The Journal of Animal & Plant Sciences, 27(3): 2017, Page: 1024-1030
ISSN: 1018-7081

HEPATIC IRON REGULATORY GENE EXPRESSION INFLUENCED DUE TO DISTURBED ESSENTIAL TRACE ELEMENTS LEVEL

N. Sheikh*, A. Farrukh and A. S. Abbas

Department of Zoology, University of the Punjab, Lahore, Pakistan.
*Corresponding author: s_nadeem77@yahoo.com

ABSTRACT

Iron and copper, the essential trace elements accomplish their numerous relevant metabolic functions appropriately in the body. Hepcidin, ferroportin-1 and transferrin are associated with iron metabolism and their expression can be affected under different physiological or pathological conditions. The aim of the current study was to investigate the changes in hepatic gene expression of these iron regulatory proteins in response to acute Fe & Cu intoxication. Male Wistar Rats were categorized in three experimental groups (G-I: $FeSO_4$, 60mg/kg; G-II: $CuSO_4$, 10mg/kg; G-III: $FeSO_4$, 30mg/kg & $CuSO_4$, 5mg/kg), against a control group. Animals were sacrificed after 24h of acute intra-peritoneal administration. Liver was excised, cut into small pieces and processed for genetic expression analysis. Hepatic hepcidin gene expression was up-regulated in G-I & G-III, whereas it was down-regulated in the G-II, when compared with the control group and with its more expression in G-I. Hepatic ferroportin-1 gene expression was down-regulated and had shown an inverse relation to the hepcidin gene expression. Hepatic transferrin gene expression was down-regulated in G-II, in comparison with the control group, whereas in G-I & G-III, no detectable level was observed. Taken together these findings it can be concluded that gene expression can significantly be affected due to disturbed levels of the essential trace elements.

INTRODUCTION

Iron (Fe) and copper (Cu); the essential trace elements; accomplish their numerous relevant metabolic functions appropriately in the body, when their concentrations are found in the standard range. Iron holds central importance in DNA synthesis, metabolic reactions and respiration (Ben-Assa *et al.,* 2009; Donovan *et al.,* 2006; Dunn *et al.,* 2007), and copper serves as a cofactor as well as structural constituent of several metalloenzymes (Stern2010); and is considered to be important in the development of bone, connective tissue and nerve coverings in humans (Fraga2005). Hepcidin (Hep), ferroportin-1(Fpn-1) and transferrin (Tf) are the important proteins associated with iron metabolism and the onset of acute phase response (APR) influences their gene expressions.

The basic system of iron metabolism involves the dietary iron absorption by the duodenal enterocytes (Ganz *et al.,* 2012) via duodenal metal transporter-1 (DMT-1) (Gunshin *et al.,* 2001), followed by delivery of iron to the tissues by means of transferrin (Dunn *et al.,* 2007); an iron-binding protein in plasma; fundamentally involved in iron transportation (Hentze *et al.,* 2004; Morgan1981; Richardson *et al.,* 1997). Transferrin then binds to diferric iron and it is transported to liver through portal blood (Hentze *et al.,* 2010) where it binds to transferrin receptor-1(Cheng *et al.,* 2004) and ultimately leads to receptor-mediated endocytosis of the whole complex and is internalized (Napier *et al.,* 2005). Iron is then utilized to carry out the cellular processes, while the surplus iron is sequestered in storage protein i.e; ferritin (Hentze *et al.,* 2004).

However, the iron from different states such as absorbed iron in duodenal enterocytes or stored within hepatocytes or recycled by macrophages; ultimately pass from cytoplasm of cells to the transferrin (Ganz *et al.,* 2012), and iron efflux from cells is facilitated via a multipass transmembrane protein i.e., iron exporter ferroportin 1 (Donovan *et al.,* 2005).

Ferroportin-1 (also termed as SLC11A3, MTP1 or IREG-1) is recognized as an important iron export protein in cells (Ganz2005; Nemeth *et al.,* 2004). Its expression is regulated by hepcidin (Ganz2005; Ramey *et al.,* 2010).

Hepcidin; an anti-microbial peptide of 25 amino acids (Park *et al.,* 2001); formerly known as LEAP-1(Liver-expressed antimicrobial peptide) (Krause *et al.,* 2000), is a negative regulator of iron metabolism (Lesbordes-Brion *et al.,* 2006; Nicolas *et al.,* 2001; Nicolas *et al.,* 2002). It is a hepatocyte-derived peptide; encoded by HAMP gene, and involves in regulating the export of ferroportin-mediated iron from macrophages and enterocytes that evaluates the total iron uptake by diet (Andrews2008; Babitt *et al.,* 2011; Ganz2011; Hentze *et al.,* 2010).

On the molecular level, hepcidin binds to ferroportin-1 and then plays a crucial role in internalizing and degrading the ferroportin-1(Nemeth *et al.,* 2004; Nemeth *et al.,* 2006) and consequently represses the iron efflux from enterocytes, macrophages and hepatocytes (Knutson *et al.,* 2003; Knutson *et al.,* 2005; Nemeth *et al.,* 2004), that in turn results in reduction of iron release

into plasma, and eventually encourages the cellular iron retention (Sun *et al.,* 2012).

Metabolic disturbance resulted due to sub-acute and sub-chronic Fe overload in rat (Adham *et al.,* 2015). Similarly, recent study evaluated oxidative stress in rat brain, induced by acute Fe overload (Piloni *et al.,* 2013) and sub-chronic Fe overload (Piloni *et al.,* 2016). Besides, extra-hepatic cells involved in inducing Hep production due to Fe loading (Sasaki *et al.,* 2014). However, another study demonstrated the highest hepatic oxidative stress in rat as a result of Cu toxicity (Kumar *et al.,* 2016). In Fe and Cu overload conditions; reduced levels of some antioxidants in liver of rats also indicated oxidative stress (Britton1996). Furthermore, studies showed the effects of acute Fe and Cu intoxication in rat i.e.; biomolecules oxidation and oxidative damage to liver and brain (Musacco-Sebio *et al.,* 2014a). Relevantly, as a result of acute Fe and Cu toxicity in rat, antioxidant responses of liver (Musacco-Sebio *et al.,* 2014b) and brain (Semprine *et al.,* 2014) were observed.

Liver toxicity is the predominant sequela of acute Fe & Cu poisoning, therefore aim behind this study was to investigate the changes in hepatic gene expression of these iron regulatory proteins (Hep, Fpn-1 & Tf) in response to acute Fe & Cu intoxication.

MATERIALS & METHODS

Experiment was conducted on Wistar Rats weighing about 200-250g. They were housed in small rat cages and were kept under normal conditions with 12-12hours light-dark cycles. They were fed on normal rat chow and were provided with fresh water *ad libitum.* Three experimental groups were established against a control group.

For dose preparation, hydrous ferrous sulphate ($FeSO_4.7H_2O$) and cupric sulphate pentahydrate ($CuSO_4.5H_2O$) were dissolved in distilled water separately and in combination as well, half an hour prior to the experiment. Route of administration was intra-peritoneal and Group-I received 60mg/kg body weight of iron, Group-II received 10mg/kg body weight of copper, Group-III received 30mg/kg and 5mg/kg body weight of iron and copper whereas the control group received distilled water only. Animals were sacrificed after 24 h. Previously, studies revealed the effects of acute Fe and Cu toxicity in rat using methods with some modifications (Boveris *et al.,* 2012; Musacco-Sebio *et al.,* 2014a; Musacco-Sebio *et al.,* 2014b; Semprine *et al.,* 2014). The liver from each animal was excised, washed with 0.9% saline solution, cut into small pieces and were immediately stored at -20˚C, till used for gene expression analysis.

Gene expression analysis was carried out by mRNA extraction from liver tissue homogenate, using Vivantis GF-1 Total RNA Extraction Kit Cat# GF-TR-100, followed by cDNA synthesis by using the Fermentas RevertAid First Strand cDNA Synthesis Kit #K1621. Selective gene amplification of cDNA was done using Thermo scientific Fermentas PCR Master Mix (2X) #K0171. Primer sequences of genes under study are shown in Table 1.

Table 1. Primer sequences of the genes under study:

Primers	Forward 5' ⟶ 3'	Reverse 5' ⟶ 3'
Hepcidin	AGGACAGAAGGCAAGATGGCA	TGTTGAGAGGTCAGGACAAGGC
Ferroportin-1	CAGACTTAAAGTGGCCCAGACG	ACAAGGCCACATTTTCGACG
Transferrin	CCGTGACCACATGAAAACCG	GGAGAGCCGAACAGTTGGAA
ß actin	TGTCACCAACTGGGACGATA	AACACAGCCTGGATGGCTAC

Then agarose gel electrophoresis was done using BIO-RAD: PowerPac basic power supply # 164-5050 EDU and Sub-Cell® GT Electrophoresis Cells. Statistical analysis was done by one-way ANOVA with post-hoc Tukey test using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA. The densitometric analysis of the gel was performed using ImageJ 1.38x software (Wayne Rasband, National Institutes of Health, USA).

RESULTS

Changes in hepcidin gene expression in the liver were found to be statistically significant in the experimental groups as compared to the control group when analyzed using one-way ANOVA in combination with post-hoc Tukey-test ($P < 0.0001$; Figure 3). Up-regulation of hepatic hepcidin expression was demonstrated in the treated groups I & III, whereas it was downregulated in the group II, when compared with the control group. Maximum expression of hepatic hepcidin was observed in group-I (Figure 1a, 2, 4a).

Ferroportin-1 gene expression in the liver showed an inverse correlation with the hepcidin as it was expressed only in the group II, in which the hepcidin was least expressed (Figure 1b, 4b).

Hepatic transferrin gene expression was downregulated in group-II, in comparison with the control group, whereas groups I & III revealed no results (Figure 1c, 4c). However, hepatic gene expression of

transferrin and ferroportin-1 were not statistically analyzed as these were the only observations from the densitometry analysis and ultraviolet images of PCR products.

DISCUSSION

In the present work, alterations in hepatic hepcidin expression were reported along with changes in other genes involved in iron metabolism as a result of acute toxicity of iron and copper. The findings in the current investigation are in concomitant with the previous studies, demonstrating the enhanced hepcidin expression in the liver following acute iron intoxication (Krijt *et al.*, 2012; Kulaksiz *et al.*, 2004; Pigeon *et al.*, 2001; Toledano *et al.*, 2009). As hepcidin is an acute-phase reactant; its expression is raised in conditions of inflammation and iron overload (Balogh *et al.*, 2004; Krijt *et al.*, 2012; Pietrangelo2011). Relevantly, augmented hepatic and non-hepatic (including brain) hepcidin expression was reported due to turpentine-oil induced acute-phase response in rat (Malik *et al.*, 2011; Sheikh *et al.*, 2007) and also the up-regulation of hepcidin expression in rat liver was indicated in response to hepatic damage *in vivo* and *in vitro* (Sheikh *et al.*, 2006).

Furthermore, the augmented expression of hepcidin mRNA was reported as a result of acute iron toxicity (Kulaksiz *et al.*, 2004). Moreover; in response to iron overload in mice, hepatic hepcidin expression (Pigeon *et al.*, 2001) and liver HAMP mRNA (Krijt *et al.*, 2012) was up-regulated. Several studies have reported increased hepatic hepcidin expression (Toledano *et al.*, 2009), enhanced hepcidin mRNA levels in substantia nigra (Sun *et al.*, 2012) and elevated serum hepcidin levels (Ben-Assa *et al.*, 2009) as a result of acute iron intoxication in rats.

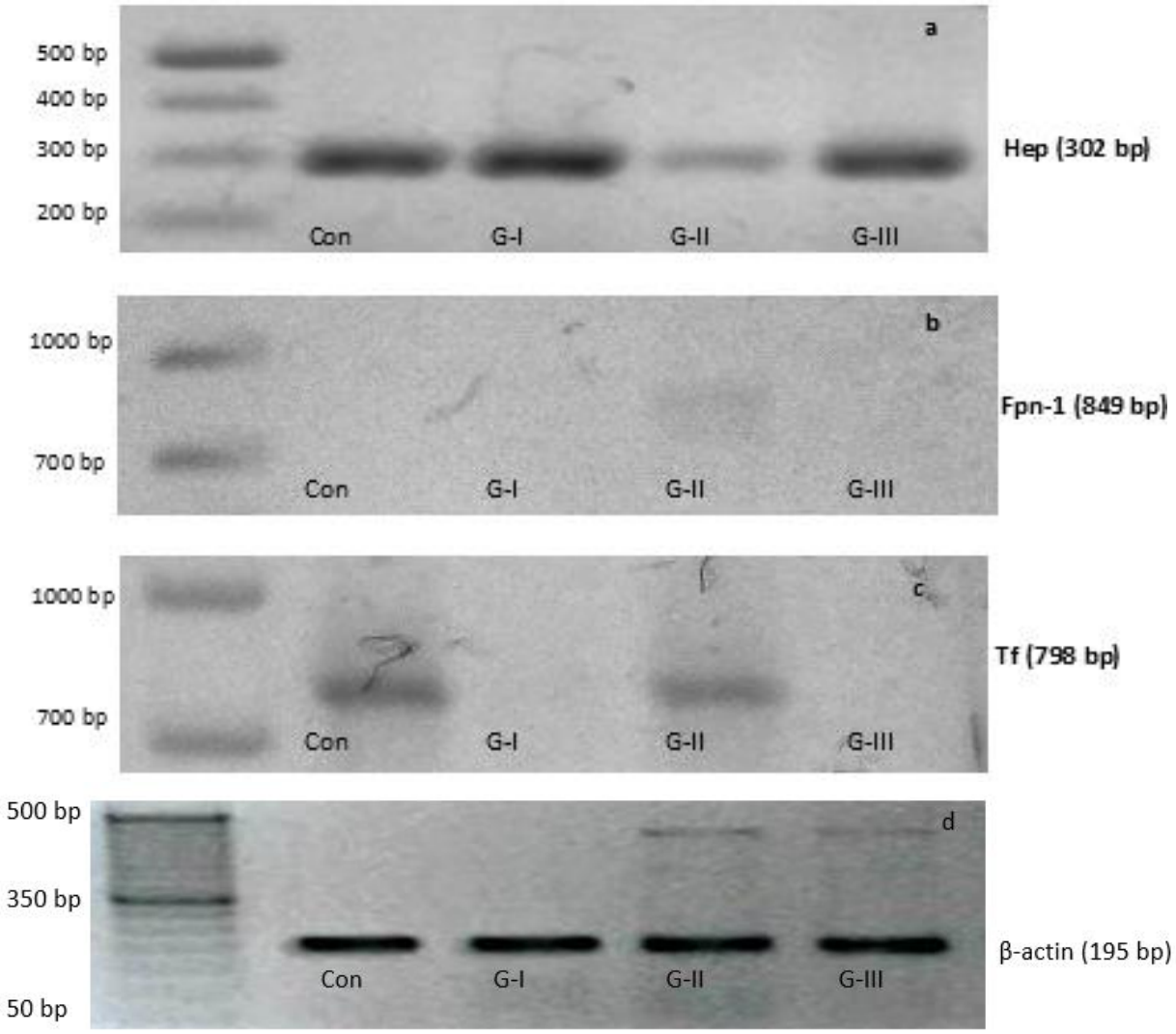

Figure 1. Ultraviolet image of 1% agarose gel showing hepatic hepcidin, ferroportin-1 and transferrin gene expression in experimental and control groups, β-actin was used to ensure equal loading of the samples.

Ferroportin-1; a negative acute-phase protein (Naz *et al.*, 2012), is regulated by hepcidin under inflammatory conditions (Fleming *et al.*, 2005; Ganz2005; Nemeth *et al.*, 2004; Sheikh *et al.*, 2007). Current study demonstrated the inverse correlation of Hep with Fpn1, showing augmented hepatic Hep expression in Fe-overload conditions, responsible for inhibition of hepatic Fpn1 whereas in Cu overload condition, Hep expression was downregulated, resulting in upregulation of Fpn1. In accordance, hepatic ferroportin-1 gene expression was markedly downregulated in rat model of acute-phase response (Malik *et al.*, 2011; Sheikh *et al.*, 2007). Concomitantly, recent study showed, that Hep suppressed the neural iron accumulation in rat by inhibiting the expression of Fe-transport proteins (TfR1, DMT1, Fpn1) under Fe-overload conditions (Du *et al.*, 2015). Accordingly, excessive iron dose resulted in enhanced hepatic hepcidin mRNA expression and decreased duodenal Fpn1 expression (Aslam *et al.*, 2014; Han *et al.*, 2008). However, in another study, similar results were reported, following Fe overload conditions; Hep inhibited the expression of TfR1, DMT1, and Fpn1 in astrocytes (Du *et al.*, 2011).

In the same way, changes in transferrin gene expression in the liver were noticed in the experimental groups. Transferrin is a negative acute-phase reactant and decreases in inflammatory conditions (Gabay *et al.*, 1999; Gruys *et al.*, 2005), thereby indicating the down-regulation of hepatic Tf expression in experimental groups. In previous studies, transferrin gene expression in the liver was slightly up-regulated at earlier points then it was down-regulated as a result of acute phase reaction (Malik *et al.*, 2011; Sheikh *et al.*, 2007). However, a recent review elucidated the concept of decreased transferrin receptor 1 (TfR1) mRNA expression, in conditions of Fe excess (Loreal *et al.*, 2014). Moreover, Hep found to be directly involved in reduced TfR1 expression, through cyclic AMP-protein kinase A pathway (Du *et al.*, 2011).

Conclusively, the variations in the hepatic gene expression of Hep, Fpn-1 and Tf had been confirmed in such inflammatory conditions, thus indicating the occurrence of APR. Hence, acute toxicity of Fe & Cu had major impact on gene expression of these proteins in liver.

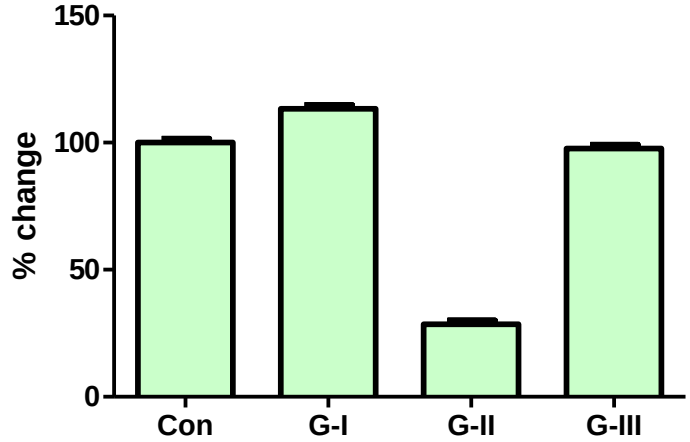

Figure 2. Percentage change in hepatic hepcidin gene expression in the experimental groups against control group.

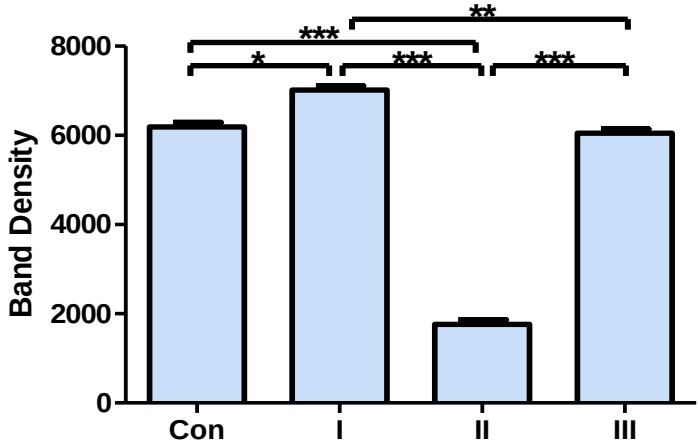

Figure 3. Hepatic hepcidin expression in experimental groups as compared to the control group. Results show mean value ± S.E.M. (Level of Significance *P<0.05, **P< 0.01, and *** P<0.001).

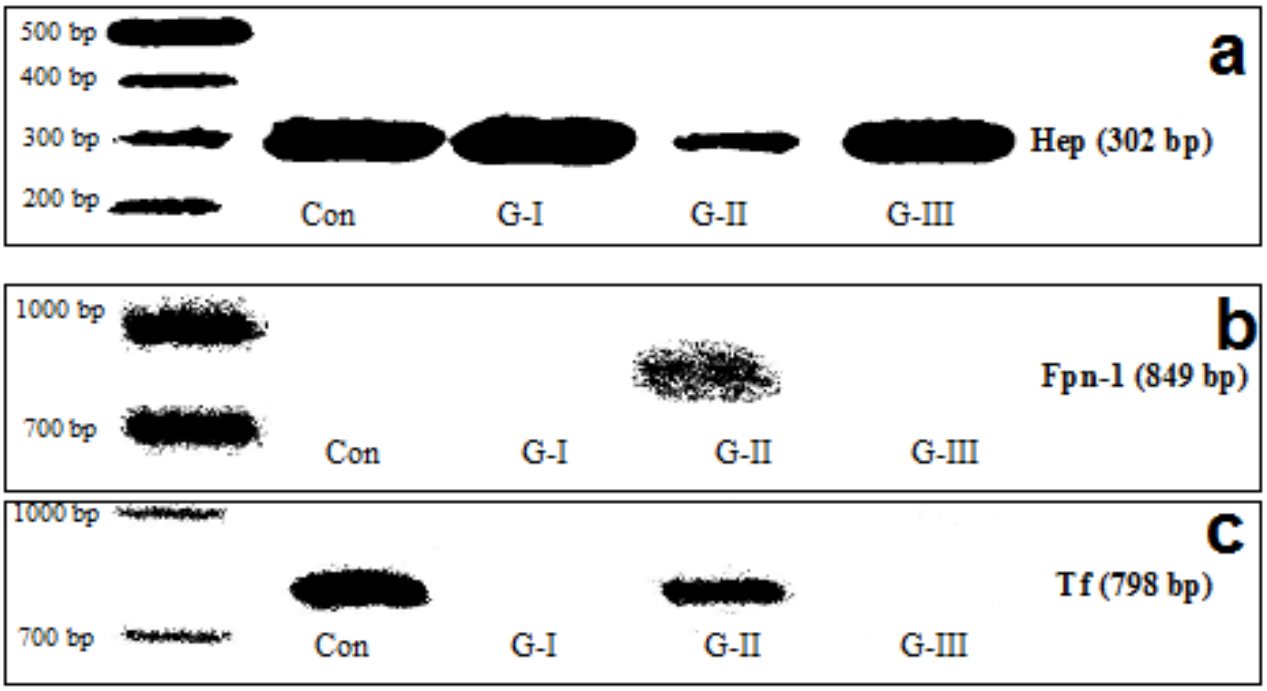

Figure 4. Densitometric analysis of hepatic hepcidin (a), ferroportin-1(b) and transferrin (c) gene expression in experimental groups against control group.

Acknowledgments: The authors are thankful to the Vice Chancellor of the University of the Punjab, for providing research grant for the study under PU R&D grant for the year 2012-13.

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