19]. In most types of cells, the coordinated control of TfR and ferritin by cellular iron occurs at the post-transcriptional level and is mediated by the cytoplasmic RNA binding

protein, known as the iron regulatory protein (IRP) [20,21]. In addition to TfR mediated transferrin-bound iron (Tf-Fe) uptake, the heart cells are able to assimilate a large amount of non-transferrin-bound iron (NTBI) [22], probably via the L-type Ca<sup>2+</sup> channel (or the L-type voltage-dependent Ca<sup>2+</sup> channel, LVDCC), the voltage-independent uptake system, and the divalent metal transporter1 (DMT1) [23-26]. In addition to iron uptake (TfR, LVDCC and DMT1) and storage (ferritin), cellular iron balance in the heart also depends on the amount of iron export from the cells. Currently, we know little about how iron is discharged from the heart cells and which molecules are involved in this process. In this study, we therefore investigated the expression of iron efflux proteins including ferroportin 1 (Fpn1), ceruloplasmin (CP) and hephaestin (Heph) and the effects of iron on their expression in the heart.

CP is an abundant plasma protein which is mainly synthesized in the hepatocytes. It is widely believed that CP and Heph (a CP homologue) have a role in iron efflux from the cells in the peripheral tissues as well as in the central nervous system [27,28]. Fpn1 (also known as IREG1 or MTP1) is a newly discovered iron transport protein that was first identified as a duodenal iron export molecule [29]. Fpn1 may work with Heph or CP in the iron transport from the enterocytes into circulation [30]. It has been suggested that they might function in the iron transport across the abluminal membrane of the blood-brain barrier cells and from other types of cells [31,32].

In this study, we demonstrated that the heart is able to express all these three proteins. Their existence in the heart suggested their potential role in iron release from the heart cells. The data also demonstrated that iron has a significant effect on the

expression of Fpn1 and CP, but not Heph, and showed that the regulation of Fpn1 and CP by iron might occur at the transcriptional level. The different effects of iron on Fpn1, Heph and CP expression implied that the Fpn1/Heph pathway might play a major role in cell iron export and that Heph might not be rate limiting for iron export from the heart cells.

## 2. Materials and methods

### 2.1. Materials

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA. Agarose, Ethidium Bromide and prestained protein marker were purchased from Bio-Rad Laboratories, Hercules, CA, USA and Trizol<sup>®</sup> Reagent was from Invitrogen., Carlsbad, CA, USA. ExpressHyb hybridization solution was obtained from Clontech, Palo Alto, CA, USA, and Prime-a-Gene labeling system was from Promega, WI, USA. Micro Spin G-50 column, Hybond-N membrane, [ $^{32}$ P]dCTP and ECL western blotting analysis system kit were purchased from Amersham Biosciences, England. The ABC Reagent and DAB kit were products of Vector, Burlingame, USA. The rabbit anti-mouse Fpn1 and Heph antiserum were purchased from Alpha Diagnostics Intl. Inc., San Antonio, USA and mouse anti rat CP IgG1 and CD71 monoclonal antibody from BD Biosciences Pharmingen, Franklin Lakes, USA. Male Sprague-Dawley (SD) rats were supplied by the Centralized Animal Facilities of The Hong Kong Polytechnic University. The Health Department of Hong Kong Government and Animal Ethics Committee of The Hong Kong Polytechnic University approved the use of animals for this study. All the animals

were housed in pairs in stainless steel cages at  $21\pm 2^{\circ}\text{C}$  with relative humidity of 60-65% and alternating 12-hour periods of light (7:00-19:00) and darkness (19:00-7:00).

### ***2.2. Experimental design and sampling of blood and tissue***

To investigate the effect of variations in dietary iron on the Fpn1, CP and Heph gene expression in the heart, male SD rats (21 days of age) in the control (n = 6), the high-iron (n = 6) and the low-iron (n = 6) groups were fed respectively with the Basal Purified Diet (containing 60 mg Fe/kg) (PMI, Catalog # 7024), the Basal Purified Diet supplemented with 2.5% carbonyl iron (PMI, Catalog # 43784) and the Low Iron Purified Diet containing no added iron (PMI, Catalog #7444) (residue of 10 mg Fe/kg diet) for 6 weeks. At the end of the period, the animals were anesthetized with 1% pentobarbital sodium (40 mg/kg body weight, i.p.) and decapitated. Blood samples were then collected into heparinized syringes and aliquots were taken immediately for the determination of hemoglobin (Hb) concentration and hematocrit (Hct). The serum samples were analyzed for serum iron, total iron-binding capacity (TIBC) and transferrin saturation (serum iron/TIBC). To remove all the blood from the hearts, the rats were intracardially perfused with heparinised PBS for 5 min. After perfusion, the hearts were collected, excised and rinsed in PBS, blotted dry and weighed according to Zaman et al [9]. Portions of the left ventricle were used immediately for total RNA extraction and protein determination. The remaining ventricle and liver were frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  for the measurement of non-heme iron.

### ***2.3. RNA purification, generation of specific probes, and northern blot assay***

Total RNA was isolated from the left ventricular myocardium using the Trizol<sup>®</sup> reagent according to the manufacturer's instructions. The relative purity of the isolated



RNA was assessed spectrophotometrically and the ratio of A260 nm to A280 nm exceeded 1.9 for all preparations. Special  $^{32}\text{P}$ -labeled probes corresponding to positions 1298-1733 of Fpn1 (Genbank, AF394785), 3659-4201 of Heph (Genbank, AF246120), 1133-1518 (Genbank, L33869) of CP and 5-574 of TfR1 (Genbank, M58040) were generated. The RNA samples (30  $\mu\text{g}$ ) were electrophoresed on 1.2% formaldehyde-agarose gels, transferred to the Hybond-N membranes and then immobilized by using an UV cross-linker (Fisher). The blots were prehybridized at 65°C in the ExpressHyb hybridization solution for 1 h, and then hybridized overnight at 65°C in the same solution containing the  $^{32}\text{P}$ -labeled probe by using the Prime-a-Gene labeling system (Promega, WI, USA). After three 10-min washes with 2 $\times$  standard sodium chloride-sodium citrate (SSC) containing 0.05% sodium dodecyl sulfate (SDS) at room temperature, the blots were washed in 0.1 $\times$  SSC containing 0.1% SDS with continuous shaking at 50-60°C for 10 min for 3-4 times. Radioactivity was then detected by a phosphorimager and quantified using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). For normalization, the blot was stripped and reprobed with  $\beta$ -actin probe corresponding to the position 474-736 of the rat  $\beta$ -actin (Genbank, NM031144). The results were expressed as the ratio to  $\beta$ -actin.

#### **2.4. Western blot analysis**

The left ventricular myocardium was homogenized in RIPA buffer containing 1% Triton X-100. After centrifugation at 10,000 $\times$ g for 30 min at 4°C, the supernatant was collected. Protein concentration in the supernatant was assayed. Aliquots of the total cell extract containing 10  $\mu\text{g}$  of protein were loaded on a single track of 10% SDS-PAGE under reducing conditions and transferred by electroblotting onto the PVDF membrane (Hybond-P) overnight at 4°C. Molecular weight standards were run

in parallel. The blots were blocked by using 5% blocking reagent in a solution of Tris-buffered salt with Tween 20 (TBS-T) (20 mM Tris-Cl, pH7.6, 137mM NaCl, 0.1% Tween 20) for 2h at room temperature, then incubated with rabbit anti-mouse Fpn1 and Heph antiserum (1:5000), mouse anti rat CP IgG1 and CD71 monoclonal antibody (1:1000) overnight at 4°C. After washing with TBS-T, the blots were incubated in anti-rabbit secondary antibody conjugated horseradish peroxidase (1:5000) (Amersham, UK) for 1 h at room temperature. Immunoreactive proteins were detected by using the enhanced chemiluminescence method (ECL kit, Amersham, UK), and quantified by transmittance densitometry using volume integration with the LumiAnalyst Image Analysis software (Roche Molecular Biochemical) to determine the enrichment of proteins in the hearts. To ensure an even loading of the samples, the same membrane was probed with rabbit anti  $\beta$ -actin polyclonal antibody (Sigma-Aldrich, MO) at a 1:5000 dilution.

### ***2.5. Analytical methods***

Hemoglobin concentration was determined by the cyanmethemoglobin method [33,34]. Hematocrit was measured using the microhematocrit centrifuge and plasma iron and total iron-binding capacity were determined using commercial kits (Sigma). Non-heme iron concentrations of tissues were measured as described previously [33,34]. The results were expressed as Means  $\pm$  SEM. Difference between the means was determined by one-way ANOVA followed by a Student-Newman-Keuls test for multiple comparisons. A probability value of  $P < 0.05$  was taken to be statistically significant.

### 3. Results

#### *3.1. Effect of iron diets on biochemical parameters*

In the high-iron rats, the Hb, Hct, Tf saturation and the non-heme iron level in the heart and liver were significantly higher than those in the control rats ( $p < 0.01$ , Table 1). The iron content in the heart increased 4.2-fold above that of the controls. These findings show that a high-iron loaded heart model was successfully developed. Rats fed with an iron-deficient diet for 6 weeks had significantly lower body weight, Hb, Hct, Tf saturation and non-heme iron levels in the heart and liver as compared to the controls ( $p < 0.01$ , Table 1). The heart weight and the heart/body weight were higher in the iron-deficient rats than in the controls ( $p < 0.01$ ). The data suggest that rats fed with the iron deficient diet did develop an iron deficiency with anaemia.

#### *3.2. Effect of iron status on expression of ferroportin1, hephaestin and ceruloplasmin mRNA and protein*

Northern blot analysis was performed to determine the effect of iron on the expression of Fpn1, Heph and CP mRNA. Representative northern blots showing the expression of Fpn1, Heph and CP mRNAs were presented in Figures 1A, 2A and 3A. Quantified mRNA levels relative to  $\beta$ -actin for each transcript from the independent samples were shown in Figures 1B, 2B and 3B. The transcript level of Fpn1 mRNA decreased significantly ( $58 \pm 3\%$ ) in the iron-deficient rats and increased ( $253 \pm 20\%$ ) in the iron-overloaded rats (Figure 1B, all  $P < 0.01$ ) as compared to the control. In contrast, the level of CP mRNA was significantly higher ( $135 \pm 2\%$ ) in the iron-deficient rats and lower ( $89 \pm 5\%$ ) in the iron-overload rats than that in the controls (Figure 3B,  $P < 0.05$ ). This finding is similar to the result reported by other studies

[35,36]. No significant differences in Heph mRNA were found among the iron-deficient, the iron-overload and the control animals (Figure 2B).

To determine the effect of iron on Fpn1, CP and Heph proteins, western blot analysis was conducted. It was found that the amount of Fpn1 protein decreased to  $74 \pm 8\%$  of the controls after the rats were fed with a low-iron diet. After the rats were fed with a high-iron diet, the FPN1 protein increased to  $230 \pm 20\%$  of the controls (Figure 1B, all  $P < 0.01$ ). As shown in Figure 3, the regulation of CP protein expression was parallel to that of the mRNA expression. After the rats were fed with a low-iron diet, the CP protein increased to  $142 \pm 3\%$  of the controls (Figure 3B,  $P < 0.05$ ); in the high-iron group the CP protein decreased to  $72 \pm 3\%$  of the controls (Fig 3B,  $P < 0.05$ ). Heph protein in the rats fed with a low-iron or high-iron diet was lower than that in the control animals, however, no significant differences were found among them. These results show that iron has a negative effect on CP, a positive effect on Fpn1 and no effect on Heph expression in the heart.

### ***3.3. Effect of iron on expression of transferrin receptor 1 mRNA and protein***

For comparison, we also analyzed the expression of TfR1 mRNA and protein in the control, low-iron and high-iron rats. In the high-iron rats, there was an increase in the iron content in the heart. The northern blotting and western blotting results showed that the expression of TfR1 mRNA and protein significantly declined about 35% and 45% to the control; respectively (Figure 4). In the iron-deficiency models, iron in the heart decreased significantly and the level of TfR1 mRNA and protein increased remarkably, being about 2.7 times(mRNA) and 2.4 times(protein) relative

to the control rats. These results demonstrated that iron has a significant effect on the expression of TfR1 in the heart.

#### 4. Discussion

In this study, we investigated the expression of iron efflux transporters including ferroportin 1 (Fpn1), ceruloplasmin (CP), and hephaestin (Heph) and the effects of iron on their expression in the heart. To our knowledge, this is the first report on the expression of Fpn1 and Heph mRNA and protein in the rat heart. Northern and western blotting results showed that the heart is able to express Fpn1 and Heph. The existence of Fpn1, Heph and CP in the heart suggests that these proteins might have a role in iron homeostasis in the heart, and is possibly involved in the iron export from the heart cells. The data obtained also demonstrated that iron has a significant effect on the expression of Fpn1 and CP, but not Heph. In the rats treated with a high-iron diet for 6 weeks, the transcript levels of Fpn1 and CP mRNA were found to be significantly increased and decreased; respectively. The control of Fpn1 and CP protein expression by iron was parallel to that of their mRNA expression, suggesting that the regulation of Fpn1 and CP by iron in the heart might occur at the transcriptional level. No significant changes in Heph mRNA and protein expression were found in the rats treated with a high- or low-iron diet.

Ferroportin 1, also known as metal transport protein 1 (MTP1), iron-regulated transporter 1 (IREG1), or Slc11a3, is a newly identified cellular iron exporter [29,37,38]. The mRNA transcript of FP1 contains an iron responsive element (IRE) in

its 5' untranslated region [29,37,38]. This structure implies that the Fpn1 expression might be regulated at the translational level in a manner similar to other 5'UTR-IRE-regulated genes, including ferritin, mitochondrial aconitase, erythroid 5-aminolevulinate synthase, and succinate dehydrogenase [38]. In an investigation on the functional role of genomic SLC40A1 elements (Fpn1 is the product of the SLC40A1 gene) in response to iron in human hepatoma (HepG2), intestinal carcinoma (Caco2) and lympho-monocytic (U937) cell lines, Lymboussaki et al [39] demonstrated directly that the IRE in Fpn1 mRNA is functional and that it controls Fpn1 expression through the cytoplasmic IRP. However, data that are not consistent with this IRE/IRP-mediated translational control have also been reported. The findings in intestinal enterocytes, Caco2 intestinal cells [37,40], murine J774 macrophages [41], and human alveolar macrophages [40] show that there is also a transcriptional mechanism that controls Fpn1 expression in these cells. The results in the present study are in agreement with the above findings, implying the existence of a transcriptional control of Fpn1 expression in the heart. Based on the published data and our results, it seems likely that both of the transcriptional and translational (IRP/IRE pathway) mechanisms of Fpn1 expression operate in a tissue-specific manner [29,38]. In addition, the Fpn1 protein level in the heart may be critically controlled by hepcidin, a newly discovered iron regulation peptide [43], through its effect on the degradation of Fpn1 [44]. The increased Fpn1 protein might be partly due to the decreased hepcidin level induced by a high-iron diet.

Fpn1 mediated-iron export has been demonstrated by the increased iron efflux in the iron-loaded *Xenopus* oocytes expressing Fpn1 [29,38], and by the cellular iron depletion in HEK293T cells transiently transfected with the full-length FPN1 cDNA

[37]. Ceruloplasmin is an abundant serum alpha-2 glycoprotein and has a molecular mass of approximately 132 kDa. It is widely accepted that CP has a role in iron efflux from cells in the peripheral tissues as well as in the central nervous system [27,28]. Heph is a transmembrane-bound CP homologue protein, which is expressed in the villus of the small intestine [30,45]. The diminished transport of iron from the mucosal cell to the circulation in *sla* mice implies that Heph has a role in the physiological iron absorption in the small intestine [30,46]. However, Heph contains only one predicted carboxy-terminal transmembrane domain. Heph itself is therefore unlikely to be a membrane iron transporter [30,47]. CP or Heph may work together with FP1 in the iron transport from the enterocytes into the circulation [30,47]. It has also been proposed that the Fpn1/Heph or Fpn1/CP systems might have a role in the iron transport across the abluminal membrane of the blood-brain barrier cells and in the iron release from other types of cells [31,32,48].

In summary, the present study provided evidence for the existence of iron efflux proteins including Fpn1, CP and their homologue Heph in the heart. This implied that these proteins might play a role in iron homeostasis in heart. Findings also showed that the regulation of Fpn1 and CP expression by iron might occur at the transcriptional level in the heart. Treatment with a high-iron (or low-iron) diet for 6 weeks led to a significant increase (or decrease) in Fpn1, a remarkable decrease (or increase) in CP, and no change in Heph mRNAs and proteins' expression. These findings suggested that the Fpn1/Heph pathway might play a major role in the iron export from the heart cells and that Heph might not play a primary regulatory role and might not be rate limiting in this process of iron export from the heart cells.

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ACCEPTED MANUSCRIPT



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Dcytb but not Ireg1 or hephaestin explains the mucosal block phenomenon of iron absorption. *Gut* 52 (2003) 340-346.

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## Legends of Figures

**Figure 1. Effects of iron on expression of ferroportin1 mRNA and protein in the heart.** Expression of Fpn1 mRNA and protein was determined by northern blot and western blot analysis in the rats fed with the control diet (CN), the iron-deficient (LF) and iron-overload (HF) diets for 6 weeks. **A:** Representative blots of Fpn1 and  $\beta$ -actin mRNAs and proteins (molecular masses of 60 and 45 kDa, respectively). **B:** Relative levels of Fpn1 mRNA and protein. Data are means  $\pm$  SEM (% control, n=6). \*\* $P$ <0.01 vs. the control.

**Figure 2. Effects of iron on expression of hephaestin mRNA and protein in the heart.** Expression of Heph mRNA and protein was determined by northern blot and western blot analysis in the rats fed with the control diet (CN), the iron-deficient (LF) and iron-overload (HF) diets for 6 weeks. **A:** Representative blots of Heph and  $\beta$ -actin mRNAs and proteins. **B:** Relative levels of Heph mRNA and protein. Data are means  $\pm$  SEM (% control, n=6). \* $P$ <0.05, \*\* $P$ <0.01 vs. the control.

**Figure 3. Effects of iron on expression of ceruloplasmin mRNA and protein in the heart.** Expression of Heph mRNA and protein was determined by northern blot and western blot analysis in the rats fed with the control diet (CN), the iron-deficient (LF) and iron-overload (HF) diets for 6 weeks. **A:** Representative blots of CP and  $\beta$ -actin mRNAs and proteins. **B:** Relative levels of CP mRNA and protein. Data are means  $\pm$  SEM (% control, n=6). \* $P$ <0.05 vs. the control.



**Figure 4. Effects of iron on expression of transferrin receptor 1 mRNA and protein in the heart.** Expression of TfR1 mRNA and protein was determined by northern blot and western blot analysis in the rats fed with the control diet (CN), the iron-deficient (LF) and iron-overload (HF) diets for 6 weeks. **A:** Representative blots of TfR1 and  $\beta$ -actin mRNAs and proteins. **B:** Relative levels of TfR1 mRNA and protein. Data are means  $\pm$  SEM (% control, n=6). \*\*P<0.01 vs. the control.

ACCEPTED MANUSCRIPT

**Abbreviations list**

CP:	Ceruloplasmin
DMT1:	Divalent metal transporter 1
Fpn1:	Ferroportin 1
Hb:	Hemoglobin
Hct:	Hematocrit
Heph:	Hephaestin
IREG1:	Iron-regulated transporter 1
IRP:	Iron regulatory protein
LVDC:	L-type voltage-dependent Ca <sup>2+</sup> channel
MTP1:	Metal transport protein 1
NTBI:	Non-transferrin-bound iron
SD rats:	Sprague-Dawley rats
SDS:	Sodium dodecylsulfate
SSC:	Sodium chloride-sodium citrate
TfR1:	Transferrin receptor 1
Tf-Fe:	Transferrin-bound iron
TIBC:	Total iron-binding capacity

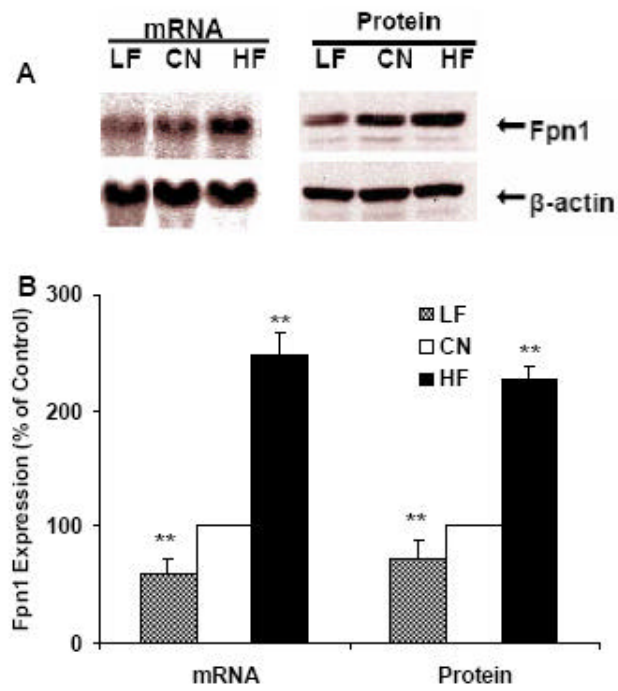
**Table 1. Hematological variables, liver non-heme iron in the control, iron deficient, and iron overload rats**

	Control (n=6)	Iron deficiency (n=6)	Iron overload (n=6)
Body weight (g)	327 ± 4.27	270 ± 4.48**	340 ± 7.11
Hct (%)	43.50 ± 2.11	17.67 ± 0.99**	48.88 ± 1.17**
Hb (g/dl)	17.96 ± 0.59	5.27 ± 0.38**	19.63 ± 0.30**
Serum iron (mmol/L)	24.67 ± 0.88	3.11 ± 0.53**	48.80 ± 2.11**
TIBC (mmol/L)	67.79 ± 1.29	109 ± 3.27**	69.69 ± 1.34
Transferrin saturation (%)	36.36 ± 0.81	2.93 ± 0.54**	70.20 ± 3.57**
Liver iron (mg/g dry weight)	0.27 ± 0.01	0.07 ± 0.01**	2.66 ± 0.13**
Heart iron (mg/g dry weight)	0.073 ± 0.008	0.032 ± 0.003**	0.379 ± 0.016**
Heart weight (g)	1.01 ± 0.05	1.33 ± 0.05**	0.92 ± 0.04
Heart:body weight ( $\times 10^{-3}$ )	3.02 ± 0.18	4.91 ± 0.26**	2.69 ± 0.13

\*\*p<0.01 vs. the controls.

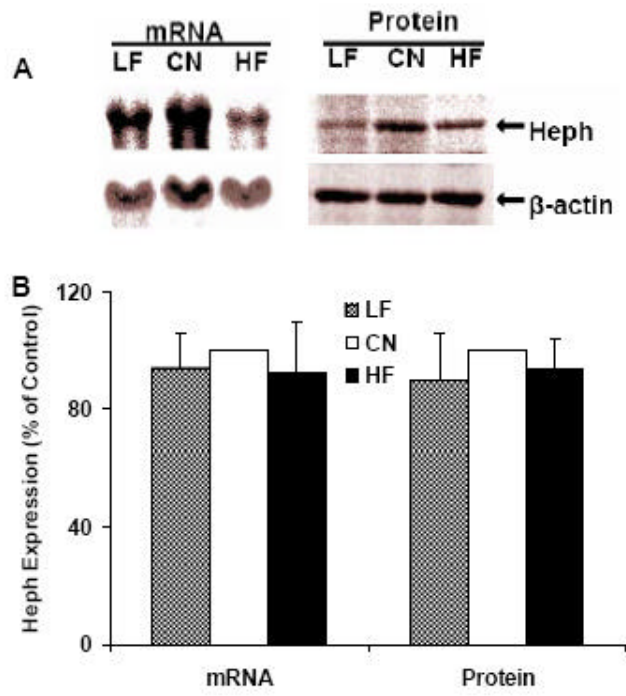
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Figure 1 (Fpn1)



AC

Figure 2 (Heph)



CRIP

Figure 3 (CP)

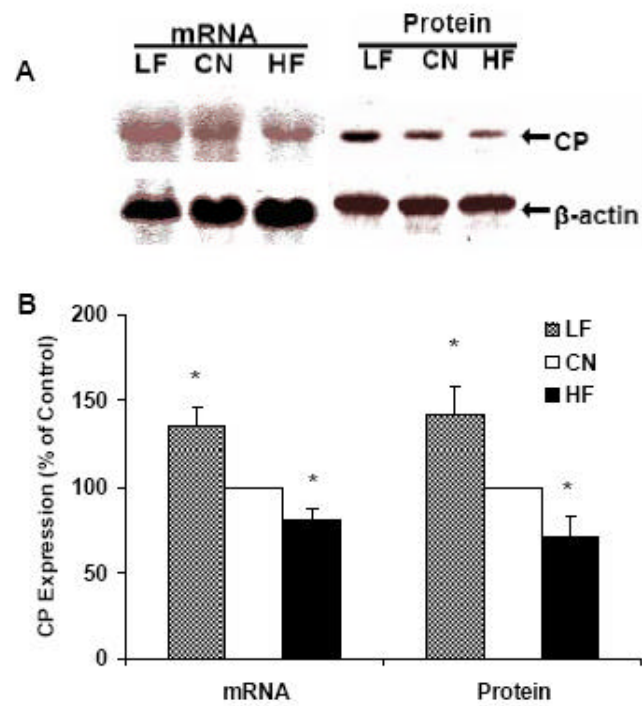


Figure 4 (TfR1)

