Regulation of gene expression of hepcidin and of other proteins of the iron metabolism in the liver and in the extrahepatic tissues: *in vivo* and *in vitro* studies in different rat models.

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Contents

ABBREVIATIONS

AMP	Adenosine monophosphate
APP	Acute-phase proteins
APR	Acute-phase response
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
bp	Base pair
BR	Bilirubin
BSA	Bovine serum albumin
cAMP	Cyclic adenosine-3´,5´-monophosphate
CCl_4	Carbon tetra chloride
cDNA	Complementary deoxyribonucleic acid
Ci	Curie
CRP	C-reactive protein
Ct.	Threshold cycle
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
Dcytb	Duodenal cytochrome B reductase
dd H_2O	Double distilled water
DEPC	Diethylpyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	Dimethylsulfoxide
DMT1	Divalent metal transporter 1
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	Ethylendiaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum

Fpn.-1	Ferroportin 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Hepc	Hepcidin
HEPES	2-(4-2-hydroxyethyl)-piperazinyl-1-ethansulfonate
Heph	Hephaestin
HFE	Hemochromatosis gene
Hjv	Hemojuvelin
IFN-γ	Interferon gamma
IL-1ß	Interleukin 1 Beta
IL-6	Interleukin 6
IRE	Iron responsive elements
IRE-BP 1	Iron responsive element binding protein 1
IRE-BP2	Iron responsive element binding protein 2
Kb	Kilobase
kDa	Kilodalton
LPS	Lipopolysaccharide
MOPS	3-(N-Morpholino)-propanesulfonic acid
NAD(P)+	Nicotinamide adenine dinucleotide (phosphate) oxidized
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate) reduced
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PH	Partial-hepatectomy
PMSF	Phenylmethyl sulfonylfluoride
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcriptase-PCR
SAA	Serum amyloid A
SEM	Standard error of the mean
TAE	Tris acetate EDTA buffer

TEMED	N, N, N′, N′-tetramethylethylenediamine
Tf	Transferrin
TfR1	Transferrin receptor 1
TfR2	Transferrin receptor 2
TNF-α	Tumor necrosis factor alpha
TO	Turpentine oil
Tris	Tris-(hydroxymethyl)-aminomethane
UTR	Untranslated region
UV	Ultraviolet

SUMMARY

Iron is an essential element required for many redox processes in all eukaryotes and most of the prokaryotes. Serum iron level decreases during microbial infection, inflammation or injury. The exact mechanism and the changes of the gene expression of the proteins involved in the iron regulation not only in the liver but also in other organs are; however, not yet fully clarified.

In the present work, we used turpentine oil (TO) injection in the hind limb muscle of the rat to stimulate an acute-phase-response (APR) and PH- and CCl_4-induced acute-liver injury models to study the changes in the gene expression of the acute-phase cytokines and of the proteins known to be involved in iron regulatory pathway.

Serum concentrations of hepcidin pro-hormone was not significantly changed as a result of sterile muscle abscess or acute-liver injury; however, significant decline in the serum iron level was observed. Similarly, serum levels of acute-phase cytokines IL-6, IL-1ß, TNF-α and IFN-γ were changed significantly. Due to elevated levels of IL-6 in the serum it could be referred as the main mediator responsible for the regulation of hepcidin gene expression along with other iron regulatory genes during the APR induced by TO. This increase in the serum concentration was associated with the increased expression of IL-6 mRNA in the injured muscle of TO injected rats and in the livers of PH rats. In CCl_4 model of acute-liver injury serum level of IL-6 was not significantly increased; however, the upregulation at transcriptional level was significant in the liver.

As a result of inflammation induced by TO injection in the hind limb muscle of the rats hepatic hepcidin gene expression was significantly increased along with the upregulation of Tf, TfR1 and TfR2, ferritin-H, IRE-BP1 and IRE-BP2 gene expression. Hjv, Fpn.-1, Dcytb, HFE and Heph gene expression was downregulated. Besides the liver, the expression of iron metabolism genes was studied in the injured muscle and extrahepatic organs. It was found that except the injured muscle and the lung hepcidin gene expression was upregulated in all the organs studied with a local upregulation of IL-6 gene expression in these organs. Similar expression patterns were observed for the Fpn.-1 gene expression in different organs but in opposite direction.

In case of acute-liver injury induced either by PH or CCl_4 iron metabolism genes behaved more or less in a similar fashion. Hepcidin gene expression was upregulated

significantly during the course of experimental study along with TfR1 and IRE-BP1 where as the other genes were downregulated as a result of liver injury. mRNA expression of acute-phase cytokines in the PH and CCl_4-administered rats demonstrated the increased expression of IL-6 in the liver. Serum concentrations of IL-6 were elevated in as a result of PH-induced injury; however, in CCl_4-administered rats IL-6 was not significantly increased in the serum. The possible explanation could be the local importance and utilization of the IL-6 in the liver. However, besides IL-6 other mediators like IL-1ß could also be involved for iron regulation during acute-phase condition.

From the present work, we can conclude that in the rat model of the sterile abscess-induced systemic APR, changes of gene expression of the main proteins involved in iron metabolism taking place in the liver are qualitatively similar to those observed in most of different other organs (with exception of lung). In the injured muscle; however, changes of the hepcidin, Hjv and Fpn.-1 gene expression may represent specific "inflammatory" changes. The changes may be compatible with local "trapping" of serum iron and of the iron taken up in the intestinal lumen possibly mediated by local hepcidin gene expression. These changes may be induced in the liver as well as in the other organs by the main acute-phase-mediators. During acute liver injury when liver is a direct target organ for the injuring noxae, acute-phase cytokines produced locally can play crucial role in iron regulation and could be responsible for the changes of the gene expression involved in iron metabolism.

1. INTRODUCTION

1.1 Iron: An essential element for the life

Iron is vital for almost every organism by participating in a wide variety of metabolic processes, including; oxygen transport, DNA synthesis, electron transport (Lieu *et al*, 2001). The metal serves as a cofactor for many heme and nonheme iron proteins (Camaschella, 2005; Hentze *et al*, 2004). The redox ability of iron; however, can lead to the production of oxygen free radicals, which can damage various cellular components. For this reason, organisms must regulate the body iron levels to provide enough for their cellular needs without developing the toxicity associated with iron excess. Unlike many other nutrients, the body lacks a defined mechanism for the active excretion of iron, so body iron levels must be tightly regulated at the point of absorption in the proximal small intestine because excessive iron leads to tissue damage because of free radical formation (Frazer *et al*, 2003). Under inflammatory conditions induced by invasion of the body by microorganisms or during liver injury a diversion of iron traffic from the circulation to storage sites of reticuloendothelial system occurs to minimize the availability of this essential element for the microbial proliferation and growth (Weiss, 2005).

1.2 Acute-phase-response

The acute-phase-response (APR) is the defense reaction of an organism against infectious agents that attack its integrity. This reaction is aimed to restrict the area of damage on one side and to eliminate, or at least isolate, the damaging agent on the other side. Every agent, which leads to the loss of the integrity of tissues, induces a local reaction, known as *inflammation* which influences the iron balance in whole organism (Roy *et al*, 2004; Ramadori and Christ, 1999). “Acute-phase” cytokines are responsible for the more generalized signs or symptoms of the APR, such as fever, loss of appetite, muscular pain, leukocytosis, hyperglycemia, hypoferraemia (Ramadori and Christ, 1999) and for the dramatic changes of the protein synthesis taking place in the liver. The purpose of these systemic and metabolic changes are to control the defense mechanisms, to maintain vital body functions during inflammation, and finally, to restore body homeostasis (Moshage, 1997).

1.3 Acute-phase cytokines

Inflammatory cells recruited to the site of injury produce and/or induce the production of acute-phase cytokines, the main soluble factors released into the vascular system and responsible for the onset, progression and resolution of the APR (Gruys *et al*, 2005; Sheikh *et al*, 2006a; Streetz *et al*, 2001; Weiss, 2005). The cytokines of the APR can be classified into two major groups on the basis of the similarities in their signal transduction via specific receptors (Table 1).

Table 1: Major types of cytokines of the acute-phase-response with major function.

IL-1-type cytokines	**Role in the APR**	**IL-6-type cytokines**	**Role in the APR**
IL-1α	Modulation	IL-6	Induction
IL-1ß	Induction	CNTF	Modulation
TNF-α	Induction	OSM	Modulation
TNF-ß	Modulation	LIF	Modulation
		Cardiotropin 1	Modulation
		IL-11	Modulation

CNTF, ciliary neurotrophic factor; **IL**, interleukin; **LIF**, leukemia inhibitory factor; **OSM** oncostatin M; **TNF**, tumor necrosis factor.

Interleukin- (IL) 6 from the group of IL-6-type cytokines and IL-1β together with tumor necrosis factor (TNF)-α from the group of IL-1-type cytokine are considered to be the major mediators of the APR. At the inflammatory sites, IL-6 is produced by macrophages, endothelial cells, and fibroblasts (Ramadori and Christ, 1999). The release of mature IL-1β by macrophages seems to take place only during or after cell death (Perregaux and Gabel, 1998). TNF-α is synthesized mainly by mononuclear phagocytes recruited at the sites of damage and by tissue macrophages (Ramadori and Christ, 1999). While IL-6, IL-1β, and TNF-α are the inducers of acute-phase protein gene expression, other cytokines (Table 1) were shown to modulate this expression (Moshage, 1997).

1.4 Acute-phase proteins and their regulation

Another important aspect of the APR is the radically *altered protein biosynthetic profile of the liver*. The liver is the central organ of the cytokine activity due to the fact that it hosts hepatocytes, which are highly susceptible to the activity of the cytokines in a variety of physiological and pathophysiological processes. Moreover, the non-parenchymal cells of the liver, in particular Kupffer cells (KCs), the resident tissue macrophages of the liver, are able to synthesize a variety of cytokines that may act systemically on any other organ of the body, or in a paracrine manner on hepatocytes and other non-parenchymal liver cells and are responsible for the induction of **acute-phase proteins** (APPs).

Under normal circumstances, the liver synthesizes a characteristic range of plasma proteins at steady state concentrations. Most of the APPs are synthesized by hepatocytes which have important functions to perform. Proteins with a transient increase in synthesis and plasma concentration are called positive acute-phase proteins (APP), e.g., α2-macroglobulin in rats. Higher plasma levels of APPs are required during the APR following an inflammatory stimulus. Synthesis of so-called the *major APPs* can increase to 1000-fold over normal levels. This group includes serum amyloid A (SAA) and either C-reactive protein (CRP) in humans or its homologue in mice, serum amyloid P component (SAP). The so-called *negative APPs* decrease in plasma concentration during the APR to allow an increase in the capacity of the liver to synthesize the induced APPs e.g., albumin, transferrin, α-1 lipoprotein (Ramadori *et al*, 1985; Ramadori and Christ, 1999).

Liver is the main organ of the reticuloendothelial system; it may probably be the organ responsible for the hypoferraemia under APR. How is iron transported into the liver, which receptors are involved and what is the real fate of the iron during inflammation is still a matter of debate.

1.5 Models of the APR

To study APR *in vivo*, two types of animal models are generally used (Ramadori and Christ, 1999).

1.5.1 LPS induced APR

In rodents the APR is induced by intraperitoneal or intravenous administration of the bacterial lipopolysaccharide (LPS). It is characterized by systemic inflammation and rapid circulatory increase of all three major pro-inflammatory cytokines: IL-6, IL-1 β and TNF-α (Lang *et al*, 2003; Ulich *et al*, 1990; Ulich *et al*, 1991).

1.5.2 Turpentine oil induced APR

In the other model of APR induction, a sterile abscess of hind limb muscle is induced by subcutaneous or intramuscular injection of turpentine oil (TO) which causes a local inflammation and subsequent systemic APR (Fey and Fuller, 1987). This model is characterized by local increase in IL-1β and TNF-α, and circulatory increase in IL-6 (Luheshi *et al*, 1997; Sheikh *et al*, 2006a).

1.6 Models of acute liver injury

Different models of acute liver injury are established to study the iron regulation in response to the liver injury. In the present study, we selected two models of acute liver injury where liver was the direct target organ for the injury.

1.6.1 Partial-hepatectomy-induced APR

The characterization of liver regeneration in mammals is of primary importance to provide better understanding of hepatic tissue repair. The APR during liver regeneration is characterized by increased expression of fibrinogen, haptoglobin and α_2-macroglobulin (Fulop *et al*, 2001). APPs have anti-inflammatory and homeostatic action. Haptoglobin is a carrier protein of globin chains of hemoglobin, it is necessary in order to avoid iron loss during haemolysis, and it reduces the free radical production of serum hemoglobin. Fibrinogen is a basic participant of blood clotting, whilst α_2-macroglobulin is protease inhibitor; however, these proteins have very diverse effects, including immunomodulatory action and cytokines binding (e.g. IL-1) (Fulop *et al*, 2001; Jakab and Kalabay, 1998; Dobryszycka, 1997). IL-6 is a key cytokine in liver regeneration. Mice with targeted disruption of the IL-6 gene had impaired liver regeneration characterized by liver necrosis and failure (Clavien, 1997).

1.6.2 CCl_4-induced acute liver injury

To study the APR as a result of acute liver injury induced by a single dose of 3 ml/kg body weight of CCl_4 corn oil mixture (1:1 v/v) administered orally by gastric catheter to the rats. In response to hepatocellular damage, inflammatory cells, mainly monocytes/macrophages, but also lymphocytes and granulocytes accumulate within the deteriorated parenchyma (Butcher and Picker, 1996; Imhof and Dunon, 1995; Knittel *et al*, 1999; Ley, 1996). As a result of CCl_4-induced APR the entire course of liver injury could be classified into three stages, the first stage for aggravation of injury until 9 h, the second from 9 to 24 h, and the third for repair of injury or regeneration of liver after 48 h (Morigasaki *et al*, 2000).

1.7 In vitro studies

Once it became obvious that the liver is a primary target organ for the APR, the individual liver cell types were introduced in culture to investigate a hierarchy of the events triggering the full APR in the liver. Besides the ability to respond to the cytokine action, different cell types within the liver are also able to express IL-1β, TNF-α, IL-6, and other modulator cytokines of the hepatic APR (Ramadori and Christ, 1999). However, despite of limited evidence on the production of cytokines by hepatic sinusoidal endothelial cells (Feder *et al*, 1993) and hepatic stellate cells (Ramadori and Armbrust, 2001), Kupffer cells are by far the most active intrahepatic "amplifiers" of the systemic APR in the liver by liberating a second wave of proinflammatory cytokines, promoting autocrine stimulation and paracrine hepatocyte stimulation (Decker, 1990). Hepatocytes express a great variety of receptors for cytokines, growth factors, and prostaglandins and therefore, represent the major target for a multiple set of mediators involved in both systemic and local host defense reactions. Hepatocytes are also known to express and secrete the cytokines of the APR, which might further stimulate adjacent hepatocytes and neighboring Kupffer cells (Rowell *et al*, 1997).

1.8 Iron regulatory proteins

1.8.1 Hepcidin antimicrobial peptide; (HAMP)

Recently discovered hormone hepcidin previously reported as LEAP-1 (liver-expressed antimicrobial peptide) (Krause *et al*, 2000); a 25-amino acid, 2–3 kDa, cationic

peptide (McGrath, Jr. and Rigby, 2004) is an acute-phase protein mainly synthesized by hepatocytes in the liver. It is a major regulator of iron balance in the intestinal mucosa which seems to have a significant role during inflammation and major contributor to the hypoferraemia associated with inflammation (Means, 2004). In addition, it regulates maternal-fetal iron transport across the placenta (Lipinski and Starzynski, 2004). It affects the release of iron from hepatic stores and macrophages involved in the recycling of iron from hemoglobin by directly binding to the cellular iron exporter Fpn.-1 (Deicher and Horl, 2006; Ganz, 2004). In mouse lack of hepcidin gene results in excess iron accumulation (Nicolas *et al*, 2001) which suggest that the peptide can repress iron absorption. Its production is increased during inflammation and iron overload condition (Balogh *et al*, 2004). During the APR; however, hepcidin expression changes more rapidly than the expression of iron transporters (Anderson *et al*, 2002).

1.8.2 Hemochromatosis genes

The haemochromatosis gene, HFE, transferrin receptor 2 (TfR2) and hemojuvelin (Hjv) potentially facilitate the transcription of hepcidin. In the majority of patients with HH a C282Y mutation in HFE was found (Feder *et al*, 1996). HFE is a transmembrane protein with a structural similarity to major histocompatibility class 1 molecules (MCH 1) (Bennett *et al*, 2000). Like other MHC1 molecules HFE associates with β2 microglobulin, which allows it to be targeted to the cell surface. It has been proposed that the phenotype of C282Y heterozygotes and homozygotes of HFE gene may be modified by heterozygosity for mutations which disrupt the function of hepcidin in iron homeostasis, with the severity of iron overload corresponding to the severity of the HAMP mutation (Merryweather-Clarke *et al*, 2003). The HH phenotype was further demonstrated in murine studies using both C282Y homozygous and HFE knock-out mutants (Zhou *et al*, 1998). Further studies showed this mutation disrupted a disulphide bond believed to be crucial to the interaction of HFE with β2 microglobulin (Feder *et al*, 1997; Waheed *et al*, 1997). This leads to HFE accumulating in the cytoplasm and being more easily degraded.

Furthermore, HFE forms complex with transferrin receptor 1 (TfR1) in duodenal crypt enterocytes (Waheed *et al*, 1997), liver homogenates and transfected cell lines (Gross *et al*, 1998). Holotransferrin competes with HFE in binding its receptor TfR1.

Following the dissociation of HFE, transferrin bound TfR1 is internalized into an acidified endosome causing iron release into the labile iron pool (Ponka and Lok, 1999; Fleming and Britton, 2006). The C282Y mutation in HFE prevents its association with TfR1 and so disrupts this process so contributing to the tissue iron overload of HH. Following the dissociation of HFE, transferrin-bound TfR1 is internalized into an acidified endosome causing iron release into the labile iron pool (Ponka and Lok, 1999).

TfR2 is a membrane glycoprotein that mediates cellular iron uptake from holotransferrin (Kawabata *et al*, 1999). TfR2 mutations developed periportal hepatic iron loading, splenic iron sparing, and elevated serum transferrin saturations by 4 weeks of age in mice homozygous for the Y245X mutation (Fleming *et al*, 2002).

In the mouse Hjv is expressed in the liver by periportal hepatocytes. Hjv deficient mice exhibit iron overload and fail to express hepcidin in response to dietary or injected iron. However, these mice retain the ability to upregulate hepcidin in response to acute inflammation induced by either lipopolysaccharide (Niederkofler *et al*, 2005) or its downstream products, IL-6 and TNF-α. Disruption of each of the four genes leads to a diminished hepatic release of hepcidin consistent with both, a dominant role of hepcidin in hereditary haemochromatosis and an upstream regulatory role of HFE, TfR2 and HJV on hepcidin expression (Deicher and Horl, 2006).

1.8.3 Transferrin “the Iron carrier”

Transferrin (Tf) is the product of an ancient intragenic duplication that led to homologous carboxyl and amino domains, each of which binds one ion of ferric iron. Tf carries iron from the intestine, reticuloendothelial system, and liver parenchymal cells to all proliferating cells in the body. The amount of iron absorbed by enterocytes is influenced by a variety of factors together with variations in body iron stores, changes in the rate of erythropoiesis, hypoxia, inflammation, and pregnancy. Circulating levels of diferric transferrin makes an ideal indicator of body iron demand because the protein is preferentially taken up by cells that require iron. Therefore, when cellular iron demand increases, diferric transferrin levels would decrease, and vice versa. Diferric Tf interacts with the TfR1 and is internalized by receptor mediated endocytosis (Frazer and Anderson, 2005). Diferric transferrin involves both HFE and TfR2 on the hepatocyte plasma membrane. HFE and transferrin bind to overlapping sites on TfR1 and diferric

transferrin out compete HFE for TfR1 binding such that higher diferric transferrin levels would lead to an increased amount of free HFE on the cell surface (Frazer and Anderson, 2003). Evidence for such a competition has been reported recently using tagged HFE constructs transfected into cell lines (Giannetti and Bjorkman, 2004). Under normal conditions, HFE is found both on the plasma membrane and in TfR1 containing endosomes; however, when treated with diferric transferrin, HFE is only detectable on the plasma membrane, indicating that diferric transferrin out competes HFE for TfR1 binding. Furthermore, the unbound HFE on the cell surface is able to stimulate a signal transduction pathway that leads to an increase in the expression of hepcidin (Frazer and Anderson, 2005).

1.8.4 Iron transporters

Different proteins are involved in the iron absorption from the brush border membrane in the intestinal lumen and transport across the basement membrane. These molecules include a brush-border ferric iron reductase known as duodenal cytochrome b (Dcytb) (McKie *et al*, 2001) a brush-border ferrous iron transporter divalent metal transporter 1 (DMT1) (Gunshin *et al*, 1997; Vulpe *et al*, 1999), the basolateral iron exporter, ferroportin-1 (Fpn.-1) (Canonne-Hergaux *et al*, 2005; McKie *et al*, 2000) and a basolateral ferroxidase hephaestin (Heph) (Vulpe *et al*, 1999). The expression of each of these molecules can be stimulated to varying degrees under iron-deficient conditions. It has been shown that Dcytb-activity in iron deficiency is stimulated via enhanced protein expression, whereas in haemochromatosis due to mutations in the HFE gene it is upregulated posttranslationally. Haemochromatosis patients with no mutations in HFE do not have increased Dcytb activity (Zoller *et al*, 2003).

Fpn.-1 is an iron transporter located on the basolateral side in duodenal enterocytes, on the macrophages and hepatocytes. This metal transporter is regulated by hepcidin during inflammatory conditions. Hepcidin binds, internalizes and causes degradation of Fpn.-1 (Figure 1). As a result no transporter is available for iron release from the store. It has been postulated that the posttranslational regulation of Fpn.-1 by hepcidin may complete a homeostatic loop regulating iron plasma levels and the tissue distribution of iron (Fleming and Bacon, 2005; Nemeth *et al*, 2004a; Sheikh *et al*, 2006b). Heph plays important role in the diminished transport of iron from the mucosal cell to the

circulation. It interacts with the Fpn.-1 and facilitates the movement of iron across the membrane. On the basis of its homology with ceruloplasmin, it has been proposed that Heph is a ferroxidase necessary for iron release from intestinal epithelial cells. However, Heph expression contrast to Cp, which is highly expressed in liver and expressed to a lesser extent in other tissues including brain and lung, but is not expressed in intestine (Vulpe *et al*, 1999).

1.8.5 Iron storage protein

Since an excess of free iron is catalyzing the fenton reaction, most of the intracellular iron is sequestered in the iron storage protein ferritin (Mehlhase *et al*) as it is the major protein involved in iron sequestration and detoxification. Ferritin is capable of storing up to 4,500 atoms of ferric iron. H-chains are important for Fe (II) oxidation as iron enters the cell in reduced form and is stored in oxidized Fe (III) form. Ferritin-H mRNA is regulated by iron at the translational leve1. Ferritin-H RNA is present as stored messengers in the cytoplasm and recruited to the ribosome when iron levels are increased.

1.8.6 Iron responsive elements and IRE- binding proteins

Translational activation of ferritin-H, TfR1 and DMT1 in response to varied iron availability is mediated by an RNA stem loop known as iron responsive elements (IREs). IREs are translational regulatory sequences in the 5`-untranslated regions (UTRs) of ferritin mRNA (Mok *et al*, 2004; Rogers, 1996) and in the 3`-UTRs of TfR1 and DMT1 (Lee *et al*, 1998) mRNA. The cytoplasmic IRE-binding protein (IRE-BP) interacts with the iron-responsive elements of mRNA. The iron status of the cell determines the ability of the IRE-BP to bind to IRE through reversible oxidation-reduction of sulfhydryl groups that are critical for the high affinity RNA/protein interaction. The IRE-BP plays a central role in cellular iron homeostasis by regulating ferritin mRNA translation and TfR1 mRNA stability (Gray and Hentze, 1994; Tacchini *et al*, 2002). DMT1 gene expression increases when intracellular iron levels are low as a result of binding of IRE-BP to DMT1 mRNA (Frazer and Anderson, 2005). IRE-BPs are of two types. IRE-BP1 is a bifunctional protein with mutually exclusive functions as an IRE RNA-binding protein or as the cytoplasmic isoform of aconitase. Aconitases are iron-sulfur proteins and a 4Fe-4S

cluster is required for their enzymatic activity (Eisenstein, 2000). The IRE-BP2 contained an inserted stretch of 73 amino acids between amino acids 37 and 38 of IREB1; the IREB2 gene product lacked a region homologous to the sequences between amino acids 436 and 470 of IREB1 (Rouault *et al*, 1990). IRE-BP2 is less abundant than IRE-BP1 in most cells (Hentze and Kuhn, 1996). The strongest expression is in intestine and brain (Henderson *et al*, 1993). Human IRE-BP2 is 57% identical to human IRP1. IRE-BP2 has a molecular mass of 105 kDa, which is slightly larger than that of IRE-BP1 due to a 73-amino acid insertion and this 73-amino acid insertion mediates IRE-BP2 degradation in iron-replete cells (Iwai *et al*, 1995).

1.9 Aim of the study

Hepcidin is a recently discovered hormone which is responsible for iron regulation and its expression increases during microbial invasion, inflammation or injury. So far most of the work to study the body response under acute-phase condition has been done in mice/rats injected with LPS. LPS induced endotoxemia of the mouse systemically simulates the infection with Gram-negative bacteria. As bacterial endotoxin LPS induces hepcidin gene expression and inhibits the Hjv gene expression (Krijt *et al*, 2004), these result let us assume that besides bacterial endotoxin, an irritation or injury induced release of acute-phase mediators can also lead to the changes in the expression of iron regulatory genes. Therefore, in the present work we selected three different models of acute-phase condition induced by different damaging noxae;

a. Turpentine oil induced sterile abscess and induction of acute-phase response.
b. Partial-hepatectomy-induced acute liver injury.
c. CCl_4-induced acute liver injury.

TO induced APR differs from the endotoxemia model in that induction of a sterile muscle abscess in the rat by injection of turpentine oil causes a local inflammation with a subsequent systemic induction of the cytokine mediated APR (Ramadori and Christ, 1999; Ramadori *et al*, 1985). TO is believed to induce aseptic local abscesses without detectable injury to other tissues (Boelen *et al*, 2005; Ramadori and Meyer zum Buschenfelde, 1990; Tron *et al*, 2005; Wusteman *et al*, 1990). This is clearly different from agents inducing an acute systemic response, like the administration of bacterial

endotoxin (lipopolysaccharide) (Boelen *et al*, 2005). Thus, the TO-induced acute-phase response model allows studying the effect of cytokines produced at distant sites on the liver. It reproduces changes observed in human disease states (Basso *et al*, 2005; Bodet *et al*, 2006; Boelen *et al*, 2005; Gabay and Kushner, 1999; Halter *et al*, 2005; Kim *et al*, 2002; Stoeck *et al*, 2006).

Induction of acute-phase-reaction by Partial-hepatectomy results the loss of the tissue as well the beginning of the cell division, healing of the wound and regulation of body iron level. CCl_4 induces hepatocellular damage, loss of hepatocellular population followed by the infiltration of inflammatory cells (Butcher and Picker, 1996; Imhof and Dunon, 1995; Knittel *et al*, 1999; Ley, 1996) and regulation of body iron level which results in hypoferraemia so as to protect the body/inflamed area from invasion of microbes by limiting the availability of iron and rendering the growth of the microbes.

Therefore, the aim of the present work is to study;

1. The regulation of hepcidin gene expression in different models of inflammation or liver injury in rats.
2. The role of interleukin-6, the principle mediator of inflammation, in the regulation of hepcidin and other iron metabolism genes expression *in vivo*.
3. The regulation of iron metabolism genes expression *in vitro* using primary cultures of isolated rat hepatocytes stimulated with different pro-inflammatory cytokines.

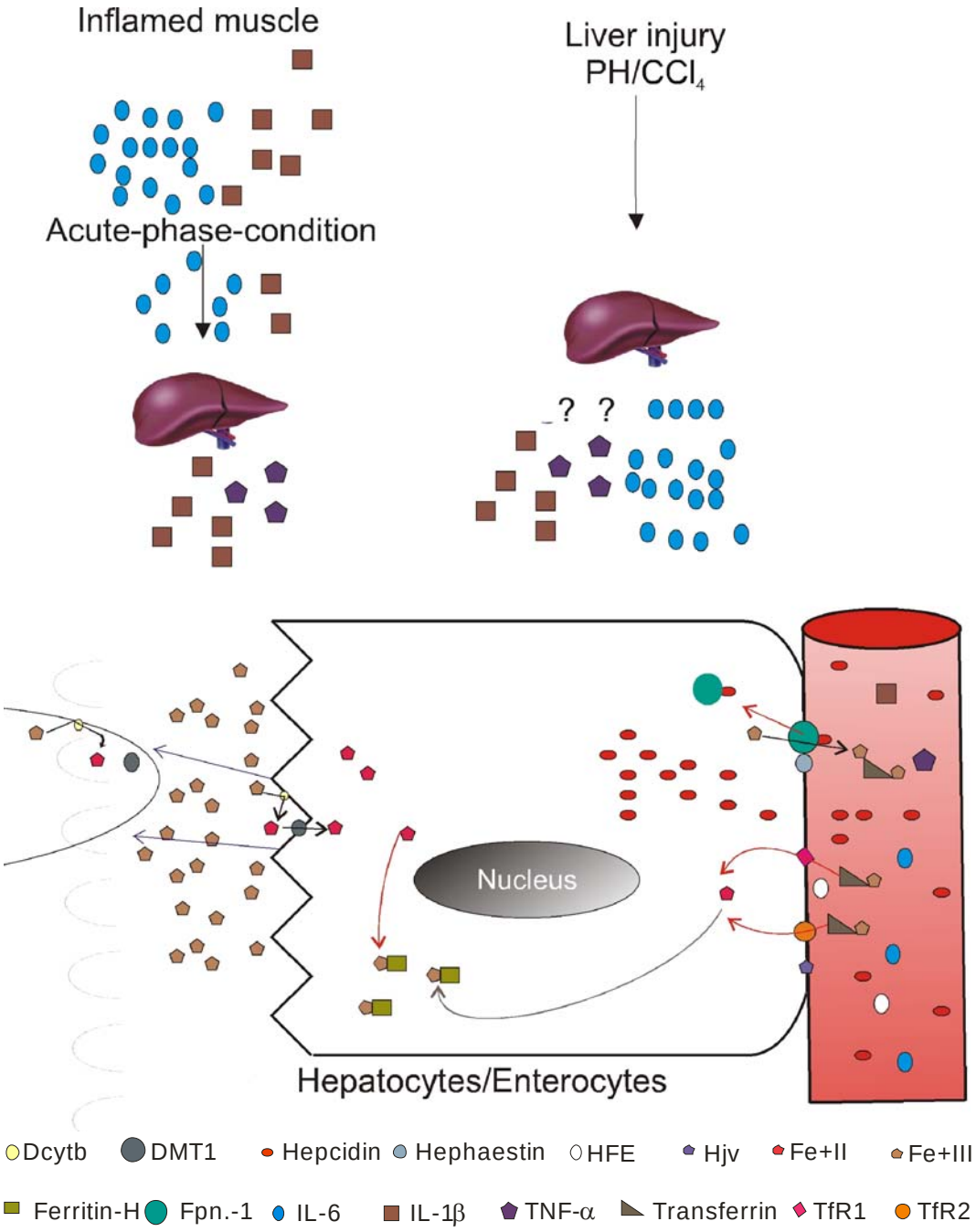


Figure 1: Expression of acute-phase cytokines during different models of acute-phase condition and interaction of different proteins known to be involved in iron regulation. Black arrows indicate the movement of iron during normal condition. Red arrows show the regulation of different proteins and movement of iron during acute-phase-condition.

2. MATERIALS

2.1 Animals

Male Wistar rats (about 200 g body weight) were purchased from Harlan-Winkelmann (Borchen, Germany) and kept under standard conditions with 12-hours light/dark cycles, access to fresh water and food pellets *ad libitum* at room temperature of 19-23°C. The rats consumed 12-15 g food (rat diet "ssniff", Spezialitäten GmbH, Soest, Germany) and 12-25 ml water per day and had a 30-40 g gain of weight per week. Animals were used for the experiments not earlier than 6 days after arrival. The preparation of hepatocytes was performed during the first 3 h of the light phase. All animals were taken care of according to the institutional guidelines, the German convention for the protection of animals and NIH guidelines.

2.1.1 Induction of acute-phase-reaction

APR was induced in ether anesthetized rats by intramuscular injection of 5mg/kg TO in both right and left hind limbs of the animals. Control animals were not given any injection. Animals were sacrificed 0.5, 1, 2, 4, 6, 12, and 24 hours after TO injection under pentobarbital anesthesia (400 mg/kg body weight). Different organs as well as hind limb muscle tissue including the TO injected area were excised, rinsed with physiological sodium saline, snap frozen in liquid nitrogen and stored at -80°C till further use.

2.1.2 Partial-hepatectomy (PH)

PH was performed under ether anesthesia by midventral laparotomy, ligation of the median anterior and left lateral hepatic lobes separately with a silk suture, and complete excision of ligated lobes (Figure 2). Control animals were subjected to sham operation (SO) by the same operator. The SO consisted of a midventral laparotomy of similar extent, gentle manipulation of the liver, followed by surgical closure of the abdomen similar to partially hepatectomized rats. Rats were sacrificed 2, 4, 8, 16, 24 and 48 hours after PH. Livers were snap frozen in liquid nitrogen and stored at -80°C.

2.1.3 CCl_4-induced liver injury

Rats were orally administered 3 ml/kg (body weight) of CCl_4 corn oil mixture (1:1 v/v) by means of gastric catheter. Control animals were given the same volume of

corn oil. Rats were sacrificed 3, 6, 12, 24 and 48 hours after CCl_4-administration. The livers were perfused with saline solution, removed, snap frozen in liquid nitrogen and stored at -80°C until further use.

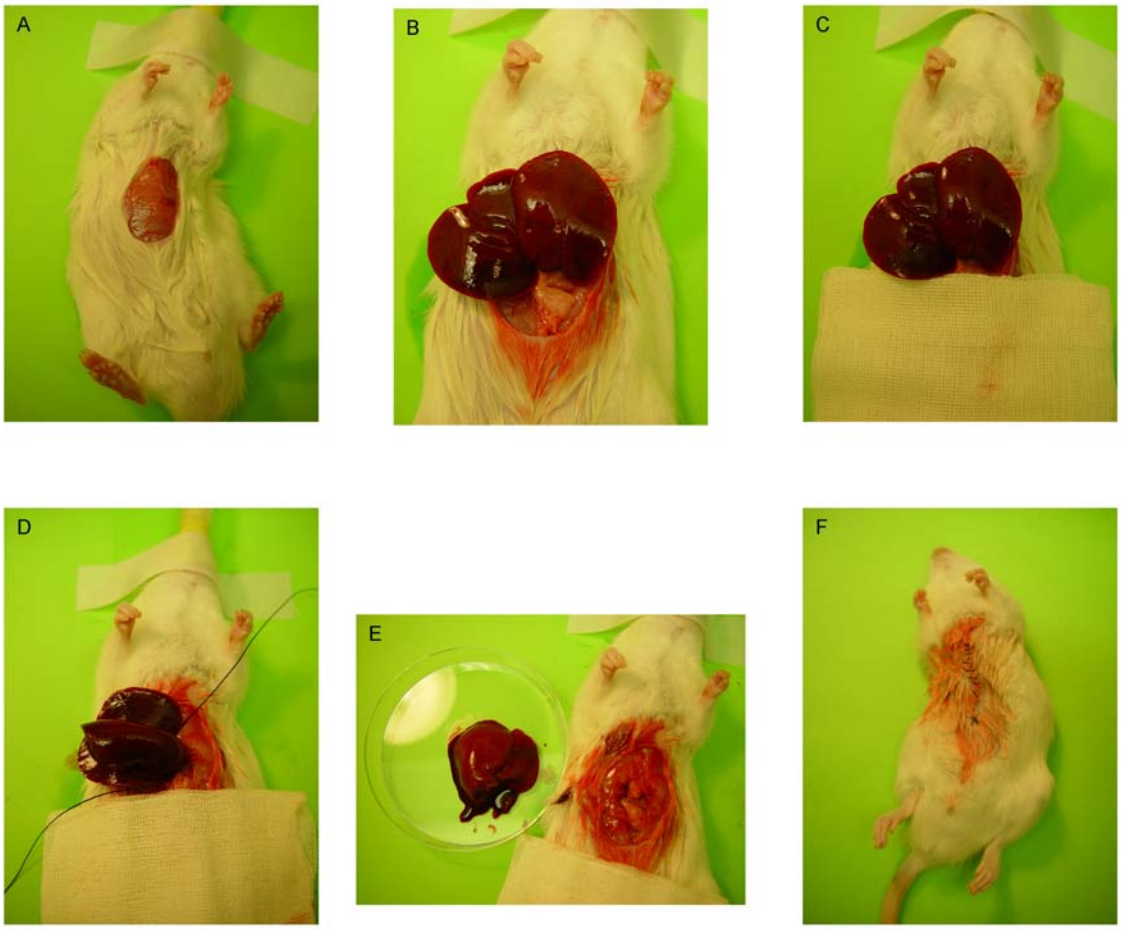


Figure 2: Partial-hepatectomy in the rat to induce acute-liver injury. PH was performed under ether anesthesia by midventral laparotomy *(A-C)*, ligation of the median anterior and left lateral hepatic lobes separately with a silk suture, and complete excision of ligated lobes *(D-F)*.

2.1.4 Blood samples

Blood samples were collected from inferior vena cava of the control and treated animals, allowed to clot overnight at 4°C and centrifuged for 20 min at 2000×g. Serum was removed and stored at –20°C.

2.2 Proteins, enzymes and protein standards

- Bovine serum albumin (BSA) (11930) Serva /Heidelberg, Germany

- Collagenase H (1 087 789) Roche /Mannheim, Germany
- Collagenase type I (35-4236), Becton Dickinson Bio Sciences /MA, USA
- DNase I (1 284 932) Roche /Mannheim, Germany
- Go Taq® Green master mix, 2× (M711B) Promega /Madison, USA
- Insulin, Bovine (977 420)Roche /Munich, Germany
- Molecular weight markers (RPN 800) Amersham Pharmacia Biotech /Freiburg, Germany
- Pronase E (1.707433) Merck /Darmstadt, Germany
- Recombinant rat IL-6 (400-06), IL-1β (400-01B), TNF-α (400-14) and IFN-γ (400-20) PeproTech Inc. /Rocky Hill, USA
- Klenow enzyme (1 008 412) Roche /Mannheim, Germany

2.3 Real-time Polymerase chain reaction:

- Platinum SYBR Green qPCR-UDG mix (11733-046) Invitrogen /Karlsruhe, Germany
- dATP (1 051 440) Roche /Mannheim, Germany
- dGTP (1 051 466) Roche /Mannheim, Germany
- dCTP (1 051 58) Roche /Mannheim, Germany
- dTTP (1 051 482) Roche /Mannheim, Germany
- Primer oligo $(dT)_{15}$, (10 814 270 001) Roche /Mannheim, Germany
- Moloney-Murine leukemia virus reverse transcriptase (M-MLV RT) (28025-013) Invitrogen /Karlsruhe, Germany
- Protector RNase inhibitor, (3 335 399) Roche /Mannheim, Germany
- 5× RT buffer (Y00146) Invitrogen /Karlsruhe, Germany
- 0.1 M DTT (Y00147) Invitrogen /Karlsruhe, Germany

2.4 Detection, purification and synthesis systems (kits)

- Hepcidin pro-hormone ELISA, (EIA4015) DRG International, Inc., USA
- NE Blot® Kit, (N1500L) New England Biolabs /Schwalbach, Germany
- Nick translation kit, (18160-010) Invitrogen /Karlsruhe, Germany.
- NucleoSpin® RNAII kit (740 955.50), Macherey-Nagel /Düren, Germany

- Quantikine® M rat IL-6 (R6000B), IL-1β (RLB00), TNF-α (RTA00) and IFN-γ (RIF00) immunoassay kit, R&D Systems /Wiesbaden, Germany.
- Nucleospin extract II, (740609.50) Macherey-Nagel /Düren, Germany

2.5 Stock solutions

All stock solutions were prepared either in double distilled water or (for experiments with RNA) in RNase-free water. The pH values of the stock solutions presented here were adjusted at 25°C.

Stock solution		Vol./weight	Final concentration
APS 10%	For final volume 10 ml.		10%
	APS	1g	
	dd H_2O	to 10ml	
	The solution was dispensed into 100 µl aliquots and stored at −20°C.		
Citric acid	For 100 ml Final volume		0.25 M
	Citric acid	4.8 g	
	RNase-free H_2O (Ampuwa®)	100ml	
	The solution was stored at room temperature.		
EDTA 0.5 M	For 100 ml Final concentration.		0.5 M
	EDTA (disodium salt)	18.61 g	
	RNase-free H_2O (Ampuwa®)	to 100 ml	
	pH was adjusted with 5N NaOH to 8.0. The solution was sterile filtered and stored at room temperature.		
Sodium acetate 2 M	For 100 ml final concentration.		2 M
	Sodium acetate	16.408 g	
	RNase-free H_2O (Ampuwa®)	to 100 ml	
	pH was adjusted with acetic acid to 5.4. The solution was stored at 4°C.		

Stock solution		Vol./weight	Final concentration
PBS 10×	For 1 l Final concentration.		
	NaCl	81.82 g	1.4 M
	KCl	2 g	27 mM
	Na_2HPO_4	14.2 g	100 mM
	KH_2PO_4	2.45 g	18 mM
	dd H_2O	to 1 l	
	pH was adjusted with HCl to 7.3. The solution was sterile filtered and stored at room temperature.		
SDS 20%	For 100 ml Final concentration		20%
	SDS	20 g	
	dd H_2O	to 100 ml	
	In the case of precipitation of SDS, the solution was warmed until clear.		
Sodium citrate 0.25 M	For 100 ml Final concentration.		0.25 M
	Sodium citrate	7.35 g	
	RNase-free H_2O (Ampuwa®)	to 100 ml	
	pH was adjusted with 0.25 M citric acid to 7.0; the solution was stored at room temperature.		
Tris-HCl 2 M	For 1 l Final concentration.		2 M
	Tris-HCl	315.2 g	
	RNase-free H_2O (Ampuwa®)	to 1 l	
	pH was adjusted with HCl to 7.4. The solution was sterile filtered and stored at 4°C.		

2.6 Chemicals

All chemicals were of analytical grade and obtained from commercial sources as indicated.

Amersham Pharmacia Biotech /Freiburg, Germany

- [α-32P]-labeled deoxy-cytidine-triphosphate (specific activity 3,000 Ci/mmol),
- Ficoll® 400

Biochrom /Berlin, Germany

- M199,
- FCS (fetal calf serum)
- Trypan blue

Bioline /Luckenwalde, Germany

- dNTP master mix

Bio-Rad /Munich, Germany

- Tween 20,
- Mixed bed resin AG 501-X8(D)

Böhringer /Mannheim, Germany

- Ampicillin

Fresenius /Bad Hamburg, Germany

- Ampuwa® water

Invitrogen /Karlsruhe, Germany

- Guanidine isothiocyanate

MBI Fermentas /Vilnius, Lithuania

- 6×loading dye solution,
- GeneRuler™ 100bp DNA Ladder Plus
- GeneRuler™ 1kb
- DNA Ladder Plus

Merck /Darmstadt, Germany

- All usual laboratory chemicals
- Acetic acid glacial
- Acetone
- Bromophenol blue
- Carbon tetra chloride
- Ethanol
- 37% formaldehyde

- Formamide
- Glucose
- Glycerol
- Kaiser's glycerol
- Gelatin
- Meyer's hemalaun
- Methanol
- β-mercaptoethanol
- Penicillin G
- streptomycin
- TEMED

Merial /Hallbergmoos, Germany

- Pentobarbital sodium (Narcoren®)

PAA /Linz, Austria

- L-Glutamine

Paesel and Lorei /Frankfurt, Germany

- Cesium chloride

Roth /Karlsruhe, Germany

- Glycine
- Sodium dodecyl sulfate (SDS)

Serva /Heidelberg, Germany

- Tris-HCl

Sigma-Aldrich /Munich, Germany

- All usual laboratory chemicals
- Ammonium persulfate
- Antifoam A
- Citric acid
- Dexamethasone
- DMSO
- DTT

- EDTA
- Ethidium bromide
- HEPES
- MOPS
- N-lauroylsarcosyl
- Sodium acetate
- Sodium citrate
- TRITON X-100

Stratagene /Heidelberg, Germany

- QuikHyb® Hybridization Solution

University Hospital Apotheke /Göttingen, Germany

- Carbon tetra chloride
- Turpentine oil

Zinsser Analytic /Frankfurt, Germany

- Scintillation liquid

2.6 Other materials

- Braunules 2G14, Braun /Melsungen, Germany.
- Culture dishes (60 mm) Falcon, Becton Dickinson /NJ, USA
- G-50 DNA grade, Amersham Pharmacia Biotech /Freiburg, Germany
- Hybond N nylon membrane, disposable NICK columns prepacked with Sephadex® Amersham Pharmacia Biotech /Freiburg, Germany
- Hybridization glass tubes, Biometra /Göttingen, Germany
- Polyallomer thin-walled centrifuge tubes (5 ml), Beckman /Munich, Germany
- Safe-Lock tubes (0.2, 0.5, 1.5 and 2 ml), Eppendorf /Hamburg, Germany
- Scintillation vials (5 ml), Zinsser Analytic /Frankfurt, Germany
- Serological pipettes (2, 5, 10, 25 ml), transfer pipettes, plastic tubes (15 and 50 ml), Sarstedt /Germany
- Sterile filter Nalgene, 0.2 µm, Sartorius /Göttingen, Germany
- Sterile filter pipette tips, Biozym /Oldendorf, Germany
- Syringes BD Discardit 2ml, 5ml, 20ml Becton Dickinson /NJ, USA

- X-ray films HyperfilmTM, Amersham Biosciences /Freiburg, Germany

2.7 Instruments

- Automatic pipettes, type Reference®, Eppendorf /Hamburg, Germany
- Automatic pipettes, type Pipetman, Gilson /Bad Camberg, Germany
- ABI Prism 7000 Thermal cycler (Applied Biosystems), USA
- Eagle Eye™ system with built-in ultraviolet emitter, video camera and frame Integrator, Stratagene Europe /Amsterdam, The Netherlands
- Electro blotting apparatus, type Mini Trans-Blot®, Bio-Rad /Munich, Germany
- Electrophoresis apparatus, type Mini-Protean® III, Bio-Rad /Munich, Germany
- Gas controlled incubators, Heraeus-Electronic /Hannover, Germany
- Hybridization oven, Biometra /Göttingen, Germany
- Ice machine, Ziegra /Isernhagen, Germany
- Incubator with shaking for cell culture, model 3-25, New Brunswick Scientific Co., Inc. /Edison, New Jersey, USA
- Liquid scintillation counter Wallac 1409 /Turku, Finland
- Magnetic mixer with warming, type M21/1 Framo-Gerätetechnik /Germany
- Microscope Axioscop with photo camera MC 100 Spot, Zeiss /Oberkochen, Germany
- Microscope Axiovert 25, Zeiss /Oberkochen, Germany
- Microwave oven, Siemens /Germany
- Peristaltic pump P-1, Amersham /Freiburg, Germany
- pH-Meter 761 Calimatic, Knick /Berlin, Germany
- Power supply, Power Pac 300, Bio-Rad /Munich, Germany
- Savant Speed Vac® concentrator, ThermoLife Sciences /Egelsbach, Germany
- Sterile bench, type Lamin Air, TL 2472, Heraeus /Hanau, Germany
- Sterile bench, type MRF 0.612-GS, Prettl Laminarflow und Prozesstechnick /Bempflingen, Germany
- Thermocycler, type Mastercycler® gradient, Eppendorf /Hamburg, Germany
- Thermomixer 5436, Eppendorf /Hamburg, Germany
- Thermostat, Heraeus /Hanau, Germany

- Ultra-Turrax TP 18/10 homogenizer, Janke & Kunkel /Staufen, Germany
- Ultraviolet emitter, 312 nm, Bachofer /Reutlingen, Germany
- UV spectrophotometer, RNA/DNA Calculator GeneQuant II, Pharmacia Biotech /Freiburg, Germany
- UV-light crosslinker, Stratalinker™ 180, Stratagene /Heidelberg, Germany
- Vortex, Genie 2™, Bender and Hobein /Zurich, Switzerland
- Vortex, with platform, Schütt Labortechnic /Göttingen, Germany
- Water bath 1083, GFL /Burgwedel, Germany
- X-ray film cassettes 10×18, Siemens /Germany
- X-ray film-developing machine SRX-101A, Konica Europe /Hohenbrunn, Germany
- Centrifuges and rotors

Bench-top, high speed and ultracentrifuges	
Beckman model J2-21 centrifuge Beckman rotor JE-6B	Beckman /Munich, Germany
Centricon T-2070 ultracentrifuge Centricon rotor TST55.5 – 55000 rpm	Kontron Instruments/Neufahrn, Germany
Eppendorf bench-top centrifuge, type MiniSpin 5415C	Eppendorf /Hamburg, Germany
Hettich Mikro Rapid/K centrifuge Hettich Rotina 3850 centrifuge Hettich Rotina 48RS centrifuge Hettich Rotixa/RP centrifuge	Hettich /Tuttlingen, Germany
Minifuge GL centrifuge	Heraeus-Christ /Osterode, Germany
Sigma 3K30 centrifuge Rotor No. 12156 – 16500 rpm Rotor No. 12153 – 22000 rpm	Sigma Laboratory, Centrifuges /Osterode, Germany

3. METHODS

3.1 Methods of cell biology

3.1.1 Isolation of rat hepatocytes

Hepatocytes were isolated from male Wistar rats by circulating perfusion with collagenase essentially as described previously (Seglen, 1973; Katz *et al*., 1979).

3.1.1.I Liver perfusion

After laparotomy, the *vena portae* was canulated, *vena cava inferior* was ligated above the diaphragm to prevent flow of the perfusion media into a whole body circulation. Finally, the *vena cava inferior* was cut beneath the liver and canulated. The liver was perfused in non-recirculative mode through the portal vein with 150-200 ml CO_2-enriched preperfusion medium at a flow rate of 30 ml/min until the liver was free from blood. To break down components of extracellular matrix, the liver was perfused in recirculative mode with collagenase perfusion medium until it started to feel soft (about 7-11 min).

3.1.1.II Preparation of the hepatocyte suspension

After perfusion, the liver was excised and transferred into a sterile glass beaker filled with culture medium M 199 with additives. Glisson's capsule, i.e. collagen tissue around the liver, was carefully removed and discarded. To obtain a cell suspension, the tissue was disrupted mechanically using sterile forceps. Connective tissue and remainder of the liver capsule as well as big cell aggregates were removed by filtration of the primary cell suspension through a nylon mesh (pore-size 79 μm). Non-parenchymal cells and cell debris were removed by numerous selective sedimentations (20 *g*, 2 min, and 4°C) in wash medium. After the last centrifugation, hepatocytes were suspended in medium M 199 with additives. 50 ml of M 199 was added per 1 g of wet weight of the sedimented cells; the cell suspension typically had a density of about 10^6/2.5 ml.

3.1.1.III Media and solutions for hepatocyte preparation and culture

All media and solutions for cell culture were prepared in double distilled water, further purified by sterile filtration and stored at 4°C. All solutions were prepared not more than one day before the isolation.

Krebs-Ringer stock solution		
	For 1l	Final concentration
NaCl	7 g	120 mM
KCl	0.36 g	4.8 mM
$MgSO_4 \times 7H_2O$	0.296 g	1.2 mM
KH_2PO_4	0.163 g	1.2 mM
$NaHCO_3$	2.016 g	24.4 mM
dd H_2O	to 1 l	
The solution was equilibrated with carbogen and pH was adjusted to 7.35		

Pre-perfusion medium		
	For 1 l	Final concentration
EGTA	95.1 mg	0.25 mM
Krebs-Ringer stock solution	to 1 l	

Collagenase perfusion medium		
	For 100 ml	Final concentration
HEPES	360 mg	15 mM
$CaCl_2 \times 2H_2O$	58.8 mg	4 mM
Collagenase	50 mg	
Krebs-Ringer stock solution	to 100 ml	
The medium was prepared directly prior to isolation, equilibrated with carbogen for 30 min and finally sterile filtered.		

Wash medium		
	For 1 l	Final concentration
HEPES/NaOH pH 7.4	4.77 g	20 mM
NaCl	7.00 g	120 mM
KCl	0.36 g	4.8 mM
$MgSO_4 \times 7H_2O$	0.30 g	1.2 mM
KH_2PO_4	0.16 g	1.2 mM
Bovine serum albumin	4.00 g	0.4%
dd H_2O	to 1 l	

Medium M 199 with additives		
	For 1 l	Final concentration
M199 with Earle's salts without $NaHCO_3$	1 l	
Glucose × H_2O	1.1 g	5.5 mM
HEPES	3.6 g	15 mM
$NaHCO_3$	1.5 g	18 mM
Bovine serum albumin	4.0 g	0.4%
The medium was equilibrated with carbogen until pH reached a value of 7.35. Finally, the medium was sterile filtered.		

3.1.2 Primary culture treatment and harvesting of rat liver cells

Rat hepatocytes cell cultures were performed on 60 mm polystyrol dishes and maintained at 37°C in a 95% air/5% CO_2 atmosphere and saturated humidity.

3.1.2.I Primary culture of rat hepatocytes

Immediately after preparation, fetal calf serum (4 ml/100 ml suspension) was added to the hepatocytes suspension in order to make the efficient cell adhesion to the polystyrol dishes. Furthermore, the antibiotics (1 ml of pen/strep stock solution per 100 ml cell suspension) together with 10^{-7} M dexamethasone and 10^{-9} M insulin as permissive

hormones were added. Rat hepatocytes were plated onto 60-mm plastic dishes at a density of 2×10^6 cells per dish.

After the initial 4 hours of attachment phase, the medium was changed, and the hepatocytes were further incubated in medium M 199 with the same concentrations of hormones and antibiotics used previously but without fetal calf serum. A volume of 2.5 ml medium per 60 mm culture dish was added. After 24 hours the medium was replaced with fresh medium.

Primary rat hepatocytes were usually stimulated with various pro-inflammatory cytokines at different concentrations on the next day of plating. The medium was changed 6 hours prior to stimulation; the stimuli were diluted to the required concentrations in the culture medium and added directly to the culture dishes by pipetting. An equal volume of the normal culture medium was added to the dishes with cells which later served as experimental controls. After addition of the stimuli, cells were incubated for 3-4 hours in the incubator prior to RNA isolation. To stop the stimulation of the culture, the culture were taken from the incubator, washed with phosphate buffered saline, pH 7.4 and frozen at –80°C for subsequent RNA isolation.

3.1.2.II Hormone and antibiotics stock solutions

All solutions were sterile filtered, aliquot and stored at –20°C.

Pen/strep stock	
	For 100 ml
Penicillin G (sodium salt)	0.64 g
Streptomycin sulfate	1.17 g
0.9% NaCl	to 100 ml

Dexamethasone (100 µM)	
	For 100 ml
Dexamethasone	3.92 g
0.9% NaCl	to 100 ml
Dexamethasone was first dissolved in 0.3 ml of ethanol and then adjusted to 100 ml with 0.9% NaCl.	

Insulin (10 µM)	
	For 100 ml
Insulin	6 mg
BSA	100 mg
0.9% NaCl	to 100 ml
Insulin was dissolved at pH 2.5, neutralized and then BSA was added.	

3.2 Methods in molecular biology

3.2.1 Real-time polymerase chain reaction

3.2.1.I Reverse transcription

The cDNA was generated by reverse transcription of 1µg of total RNA with 100 nM of dNTPs, 50 pM of primer oligo $(dT)_{15}$, 200 U of moloney-murineleukemia virus reverse transcriptase (M-MLV RT), 16 U of protector RNase inhibitor, 1× RT buffer and 2.5 µl of 0.1 M DTT for 1 hour at 40°C. Expression of Hepc, Hjv, Tf, TfR1, TfR2 ferritin-H, Fpn.-1, DMT1, Dcytb, Heph, HFE, IRE-BP1, IRE-BP2, IL-6, IL-1ß, TNF-α and IFN-γ genes was analysed using Platinum SYBR Green qPCR mix UDG (Invitrogen). GAPDH and ß-actin were used as housekeeping genes. Primer sequences used are given in Table 1.

The cDNA samples are analysed by the real-time PCR using the following ingredients for each PCR reaction:

	Volume per reaction
"X" primer-forward (5mM)	1.5µl
"X" primer-reverse (5mM)	1.5 µl
H_2O	6.5µl
SYBR Green master mix *Taq* polymerase	13.0 µl

2.5µl of the cDNA sample or 2.5µl of H_2O for the negative control was added to each PCR reaction.

Table 1: Primer sequences used for real-time PCR analysis.

Primer	Forward 5' ⟶ 3'	Reverse 5' ⟶ 3'
Dcytb	TCCTGAGAGCGATTGTGTTG	TTAATGGGGCATAGCCAGAG
DMT1	GCTGAGCGAAGATACCAGCG	TGTGCAACGGCACATACTTG
Ferritin-H	GCCCTGAAGAACTTTGCCAAAT	TGCAGGAAGATTCGTCCACCT
Fpn.-1	TTCCGCACTTTTCGAGATGG	TACAGTCGAAGCCCAGGACTGT
GAPDH	TCCTGCACCACCAACTGCTTAG	TTCTGAGTGGCAGTGATGGCA
Hepc	GAAGGCAAGATGGCACTAAGCA	TCTCGTCTGTTGCCGGAGATAG
Heph	CACATTTTTCCAGCCACCTT	TGACGAACTTTGCCTGTGAG
HFE	ATCAGCCTCTCACTGCCACT	CAAGTGTGTCCCCTCCAAGT
Hjv	ATGCCGTGTCCAAGGAGCTT	TCCACCTCAGCCTGGTAGAC
IFN-γ	AGTCTGAAGAACTATTTTAACTCAAGTAGCAT	CTGGCTCTCAAGTATTTTCGTGTTAC
IL-1ß	TACCTATGTCTTGCCCGTGGAG	ATCATCCCACGAGTCACAGAGG
IL-6	GTCAACTCCATCTGCCCTTCAG	GGCAGTGGCTGTCAACAACAT
IRE-BP1	GAGTCATGCCTTACCTGTCCCA	TGATAGCCTCCACCACAGGTTC
IRE-BP2	CTGCATCCCAGCCTATTGAAAA	GCACTGCTCCTAGCAATGCTTC
ß-actin	TGTCACCAACTGGGACGATA	AACACAGCCTGGATGGCTAC
TfR1	ATACGTTCCCCGTTGTTGAGG	GGCGGAAACTGAGTATGGTTGA
TfR2	AGCTGGGACGGAGGTGACTT	TCCAGGCTCACGTACACAACAG
TNF-α	ACAAGGCTGCCCCGACTAT	CTCCTGGTATGAAGTGGCAAATC
Tf	GGCATCAGACTCCAGCATCA	GCAGGCCCATAGGGATGTT

3.2.1.II Thermal cycler amplification program

The amplification was performed at 50 °C for 2 min., 95°C for 2 min., 95°C for 15 sec to 60°C for 30 sec for 45 cycles (Figure 2) in an ABI prism 7000 sequence detection system. All samples were assayed in duplicate. Expression of different genes was analysed using Platinum SYBR Green qPCR mix UDG. The PCR amplification program was followed by dissociation curve protocol for controlling the specificity of the PCR products. Specific temperature of dissociation of the PCR product was calculated by the Primer Express software. Curves of amplification were analysed to measure the Ct value in the linear range of the amplification. The results were normalized to the

housekeeping gene and fold change expression was calculated using Ct values by Prism Graph Pad 4 software.

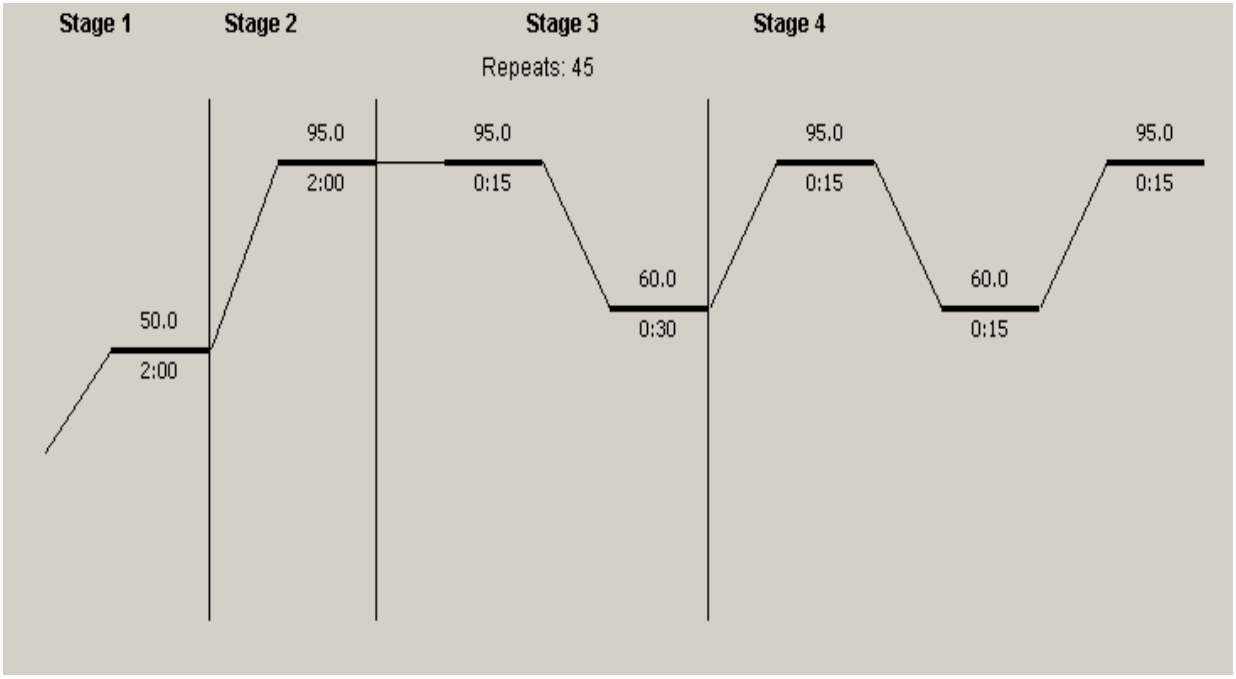


Figure 3: Thermal cycler amplification program for the quantitative real-time PCR amplification of the mRNA using Platinum® SYBR® Green qPCR SuperMix UDG, specific forward, reverse primer pairs and template cDNA in ABI Prism® 7000 Sequence Detection System by Applied Biosystems. Stage 1; 2 min. incubation at 50°C, Stage 2; 2 min. incubation at 95°C for hot start, Stage 3; 15 sec at 95°C and 30 sec at 60°C for 45 repeats. Stage 4; 15 sec at 95°C; 15 sec at 60°C and 15 sec at 95°C to get dissociation curve.

3.2.1.III Standard Curve

Serially diluted PCR products, of the gene of interest were amplified by Real Time PCR and Ct values were calculated. The standard curves were obtained graphically by using the following parameters: Ct values and the logarithm of the number of copies. Standard curve were calculated for the gene of interest and the housekeeping genes. Number of copies of gene in the sample was obtained by extrapolation of the measured Ct value to the relative standard curve. Normalization of gene expression in each sample was performed by calculating the ratio of number of copies of the gene of interest with respect to the number of copies of the housekeeping gene.

3.2.1.IV Primers designing

Primers for different genes were designed using the program "Primer Express" (ABI System) and the gene bank data (http://www.ncbi.nlm.nih.gov). All the primer sets used for real-time PCR are listed in the Table 1.

3.2.2 Isolation of total RNA

3.2.2.I RNA isolation procedure using silica columns

The isolation of total RNA from cultured rat hepatocytes was conducted using the NucleoSpin® RNAII kit (Macherey-Nagel) in accordance to the protocol for cultured animal cells.

3.2.2.II Principle

NucleoSpin® RNA method utilizes the silica membrane which adsorbs the RNA from the cell lysates. Contaminating DNA, which also binds to the membrane, is removed with a solution containing DNase. Salts, metabolites and macromolecular cellular components are washed away in two washing steps. Pure RNA is finally eluted under low ionic strength conditions with RNase-free water.

3.2.2.III Procedure

Cell lyses

The cells frozen on the culture dishes (2×10^6 cells per 6 cm culture dish) were thawed on ice. 350 µl of RA1 buffer with freshly added β-mercaptoethanol was applied to the dish, and cells were scraped with a disposable scraper, transferred to RNase-free 1.5 ml tubes and homogenized by passing 5 times through a 22 G injection needles connected to a syringe rapidly to prevent degradation of the RNA.

Filtration of the lysates

Cell lysates were pipette directly onto NucleoSpin® filter unit, placed in 2 ml collection tubes, and centrifuged for 1 min at 11,000 *g*. This step was performed to reduce viscosity and clear the cell lysates.

Adjustment of RNA binding conditions

To adjust the RNA binding conditions 350 µl of 70% ethanol was added to the filtered lysates and mixed by vortex. 700 µl of each sample was applied to a NucleoSpin® RNA II column placed in a 2 ml collection tube, and centrifuged for 30 sec at 8,000 *g*.

Desalt silica membrane

To desalt the columns prior to DNA digest, 350 µl of MDB buffer was pipette onto NucleoSpin® RNA II column, followed by centrifugation for 1 min at 11,000 *g*.

DNA digestion

To digest the DNA bound to the membrane, 95 µl of DNase reaction mixture was applied directly onto the center of the silica membrane of the column, followed by incubation at room temperature for 15 min.

Washings

To wash the silica membrane, 200 µl of RA2 buffer was added to the NucleoSpin® RNA II column followed by centrifugation for 30 sec at 8,000 *g*. To continue washing, 600 µl of RA3 buffer was applied and columns were centrifuged for 30 sec at 8,000 *g*. The last washing step was performed with 250 µl of RA3 buffer, followed by centrifugation for 2 min at 11,000 *g* to dry the membrane completely.

Elution of highly pure RNA

To elute the highly pure RNA, the NucleoSpin® RNA II columns were placed into nuclease-free 1.5 ml eppendorf tubes, the RNA was eluted with 60 µl of RNase free H_2O pipette directly onto the silica membrane and columns were centrifuged at 11,000 *g* for 1 min.

RNA concentration

To determine the RNA concentration and purity, the aliquot of RNA sample was diluted 1:100 in RNase-free H_2O and the concentration was measured at 260 nm and 280 nm by spectrophotometer (GeneQuant II, Pharmacia Biotech). The ratio of the OD at 260 nm and at 280 nm served as a measure of RNA purity. In a protein-free solution, the ratio OD260/OD280 is 2. Due to minor protein contaminations this coefficient is usually lower. In our experiments it was typically higher than 1.8.

Solutions used for RNA isolation

All solutions used for RNA isolation were provided in the NucleoSpin® RNAII kit; however, their detailed composition were not provided. Buffers RA1, RA2 and MD contain thiocyanate.

RA1 buffer

- RA1 buffer (Macherey-Nagel) 1000 µl
- β-Mercaptoethanol 10 µl

DNase reaction mixture (for 1 sample)

- DNase I (Macherey-Nagel) 10 µl
- DNase reaction buffer (Macherey-Nagel) 90 µl

RA3 buffer

- RA3 buffer (concentrate, Macherey-Nagel) 12.5 ml
- 100% ethanol 50 ml

3.2.2.IV Isolation of RNA by density-gradient ultracentrifugation

Total RNA was isolated from the liver, the skeletal muscle and extrahepatic organs by means of guanidine isothiocyanate extraction, cesium chloride density-gradient ultracentrifugation and ethanol precipitation according to method of Chirgwin (Chirgwin *et al*, 1979). This method is a versatile and efficient way to extract intact RNA from most tissues and cultured cells, even if the endogenous level of RNase is high.

Cell lysis

The cells were rapidly lysed in guanidine isothiocyanate-containing buffer, which ensures inactivation of RNases. The lysates were layered onto a CsCl gradient and spun in an ultracentrifuge. Proteins remain in the aqueous guanidine portion, DNA bands in the CsCl, and RNA settle down at the bottom of the tubes as a pellet. The RNA was recovered by dissolving the pellet. The recovery of RNA was usually excellent if the capacity of the gradient does not exceed.

Homogenization of the tissue sample

About 100 mg of frozen tissue was homogenized with ultra-turrax TP 18/10 homogenizer 3 times for 10 sec each in 3 ml of ice-cold GITC buffer with freshly added antifoam A (Sigma). The homogenates were centrifuged for 10 min at 3,500 rpm in a Rotixa/RP centrifuge (Hettich) at 4°C to pellet connective tissue and large cell debris.

CsCl gradient and ultra centrifugation

To prepare the gradient 2 ml of CsCl buffer was poured into 5-ml polyallomer ultracentrifuge tubes (6 per preparation). The cleared guanidine lysed samples were carefully layered on top of the CsCl buffer. The samples were centrifuged overnight (21 h) at 35,000 rpm in a Kontron TST55 rotor at 20°C. The supernatants were carefully removed by aspiration and the transparent gelatin-like RNA pellets were gently washed (preserving undisturbed) with 200 µl of 70% ethanol at room temperature. The pellets were reconstituted in 200 µl of RNase-free water by pipetting and transferred into sterile 1.5 ml eppendorf tubes and the procedure was immediately continued to RNA precipitation.

RNA precipitation

The RNA was precipitated with 450 µl of 100% ethanol in the presence of sodium acetate, pH 5.4 (20 µl of 2 M solution per pellet) overnight at –20°C. The RNA precipitates were centrifuged for 30 min at 12,000 rpm in an Eppendorf bench-top centrifuge at 4°C to get RNA pellet.

Washing of the RNA pellet

Supernatants were discarded and pellets were washed with 200 µl of ice-cold 70% ethanol to remove all traces of sodium acetate. The RNA precipitates were centrifuged as described above, the supernatants were discarded and the pellets were dried for 30 minutes at room temperature.

Reconstitution of RNA

The pellets were reconstituted in 100 µl of RNase-free water. To determine the concentration and purity of the RNA obtained, the aliquot of RNA sample was diluted 1:100 in RNase-free H_2O and the concentration was measured at 260 nm and 280 nm by spectrophotometer (GeneQuant II, Pharmacia Biotech). The ratio of the OD at 260 nm and at 280 nm served as a measure of RNA purity. In a protein-free solution, the ratio OD260/OD280 is 2. Due to minor protein contaminations this coefficient is usually somewhat lower. In our experiments it was typically higher than 1.8.

Solutions used for Ultracentrifugation

Guanidine isothiocyanate (GITC) buffer		
	For 200 ml	Final concentration
Guanidine isothiocyanate	94.53 g	4 M
0.25 M sodium citrate	20 ml	25 mM
N-lauroylsarcosyl	1 g	0.5%
RNase-free H_2O	to 200 ml	
The solution was sterile filtered and stored in the dark at 4°C. β-Mercaptoethanol was added just prior to use at a ration of 1 to 100 µl of GITC buffer.		

Cesium chloride (CsCl) buffer		
	For 200 ml	Final concentration
Cesium chloride	192 g	5.7 M
0.25 M sodium citrate	20 ml	25 mM
0.5 M EDTA	40 ml	100 mM
RNase-free H_2O	to 200 ml	5.7 M
pH was adjusted with 0.25 M citric acid to 7.5; the solution was sterile filtered and stored at room temperature.		

3.2.3 Northern blot analysis

Northern blot analysis is a method to quantify RNA expression. The RNA is separated in a denaturing formaldehyde/agarose gel, transferred by capillary transfer to a nylon membrane and fixed by UV cross linking. The RNA of interest is identified by hybridization with a specific radiolabeled cDNA probe. All solutions used for the northern blot were autoclaved, the electrophoresis and blot chambers, gel plates and combs were kept in freshly prepared 0.1% DEPC solution for 10-20 min before use to inactivate RNases.

3.2.3.I Preparation of RNA samples

Each RNA probe (5-10 µg of total RNA) in a volume not more than 5 µl was mixed with 7.5 µl of sample buffer. In case of dilute sample where the volume of the

probe exceeded 5 µl, the sample was concentrated in a vacuum centrifuge until the volume was reduced to 5 µl. RNA probes mixed with sample buffer were denatured by heating at 65°C for 10 min. Afterwards, the samples were briefly cooled on ice and centrifuged for 1 min at 10,000 rpm in an Eppendorf bench-top centrifuge. Each sample was mixed with 3 µl of loading buffer and centrifuged for 1 min at 10,000 rpm in an Eppendorf bench-top centrifuge.

3.2.3.II Recipe of the denatured formaldehyde/agarose gel (1%)

1 g of agarose was dissolved in 72.2 ml of RNase-free water (Ampuwa®) by heating in a microwave oven and then slightly cooled to approximately 70°C, followed by sequential addition of 10 ml of 10×running buffer, 16.7 ml of 37% formaldehyde, 8 µl of ethidium bromide (10 mg/ml). Ethidium bromide helps to visualize the 28S and 18S rRNA bands which serve to estimate the apparent molecular weights of the RNAs of interest in further hybridizations. The mixture was poured into the prepared gel casting chamber and let to polymerize under a fume hood for 20-30 minutes. Immediately after preparation, the RNA samples were loaded onto the gel.

3.2.3.III Electrophoresis

The electrophoresis was performed at constant voltage of 80 V for 1-1.5 h. After electrophoresis, the quality of RNA was estimated under UV transilluminator built-in Eagle Eye™ system (Stratagene); the gel was photographed, and the procedure was immediately continued to blotting.

3.2.3.IV Transfer of RNA to nylon membrane

After the separation of RNA in the gel, it was transferred to a nylon membrane by capillary transfer method. The system for transfer was assembled as depicted in Figure 4. A plastic tray was filled with 500 ml of 20×SSC. A piece of Whatman 3MM filter was soaked in 20×SSC and swaddled over the glass plate placed over the tray with both ends hanging into buffer to act as wick. Any bubbles between the filter and glass plate were smoothed out. The gel was carefully placed upside down over the filter. The bubbles between the gel and wick were carefully removed. The nylon membrane wetted in 2×SSC was placed on top of the gel and smoothed. Two other pieces of Whatman 3MM filters soaked in 2×SSC were sequentially flat laid on top of nylon filter and smoothed. A stack

of paper towels was placed on top, covered with another glass plate and pressed with 1 kg blotting weight. The transfer was carried out overnight. After the transfer, RNA was fixed on the membrane by UV cross linking for 2 min from both sides using Stratalinker™ 180 (Stratagene) set at "autocross link" mode.

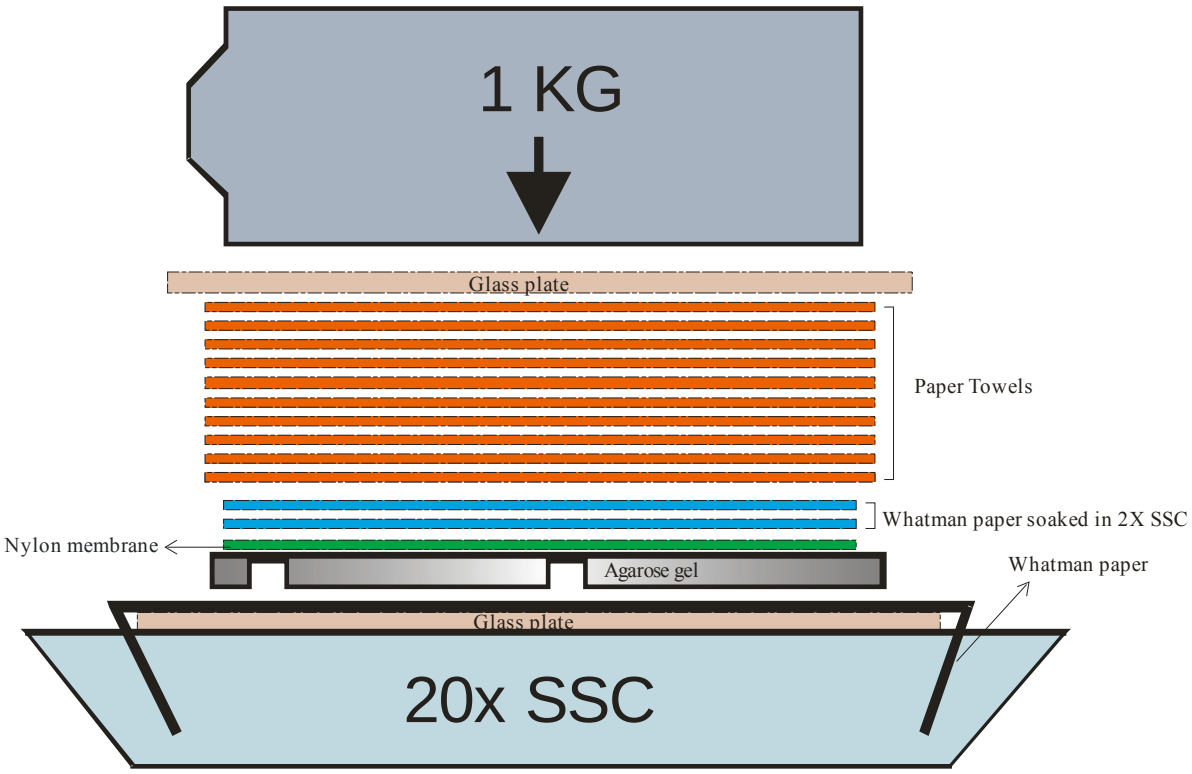


Figure 4: Scheme of the system for capillary transfer of RNA on nylon membrane. (For explanations, see the text).

3.2.3 V Hybridization of RNA with radiolabeled cDNA probe

After crosslinking, the nylon membrane was rinsed with 1×TE buffer to wash away any traces of agarose.

Pre-hybridization

Afterwards, the membrane was placed into a hybridization tube, and any bubbles between the membrane and internal wall of the tube were carefully removed. The pre-hybridization which is necessary to prevent unspecific binding was performed for 2 h with 10 ml of QuikHyb® hybridization solution at 68°C in a hybridization oven.

Hybridization

The volume of the radiolabel cDNA used for an appropriate volume of QuickHyb (Stratagene, TX, USA) solution was calculated as follows;

$$V[\alpha\text{-}^{32}P]cDNA = V_{quickhyb} \times 1{,}000{,}000/\text{ß-radioactivity of cDNA in 1 µl}$$

$$V_{salmon\ sperm}\ DNA = 2 \times V[\alpha\text{-}^{32}P]cDNA$$

If radiolabel probe is used after 1 day of labeling the activity was adjusted as follows;

$$\text{ß-radioactivity of cDNA in 1 µl} \times Y = Z\ cpm/\mu l$$

Where Y is the value from the α-^{32}P decay table (Table2).

Radiolabeled probe prepared as described in 3.2.6 was mixed with double volume of salmon sperm DNA. The cDNA and salmon sperm DNA mix was denatured for 5 min at 95°C and chilled on ice for 3-5 minutes. The probe was centrifuged shortly and mixed with QuikHyb® solution in the hybridization tube. The hybridization was carried out for 2 h at 68°C in a hybridization oven.

Table 2: α-^{32}Phosphorous decay table (Physical half life 14.29 days)

Days	**0.0**	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**	**4.5**
0	1	0.976	0.953	0.963	0.908	0.866	0.865	0.844	0.824	0.804
5	0.785	0.466	0.748	0.730	0.712	0.695	0.678	0.662	0.646	0.631
10	0.616	0.601	0.587	0.573	0.559	0.545	0.532	0.520	0.507	0.495
15	0.483	0.472	0.460	0.0.449	0.438	0.428	0.418	0.408	0.398	0.388
20	0.379	0.37	0.361	0.353	0.344	0.336	0.328	0.320	0.312	0.305
25	0.297	0.29	0.283	0.277	0.270	0.264	0.257	0.251	0.245	0.239
30	0.233	0.228	0.22	0.217	0.212	0.207	0.202	0.197	0.192	0.188
35	0.183	0.179	0.174	0.170	0.166	0.162	0.158	0.155	0.151	0.147
40	0.144	0.14	0.137	0.134	0.130	0.127	0.124	0.121	0.118	0.116
45	0.113	0.11	0.107	0.105	0.102	0.100	0.098	0.095	0.093	0.091
50	0.088	0.086	0.084	0.082	0.080	0.078	0.077	0.075	0.073	0.071
55	0.069	0.068	0.066	0.065	0.063	0.062	0.060	0.059	0.057	0.056
60	0.054	0.53	0.052	0.051	0.049	0.048	0.047	0.046	0.045	0.040

Washing

After hybridization, the membrane was rinsed in the tube with 30 ml of 2×SSC/0.1% SDS, next portion of 2×SSC/0.1% SDS was added followed by washing for 10 min at RT and two washings at 55°C with 0.1×SSC/0.1% SDS in the hybridization oven. Afterwards, the membrane was transferred to a plastic tray and further washed in 2×SSC/0.1% SDS with shaking at room temperature. The radioactivity was controlled to 100 cpm, the washing was stopped. The membrane was packed in a cling film, placed in an X-ray film cassette and exposed to X-ray film, which was developed afterwards.

3.2.3 VI Solutions

10×running buffer		
	For 500 ml	Final concentration
MOPS	20 g	200 mM
2M sodium acetate	12.5 ml	50 mM
0.5 M EDTA	10 ml	10 mM
RNase-free H_2O (Ampuwa®)	to 500 ml	
pH was adjusted with 5N NaOH to 7.0; the solution was sterile filtered and stored in the dark at 4°C.		

1×running buffer	
	For 1 l
10×running buffer	100 ml
RNase-free water (Ampuwa®)	to 1 l
The solution was stored at room temperature for 1-2 weeks.	

Sample buffer	
Deionized formamide	500 µl
37% formaldehyde	169 µl
10×running buffer	100 µl
The solution was dispensed into 1 ml aliquots and stored at –20°C for 2-3 months	

Deionized formamide	
Formamide	100 ml
Mixed Bed Resin AG 501-X8 (D)	10 g
The mixture was stirred for 30 min in the dark at room temperature, afterwards it was sterile filtered, dispensed into 50 ml aliquots and stored in the dark at –20°C.	

Loading buffer	
10×running buffer	5 ml
RNase-free water (Ampuwa®)	3 ml
Ficoll 400	1.5 g
Bromophenol blue	10 mg
The solution was dispensed into 0.5 ml aliquots and stored for 2-3 months at –20°C.	

20×SSC	
	For 2 l
NaCl	350.6 g
Sodium citrate×$2H_2O$	176.4 g
dd H_2O	to 1 l
The solution was autoclaved and stored at room temperature.	

2×SSC	
	For 500 ml
20×SSC	50 ml
dd H_2O	to 500 ml

10×TE		
	For 1 l	Final concentration
2 M Tris-HCl, pH 7.4	50 ml	100 mM
0.5 M EDTA	20 ml	10 mM
RNase-free H_2O	to 1 l	
pH was controlled and if necessary adjusted with NaOH or HCl to 7.6. The solution was sterile filtered and stored at 4°C.		

1×TE	
	For 500 ml
10×TE	50 ml
RNase-free H_2O	to 500 ml

2×SSC/0.1% SDS	
	For 500 ml
20×SSC	50 ml
20% SDS	2.5 ml
dd H_2O	to 500 l
In the case of precipitation of the SDS, the solution was warmed until clear.	

3.2.4 Amplification of DNA by polymerase chain reaction (PCR)

The polymerase chain reaction allows amplifying fragments due to repetitive cycles of DNA synthesis (Figure 4). The reaction uses two specific synthetic oligonucleotides (primers) (Table 3), that hybridize to sense and antisense DNA strands of the DNA fragment to be amplified, four deoxyribonucleotide triphosphates (dNTPs) and a heat-stable DNA polymerase. Each cycle consists of three reactions that take place under different temperatures. An initial denaturation step at 95°C for 2 minutes is necessary to ensure the complete denaturation of the target DNA. The two strands of denatured DNA serve as templates for the synthesis of new DNA. After heating, the

reaction is cooled (45- 60°C) to allow the annealing of primers to the complementary DNA strands. Starting from the primers, DNA polymerase extends both DNA strands at 72°C (DNA synthesis) (Figure 6 A). Since the DNA molecules synthesized in each cycle can serve as a template in the next cycle, the number of target DNA copies approximately doubles every cycle. Already after the third cycle, double stranded DNA molecules of the size corresponding to the distance between two primers are synthesized (Figure 6B). The repeating cycles of heating and cooling take place in a thermocycler. The PCR reaction was performed with specific forward and reverse primers to amplify rat Hepc, Hjv, Fpn.-1, IL-6, IL-1ß and 28S rRNA. The PCR lasted for 45 cycles under the conditions as shown in Figure 3. After the last cycle the synthesis step was prolonged for 10 min at 72°C to finish synthesis of incompletely synthesized DNA strands.

The PCR amplification was performed using the Go Taq ® Green master mix, which is premixed ready to use solution containing a non-recombinant modified form of *Taq* DNA polymerase that lacks 5`→3` exonuclease activity. The master mix contains dNTPs, $MgCl_2$ and reaction buffer at optimal concentrations for efficient amplification of DNA templates by PCR. Go Taq ® Green master mix contains blue and yellow dyes that allow monitoring the progress during electrophoresis.

To verify the specificity and purity of the PCR product, the size of the product was determined by agarose gel electrophoresis. For this purpose, an aliquot of 10 µl from the PCR reaction mix was used along with 3 µl of 6× loading dye.

Go Taq ® Green master mix composition		PCR reaction mix (Volume for one reaction)	
dATP	400 µM	Go Taq ® Green Master	12.5 µl
dCTP	400 µM	(5 pmol) Forward primer	1.5 µl
dTTP	400 µM	(5 pmol) Reverse primer	1.5 µl
dGTP	400 µM	Ampuwa ® to adjust the volume	7.5 µl
$MgCl_2$	3 mM	template	2.5 µl
Reaction buffer (composition was not provided by the manufacturer)			

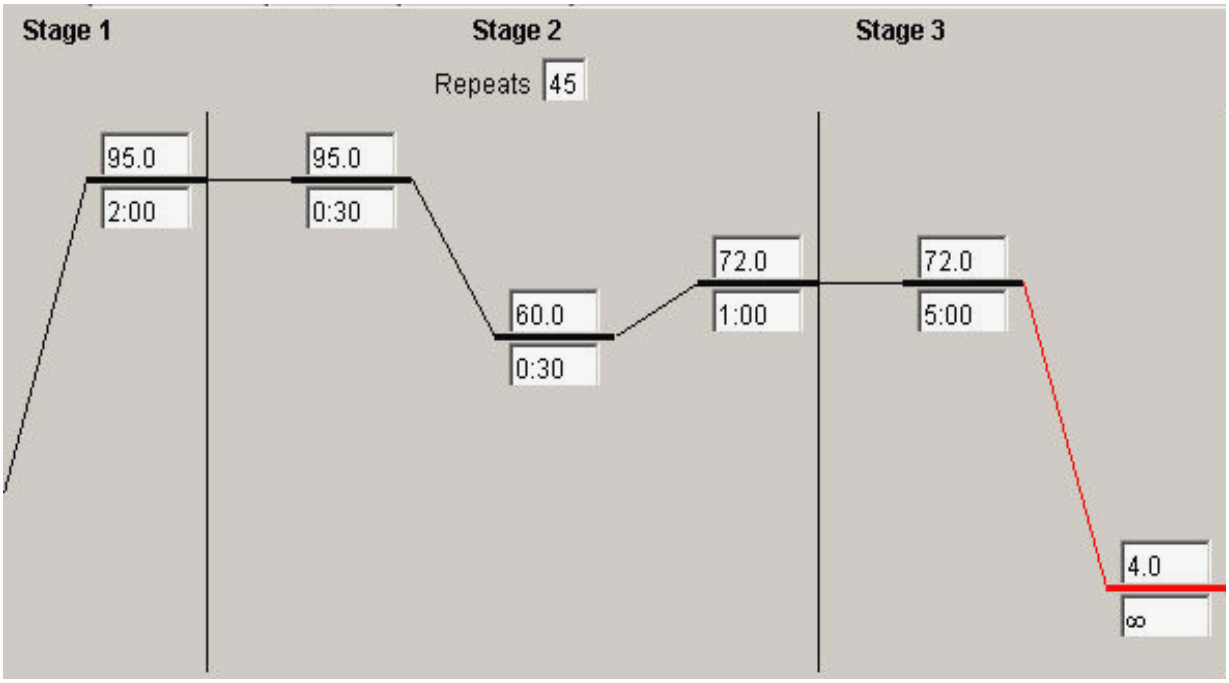


Figure 5: PCR amplification. Steps for cDNA synthesis used for northern blot analysis. cDNA was amplified in a thermocycler type Mastercycler® gradient, (Eppendorf) using Go Taq® Green master mix, 2× (Promega) with the PCR program as shown above. The DNA was denatured at high temperature (Stage 1; 2 min. incubation at 95°C to ensure complete denaturation of the target DNA). Step 1 was followed by repetitive cycles of denaturation, annealing and extension for 45 repeats (stage 2; denaturation for 30 sec at 95°C, annealing for 30 sec at 60°C and extension for 1 min. at 72°C). Final extension of the DNA was performed in stage 3 at 72°C for 5 min. Finally, the PCR product was soaked at 4°C for infinity.

Table 3: Primer sequences used for northern blot analysis.

Primer	Forward 5' ⟶ 3'	Reverse 5' ⟶ 3'
Hepcidin	AGG ACA GAA GGC AAG ATG GCA	TGT TGA GAG GTC AGG ACA AGG C
Hjv	CCATGGCAGTCCTCCAACTCTA	AGACGCAGGATTGGAAGTAGGC
Fpn.-1	CAG ACT TAA AGT GGC CCA GAC G	ACA AGG CCA CAT TTT CGA CG
IL-6	ATGTTGTTGACAGCCACTGCC	TGGTCCTTAGCCACTCCTTCTG
IL-1ß	AGCAGCTTTCGACAGTGAGGAG	GCTCTGCTTGAGAGGTGCTGAT

3.2.5 Agarose gel electrophoresis of DNA

The amplified cDNA was subjected to electrophoresis to verify the amplification of specific probe. Electrophoresis was performed in 1% agarose gel. For this purpose,

1.00 g of agarose was dissolved by heating in 100 ml of 1×TAE buffer. For visualization of the bands, 8 µl of ethidium bromide (10 mg/ml) was added to the mixture. After mixing, the gel was poured into the gel casting chamber. The samples were prepared for loading by mixing of 8 µl of DNA probe with 2 µl of 6×Loading Dye Solution (MBI, Fermentas). After polymerization, the gel was placed into an electrophoresis chamber filled with 1×TAE buffer, the samples were loaded, and the electrophoresis was performed at 80 V for 1 hour. DNA bands, intercalating ethidium bromide, were visualized by UV detection. Ethidium bromide is a fluorescent dye which contains a planar group that intercalates between the stacked bases of the DNA. The fixed position of this group and its close proximity to the bases cause dye, bound to DNA, to display an increased fluorescence yield compared to that of the dye in free solution.

Ultraviolet radiation at 254 nm is absorbed by the DNA and transmitted to the dye; radiation at 302 nm and 366 nm is absorbed by the bound dye itself. In both cases, the energy is reemitted at 590 nm in the red orange region of the visible spectrum. Hence, DNA can be visualized under a UV transilluminator. The gel was photographed using Eagle Eye™ system (Stratagene), a setup containing UV transilluminator, intensity control unit, video camera and printer.

20×Tris/acetate/EDTA (TAE) buffer

	For 1l	Final concentration
Tris base	122 g	1 M
Sodium acetate	32 g	0.4 M
EDTA	14 g	40 mM
dd H_2O	to 1 l	

After EDTA was completely dissolved, pH was adjusted with acetic acid to 7.4; the buffer was autoclaved and stored at room temperature.

1×TAE buffer

	For 1 l
20×TAE buffer	50 ml
dd H_2O	To 1 l

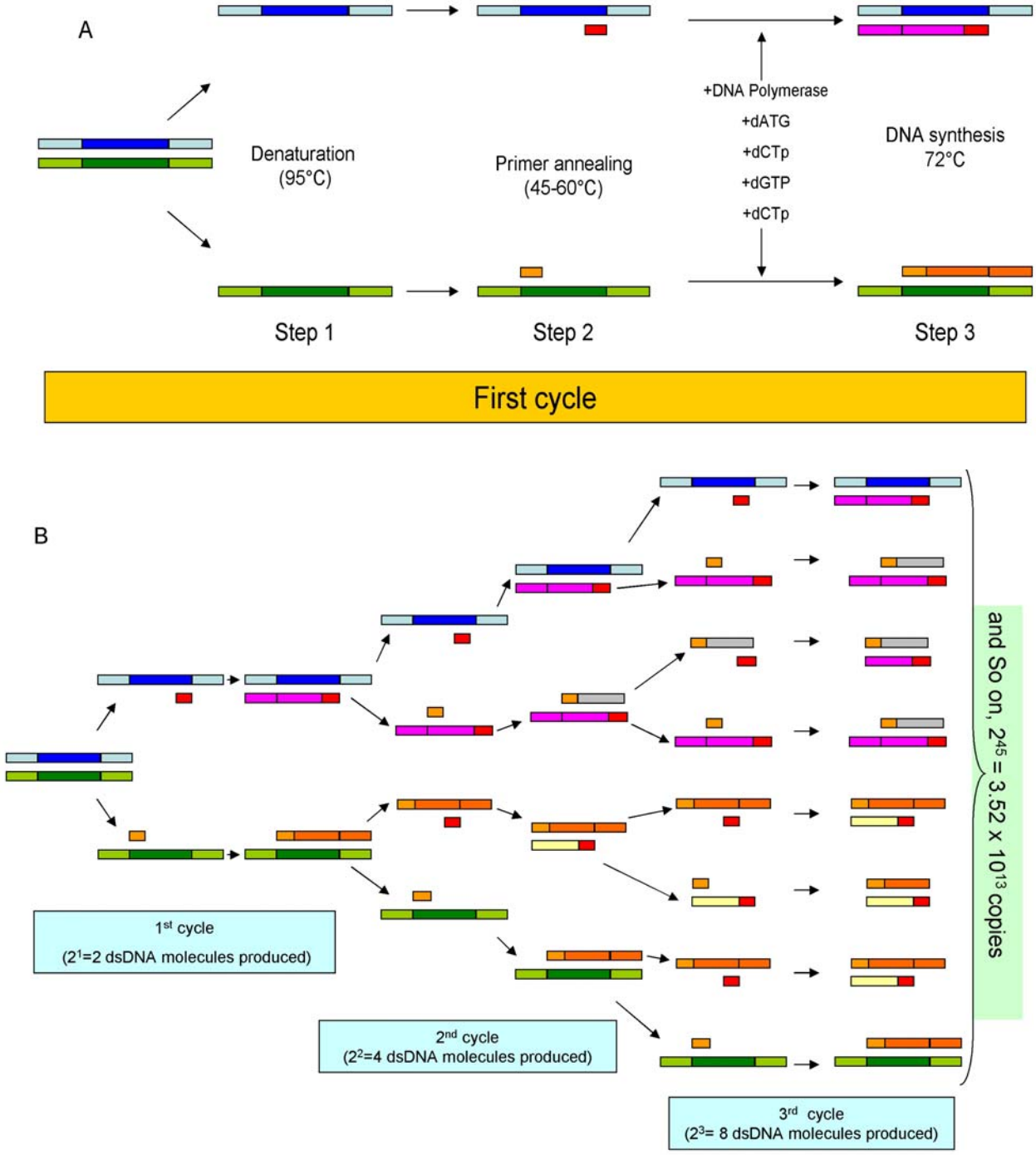


Figure 6: Schematic representation of PCR Amplification of DNA used for northern blot analysis. After denaturation (step1), cooling of the DNA allows annealing of the primers to the complementary sequences in the template DNA strands (step 2). In the presence of primers (forward and reverse), DNA polymerase and the four dNTPs, two complementary strands of DNA are synthesized from one DNA molecule two primers

(step 3) **(A)**. PCR results in exponential increase in the number of copies of the DNA (2^n= No. of copies after specific cycle where "n" is the number of cycle). Figure **(B)** illustrates three cycles of PCR reaction which results in the synthesis of 8 DNA molecules (2^3 = 8 DNA molecules after 3 PCR cycles). These 8 molecules contain 8 DNA strands which are of equal length and represent the segment of parent DNA molecule which lies in between the two Primer binding sites. Other 8 strands of the DNA molecules have flanking DNA segment, these are the strands originated in first 2 cycles from the parent DNA molecule. Essentially after several cycles, all of the DNA strands have unique equal length with specific base composition.

3.2.6 Radioactive labeling of DNA

Two methods were used to label the cDNA of interest;

I) Nick translation method

II) Random primed labeling (for short specific cDNA fragments)

3.2.6.I DNA labeling by nick translation method

Nick Translation System kit (Invitrogen) was used to label the DNA by nick translation method. Nick translation requires the activity of two different enzymes. DNase I was used to cleave (nick) phosphodiester bonds at random sites in both strands of a double stranded target DNA. *E. coli* DNA polymerase I was used to add deoxynucleotides to the 3′-hydroxyl termini created by DNase I. In addition to its polymerizing activity, DNA polymerase I carries a 5′→3′ exonuclease activity that removes nucleotides from the 5′ side of the nick. The simultaneous elimination of nucleotides from the 5′ side and the addition of radiolabeled nucleotides to the 3′ side resulted in the movement of the nick along the DNA hence labeled to highly specific activity (Kelly *et al*., 1970). The reaction was carried out at low temperature in a sterile 1.5 ml tube placed in the ice cold water with controlled temperature to 15°C. The reaction mix for the nick translation labeling is;

- cDNA (1 µg) with the final volume brought to 35 µl using Ampuwa®.
- 5 µl dNTP mix (dATP, dTTP and dGTP)
- 5 µl α-32P-dCTP (3,000 ci/mmol, 50 µCi)
- 5 µl Pol I/DNase I mix

The components were mixed gently but thoroughly and centrifuged briefly in an eppendorf bench-top centrifuge. The mixture was incubated at 15°C for 1 hour. 5 µl of stop buffer was added to terminate the reaction.

3.2.6.II DNA labeling by random priming reaction

Random primed DNA labeling was performed using NE Blot® Kit (New England Biolabs) designed to produce labeled DNA probes using the method of Feinberg and Vogelstein (Feinberg and Vogelstein, 1983), where random sequence octadeoxynucleotides serve as primers for DNA synthesis *in vitro* from denatured double-stranded template DNA by the Klenow Fragment of *E. coli* polymerase I. One labeled deoxyribonucleotide is used in the dNTP reaction mixture and is incorporated during primer directed DNA synthesis by DNA polymerase. The resulting labeled DNA is used as hybridization probe in northern blot. 50-100 ng of template DNA was dissolved in nuclease free H_2O (the volume of added water should not exceed 33 µl). The DNA was denatured by heating at 95°C for 5 min and subsequently chilled on ice for 5 min. The following reagents were added to the DNA in the indicated order:

- 5 µl 10×labeling buffer (includes Random Octadeoxyribonucleotides)
- 6 µl dNTP mixture (2 µl of dATP, dTTP and dGTP were mixed previously)
- 5 µl α-32P-dCTP (3,000 ci/mmol, 50 µCi)
- 1 µl DNA Polymerase I − Klenow Fragment (3′→5′ exo-) (5 units)

The mixture was incubated at 37°C for 30 min. followed by termination of the reaction by adding 5 µl of 0.2 M EDTA, pH 8.0.

3.2.6 III Purification of labeled DNA

Labeled DNA probe was separated from unincorporated nucleotides by gel filtration on Sephadex® G-50 using Pharmacia NICK Column (Pharmacia Biotech). A column was opened according to the manufacturer's instructions and equilibrated with 3 ml of 1×TE buffer, pH 8.0. After the entire volume of buffer had entered the gel, random priming reaction mixture was applied onto the column. An addition of 400 µl of 1×TE buffer was enough to remove the unincorporated nucleotides. The flow through was collected in the tube placed under the column and kept for further measurement of radioactivity. The labeled cDNA was eluted with 400 µl of 1×TE. The sample obtained

was subjected to measurement of radioactivity and stored at −20°C until use for Northern blot hybridization.

3.2.6.IV Measurement of β-radioactivity

After the purification step as described above, the radioactivity of labeled cDNA samples was measured using Wallac 1409 liquid scintillation β-counter (Turku /Finland). 2 µl aliquots from the flow through and elution fractions were transferred to screw-lid plastic tubes containing 5 ml of scintillation liquid, mixed by inverting and subjected to radioactivity measurements. β-Radioactivity of the samples was expressed in counts per minute (cpm). The activity value in flow through fraction was used as means to assess the efficiency of radioactive nucleotide incorporation. For effective labeling this value should not exceed 10% of the radioactivity value in the labeled cDNA sample.

10×TE		
	For 1l	Final concentration
2 M Tris-HCl, pH 7.4	50 ml	100 mM
0.5 M EDTA	20 ml	10 mM
RNase-free H_2O	to 1 l	
pH was controlled and if necessary adjusted with NaOH or HCl to 7.6. The solution was sterile filtered and stored at 4°C.		

1 ×TE	
	For 500ml
10×TE	50 ml
RNase-free H_2O	to 500ml

3.3 Biochemical methods

3.3.1 Enzyme-Linked Immunosorbent Assay (ELISA)

To measure IL-6, IL-1β, TNF-α and IFN-γ concentration in rat serum, the Quantikine® rat IL-6, IL-1β, TNF-α and IFN γ immunoassay kit (R&D Systems, Wiesbaden, Germany), based on solid phase ELISA, was used.

3.3.1.I Principle

This assay employs the quantitative sandwich enzyme immunoassay technique. A microplate is pre-coated with monoclonal antibody specific for rat pro-inflammatory cytokine. Serum samples are pipette into the wells and any IL-6 IL-1β, TNF-α or IFN γ binds by immobilized antibody. After washing away any unbound material, an enzyme-linked polyclonal antibody specific for rat IL-6 IL-1β, TNF-α or IFN γ is added. Any unbound antibody-enzyme reagent is washed away, and a substrate solution is added to the wells. The enzymatic reaction yields a blue-colored product that turns yellow when the stop solution is added. The intensity of the color measured by colorimetric method is proportional to the amount of protein bound in the initial step.

3.3.1.II Reagent preparation

Since all samples should be pipette within 15 min, reagents needed for the assay were prepared prior to assay procedure. All reagents were provided with Quantikine® immunoassay kit.

3.3.1.III Rat IL-6, IL-1β, TNF-α and IFN-γ control

The control provided with kit, was reconstituted with 1 ml double distilled water.

3.3.1.IV Rat IL-6 IL-1β, TNF-α and IFN-γ conjugate concentrate

	For 96 wells
Conjugate concentrate	0.5 ml
Conjugate diluent	11 ml

3.3.1.V Washing buffer

	For 96 wells
Washing buffer concentrate	25 ml
dd H_2O	to 625 ml

3.3.1.VI Substrate solution

Equal volumes of color reagents A and B, provided by kit, were mixed together, and solution was used with 15 min.

3.3.1.VII Standard and sample preparation

The standards were reconstituted with 2 ml of calibrator diluent. This stock solution (2000 pg/ml) was used to prepare a serial dilution ranging from 31.2 pg/ml to 2000 pg/ml. Calibrator diluent served as zero standard. Prior to assay, serum samples were diluted 2 fold into calibration diluent.

3.3.1.VIII Assay procedure

The whole procedure was performed at room temperature. All samples, standards and controls were brought to the RT and assayed in duplicates. To synchronize the reaction in each well, all reagents were pipette using a multi-channel pipette. 50 µl of assay diluent was added to each well. Standards, control and samples were added in a quantity of 50 µl per well. The components were mixed by gentle tapping the plate frame for 1 min. After that, the plate was covered with the adhesive strip provided and incubated for 2 h at room temperature. After the incubation period each well was aspirated and washed with 400 µl of wash buffer using a manifold dispenser, and procedure was repeated four times for a total of five washes. After washing, 100 µl of rat IL-6, IL-1β, TNF-α or IFN γ conjugate was added to each well. The plate was covered with a new adhesive strip and incubated for another 2 hours at room temperature. The aspiration and washing procedure was performed as described above. Subsequently, 100 µl of substrate solution was added to each well to start enzymatic reaction and plate was incubated in the dark for 30 min at room temperature. To stop the enzymatic reaction, 100 µl of stop solution was added to each well, followed by determination of optical density of each well using a microplate reader (Dynatech Laboratories) set to dual wavelength mode (test filter 450 nm, reference filter 570 nm). The calculation of results was performed with a program (Dynatech MRX software, version 1.33) created in accordance to the manual instructions (Quantikine® immunoassay kit).

3.3.2 Hepcidin pro-hormone ELISA

The DRG® hepcidin pro-hormone enzyme immunoassay kit is used for the quantitative determination of hepcidin pro-hormone in the serum. Hepcidin is a small cystein-rich peptide produced in the liver. This molecule regulates the absorption of iron in the body similarly to the correlation of insulin and sugar. Hepcidin was initially isolated as a 25 amino acid peptide in human plasma and urine exhibiting antimicrobial

activity. Application of the present ELISA allows the detection and determination of hepcidin pro-hormone (pro-hepcidin) in the serum.

3.3.2.I Principle

The DRG® hepcidin pro-hormone ELISA kit is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the principle of competitive binding. The microtiter wells are coated with a polyclonal antibody directed towards an antigenic site on the hepcidin pro-hormone molecule (28-47 amino acids). Endogenous hepcidin pro-hormone in the sample competes with a hepcidin pro-hormone-biotin conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off. The amount of bound biotin conjugate is reverse proportional to the concentration of pro-hormone in the sample. After addition of the substrate solution, the intensity of colour developed is reverse proportional to the concentration of pro-hormone in the serum sample.

3.3.2.II Microtiterwells

12x8 (break apart) strips, 96 wells; wells coated with anti pro-hepcidin antibody (polyclonal).

3.3.2.III Standard

7 vials (lyophilized), 1 ml; concentrations: 10, 50, 100, 250, 500, 1000 ng/ml of synthetic peptide hepcidin (28-47). Contains < 0.3% proclin as a preservative. Reconstitute the lyophilized contents of the standard vial with 1.0 ml Ampuwa.

3.3.2.IV Control

1 vial (lyophilized), 1 ml, Contains < 0.3% proclin as a preservative. Reconstitute the lyophilized content with 1.0 ml Ampuwa ®. Wait for 10 minutes and mix the control several times before use

3.3.2.V Assay Buffer

1 vial, 14 ml, ready to use, contains < 0.3% proclin as a preservative.

3.3.2.VI Biotin Conjugate

1 vial, 14 ml, ready to use, Pro-Hepcidin fragment conjugated to biotin; contains < 0.3% proclin as a preservative.

3.3.2.VII Enzyme Complex

1 vial, 14ml, ready to use, contains horseradish peroxidase, 0.01% methylisothiazolone and 0.02% bromonitrodioxane as a preservative.

3.3.2.VIII Substrate Solution

1 vial, 14 ml, ready to use. Tetramethylbenzidine (TMB).

3.3.2.IX Stop Solution

1 vial, 14 ml, ready to use, contains 0.5M H_2SO_4. Avoid contact with the stop solution. It may cause skin irritations and burns.

3.3.2.X Wash Solution

1 vial, 30 ml (40X concentrated). Dilute 30 ml of concentrated wash solution with 1170 ml deionised water to a final volume of 1200 ml

3.3.2.XI Specimen Dilution

In case of concentrated samples above the highest standard, the specimens were diluted with zero standard and reassayed as described in assay procedure. For the calculation of the concentrations this dilution factor has to be taken into account.

Example:

a) Dilution 1:10: 10 µl Serum + 90 µl *assay buffer* (mix thoroughly)

b) Dilution 1:100: 10 µl dilution a) 1:10 + 90 µl *assay buffer* (mix thoroughly).

3.3.2.XII Assay Procedure

The whole procedure was performed at room temperature. All samples, standards and controls were brought to the RT and assayed in duplicates. To synchronize the reaction in each well, all reagents were pipette using a multi-channel pipette. 100 µl of assay buffer was added to each well. Standard, control and samples were added in a quantity of 50 µl per well using new disposable pipette tip. 100 µl of biotin conjugate was pipette into each well. The components were mixed thoroughly for 10 seconds by gentle

tapping the plate frame. The plate was then incubated for 2 hours at RT without covering by any adhesive strip. The contents of the wells were briskly shacked out by striking the wells sharply on absorbent paper to remove residual droplets. The wells were rinsed 5 times with the wash solution (400 µl per well). The sensitivity and precision of the assay is markedly influenced by the correct performance of the washing procedure.

After the washing steps, 100 µl of enzyme complex was added to each well and incubated for 60 minutes at RT. After one hour the contents in the wells were washed as described previously. Next 100 µl of substrate solution was added to each well and incubated at RT for 30 minutes. 100 µl of the stop solution was added in each well to stop the enzymatic reaction after incubation period. The OD was read at 450 ± 10 nm using a microplate reader (Dynatech Laboratories). The calculation of results was performed with a program (Dynatech MRX software, version 1.33) according to the manufacturer's instruction.

3.4 Methods in clinical chemistry

3.4.1 Iron measurement

3.4.1.I Principle

Iron bound to transferrin is released in an acidic medium as ferric iron and is then reduced to ferrous iron in the presence o ascorbic acid. Ferrous iron forms a blue complex with ferene. The absorbance at 595 nm is directly proportional to the iron concentration.

$$\text{Transferrin } (Fe^{3+})_2 \xrightarrow{\text{Ascorbic acid, Buffer}} 2\ Fe^{2+} + \text{transferrin}$$

$$Fe^{2+} + 3\ \text{Ferene} \longrightarrow \text{Ferrous Ferene (blue complex)}$$

3.4.1.II Reagents

			Final concentration	
R1:	Acetate buffer	pH 4.5	800 mM/l	
	Thiourea		90 mM/l	
R2:	Ascorbic acid		45 mM/l	
	Ferene		0.6 mM/l	
	Thiourea		20mM/l	
Standard			100µg/dl	(17.9 µM/l)

(Should be protected from light)

Reagents are stable at 2-25°C until the expiry date).

3.4.1.III Assay Procedure

To determine the serum iron level, 100 µl of the serum sample along with 1000 µl of the reagent 1 was taken in the reaction tube. For blank 100 µl of dd H_2O was used. The sample and reagent 1 was mixed thoroughly, incubated for 5 minutes and read the absorbance (A1). After that 250 µl of the Reagent 2 was added, mixed thoroughly and the absorbance (A2) was read after 10 minutes of incubation.

3.4.1.IV Calculations

$$\Delta A = [(A2- 0.82\ A1)\ sample/Std.] – [(A2-0.82A1)\ blank]$$

The factor 0.82 compensates the decrease of the absorbance by addition of reagent 2. The factor is calculated as follows:

(Sample +R1)/Total volume.

This compensation is necessary as a high sample volume is used.

$$Iron\ [\mu g/dl] = \Delta A\ Sample/\Delta A\ Std/Cal \times Conc.\ Std.\ /Cal\ [\mu g/dl]$$

$$Conversion\ factor \qquad Iron\ [\mu g/dl] \times 0.1791\ [\mu M/l]$$

3.4.1.V Measuring range

The test has been developed to determine iron concentrations within a measuring range from 5-1000 µg/dl (0.9-179 µM/l). When values exceed this value samples should be diluted 1 + 2 with NaCl solution (9 g/l) and the results multiplied by 3.

3.4.1.VI Specificity and sensitivity

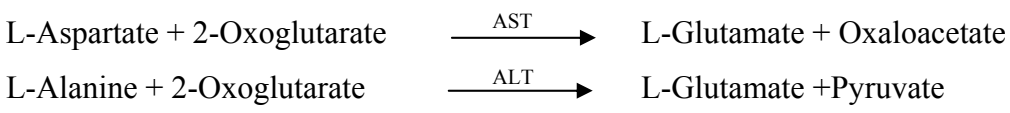
The test was specific and no interference was observed by conjugated and free bilirubin up to 60 mg/dl, hemoglobin up to 100 mg/dl, lipemia up to 2000 mg/dl triglycerides, and copper up to 200 µg/dl. The lower limit of detection for the test is 2 µg/dl (0.4 µM/l).

3.4.2 Transaminases

Transminases (ALT and AST) are the most important representatives of a group of enzymes, the aminotransferases or transaminases, which catalyze the conversion of α-keto acids into amino acids by transfer of amino groups. As a liver specific enzymes ALT is significantly elevated in hepatobiliary disease, increased AST levels however, can occur in connection with damages of heart or skeletal muscle as well as of liver parenchyma. Transminases (ALT and AST) are the most important representatives of a group of enzymes, the aminotransferases or transaminases, which catalyze the conversion of α-keto acids into amino acids by transfer of amino groups. As liver specific enzymes ALT is significantly elevated in hepatobiliary disease, increased AST levels; however, can occur in connection with damages of heart or skeletal muscle as well as of liver parenchyma. Parallel measurements of ALT and AST are therefore applied to distinguish liver from heart or skeletal muscle damages. The AST/ALT ratio is used for differential diagnosis in liver disease, while ratio < 1 indicate mild liver damage; ratios > 1 are associated with severe, often chronic liver disease. Serum level of transaminases were determined by routine clinical laboratory test using diasys kit (diagnostic systems international Holzheim Germany)

3.4.2.I Principle of transaminase action

Transamination is the process in which an amino group is transferred from amino acid to an α-keto acid. The enzymes responsible for transamination are called transaminases. The substrates in the reaction are α-ketoglutaric acid (α-KG) plus L-aspartate for AST, and α-KG plus L-alanine for ALT. The products formed by enzyme action are glutamate and oxaloacetate for AST and glutamate and pyruvate for ALT. Addition of 2, 4, dinitrophenyl hydrazine results in the formation of hydrazone complex with the ketoacids. A red color is produced on the addition of sodium hydroxide. The intensity of color is related to enzymatic activity.



Addition of pyridoxal-5-phosphate (P-5-P) stabilizes the transaminases and avoids falsely low values in the samples containing insufficient endogenous P-5-P, e.g. from patients with myocardial infarction, liver disease and intensive care patients.

3.4.2.II Reagents

R1:	Tris	pH 7.15	100 mM/l
	L-alanine		500 mM/l
	LDH (lactate dehydrogenase)		≥ 1700 U/l
R2:	2-Oxoglutarate		15 mM /l
	NADH		0.18 mM /l
Pyridoxal-5-Phosphate FS			
	Good's buffer	pH 9.6	0.7 mM /l
	Pyridoxal-5-phosphate		0.09 mM/l

(Should be protected from light)

3.4.2.III Procedure

To determine the transaminase activity in the serum samples, 100 µl of the serum sample along with 1000 µl of the reagent 1 was taken in the reaction tube. The sample and reagent 1 was mixed thoroughly and incubated for 5 minutes. After the incubation period 250 µl of the Reagent 2 was added and mixed. The absorbance was read at 340 nm for four times after every 1 minute to get four absorbance readings.

3.4.2.IV Calculations

From the absorbance readings the activity of the transaminases was calculated. In the first step the rate of change of absorbance i.e. ΔA/min was calculated and then multiplied by the corresponding factor from the following table:

ΔA/min × factor = Transaminase activity (U/l)

	Substrate Start	Sample start
340 nm	2143	1745
334 nm	2184	1780
365 nm	3971	3235

3.4.2.V Measuring range

The test has been developed to determine the transaminase activities which correspond to a maximal ΔA/min of 0.16 at 340 and 334 nm or 0.08 at 365 nm. In case of concentrated samples 1:10 dilution should be performed with saline solution and results should be multiplied by 10.

3.4.2.VI Specificity and Sensitivity

The test was specific and no interference was observed by ascorbic acid up to 30 mg/dl, bilirubin up to 40 mg/dl, hemoglobin up to 400 mg/dl, and lipemia up to 2000 mg/dl triglycerides. The lower limit of detection of transaminases by this method is 4 U/l.

3.5 Statistical analysis

The data were analysed using Prism Graph pad 4 software (San Diego, USA). All experimental errors are shown as SEM. Statistical significance was calculated by Student's t test and one way ANOVA with Dunnett post hoc test. Significance was accepted at $P < 0.05$.

3.6 Safety measures

All operations with genetically modified organisms and plasmid DNA were performed in accordance to the ''Gentechnikgesetz'' of 1990 and to the rules prescribed by the ''Gentechnik-Sicherheitsverordnung'' of 1990. Ethidium bromide, formaldehyde, DEPC and other chemicals deleterious for the environment, when used in the course of the work, were carefully managed and disposed properly in accordance with institutional guidelines. All the operations with radioactive chemicals were performed in a radioactivity class II laboratory and the radioactive waste was disposed off according to the institutional instructions.

4 RESULTS

4.1 Turpentine oil-induced APR

4.1.1 Serum and blood analysis

Sera from TO injected rats were analysed to study different parameters during the acute-phase condition like, serum iron level, hepcidin pro-hormone level and tissue injury marker enzymes.

4.1.1.I Serum iron levels

Changes in the serum iron levels were studied at different time points after the injection of the TO. A decrease in the serum iron level was measured early after TO injection. This decline in serum iron level was highly significant at the later time points by Student's *t*-test ($P < 0.001$) and during the whole course of study by one way ANOVA ($P=0.0008$; Figure 7).

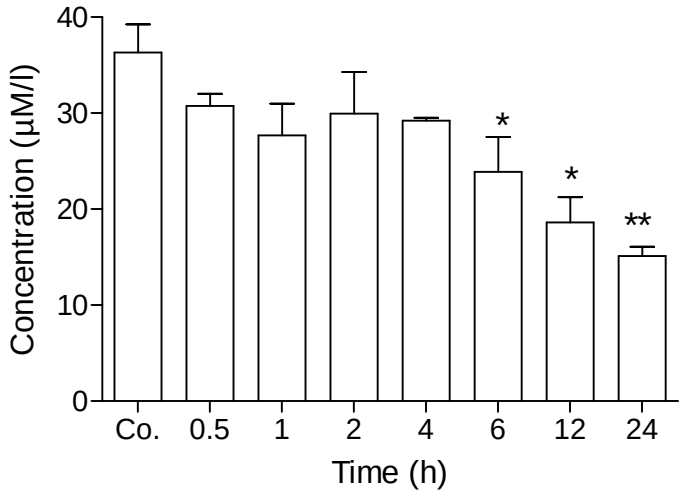


Figure 7: Serum iron levels determined by iron ferene based assay (as described in material and methods). Statistically significant decline in serum iron levels was found after the TO injection. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).

4.1.1.II Serum hepcidin pro-hormone

Serum concentrations of hepcidin pro-hormone were studied by hepcidin pro-hormone specific ELISA. Pro-hepcidin concentration was not significantly changed in response to TO injection; however, the concentrations were slightly depressed at different time points compared to control values (Figure 8).

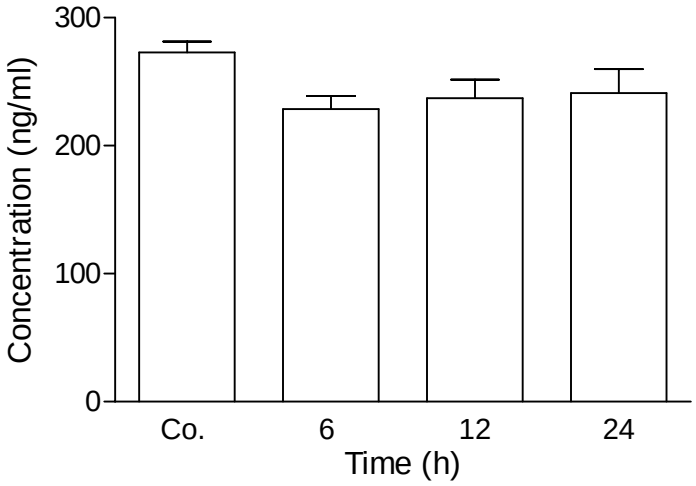


Figure 8: Serum hepcidin pro-hormone concentration was measured by hepcidin pro-hormone ELISA. No significant changes in the pro-hepcidin concentration were observed in the serum of TO injected rats. The concentration was slightly lower than the basal values in the sera of the TO injected rats. Results represent the mean value ± SEM (n=4).

4.1.1.III Serum tissue injury markers

The serum levels of creatine kinase (CK), the marker of muscle tissue damage, were strongly elevated above the control value as early as 30 min after administration of TO, returning below the baseline by 2 hours. A secondary peak at 24 hours was observed (Figure 9A). This increase was statistically significant at the early time point with Student's *t*-test ($P = 1.77 \times 10^{-5}$) analysis, and during the whole time-course by one way ANOVA ($P < 10^{-4}$).

Serum activity of aspartate aminotransferase (AST) was found to be elevated 24 hours after TO administration ($P < 10^{-4}$), while alanine aminotransferase (ALT) and alkaline phosphatase (AP) serum levels remained constantly normal during the experiment (Figure 9 B).

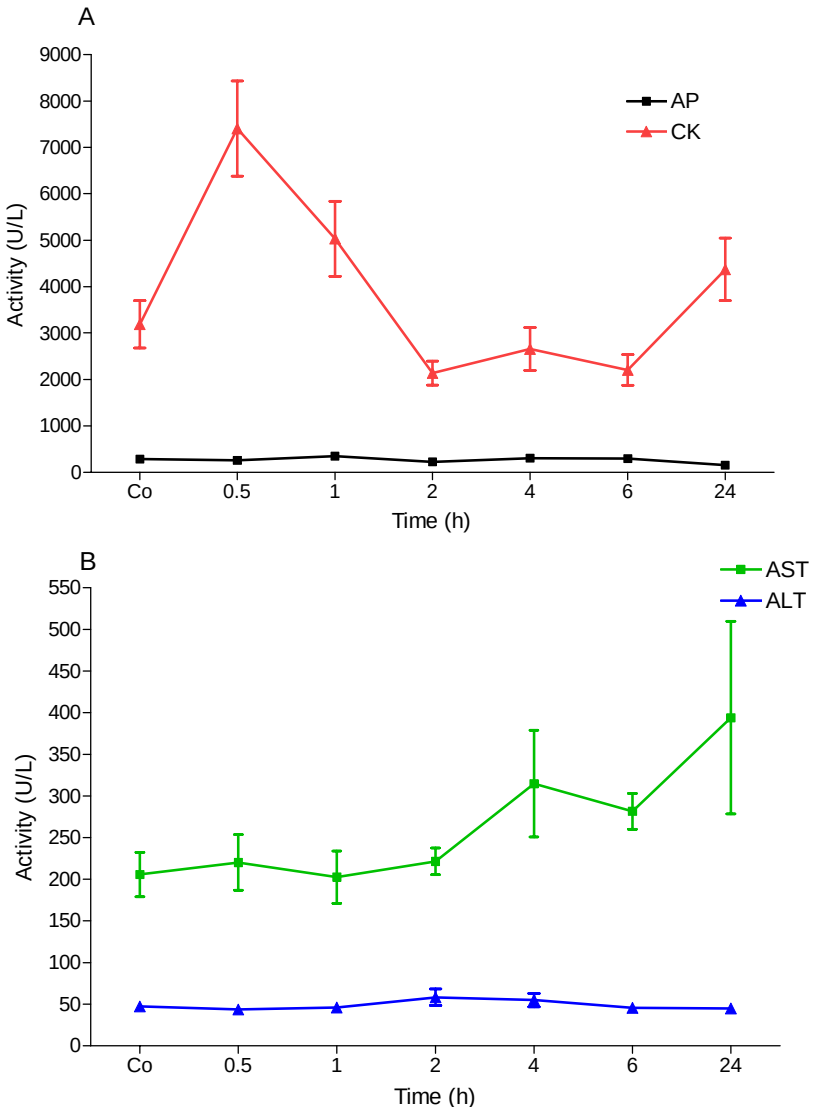


Figure 9: Serum alkaline phosphatase (AP), creatine kinase CK *(A)* and transaminases *(B)* levels in TO-treated rats. Serum CK levels were elevated significantly above the control levels 30 min after TO injection. AST increased to double the control levels in the sera of TO-injected rats by 24 hours ($P < 0.05$). ALT and AP did not change during the treatment. Results represent the mean value ± SEM (n=3).

4.1.1.IV Serum levels of acute-phase cytokines

In the serum of the TO-treated rats high concentrations of both IL-6 and IL-1ß were found. Serum concentration of IL-6 was 2018 pg/ml when compared to control rats (196 pg/ml) by 6 hours. IL-1ß concentration in the serum was 150 pg/ml compared to 55 pg/ml in the control animals by 12 hours of the onset of APR (Figure 10 A) with similar

kinetics of their mRNA expressions in the muscle (Figure 12 A). This increase of serum IL-6 was significant ($P = 13\times10^{-4}$); however, the increase in IL-1ß concentrations was significant only at later time points when analysed by Student's *t*-test; however, it was not significant when analysed by one way ANOVA ($P = 0.15$).

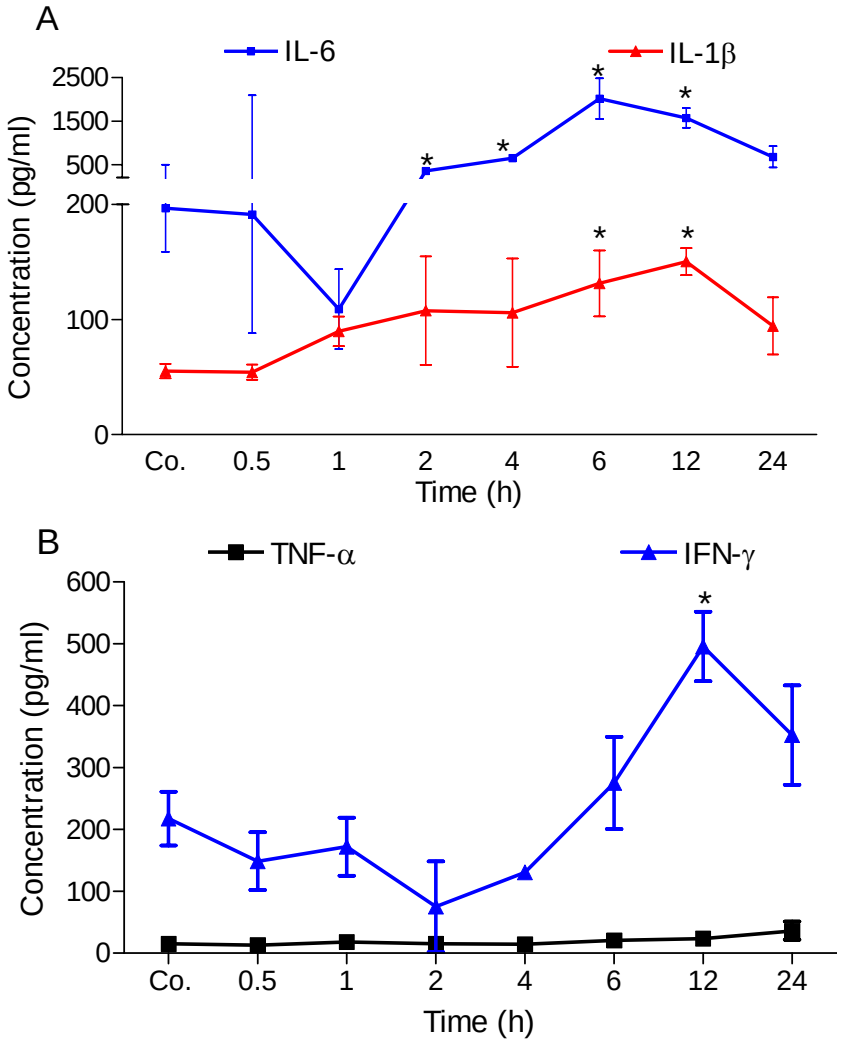


Figure 10: Serum concentration of IL-6, IL-1ß *(A),* TNF-α and IFN-γ *(B)* were measured by rat specific Quantikine® ELISA as per manufacturer's instruction. Serum IL-6 concentration was significantly increased; however, the changes in the serum IL-1ß concentrations were not statistically significant *(A)*. Serum IFN-γ concentration was increased significantly but this increase was delayed to 12 hours *(B)*. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).

Serum levels of TNF-α and IFN-γ was also measured and a delayed but significant increase in serum IFN-γ concentration was found ($P = 0.02$) through the experimental

study. On the other hand, the changes in the serum level of TNF-α were not statistically significant (Figure 10 B).

4.1.2 Real-time PCR analysis of acute-phase cytokines

4.1.2.I Acute-phase cytokines gene expression in the liver

As revealed by real-time PCR analysis, gene expression of IL-1β and TNF-α was significantly upregulated in the liver reaching to the maximum of 3.24- and 3.83-fold, respectively (4-6 hours), whereas IL-6 and IFN-γ gene expression was downregulated; however, the downregulation of IFN-γ was statistically significant but not of the IL-6, when analysed by one way ANOVA (Figure 11 A, B).

4.1.2.II Acute-phase cytokines gene expression in the injured muscle

In the hind limb muscle, the site of injury, IL-6 gene expression was significantly upregulated to 3427-fold by 6 hours after the onset of the APR as revealed by real-time PCR. At the same time IL-1ß gene expression was significantly upregulated to 400-fold. IFN-γ gene expression was significantly upregulated to 5.71 fold (Figure 12 A, B) earlier at 4 hours whereas, TNF-α gene expression was not significantly changed.

4.1.2.III Acute-phase cytokines gene expression in the extrahepatic tissues in TO injected rats

Real-time PCR analysis has shown significantly increased expression of IL-6 mRNA in the heart, small intestine, colon, spleen, kidney and lung from 6–12 hours (Figure 13 A-F). Changes of IL-1ß gene expression were weak in different organs but statistically significant in the lung and kidney when analysed by one way ANOVA. IFN-γ gene expression was downregulated in the extrahepatic organs from 2-12 hours with the exception of kidney where an upregulation was observed at 6 hours; however, these changes were not significant when analysed by one way ANOVA except in small intestine. On the other hand, TNF-α gene expression was variable and non-significant (Figure 13A-F). Above all it is interesting to note that the base line of IL-1ß and TNF-α mRNA in the liver and in the injured muscle was quantitatively much more abundant than IL-6 and IFN-γ as determined by comparing the Ct-values obtained by the real-time PCRs (Table 4).

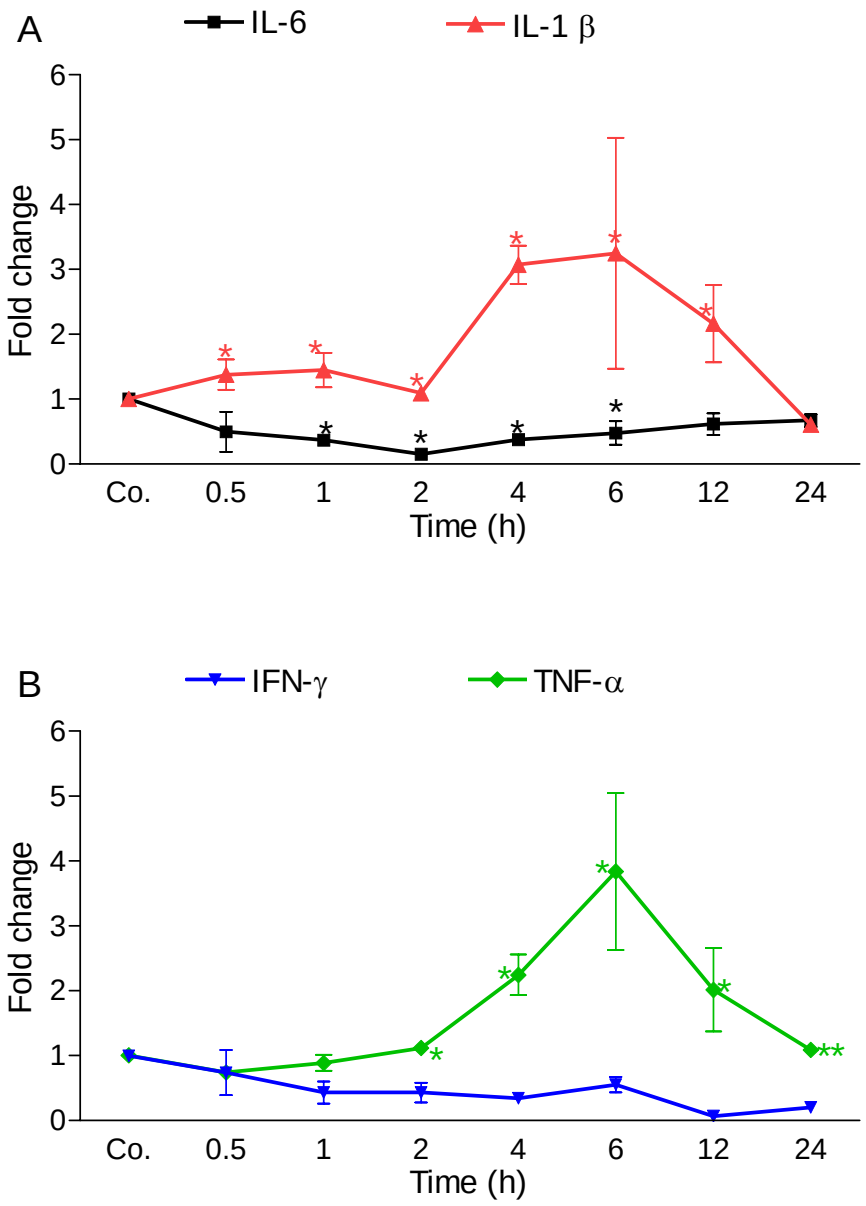


Figure 11: mRNA expression of acute-phase cytokines in the liver at different time points after intramuscular injection of TO. The mRNA expression was quantified by real-time PCR. IL-6 mRNA was downregulated in the liver; however, IL-1ß mRNA was induced to a maximum from 4-6 hours after TO injection *(A)*. TNF-α mRNA was upregulated to its maximum by 6 hours; however, IFN-γ gene expression was significantly downregulated in the liver after the onset of the APR. Results represent mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).

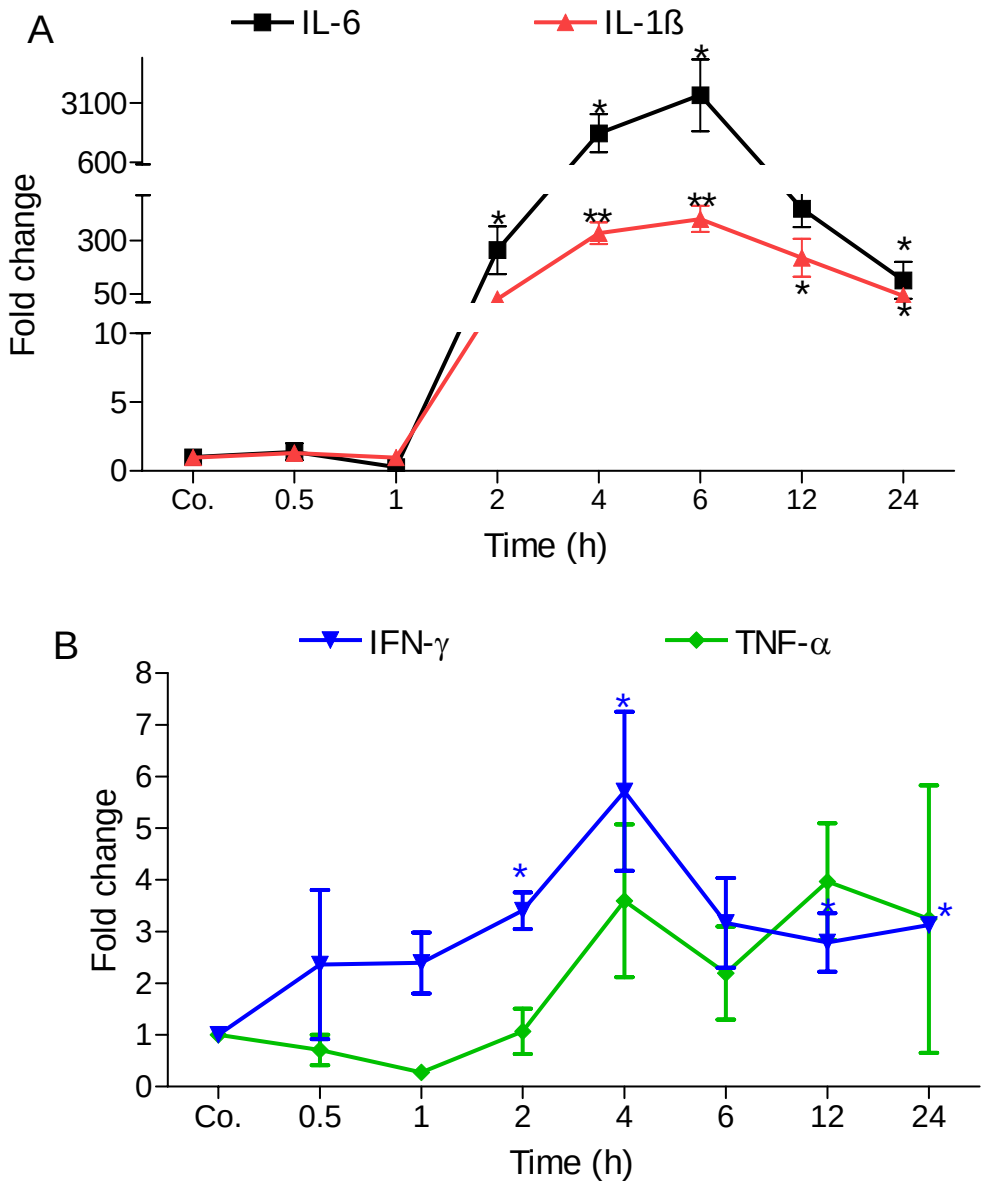


Figure 12: mRNA expression of acute-phase cytokines in the injured muscle. The mRNA expression was quantified by real-time PCR. IL-6 and IL-1ß mRNA was induced in the muscle already two hours after TO injection *(A)*. IFN-γ and TNF-α gene expression was also found to be upregulated; however, the changes in the TNF-α gene expression were not significant. Results represent mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).

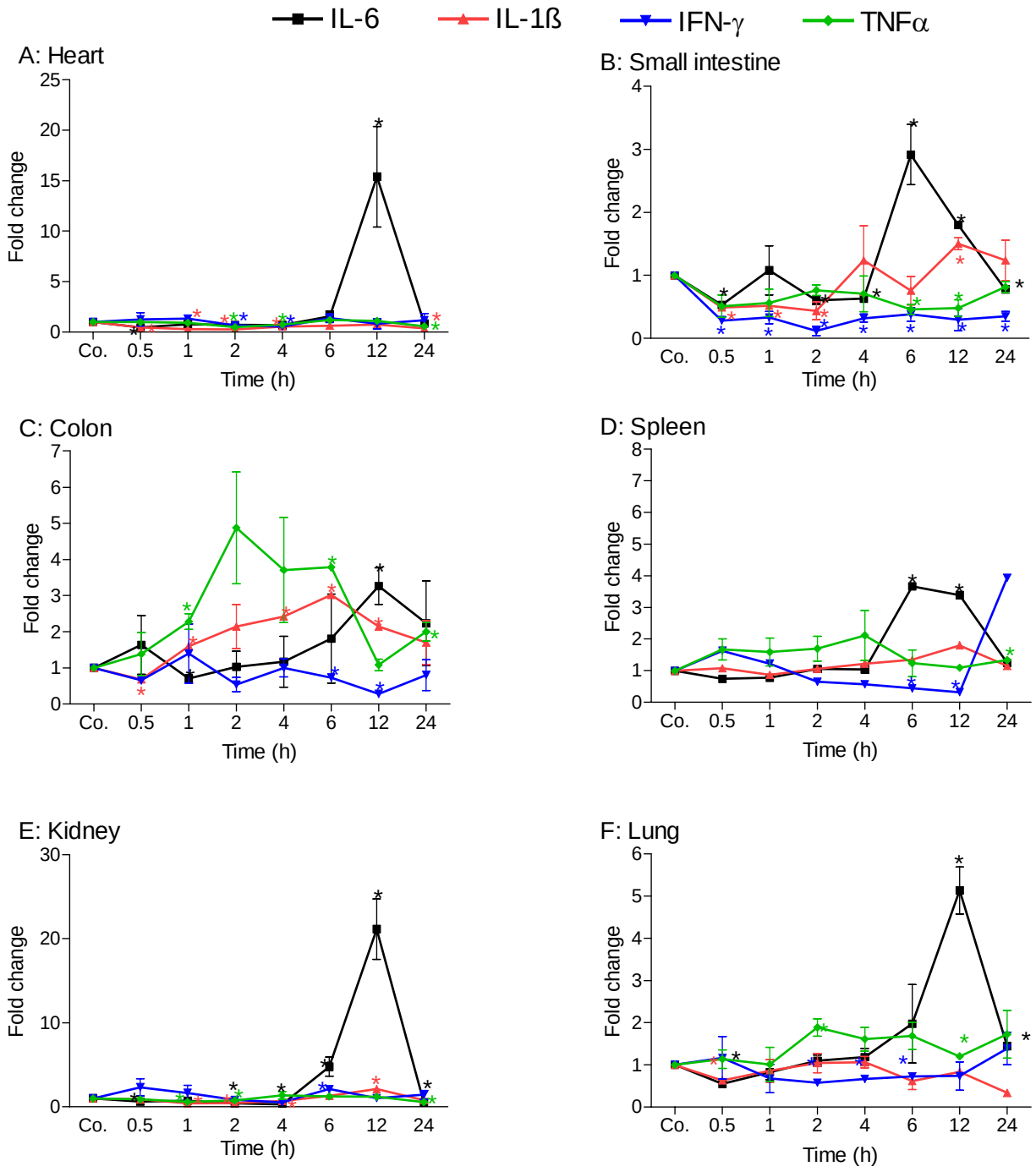


Figure 13: mRNA expression of acute-phase cytokines in extrahepatic organs. IL-6, IL-1ß, IFN-γ and TNF-α mRNA expression was analysed by real-time PCR. An upregulation of IL-6 mRNA expression was found in the extrahepatic organs from 6-12 hours *(A-F)*. IL-1ß mRNA expression was variable but significant in the kidney and lung *(E, F)*. IFN-γ gene expression was downregulated in the extrahepatic organs; however, the downregulation was only significant in the small intestine (*$P < 0.05$, analysed by Student's *t*-test; n=4).

4.1.3 Real-time PCR analysis of iron regulatory genes

4.1.3.I Expression of iron regulatory genes in the liver

Hepc gene expression was upregulated (7 ± 0.72 fold; $P < 0.05$) by 6 hours after the onset of the APR. This upregulation was highly significant ($P < 10^{-4}$). Tf gene expression was upregulated at earlier time points but later on the gene expression was downregulated (0.70 ± 0.08 fold; Figure 14 A, B). The changes in the Tf gene expression were highly significant during the whole-course of study analysed by one way ANOVA ($P = 0.0002$). Both TfR1 and TfR2 responsible for Tf bound iron uptake gene expression was significantly upregulated. An early upregulation of TfR1 gene expression was observed (1.7 ± 0.24 fold; $P < 0.05$) 30 min. after the TO injection; however, TfR2 upregulation was delayed (1.76 ± 0.03 fold; $P < 0.05$) to 6 hours (Figure 14 C).

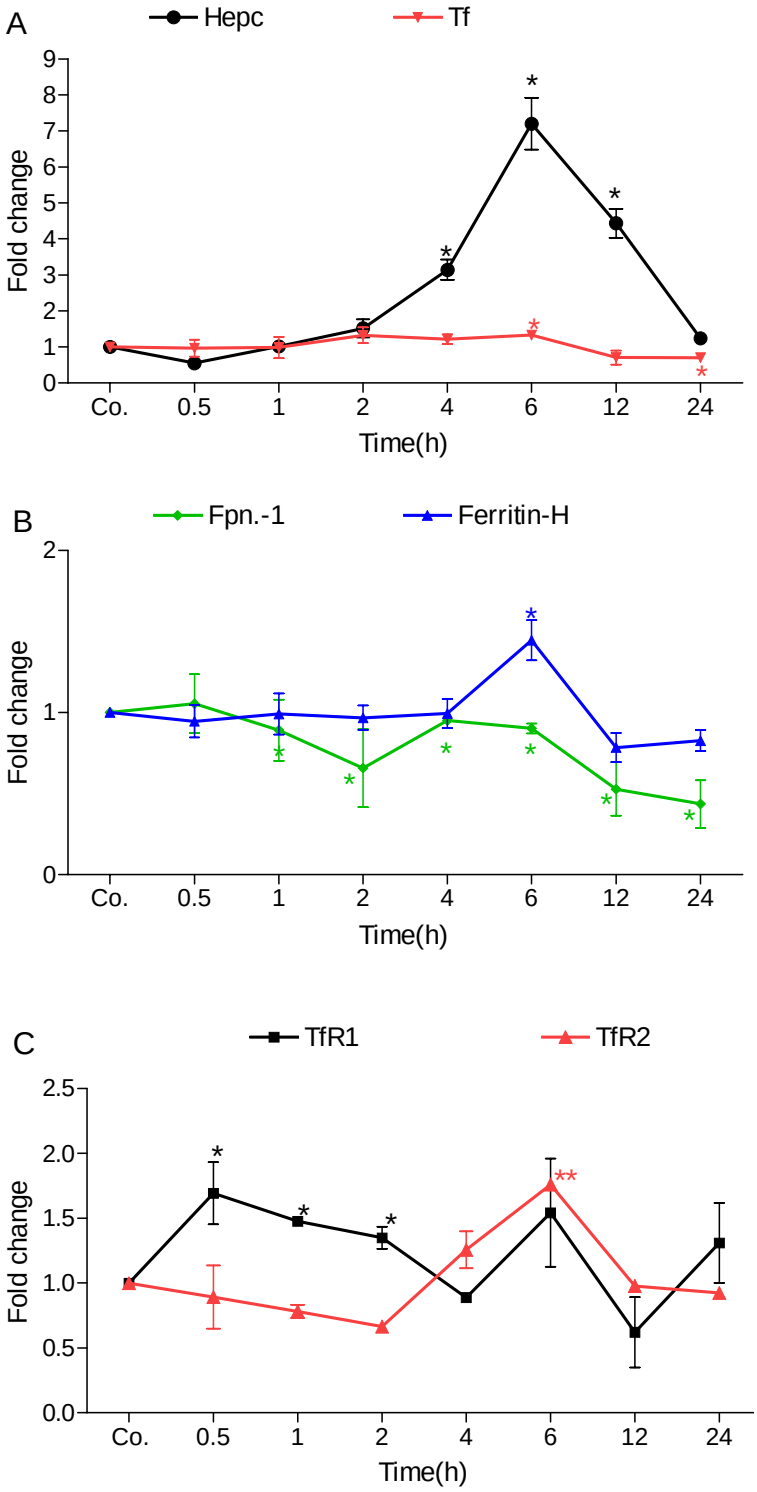
Ferritin-H and DMT1 gene expression was upregulated (1.44 ± 0.12, 2.85 ± 0.27 fold, respectively) by 6 hours (Figure 14 B, D). DMT1 upregulation was not significant through the experimental study when analysed by one way ANOVA; however, the upregulation at a specific time point was significant when analysed by Student's *t*-test. A statistically significant upregulation of IRE-BP1 (1.7 ± 0.08 fold, $P < 0.05$) was found after 6 hours of TO injection; however, IRE-BP2 upregulation was statistically non significant at the same time ($P = 0.06$; Figure 14 F).

Hjv gene expression was significantly downregulated (0.12 ± 0.05 fold; $P < 0.001$; Figure 14 C) at 6 hours while Fpn.-1, Dcytb, HFE and Heph gene expression (0.30 ± 0.10, 0.30 ± 0.07, 0.18 ± 0.03 and 0.27 ± 0.03 fold respectively; $P < 0.05$) was downregulated at 12 hours (Figure 14). The downregulation was statistically highly significant when analysed by one way ANOVA ($P < 0.0005$).

4.1.3.II Expression of iron regulatory genes in the injured muscle

Hepc, TfR2 and HFE gene expression was significantly downregulated at the site of injury in the muscle (0.28 ± 0.08, 0.55 ± 0.03 and 0.12 ± 0.01 fold respectively; Figure 15 A, C, E). Hjv gene expression was also downregulated quite early (0.40 ± 0.01 fold) at 2 hours while Dcytb gene expression was significantly upregulated (2.31 ± 0.24 fold) at the same time (Figure 14 D, E). Ferritin-H, Fpn.-1, DMT1, Heph, Tf, IRE-BP1 and IRE-BP2 gene expression was significantly upregulated (1.98 ± 0.004, 2.34 ± 0.05, 4.50 ±

0.32, 10.0 ± 0.40, 4.62 ± 1.96, 1.29 ± 0.01 and 1.88 ± 0.20 fold respectively; Figure 15 A, B, D, E). The data obtained for Hjv and for Fpn.-1 were confirmed by northern blot analysis (Figure 22 B).



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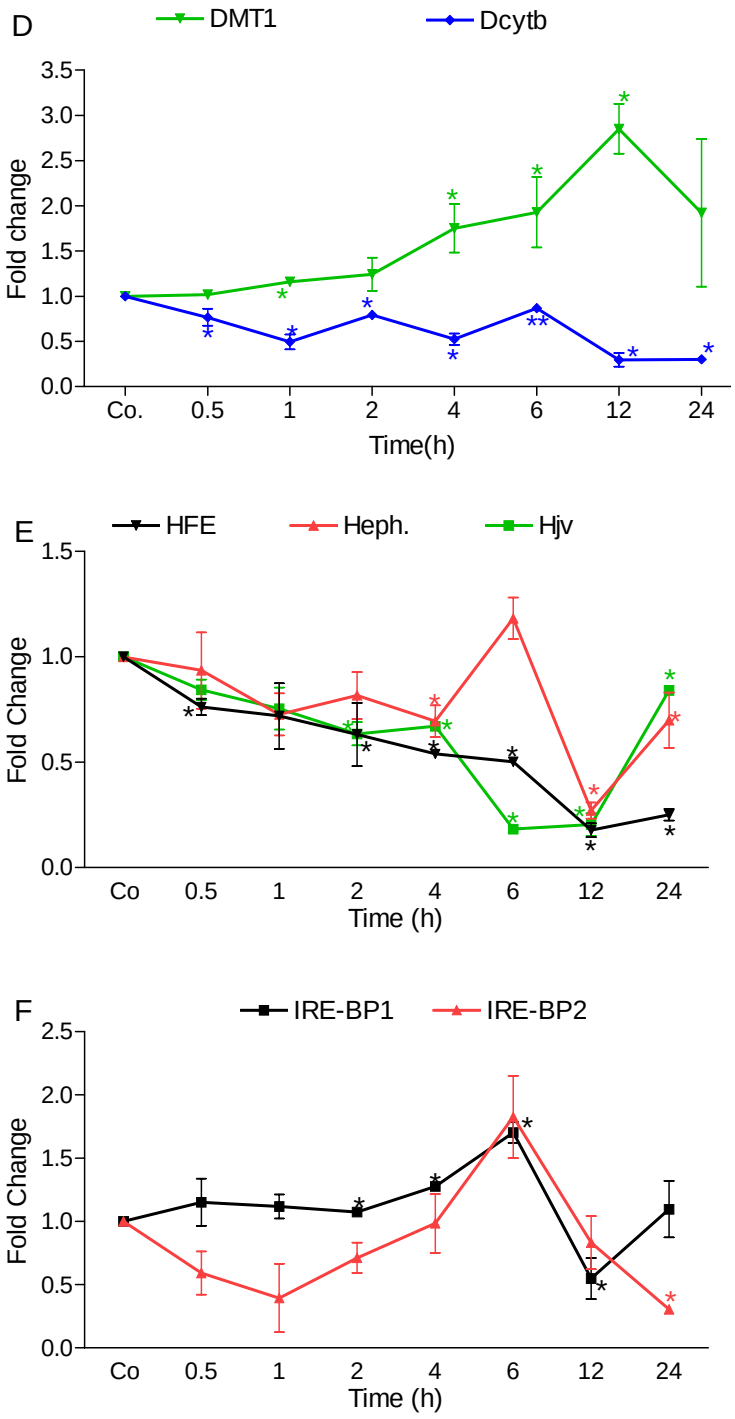


Figure 14: mRNA expression of Hepc, Tf *(A),* Fpn.-1, Ferritin-H *(B),* TfR2, TfR1 *(C),* DMT1, Dcytb *(D)*, HFE, Heph, Hjv *(E),* and IRE-BP1, IRE-BP2 *(F)* in the liver of the rats after 5mg/kg administration of TO in the hind limb muscle. The animals were sacrificed at given time points, RNA was isolated, reverse transcribed and subjected to real-time PCR as described in materials and methods. Results represent mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).

Table 4: Ct values (Threshold cycle) is defined as "the fractional cycle number at which the fluorescence passes the fixed threshold". This table includes the ct values of iron metabolism genes (real time PCR analysis of total RNA) in the liver, in the Injured muscle, in the heart, in the Small intestine, in the colon, in the spleen, in the kidney and in the lung taken from the rats after the TO injection. The Higher ct value for the specific gene at a given time the lower is the abundance of the specific mRNA.

	Time(h)	Liver	Injured muscle	Heart	Lung	Small intestine	Colon	Spleen	Kidney
Hepcidin	Co.	20.03 ± 0.01	28.46 ± 2.63	23.14 ± 0.01	22.81 ± 0.39	31.14 ± 0.19	32.28 ± 0.17	31.70 ± 0.05	31.72 ± 0.23
	0.5	20.80 ± 0.02	28.47 ± 0.15	23.19 ± 0.08	23.90 ± 0.41	30.88 ± 0.13	31.94 ± 0.07	30.62 ± 0.14	32.26 ± 0.26
	1	19.57 ± 0.07	29.18 ± 0.38	22.26 ± 0.03	24.04 ± 0.29	31.20 ± 0.73	32.27 ± 0.05	30.90 ± 0.29	32.14 ± 0.09
	2	19.58 ± 0.03	30.12 ± 0.08	24.96 ± 0.20	22.73 ± 0.01	30.70 ± 0.08	31.03 ± 0.08	30.75 ± 0.41	31.03 ± 0.45
	4	18.08 ± 0.02	27.94 ± 0.11	25.82 ± 0.38	24.32 ± 0.80	30.28 ± 0.80	30.16 ± 0.05	31.22 ± 0.38	31.05 ± 0.01
	6	17.34 ± 0.11	29.14 ± 0.13	20.77 ± 0.46	24.94 ± 0.65	30.70 ± 0.58	32.65 ± 0.01	31.28 ± 0.09	30.80 ± 0.20
	12	17.74 ± 0.28	30.81 ± 0.16	21.25 ± 0.01	23.16 ± 0.12	30.99 ± 0.16	32.06 ± 0.14	30.18 ± 0.10	29.39 ± 0.22
	24	18.43 ± 0.05	29.90 ± 0.83	22.17 ± 0.10	24.62 ± 0.11	31.36 ± 0.21	31.97 ± 0.20	30.87 ± 0.20	32.07 ± 0.24
Hjv	Co.	21.88 ± 0.10	18.59 ± 0.23	21.18 ± 0.05	26.40 ± 0.02	33.67 ± 0.16	35.01 ± 0.62	31.89 ± 0.05	26.36 ± 0.07
	0.5	21.49 ± 0.23	18.80 ± 0.11	20.18 ± 0.20	27.24 ± 0.23	33.88 ± 0.47	34.00 ± 0.54	31.22 ± 0.56	25.87 ± 0.38
	1	21.94 ± 0.43	19.36 ± 0.45	20.62 ± 0.16	28.19 ± 0.31	34.07 ± 0.12	34.40 ± 0.49	31.77 ± 0.48	26.39 ± 0.37
	2	22.35 ± 0.08	19.58 ± 0.38	20.94 ± 0.11	27.06 ± 0.00	33.25 ± 0.28	33.86 ± 0.28	30.53 ± 0.26	26.37 ± 0.08
	4	22.09 ± 0.03	19.31 ± 0.01	20.22 ± 0.63	27.13 ± 0.26	34.00 ± 0.96	34.60 ± 0.43	30.28 ± 0.09	25.30 ± 0.38
	6	24.07 ± 0.17	19.13 ± 0.02	20.08 ± 0.86	28.34 ± 0.47	34.40 ± 0.79	35.13 ± 0.36	31.86 ± 0.40	26.79 ± 0.49
	12	23.45 ± 0.16	19.55 ± 0.09	21.59 ± 0.10	27.29 ± 0.29	33.74 ± 0.65	34.58 ± 0.64	31.24 ± 0.40	28.48 ± 0.15
	24	21.14 ± 0.07	20.20 ± 0.16	20.73 ± 0.09	27.91 ± 0.47	33.63 ± 0.62	33.61 ± 0.36	30.72 ± 0.10	26.30 ± 0.39

	Time (h)	Liver	Injured muscle	Heart	Lung	Small intestine	Colon	Spleen	Kidney
DMT1	Co.	29.73 ± 0.13	31.14 ± 0.58	27.37 ± 0.04	28.60 ± 0.16	24.34 ± 0.07	30.01 ± 0.03	27.25 ± 0.02	23.67 ± 0.11
	0.5	29.00 ± 0.33	30.82 ± 0.10	26.97 ± 0.17	27.64 ± 0.50	23.56 ± 0.38	28.84 ± 0.16	26.48 ± 0.24	24.15 ± 0.68
	1	29.11 ± 0.28	30.88 ± 0.12	27.43 ± 0.17	28.25 ± 0.36	26.50 ± 0.41	29.24 ± 0.12	27.31 ± 0.21	24.58 ± 0.73
	2	29.28 ± 0.25	31.44 ± 0.29	27.46 ± 0.20	27.00 ± 0.37	25.28 ± 0.10	29.56 ± 0.20	27.96 ± 0.24	24.57 ± 0.22
	4	28.69 ± 0.17	30.07 ± 0.27	27.77 ± 0.43	26.90 ± 0.22	24.38 ± 0.52	29.10 ± 0.11	27.59 ± 0.04	24.59 ± 0.49
	6	28.86 ± 0.21	29.29 ± 0.12	28.49 ± 0.05	28.46 ± 0.57	26.77 ± 0.64	28.71 ± 0.21	27.40 ± 0.29	24.37 ± 0.33
	12	27.37 ± 0.05	30.05 ± 0.27	28.06 ± 0.10	28.18 ± 0.42	28.05 ± 0.02	30.52 ± 0.01	27.89 ± 0.41	25.05 ± 0.13
	24	27.99 ± 0.30	30.74 ± 0.18	27.94 ± 0.18	27.46 ± 0.32	25.35 ± 0.59	30.12 ± 0.05	28.48 ± 0.22	23.68 ± 0.13
TfR1	Co.	25.89 ± 0.10	26.27 ± 0.12	26.15 ± 0.60	26.86 ± 0.20	25.17 ± 1.05	25.01 ± 0.78	22.61 ± 0.05	24.37 ± 0.49
	0.5	24.36 ± 0.18	25.47 ± 0.02	24.42 ± 1.02	26.52 ± 0.05	24.81 ± 0.81	25.52 ± 1.02	22.60 ± 0.49	23.49 ± 0.59
	1	24.85 ± 0.22	24.73 ± 0.10	25.06 ± 1.02	26.32 ± 0.15	25.52 ± 0.79	25.71 ± 0.64	22.04 ± 0.06	24.40 ± 0.63
	2	25.24 ± 0.32	26.58 ± 0.23	24.72 ± 0.94	26.17 ± 0.30	25.51 ± 0.14	25.34 ± 0.92	23.21 ± 0.25	24.14 ± 0.64
	4	25.75 ± 0.10	26.26 ± 0.13	25.26 ± 0.61	26.28 ± 0.12	25.42 ± 0.22	25.39 ± 0.61	23.48 ± 0.86	24.35 ± 0.73
	6	25.30 ± 0.10	25.86 ± 0.14	26.39 ± 0.64	26.41 ± 0.11	25.71 ± 1.07	24.03 ± 0.04	22.96 ± 0.49	24.28 ± 0.49
	12	25.80 ± 0.23	28.17 ± 0.12	27.80 ± 0.58	26.63 ± 0.37	25.41 ± 0.96	24.57 ± 0.12	23.74 ± 0.70	24.71 ± 0.58
	24	24.53 ± 0.17	28.51 ± 0.29	26.17 ± 0.61	26.02 ± 0.07	25.75 ± 0.98	25.66 ± 0.40	24.99 ± 0.59	23.67 ± 0.35
TfR2	Co.	20.92 ± 0.07	31.44 ± 0.32	31.86 ± 0.38	30.37 ± 0.00	31.01 ± 0.13	32.29 ± 0.20	24.02 ± 0.09	28.25 ± 0.14
	0.5	20.38 ± 0.04	31.91 ± 0.29	32.28 ± 0.46	30.94 ± 0.16	31.96 ± 0.21	32.20 ± 0.26	24.08 ± 0.30	28.89 ± 0.38
	1	20.82 ± 0.24	31.67 ± 0.05	32.49 ± 0.28	30.92 ± 0.32	31.78 ± 0.32	32.15 ± 0.20	24.41 ± 0.18	28.51 ± 0.21
	2	21.31 ± 0.30	32.51 ± 0.10	32.05 ± 0.17	30.49 ± 0.19	32.96 ± 0.07	32.35 ± 0.19	25.54 ± 0.12	29.09 ± 0.33
	4	20.31 ± 0.05	32.47 ± 0.20	32.55 ± 0.14	29.88 ± 0.18	34.24 ± 0.57	32.12 ± 0.65	24.10 ± 0.06	28.51 ± 0.17
	6	20.07 ± 0.17	32.49 ± 0.03	31.90 ± 0.22	30.84 ± 0.32	32.37 ± 0.59	32.86 ± 0.26	24.70 ± 0.35	27.34 ± 0.52
	12	20.05 ± 0.18	33.04 ± 0.44	32.67 ± 0.48	31.07 ± 0.48	32.02 ± 0.38	32.08 ± 0.17	25.30 ± 0.27	28.35 ± 0.69
	24	20.04 ± 0.18	29.49 ± 0.64	32.53 ± 0.30	30.74 ± 0.22	30.93 ± 0.34	32.67 ± 0.26	26.36 ± 0.05	28.49 ± 0.28

	Time (h)	Liver	Injured muscle	Heart	Lung	Small intestine	Colon	Spleen	Kidney
HFE	Co.	20.52 ± 0.15	22.65 ± 0.03	26.33 ± 0.08	23.39 ± 0.07	26.33 ± 0.37	26.19 ± 0.13	25.38 ± 0.17	25.30 ± 0.08
	0.5	20.41 ± 0.20	24.10 ± 0.09	26.47 ± 0.25	23.50 ± 0.02	26.81 ± 0.15	25.85 ± 0.22	25.20 ± 0.14	25.44 ± 0.27
	1	20.51 ± 0.34	24.61 ± 0.75	26.37 ± 0.19	23.44 ± 0.08	26.68 ± 0.29	26.20 ± 0.18	25.56 ± 0.10	25.49 ± 0.21
	2	20.73 ± 0.17	23.51 ± 0.12	26.58 ± 0.06	23.21 ± 0.12	26.85 ± 0.09	25.87 ± 0.43	25.38 ± 0.07	26.03 ± 0.06
	4	21.15 ± 0.25	25.46 ± 0.08	26.89 ± 0.19	23.56 ± 0.17	26.53 ± 0.25	26.14 ± 0.17	25.52 ± 0.10	25.83 ± 0.14
	6	21.10 ± 0.24	26.01 ± 0.06	26.79 ± 0.14	23.85 ± 0.10	26.77 ± 0.30	25.76 ± 0.07	25.94 ± 0.27	25.57 ± 0.17
	12	22.12 ± 0.16	25.57 ± 0.10	27.11 ± 0.10	24.20 ± 0.13	27.29 ± 0.31	26.98 ± 0.06	25.98 ± 0.13	26.47 ± 0.37
	24	21.53 ± 0.28	23.46 ± 0.26	26.91 ± 0.25	23.84 ± 0.26	26.37 ± 0.28	25.94 ± 0.06	25.46 ± 0.27	25.76 ± 0.05
Dcytb	Co.	26.02 ± 0.20	28.91 ± 0.25	26.91 ± 0.19	22.65 ± 0.12	23.98 ± 0.12	24.72 ± 0.19	22.99 ± 0.17	27.29 ± 0.08
	0.5	26.08 ± 0.42	28.21 ± 0.02	27.08 ± 0.20	22.56 ± 0.04	23.20 ± 0.30	24.76 ± 0.36	23.07 ± 0.00	27.25 ± 0.44
	1	26.76 ± 0.37	28.54 ± 0.09	27.00 ± 0.22	22.81 ± 0.10	24.54 ± 0.06	24.40 ± 0.10	23.32 ± 0.14	26.94 ± 0.07
	2	26.60 ± 0.23	27.55 ± 0.06	27.33 ± 0.10	22.50 ± 0.17	25.02 ± 0.31	24.83 ± 0.13	22.91 ± 0.11	27.09 ± 0.11
	4	26.79 ± 0.26	28.49 ± 0.09	27.51 ± 0.08	22.80 ± 0.21	22.87 ± 0.25	25.25 ± 0.10	23.20 ± 0.22	27.07 ± 0.39
	6	26.86 ± 0.36	29.10 ± 0.02	27.01 ± 0.06	22.99 ± 0.27	24.65 ± 0.10	24.74 ± 0.03	23.25 ± 0.31	26.72 ± 0.19
	12	27.17 ± 0.35	28.94 ± 0.06	27.37 ± 0.22	23.31 ± 0.39	24.90 ± 0.23	24.50 ± 0.11	23.27 ± 0.13	27.11 ± 0.12
	24	27.01 ± 0.22	27.86 ± 0.11	27.10 ± 0.08	23.39 ± 0.45	23.55 ± 0.16	24.63 ± 0.16	23.19 ± 0.05	27.14 ± 0.05
Hephaestin	Co.	28.43 ± 0.13	32.05 ± 0.76	26.73 ± 0.10	24.50 ± 0.21	24.23 ± 0.53	24.29 ± 0.23	24.71 ± 0.16	26.44 ± 0.10
	0.5	28.27 ± 0.31	29.34 ± 0.23	27.60 ± 0.49	24.19 ± 0.08	22.44 ± 0.32	24.44 ± 0.75	24.86 ± 0.14	26.42 ± 0.37
	1	28.52 ± 0.16	28.91 ± 0.06	27.12 ± 0.68	24.38 ± 0.13	23.65 ± 0.10	25.74 ± 0.68	24.15 ± 0.66	26.54 ± 0.32
	2	28.68 ± 0.28	32.05 ± 0.76	27.57 ± 0.51	23.96 ± 0.16	25.39 ± 0.97	24.73 ± 1.17	23.36 ± 1.00	26.35 ± 0.16
	4	28.71 ± 0.23	29.34 ± 0.23	27.66 ± 0.51	23.94 ± 0.03	24.48 ± 0.62	24.33 ± 0.46	24.79 ± 1.17	26.85 ± 0.15
	6	28.32 ± 0.12	28.91 ± 0.06	27.43 ± 0.47	24.31 ± 0.13	24.29 ± 0.23	23.66 ± 0.11	24.79 ± 0.84	26.01 ± 0.20
	12	28.97 ± 0.39	29.34 ± 0.23	27.64 ± 0.50	24.64 ± 0.35	24.04 ± 0.07	23.37 ± 0.05	24.54 ± 1.26	26.64 ± 0.33
	24	28.17 ± 0.21	32.05 ± 0.76	27.46 ± 0.48	24.32 ± 0.06	24.23 ± 0.11	24.57 ± 0.16	24.83 ± 1.31	26.17 ± 0.14

	Time (h)	Liver	Injured muscle	Heart	Lung	Small intestine	Colon	Spleen	Kidney
Ferritin-H	Co.	16.76 ± 0.12	16.66 ± 0.04	16.46 ± 0.05	15.60 ± 0.10	15.30 ± 0.04	15.40 ± 0.01	16.04 ± 0.02	14.74 ± 0.02
	0.5	16.24 ± 0.11	16.46± 0.05	16.41 ± 0.04	15.55 ± 0.10	15.16 ± 0.07	15.24 ± 0.03	15.77 ± 0.05	14.82 ± 0.10
	1	16.48 ± 0.06	16.46± 0.23	16.53 ± 0.11	15.91 ± 0.13	15.58 ± 0.21	15.52 ± 0.25	16.33 ± 0.21	14.93 ± 0.13
	2	16.77 ± 0.23	16.19± 0.44	16.53 ± 0.07	15.49 ± 0.12	15.59 ± 0.14	15.21 ± 0.10	15.75 ± 0.08	14.93 ± 0.05
	4	16.60 ± 0.05	16.25± 0.05	16.20 ± 0.17	15.55 ± 0.13	15.62 ± 0.27	15.31 ± 0.09	15.90 ± 0.10	14.75 ± 0.03
	6	16.39 ± 0.12	15.77± 0.01	16.37 ± 0.12	15.57 ± 0.12	15.68 ± 0.16	15.14 ± 0.02	15.96 ± 0.06	14.65 ± 0.09
	12	16.37 ± 0.06	15.84± 0.19	16.43 ± 0.11	15.59 ± 0.14	16.20 ± 0.08	15.17 ± 0.03	15.78 ± 0.07	14.82 ± 0.03
	24	16.20 ± 0.02	15.82± 0.27	16.42 ± 0.04	15.52 ± 0.02	15.47 ± 0.23	15.67 ± 0.10	15.86 ± 0.06	14.80 ± 0.05
Transferrin	Co.	15.16 ± 0.02	24.77 ± 0.28	23.23 ± 0.16	22.43 ± 0.06	25.10 ± 0.48	22.15 ± 0.07	20.28 ± 0.10	21.89 ± 0.05
	0.5	15.09 ± 0.15	24.78 ± 0.86	23.33 ± 0.12	22.30 ± 0.27	25.19 ± 0.14	21.76 ± 0.11	20.15 ± 0.08	22.60 ± 0.33
	1	15.22 ± 0.13	24.32 ± 0.21	23.20 ± 0.17	22.38 ± 0.12	25.33 ± 0.29	21.81 ± 0.13	20.61 ± 0.02	22.29 ± 0.10
	2	15.05 ± 0.11	23.93 ± 0.13	23.46 ± 0.07	22.19 ± 0.10	25.07 ± 0.29	21.62 ± 0.30	20.17 ± 0.03	22.04 ± 0.03
	4	15.06 ± 0.13	23.98 ± 0.58	24.15 ± 0.23	21.30 ± 0.11	24.68 ± 0.31	22.57 ± 0.17	20.54 ± 0.03	23.34 ± 0.11
	6	15.02 ± 0.10	23.57 ± 0.70	23.64 ± 0.14	21.61 ± 0.17	24.77 ± 0.26	22.62 ± 0.09	20.74 ± 0.22	22.30 ± 0.14
	12	15.49 ± 0.05	22.29 ± 0.57	23.27 ± 0.09	20.68 ± 0.36	24.93 ± 0.28	22.01 ± 0.18	20.43 ± 0.07	21.81 ± 0.18
	24	15.25 ± 0.14	22.41 ± 0.28	23.24 ± 0.40	21.64 ± 0.07	24.69 ± 0.32	22.60 ± 0.28	20.41 ± 0.24	22.19 ± 0.26
Fpn.-1	Co.	21.95 ± 0.24	27.41 ± 0.21	24.84 ± 0.06	24.87 ± 0.07	21.95 ± 0.02	22.69 ± 0.11	19.70 ± 0.06	23.16 ± 0.23
	0.5	21.54 ± 0.35	28.42 ± 0.20	24.82 ± 0.04	24.80 ± 0.23	21.49 ± 0.09	22.61 ± 0.15	20.03 ± 0.10	23.33 ± 0.51
	1	21.81 ± 0.37	27.11 ± 0.11	25.00 ± 0.08	24.86 ± 0.06	22.98 ± 0.23	22.27 ± 0.04	20.29 ± 0.09	23.10 ± 0.28
	2	22.18 ± 0.21	25.83 ± 0.25	25.31 ± 0.05	24.76 ± 0.03	22.75 ± 0.21	22.94 ± 0.24	19.71 ± 0.21	23.27 ± 0.15
	4	22.09 ± 0.35	26.13 ± 0.14	25.05 ± 0.20	23.88 ± 0.05	21.26 ± 0.72	22.62 ± 0.21	20.28 ± 0.08	23.59 ± 0.12
	6	22.64 ± 0.22	26.31 ± 0.00	25.15 ± 0.03	24.70 ± 0.12	22.36 ± 0.04	21.91 ± 0.18	20.74 ± 0.22	23.35 ± 0.39
	12	22.77 ± 0.20	26.16 ± 0.26	25.06 ± 0.27	24.98 ± 0.06	23.44 ± 0.26	22.16 ± 0.02	19.99 ± 0.18	23.62 ± 0.01
	24	21.87 ± 0.27	25.25 ± 0.05	24.89 ± 0.05	24.90 ± 0.14	21.91 ± 0.57	22.86 ± 0.24	20.35 ± 0.19	22.96 ± 0.10

	Time(h)	Liver	Injured muscle	Heart	Lung	Small intestine	Colon	Spleen	Kidney
IRE-BP1	Co.	23.04 ± 0.03	25.02 ± 0.12	23.82 ± 0.06	24.43 ± 0.05	21.38 ± 0.02	22.26 ± 0.03	23.07 ± 0.03	20.61 ± 0.21
	0.5	22.09 ± 0.12	24.19 ± 0.05	23.84 ± 0.09	22.47 ± 0.22	21.10 ± 0.09	21.56 ± 0.19	22.22 ± 0.24	20.43 ± 0.28
	1	22.43 ± 0.23	24.37 ± 0.14	23.85 ± 0.07	22.48 ± 0.26	21.80 ± 0.46	22.53 ± 0.37	22.60 ± 0.19	20.55 ± 0.37
	2	22.74 ± 0.33	24.77 ± 0.05	24.30 ± 0.07	22.54 ± 0.26	21.80 ± 0.16	22.52 ± 0.34	22.40 ± 0.26	20.68 ± 0.35
	4	22.39 ± 0.05	24.73 ± 0.22	24.61 ± 0.11	22.37 ± 0.57	21.69 ± 0.19	22.07 ± 0.18	22.33 ± 0.11	20.46 ± 0.21
	6	22.27 ± 0.21	24.71 ± 0.04	24.46 ± 0.17	22.45 ± 0.43	20.99 ± 0.26	20.94 ± 0.07	22.57 ± 0.32	19.80 ± 0.26
	12	23.07 ± 0.11	23.84 ± 0.10	24.37 ± 0.09	22.91 ± 0.27	21.37 ± 0.17	21.84 ± 0.06	22.41 ± 0.23	20.00 ± 0.15
	24	22.94 ± 0.06	23.71 ± 0.28	24.25 ± 0.11	22.39 ± 0.06	21.36 ± 0.08	22.16 ± 0.24	22.41 ± 0.28	20.59 ± 0.11
IRE-BP2	Co.	24.45 ± 0.01	26.60 ± 0.12	26.24 ± 0.15	24.43 ± 0.05	22.94 ± 0.10	24.91 ± 0.05	24.24 ± 0.12	23.86 ± 0.18
	0.5	24.50 ± 0.08	26.32 ± 0.12	26.57 ± 0.10	24.15 ± 0.11	22.14 ± 0.08	24.47 ± 0.14	23.63 ± 0.06	23.67 ± 0.29
	1	25.79 ± 1.57	26.66 ± 0.00	26.51 ± 0.46	24.26 ± 0.10	23.33 ± 0.19	25.24 ± 0.17	24.39 ± 0.18	23.78 ± 0.33
	2	24.75 ± 0.21	27.23 ± 0.06	26.13 ± 0.12	23.95 ± 0.30	23.51 ± 0.20	25.36 ± 0.45	24.23 ± 0.14	23.69 ± 0.37
	4	24.22 ± 0.16	26.57 ± 0.14	26.62 ± 0.19	24.04 ± 0.17	23.47 ± 0.01	24.81 ± 0.12	24.35 ± 0.05	23.72 ± 0.20
	6	23.60 ± 0.35	25.78 ± 0.23	26.74 ± 0.07	24.44 ± 0.13	23.97 ± 0.54	24.78 ± 0.07	24.83 ± 0.32	23.82 ± 0.51
	12	23.85 ± 0.07	25.41 ± 0.17	26.44 ± 0.08	24.74 ± 0.41	23.82 ± 0.15	24.86 ± 0.06	24.05 ± 0.12	23.87 ± 0.24
	24	25.16 ± 0.19	24.90 ± 0.12	26.22 ± 0.10	24.34 ± 0.04	23.30 ± 0.00	25.52 ± 0.55	24.15 ± 0.02	23.41 ± 0.41

	Time (h)	Liver	Heart	Lung	Small intestine	Colon	Spleen	Kidney
ß-Actin	Co.	19.27 ± 0.05	17.90 ± 0.31	15.07 ± 0.09	15.58 ± 0.38	15.86 ± 0.17	15.71 ± 0.01	16.48 ± 0.03
	0.5	18.51 ± 0.25	17.50 ± 0.26	15.07 ± 0.02	15.28 ± 0.37	15.63 ± 0.17	15.23 ± 0.09	16.38 ± 0.08
	1	18.81 ± 0.18	17.88 ± 0.30	15.39 ± 0.02	15.65 ± 0.22	15.73 ± 0.19	15.41 ± 0.15	16.71 ± 0.16
	2	19.07 ± 0.35	17.69 ± 0.37	15.13 ± 0.05	15.87 ± 0.44	15.86 ± 0.26	15.52 ± 0.09	16.93 ± 0.14
	4	18.98 ± 0.06	17.87 ± 0.46	15.15 ± 0.03	15.95 ± 0.37	16.07 ± 0.15	15.53 ± 0.17	16.65 ± 0.09
	6	19.27 ± 0.16	17.80 ± 0.36	15.31 ± 0.06	15.77 ± 0.30	15.77 ± 0.09	15.44 ± 0.14	16.69 ± 0.10
	12	18.36 ± 0.18	17.32 ± 0.02	15.52 ± 0.18	15.77 ± 0.43	15.70 ± 0.06	15.51 ± 0.21	16.61 ± 0.13
	24	18.28 ± 0.14	17.65 ± 0.27	15.35 ± 0.04	15.69 ± 0.26	16.12 ± 0.03	15.37 ± 0.12	16.33 ± 0.10

GAPDH	Injured muscle
Co.	15.44 ± 0.04
0.5	15.34 ± 0.07
1	14.98 ± 0.02
2	15.13 ± 0.01
4	15.40 ± 0.02
6	15.53 ± 0.08
12	15.46 ± 0.04
24	15.66 ± 0.06

Acute phase cytokines

	Time (h)	Liver	Injured muscle	Heart	Lung	Small intestine	Colon	Spleen	Kidney
IL-6	Co.	35.55 ± 0.18	32.76 ± 0.66	33.68 ± 0.08	32.21 ± 0.43	32.30 ± 0.37	31.47 ± 0.97	31.10 ± 0.06	31.69 ± 0.27
	0.5	36.34 ± 0.43	32.23 ± 0.05	32.71 ± 0.83	32.45 ± 0.34	32.60 ± 0.30	31.86 ± 0.72	31.48 ± 0.46	31.93 ± 0.29
	1	36.58 ± 0.33	34.62 ± 0.90	33.04 ± 0.42	32.20 ± 0.24	32.04 ± 0.30	32.98 ± 0.51	31.72 ± 0.52	32.06 ± 0.50
	2	38.28 ± 0.07	24.28 ± 0.07	32.88 ± 0.54	31.51 ± 0.18	33.01 ± 0.27	32.71 ± 0.12	31.06 ± 0.11	33.02 ± 0.29
	4	36.64 ± 0.43	20.58 ± 1.07	33.18 ± 0.56	31.43 ± 0.13	33.02 ± 0.18	32.90 ± 0.48	31.21 ± 0.28	33.58 ± 1.20
	6	36.72 ± 0.87	19.33 ± 0.04	32.24 ± 0.34	31.19 ± 0.29	30.65 ± 0.43	32.13 ± 0.84	29.48 ± 0.19	29.31 ± 0.11
	12	35.39 ± 0.27	22.92 ± 0.43	29.29 ± 0.51	29.81 ± 0.46	31.59 ± 0.20	30.42 ± 0.14	29.42 ± 0.13	27.05 ± 0.32
	24	35.15 ± 0.00	25.21 ± 0.67	32.59 ± 0.61	31.33 ± 0.0	32.46 ± 0.12	31.93 ± 0.66	30.87 ± 0.39	32.13 ± 0.37
IL-1ß	Co.	27.54 ± 0.13	29.24 ± 0.12	29.10 ± 0.15	24.61 ± 0.14	24.91 ± 0.19	27.76 ± 0.06	22.47 ± 0.21	26.68 ± 0.02
	0.5	27.03 ± 0.02	28.52 ± 0.13	29.12 ± 0.12	24.38 ± 0.48	25.15 ± 0.12	27.78 ± 0.23	21.98 ± 0.16	26.81 ± 0.13
	1	26.42 ± 0.04	29.09 ± 0.03	30.13 ± 0.18	25.16 ± 0.18	25.45 ± 0.13	26.66 ± 0.16	22.75 ± 0.18	28.09 ± 0.16
	2	27.57 ± 0.05	23.76 ± 0.13	29.94 ± 0.30	24.57 ± 0.06	25.99 ± 0.19	26.19 ± 0.15	22.31 ± 0.12	28.22 ± 0.10
	4	25.73 ± 0.27	19.22 ± 0.00	29.08 ± 0.11	24.16 ± 0.25	25.18 ± 0.61	26.52 ± 0.10	22.13 ± 0.14	27.28 ± 0.17
	6	25.49 ± 0.08	18.62 ± 0.03	28.90 ± 0.08	25.54 ± 0.11	25.08 ± 0.17	25.78 ± 0.03	21.84 ± 0.26	26.46 ± 0.18
	12	26.29 ± 0.07	21.08 ± 0.76	29.14 ± 0.15	25.18 ± 0.21	24.30 ± 0.15	26.21 ± 0.06	21.53 ± 0.20	25.83 ± 0.20
	24	27.40 ± 0.04	23.55 ± 0.85	29.70 ± 0.24	26.40 ± 0.11	24.28 ± 0.32	27.05 ± 0.31	22.07 ± 0.02	26.77 ± 0.21
TNF-α	Co.	22.70 ± 0.05	30.06 ± 0.32	30.80 ± 0.02	28.73 ± 0.18	29.74 ± 0.63	32.11 ± 0.07	27.58 ± 0.05	28.87 ± 0.18
	0.5	21.74 ± 0.88	30.32 ± 0.23	29.77 ± 0.10	28.75 ± 0.17	30.40 ± 0.23	31.25 ± 0.25	26.38 ± 0.09	29.13 ± 0.17
	1	21.51 ± 0.68	31.83 ± 0.84	30.28 ± 0.21	29.33 ± 0.10	30.65 ± 0.08	30.50 ± 0.30	26.91 ± 0.05	29.99 ± 0.23
	2	21.55 ± 0.73	29.28 ± 0.20	30.85 ± 0.29	28.06 ± 0.43	29.94 ± 0.11	29.60 ± 0.05	26.70 ± 0.29	29.82 ± 0.25
	4	20.46 ± 0.41	26.68 ± 0.15	30.33 ± 0.21	28.32 ± 0.17	30.56 ± 0.16	30.37 ± 0.28	26.43 ± 0.24	28.72 ± 0.45
	6	19.91 ± 0.28	27.06 ± 0.49	29.91 ± 0.12	28.35 ± 0.15	30.97 ± 0.34	29.80 ± 0.12	26.92 ± 0.43	28.74 ± 0.39
	12	20.39 ± 0.51	27.04 ± 0.61	30.09 ± 0.19	29.01 ± 0.42	31.20 ± 0.09	31.48 ± 0.15	27.25 ± 0.17	28.95 ± 0.10
	24	20.49 ± 0.35	27.07 ± 0.72	30.65 ± 0.16	28.48 ± 0.01	30.37 ± 0.59	31.08 ± 0.10	26.82 ± 0.13	29.66 ± 0.04

	Time (h)	Liver	Injured muscle	Heart	Lung	Small intestine	Colon	Spleen	Kidney
IFN-γ	Co.	34.21 ± 0.18	38.91 ± 0.26	32.60 ± 0.39	32.56 ± 0.23	33.47 ± 0.24	35.62 ± 0.20	30.80 ± 0.24	34.53 ± 0.29
	0.5	34.36 ± 0.46	37.74 ± 0.60	32.31 ± 0.67	32.53 ± 0.32	34.36 ± 0.19	35.65 ± 0.20	30.62 ± 0.31	33.50 ± 0.27
	1	35.06 ± 0.42	37.65 ± 0.42	32.08 ± 0.16	33.69 ± 0.62	34.56 ± 0.44	34.97 ± 0.48	30.58 ± 0.18	34.35 ± 0.52
	2	35.43 ± 0.17	37.07 ± 0.18	32.81 ± 0.25	33.46 ± 0.32	35.48 ± 0.10	36.28 ± 0.40	31.42 ± 0.25	35.27 ± 0.28
	4	35.33 ± 0.26	37.06 ± 0.38	33.23 ± 0.11	33.27 ± 0.32	34.86 ± 0.56	35.65 ± 0.52	32.43 ± 1.29	35.81 ± 0.35
	6	35.09 ± 0.27	37.81 ± 0.23	32.09 ± 0.34	33.28 ± 0.26	34.47 ± 0.31	35.64 ± 0.48	31.75 ± 0.20	33.68 ± 0.17
	12	37.14 ± 0.27	37.92 ± 0.47	33.25 ± 0.29	33.58 ± 0.63	35.32 ± 0.76	37.09 ± 0.03	32.43 ± 0.19	34.89 ± 0.32
	24	35.11 ± 0.37	37.43 ± 0.20	32.27 ± 0.39	32.46 ± 0.19	34.49 ± 0.19	36.09 ± 0.63	29.57 ± 0.91	33.96 ± 0.33

4.1.3.III Expression of iron-metabolism genes in extrahepatic organs

Besides liver the expression of the genes known to be involved in iron regulation was studied in different organs. Besides comparing the gene expression to the housekeeping gene in different organs, the quantitative comparison of the gene expression in different organs was made by comparing the Ct values (Table 4).

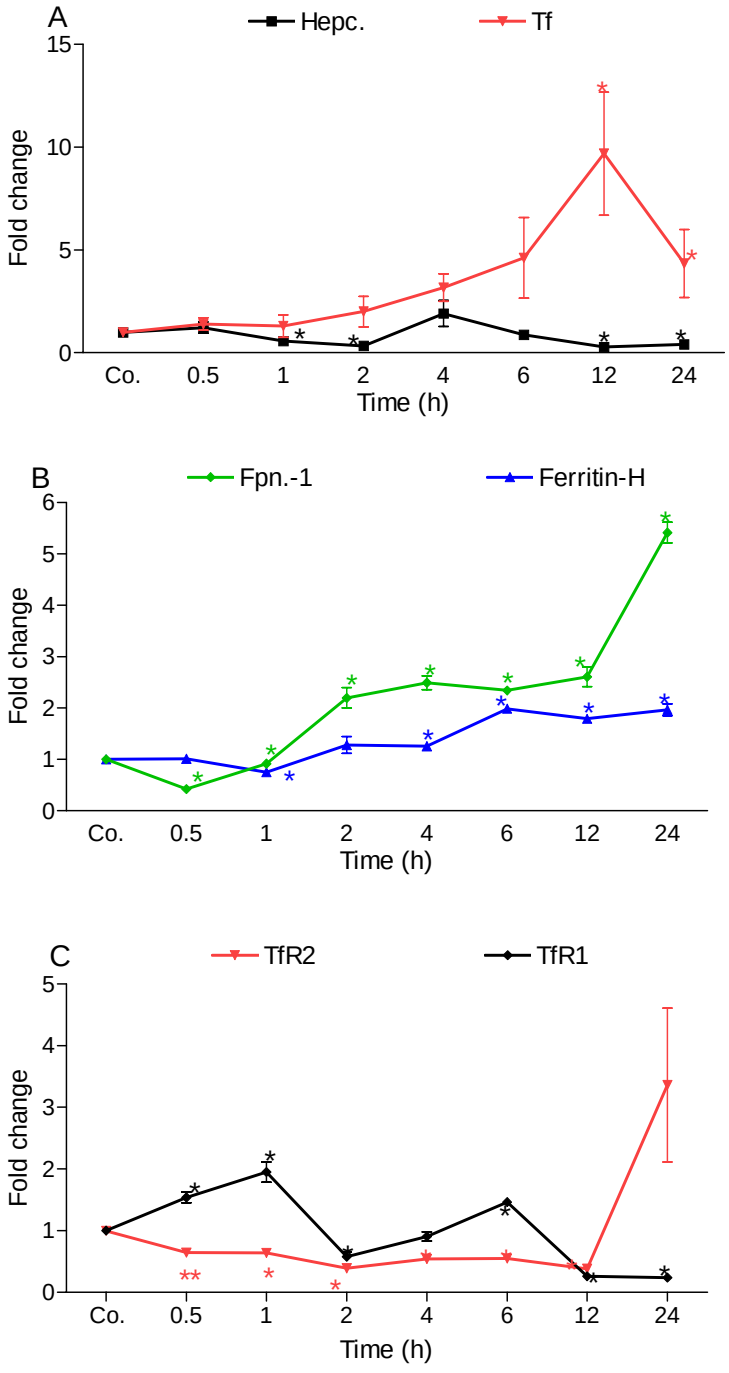
We not only found the expression of Hepc gene in the extrahepatic organs but also an upregulation of Hepc gene expression was observed in these organs with the exception in the lung. Hepc gene expression was significantly upregulated in these organs from 4-12 hours, on the other hand; in the lung, Hepc gene expression was significantly downregulated. However, this expression was not statistically significant in small intestine, spleen and lung when analysed by one way ANOVA. Tf gene expression was regulated variably in the extrahepatic organs. A significant upregulation was observed in the lung and small intestine. On the other hand, it was downregulated in the heart and kidney significantly whereas the downregulation was not significant in the colon and spleen when analysed by one way ANOVA (Figure 16 A-21 A).

Ferritin-H gene expression was significantly downregulated in the heart, small intestine, and colon while in the lung and kidney an upregulation was found. Fpn.-1 gene expression was significantly downregulated in the heart, colon and spleen at 6 hours like in the liver; however, in the small intestine and kidney the downregulation was not statistically significant when analysed by one way ANOVA. On the other hand, in the lung, Fpn.-1 gene expression was significantly upregulated ($P = 0.0032$; Figure 16 B-21B).

An early upregulation of TfR1 gene expression 30 min. after the TO injection was seen in the heart and kidney; however, upregulation was delayed in the colon. TfR1 gene expression was only significantly downregulated in the spleen ($P = 0.013$) whereas, the changes in the other organs were not statistically significant (Figure 16 C-21 C).

DMT1 gene expression was significantly downregulated in the heart, small intestine, spleen and kidney from 4-12 hours. In the colon, DMT1 gene expression was upregulated initially whereas downregulated later on. On the other hand, a sustained upregulation of DMT1 gene expression was observed in the lung which was statistically non-significant by one way ANOVA ($P = 0.22$). Dcytb gene expression in the heart,

colon, spleen, and lung was decreased while in the small intestine Dcytb gene expression was significantly increased at 4 hours ($P < 0.005$). In the kidney, the Dcytb gene expression was continuously upregulated during the course of study (Figure 16 D-21 D).



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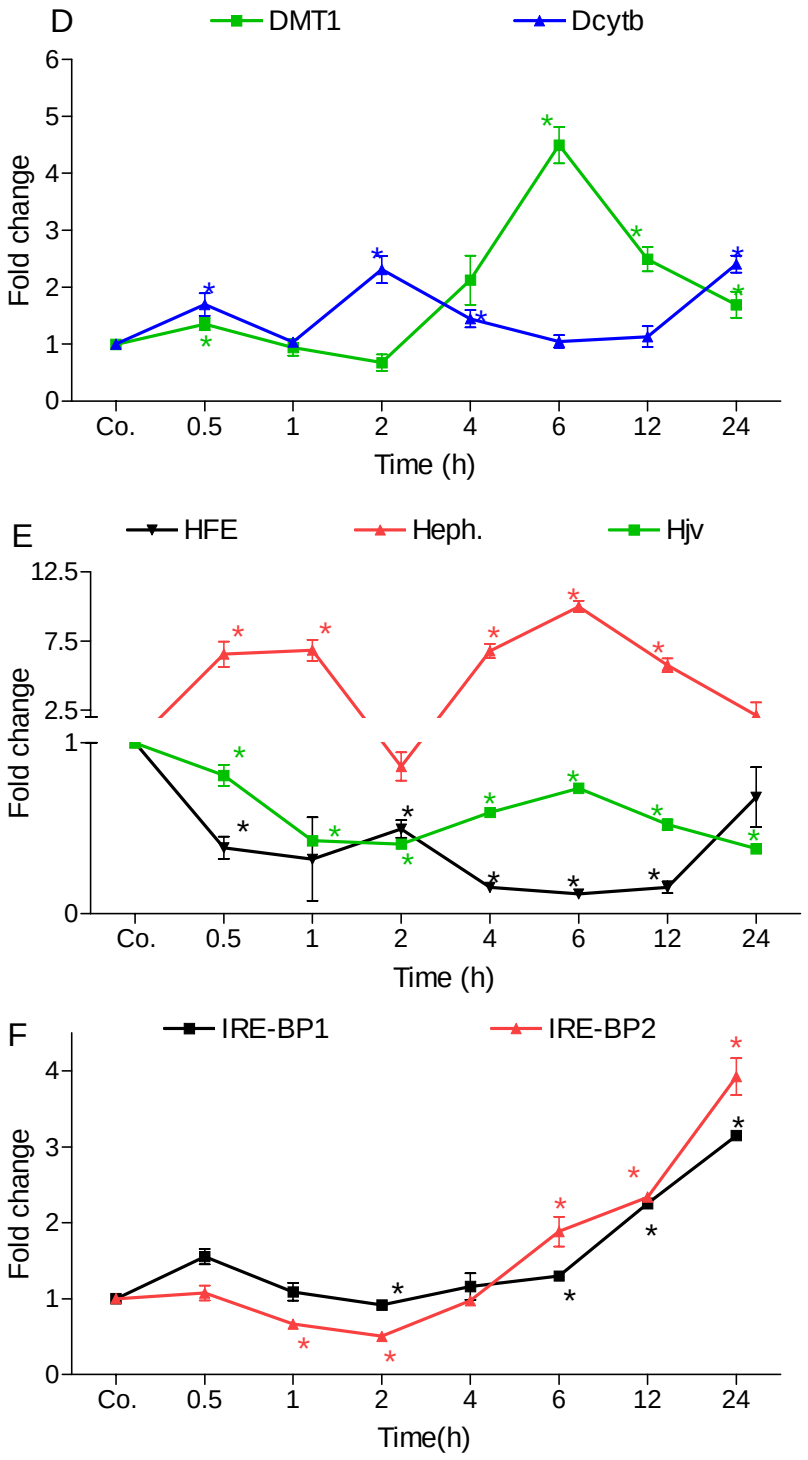
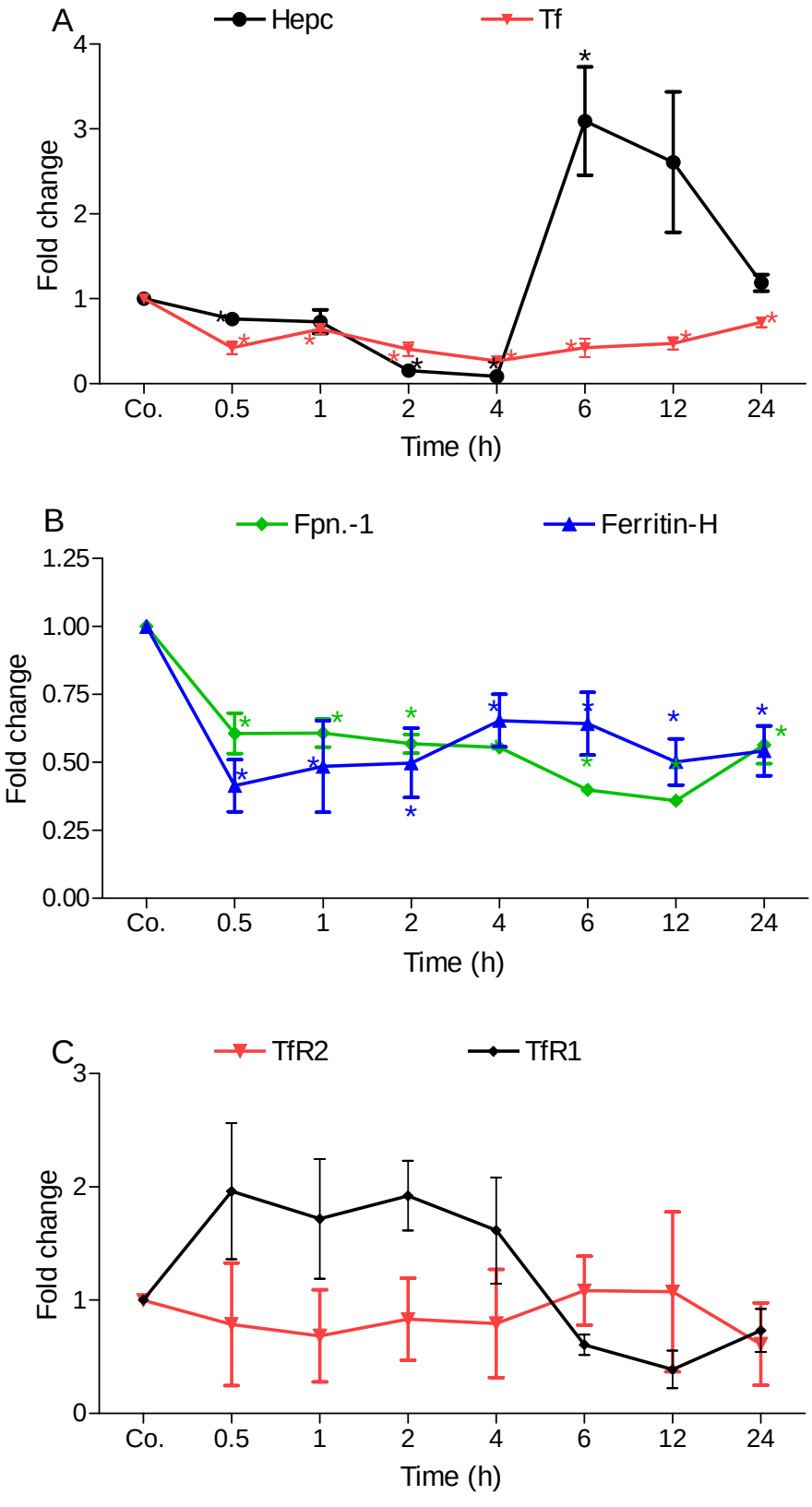


Figure 15: mRNA expression of Hepc, Tf *(A),* Fpn.-1, Ferritin-H *(B),* TfR2, TfR1 *(C),* DMT1, Dcytb *(D)*, HFE, Heph, Hjv *(E),* and IRE-BP1, IRE-BP2 *(F)* in the injured muscle of the rats after 5mg/kg administration of TO in the hind limb muscle. Results represent mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=3).

Heph gene expression was downregulated in the heart, small intestine, colon and spleen. However, in the kidney and lung Heph gene expression was upregulated. The changes in the Heph gene expression were significant only in the spleen *(P = 0.0042)* but not in other organs studied when analysed by one way ANOVA.

Hjv gene expression was quite strong in the heart as it was evident by Ct values (Table 4); however, this upregulation was not statistically significant. In contrast to the heart, Hjv gene expression was significantly upregulated at 4 hours in the kidney; whereas, at later time points it was significantly downregulated when the Hepc gene expression was upregulated significantly. An overall significant change in the Hjv gene expression was found in the kidney by one way ANOVA through the course of study *(P = 0.007)*. In the colon and lung Hjv gene expression was significantly downregulated. HFE gene expression was significantly downregulated in the heart, colon, spleen, kidney and lung from 6 to 12 hours. In the small intestine; however, this downregulation was not statistically significant (Figure 16 E-21 E).

IRE-BP1 gene expression was significantly upregulated in the colon and kidney ($P < 0.025$) but not in the other organs. The expression of IRE-BP2 gene was significantly changed in the small intestine but not in any other organ under study when evaluated by one way ANOVA (Figure 16 F-21 F).



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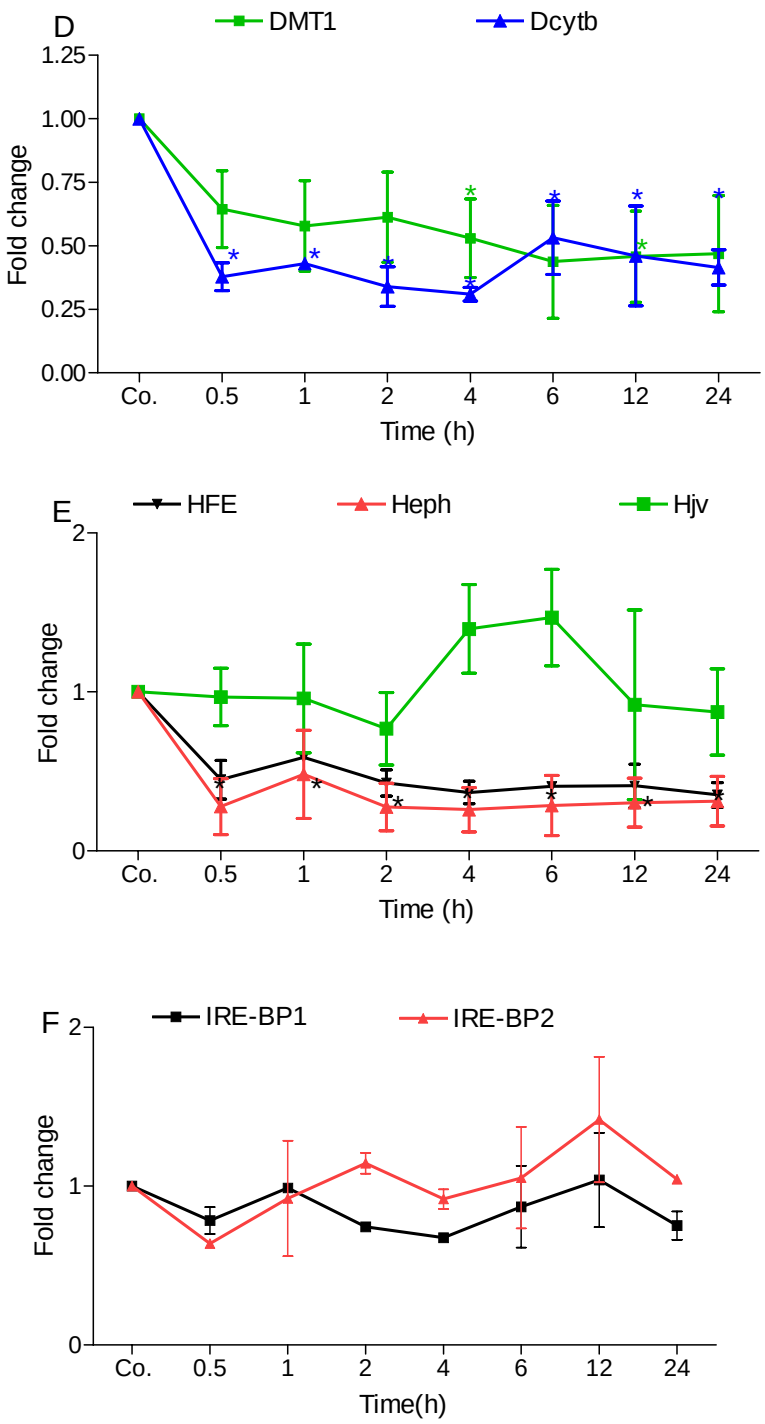
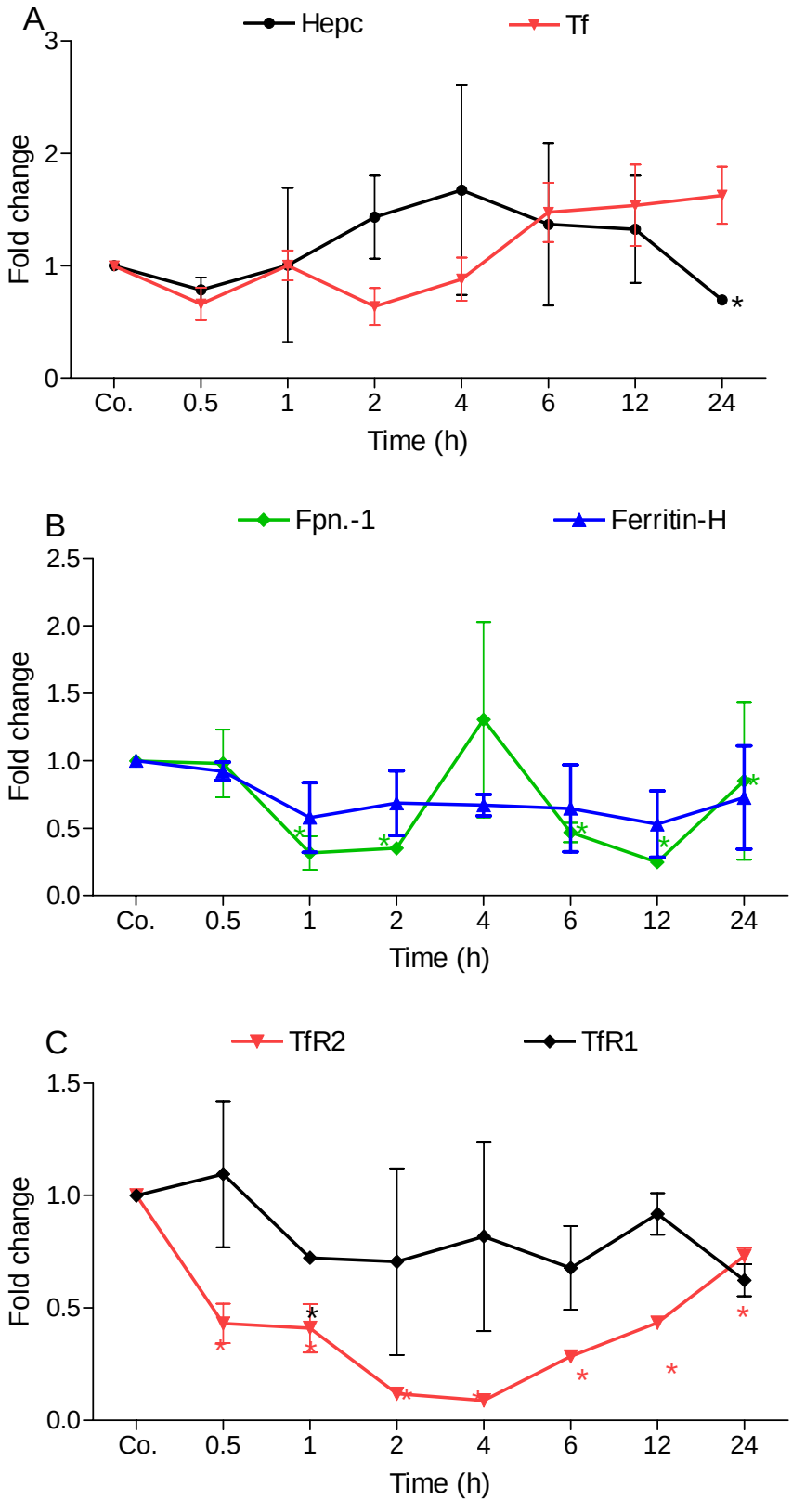


Figure 16: mRNA expression of Hepc, Tf *(A),* Fpn.-1, Ferritin-H *(B),* TfR2, TfR1 *(C),* DMT1, Dcytb *(D)*, HFE, Heph, Hjv *(E),* and IRE-BP1, IRE-BP2 *(F)* in the heart determined by real-time PCR as described in materials and methods. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).



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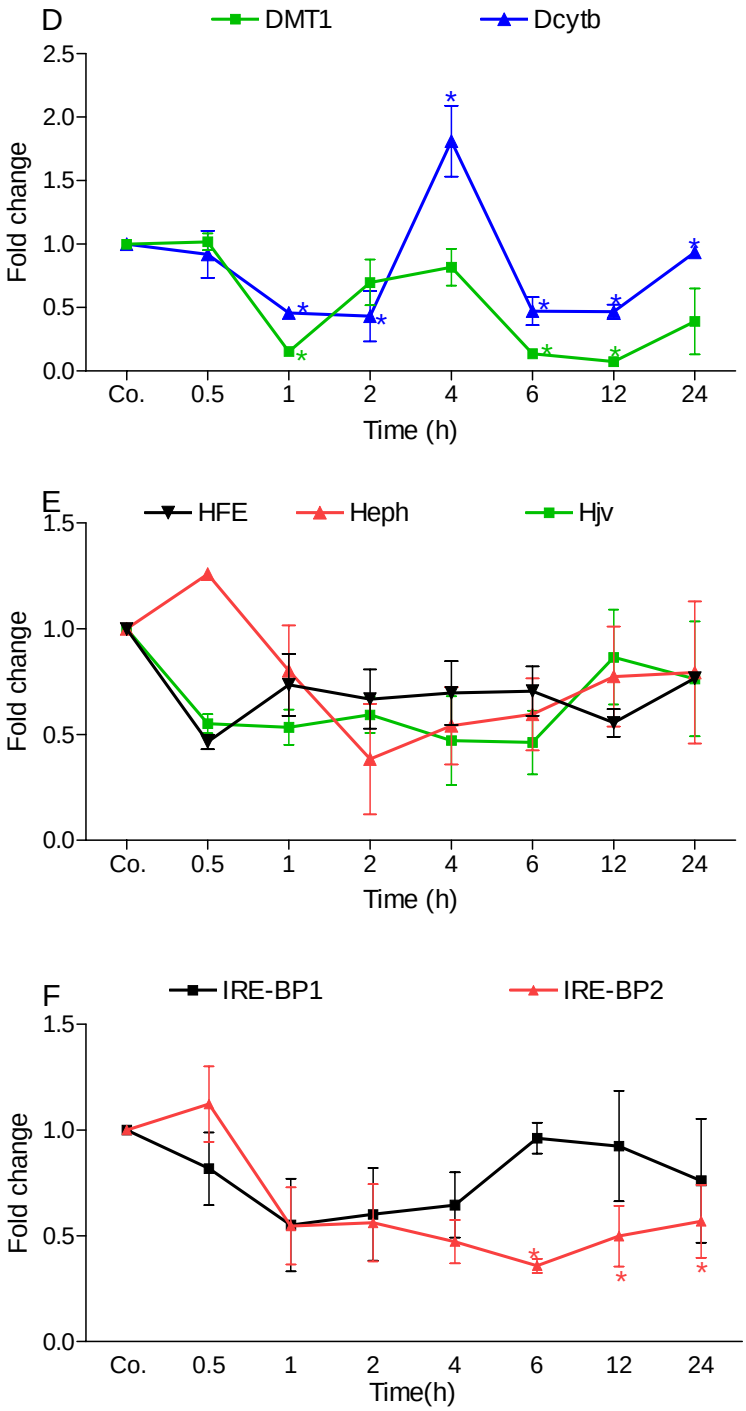
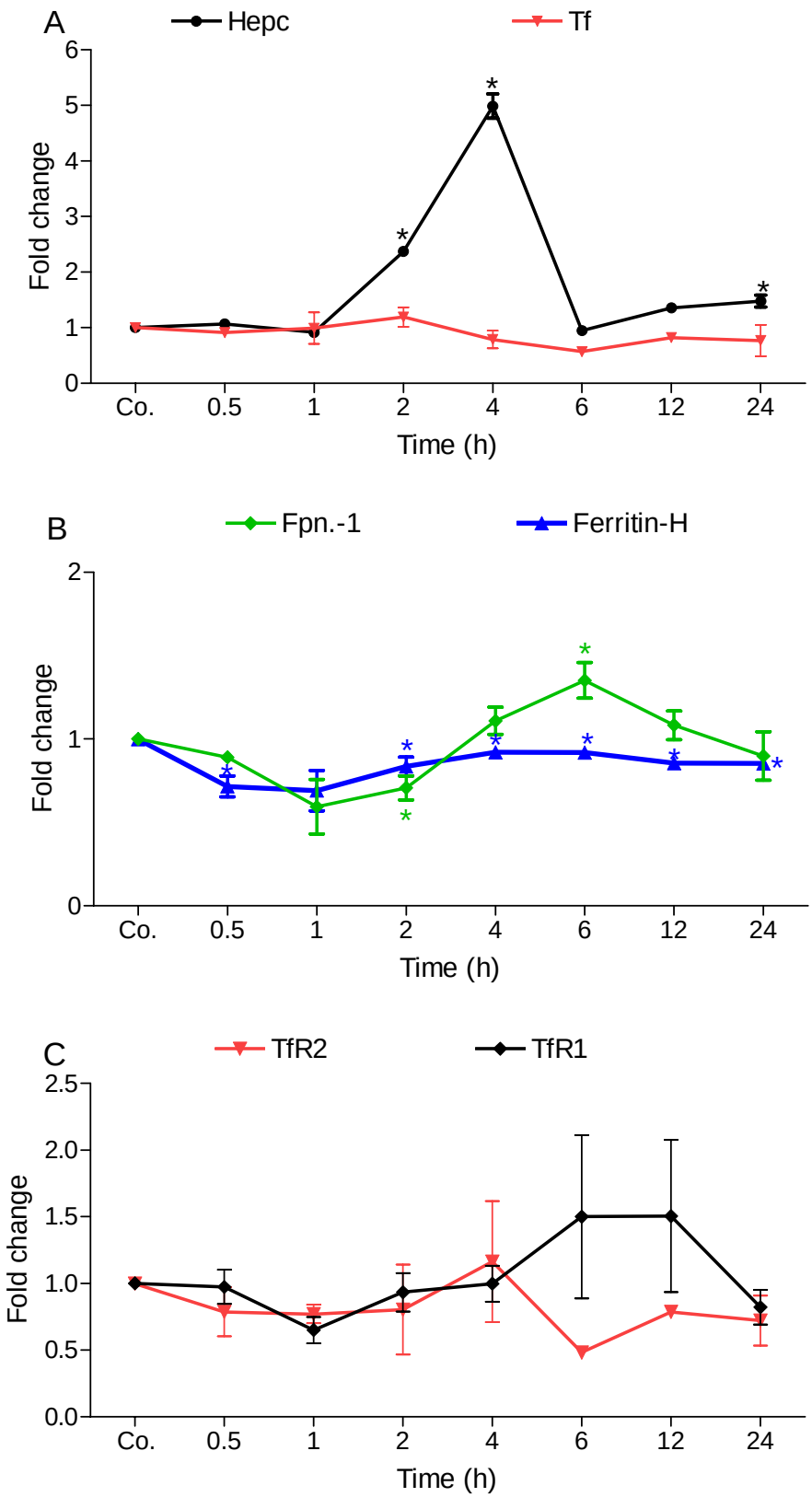


Figure 17: mRNA expression of Hepc, Tf *(A),* Fpn.-1, Ferritin-H *(B),* TfR2, TfR1 *(C),* DMT1, Dcytb *(D)*, HFE, Heph, Hjv *(E),* and IRE-BP1, IRE-BP2 *(F)* in the small intestine studied by real-time PCR. Significant downregulation of Fpn.-1, Ferritin-H, DMT1, TfR2, Hjv and Heph gene was observed from 4-12 hours; however, Dcytb gene expression was significantly upregulated. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).



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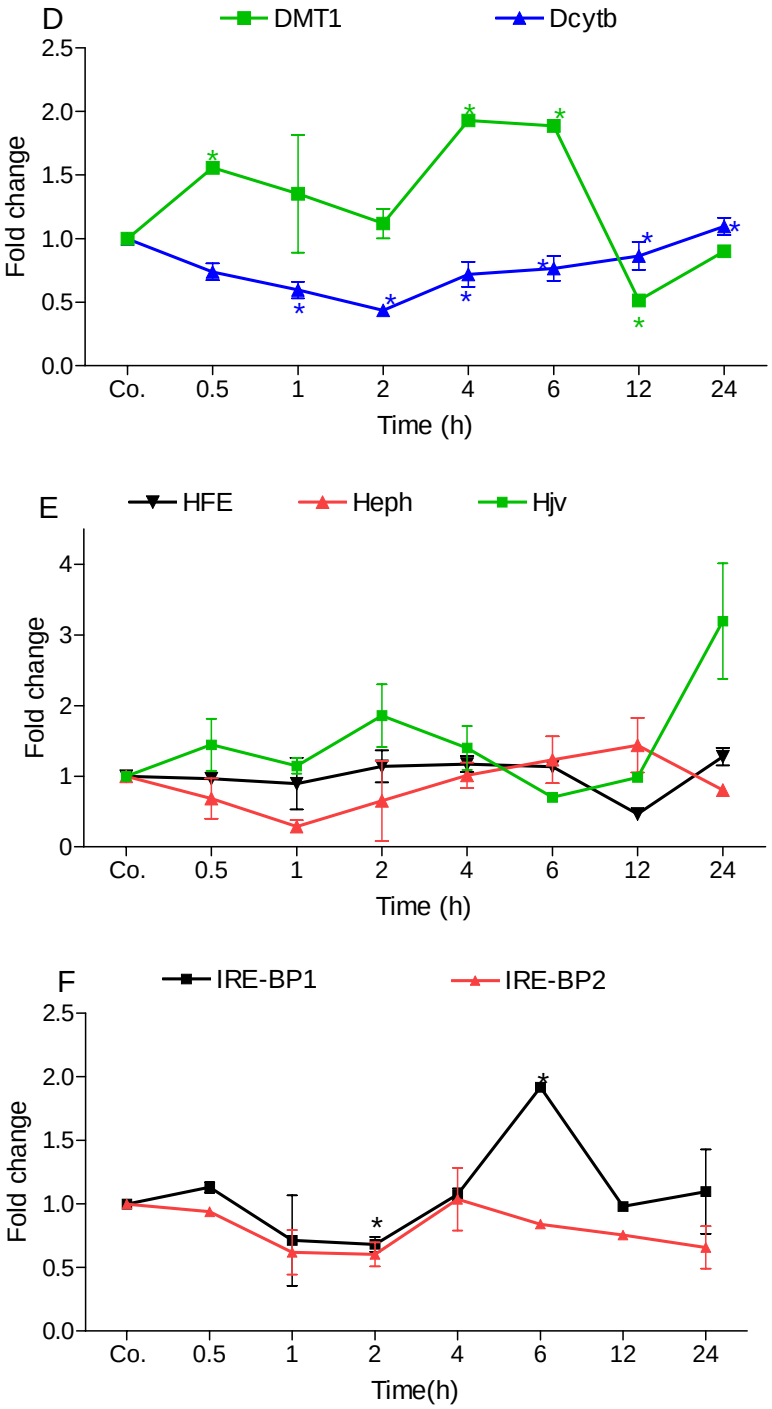
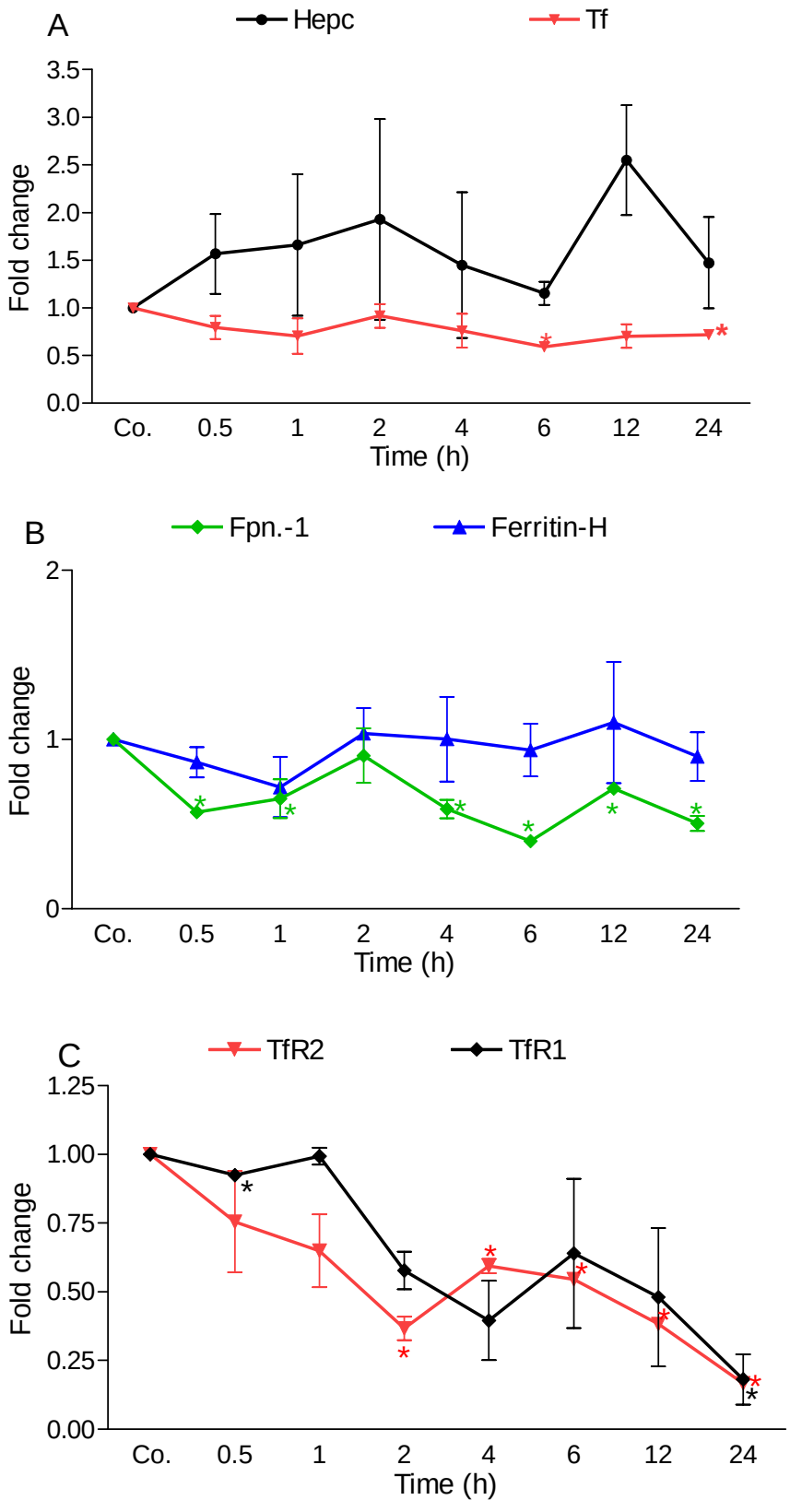


Figure 18: Real-time PCR analysis of mRNA expression of Hepc, Tf *(A),* Fpn.-1, Ferritin-H *(B),* TfR2, TfR1 *(C),* DMT1, Dcytb *(D)*, HFE, Heph, Hjv *(E),* and IRE-BP1, IRE-BP2 *(F)* in the colon. Hepc, DMT1 and HFE gene expression was significantly upregulated; however, Tf, Ferritin-H, Fpn.-1, Dcytb, TfR2, Hjv and Heph gene expression was significantly downregulated at 1-12 hours. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).



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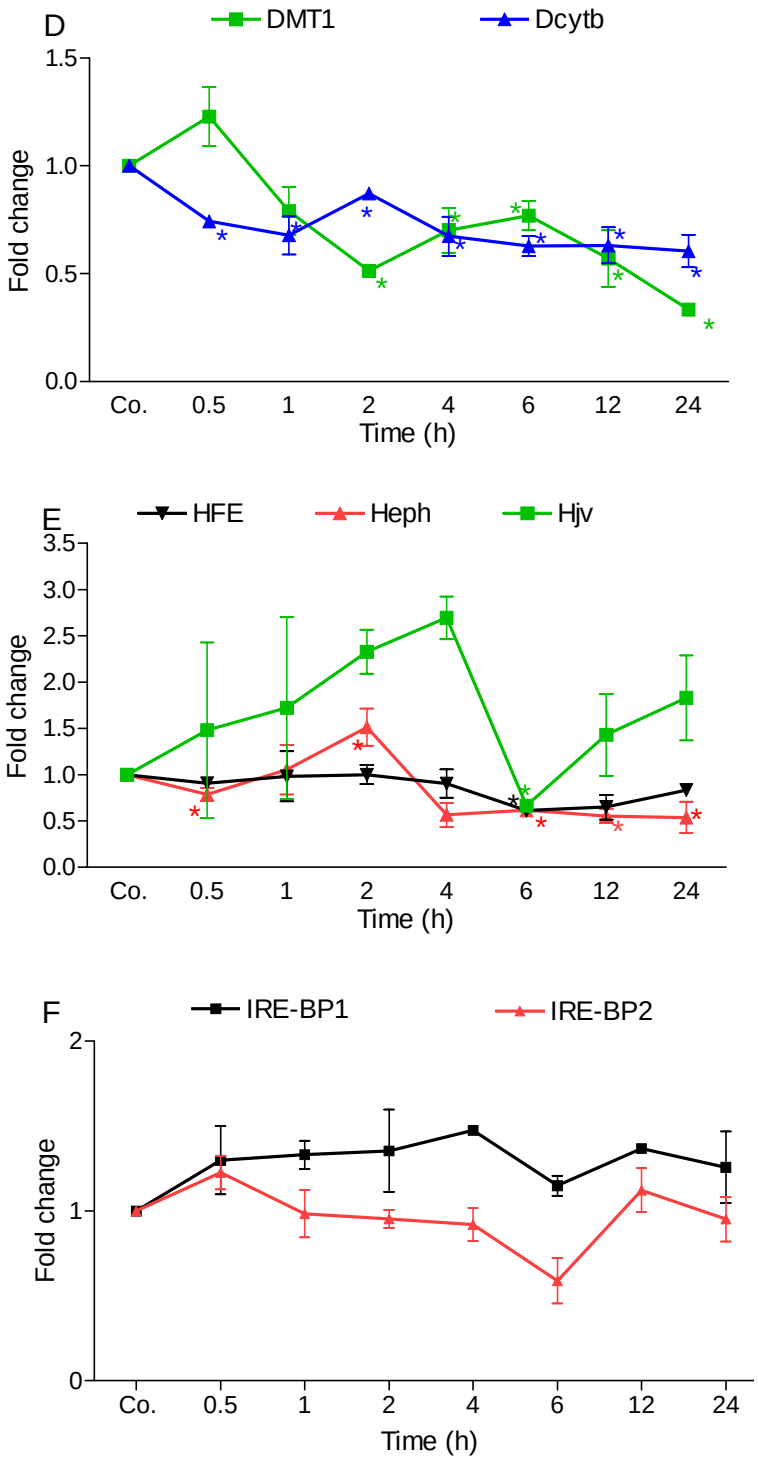
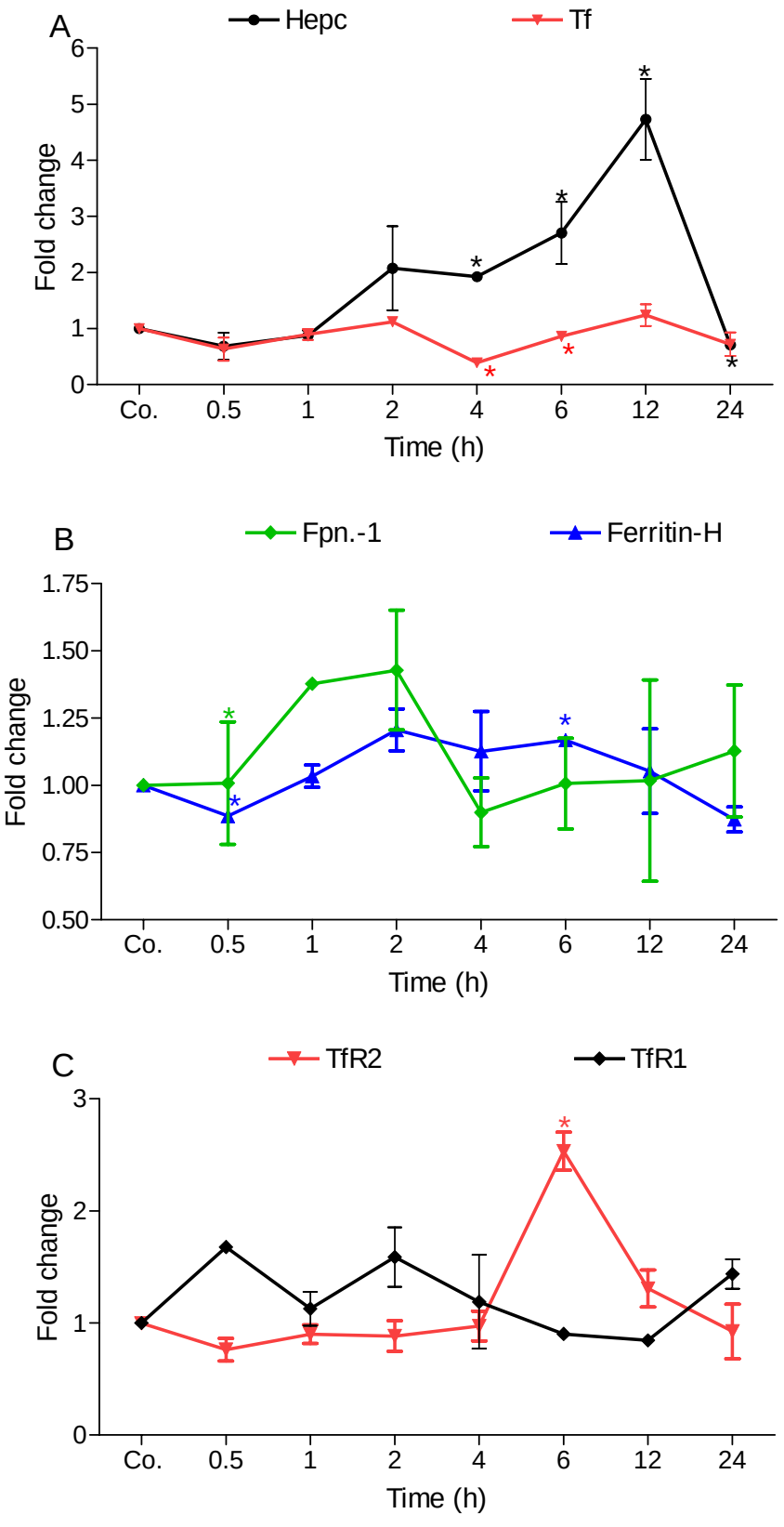


Figure 19: Real-time PCR analysis of mRNA expression of Hepc, Tf *(A),* Fpn.-1, Ferritin-H *(B),* TfR2, TfR1 *(C),* DMT1, Dcytb *(D)*, HFE, Heph, Hjv *(E),* and IRE-BP1, IRE-BP2 *(F)* in the spleen. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).



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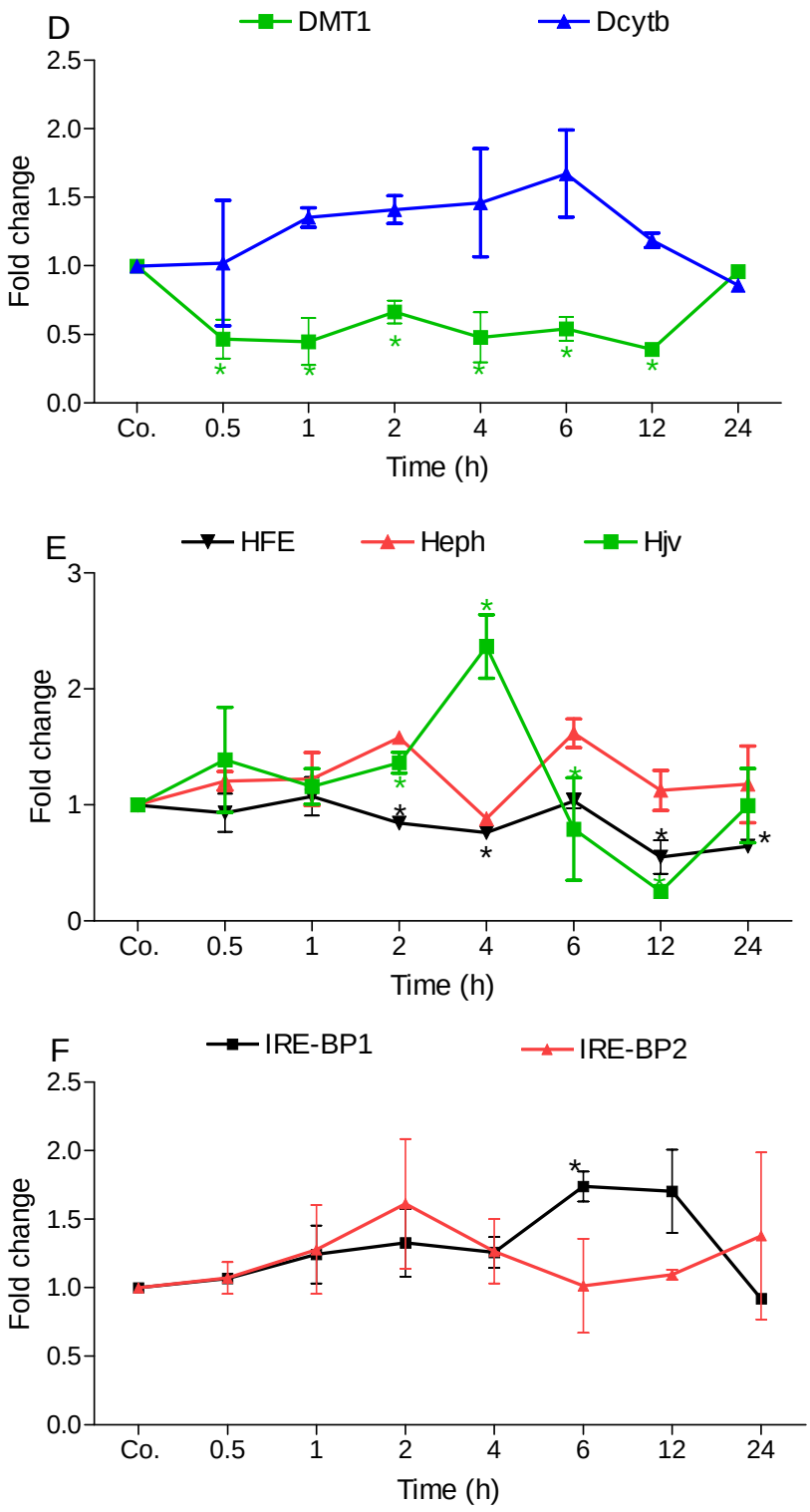
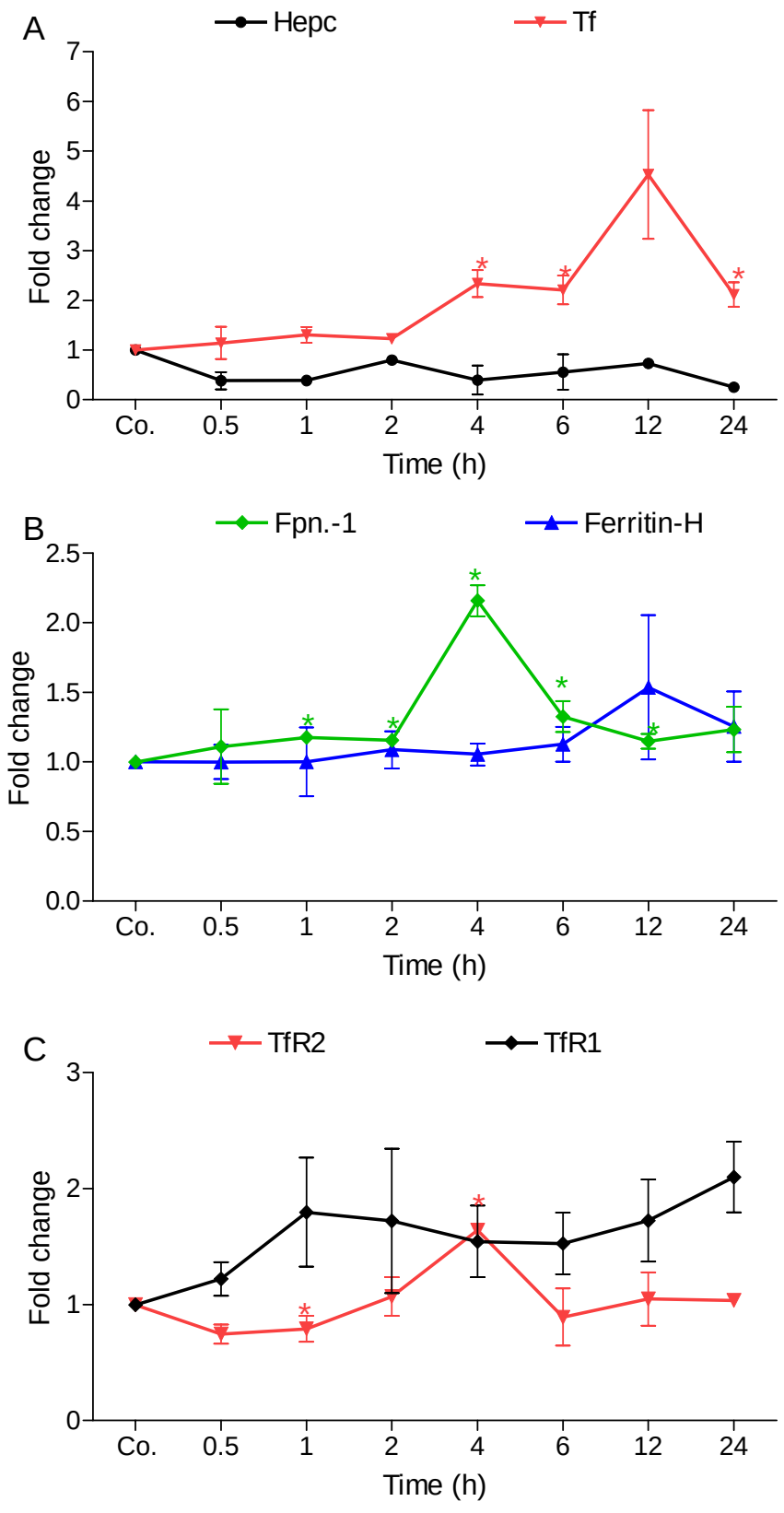


Figure 20: mRNA expression of Hepc, Tf *(A),* Fpn.-1, Ferritin-H *(B),* TfR2, TfR1 *(C),* DMT1, Dcytb *(D)*, HFE, Heph, Hjv *(E),* and IRE-BP1, IRE-BP2 *(F)* in the kidney studied by real-time PCR. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).



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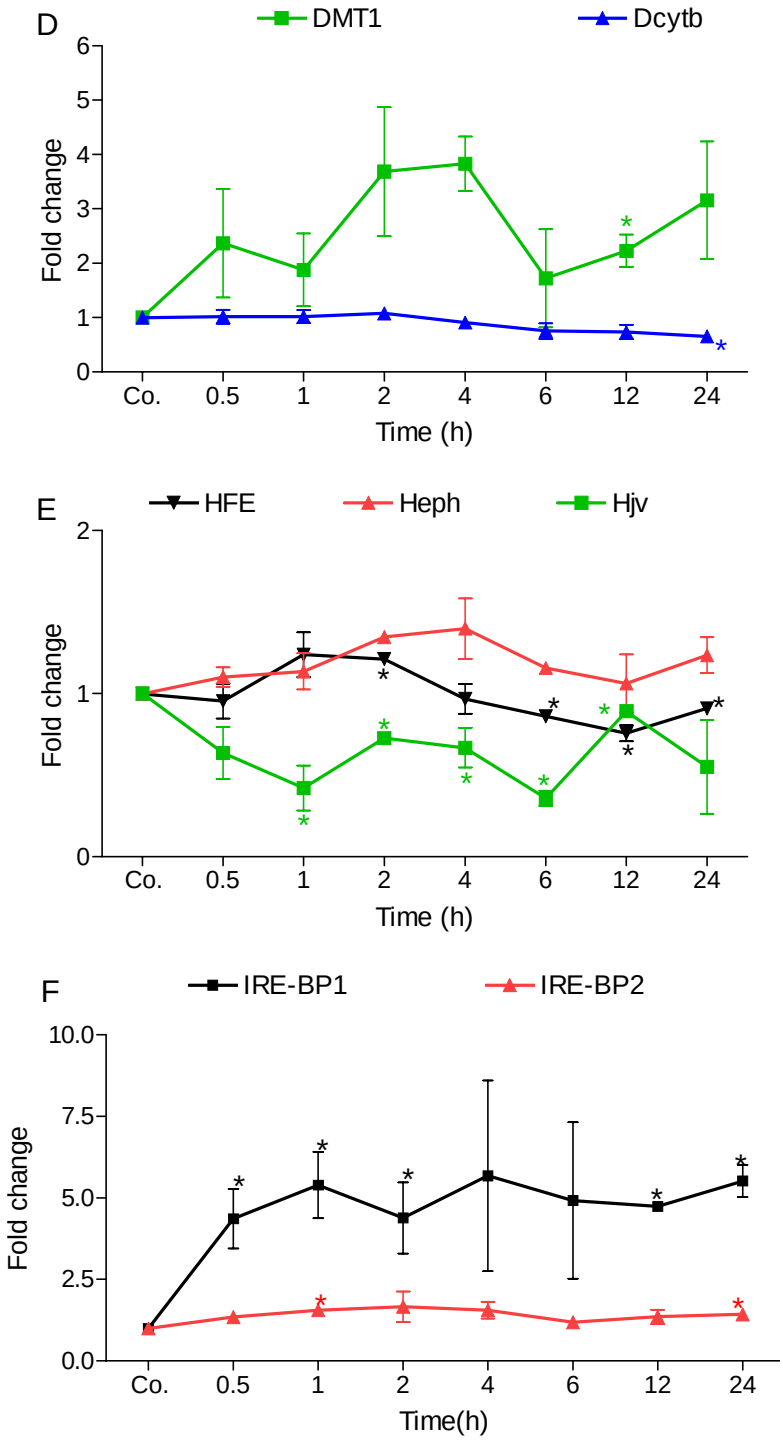


Figure 21: mRNA expression of Hepc, Tf *(A),* Fpn.-1, Ferritin-H *(B),* TfR2, TfR1 *(C),* DMT1, Dcytb *(D)*, HFE, Heph, Hjv *(E),* and IRE-BP1, IRE-BP2 *(F)* in the lung. Iron regulatory genes behaved in a different way in lung. Real-time PCR analysis has shown a significant downregulation of Hepc gene expression at RNA level; however, Fpn.-1 gene expression was significantly upregulated. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).

4.1.4 Northern Blot analysis

4.1.4.I Liver

Total RNA was analysed by northern blot analysis to verify real-time PCR findings. The filters containing the total RNA from the liver of TO injected rats, size fractionized by electrophoresis (as described in 3.2.3) were hybridized with Hepc, Hjv and Fpn.-1 specific cDNA probes and the intensity of the radioactive label was recorded on the autoradiographic films after incubating at -80°C for different time periods. We found an increase in the Hepc gene expression with a maximum expression at 6 hours after the TO injection; however, Hjv and Fpn.-1 gene expression was downregulated at this time (Figure 22A).

4.1.4.II Injured muscle

Filters with total RNA from the injured muscle were hybridized with Hepc, Hjv, Fpn.-1, IL-6, and IL-1ß specific cDNA probes and the specific activity of the radioactive label was recorded on the autoradiographic films after incubating at -80°C. Hepc gene expression was not detectable by northern blot in the injured muscle. A strong expression of Hjv in the skeletal muscle with a downregulation during APR was found. However, the expression of Fpn.-1 gene was upregulated. IL-6 and IL-1ß gene expression was strongly upregulated from 4–6 hours after the induction of APR (Figure 22 B). Northern blot results were in agreement with the real-time PCR results.

4.1.4.III Extrahepatic organs

Total RNA was size fractionized and blotted onto the nylon membrane from the extrahepatic organs. These filters were then used to hybridize with Hepc, Hjv and Fpn.-1 specific cDNA. Fpn.-1 gene expression was detectable in all the organs studied. Fpn.-1 gene expression was significantly downregulated in the heart, small intestine, colon, spleen and kidney at 6 hours as in the liver; however, in the lung Fpn.-1 gene expression was upregulated. Detectability of Hepc, Hjv and Fpn.-1 mRNA expression by northern blotting was somehow related to Ct-values. Hepc and Hjv mRNA could not be detected by northern blotting because of the low abundance of the specific mRNA (high Ct values determined by real-time PCR). Except the lung, in all other organs the Fpn.-1 gene expression qualitatively behaved similarly; however, quantitatively Fpn.-1 gene

expression was different. In the injured muscle and in the lung increased expression of Fpn.-1 gene was found (Figure 23).

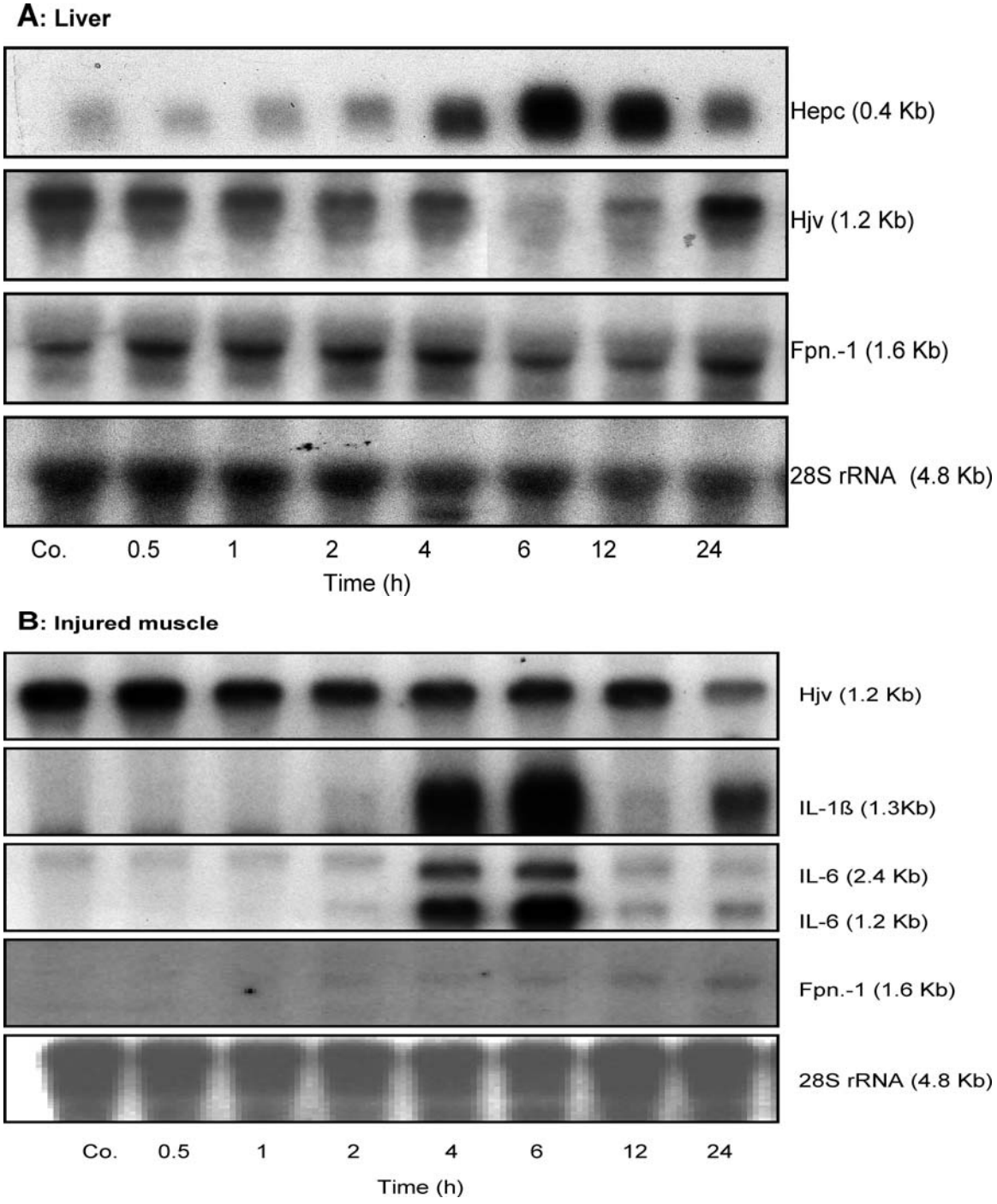
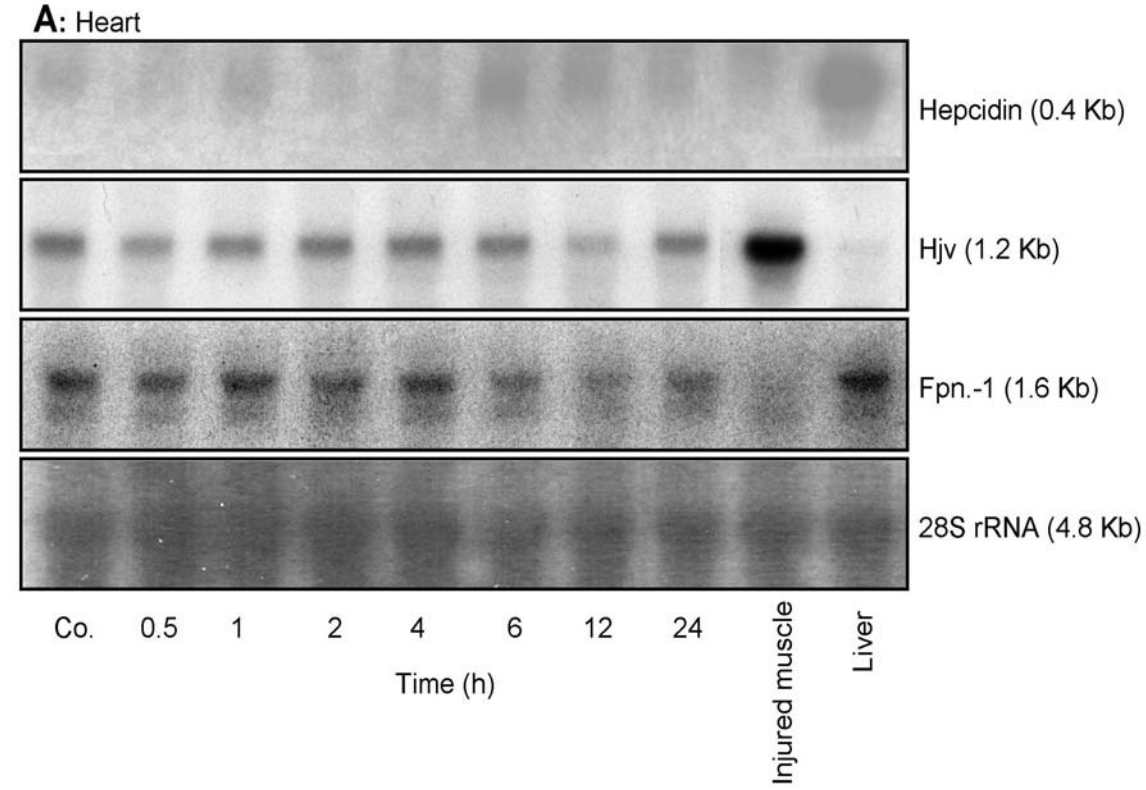
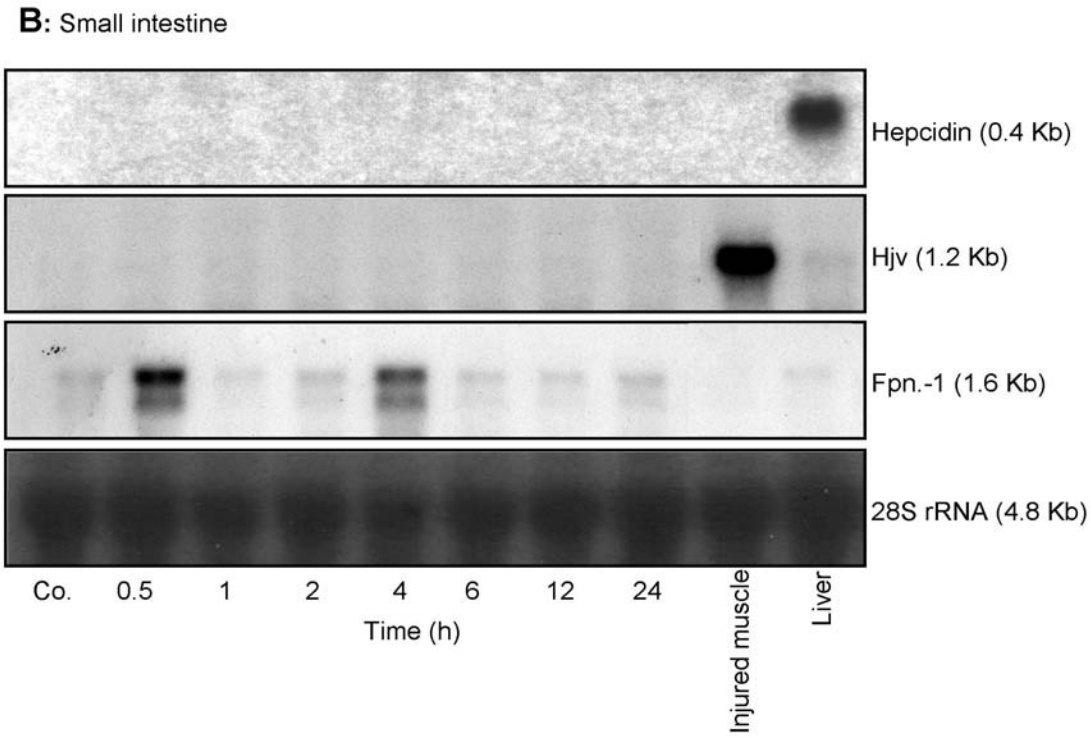


Figure 22: Northern blot analysis of total RNA extracted from liver *(A)* and injured muscle *(B)*. Specific cDNA probes were developed using [α-$_{32}$P] labeled deoxy-cytidin triphosphate (specific activity 3000 ci/mmol) for Hepc, Hjv and Fpn.-1 specific RNA *(A)* and Hjv, IL-1ß and IL-6 specific RNA *(B)*. The filters with size fractionized total RNA from the liver or injured muscle were hybridized with these radiolabeled cDNA.





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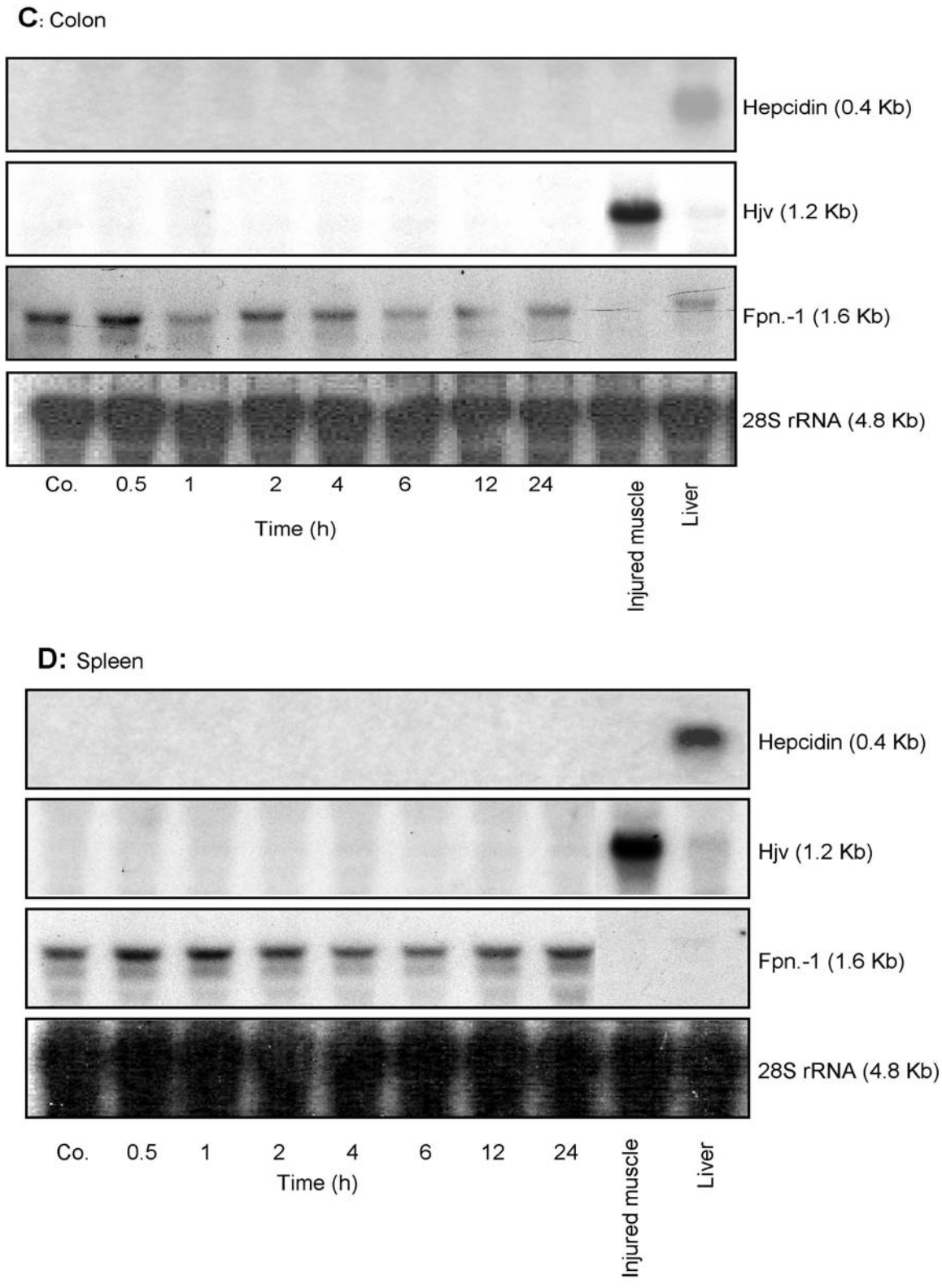

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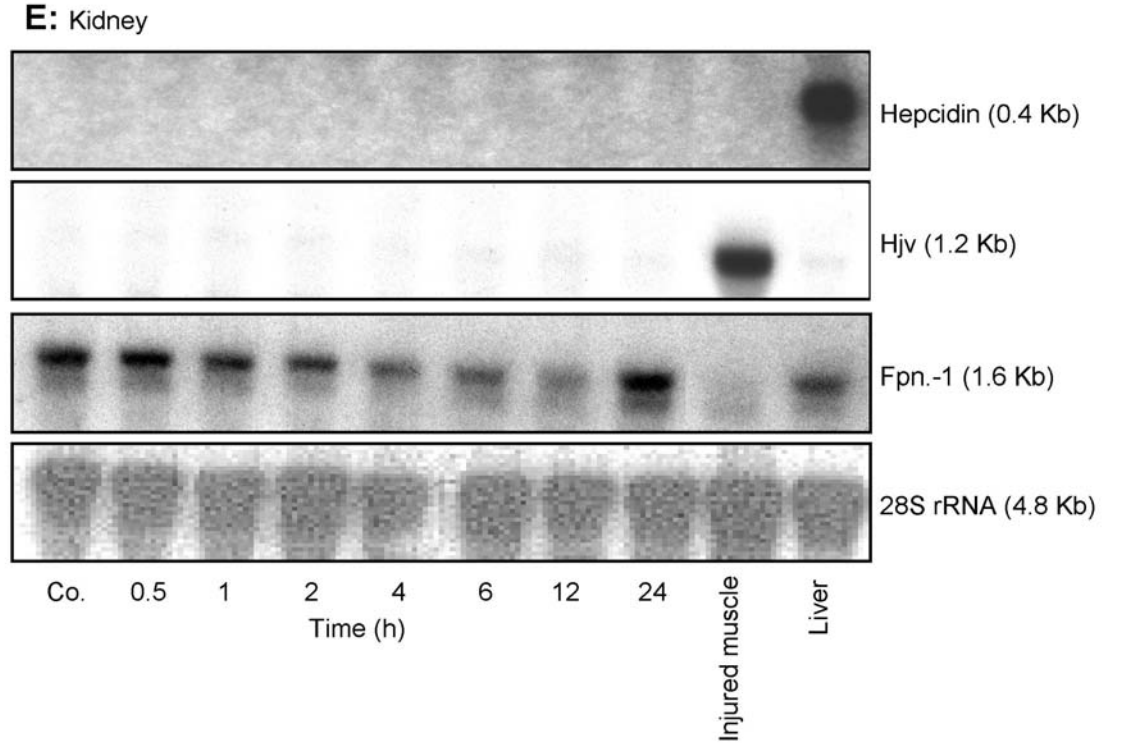

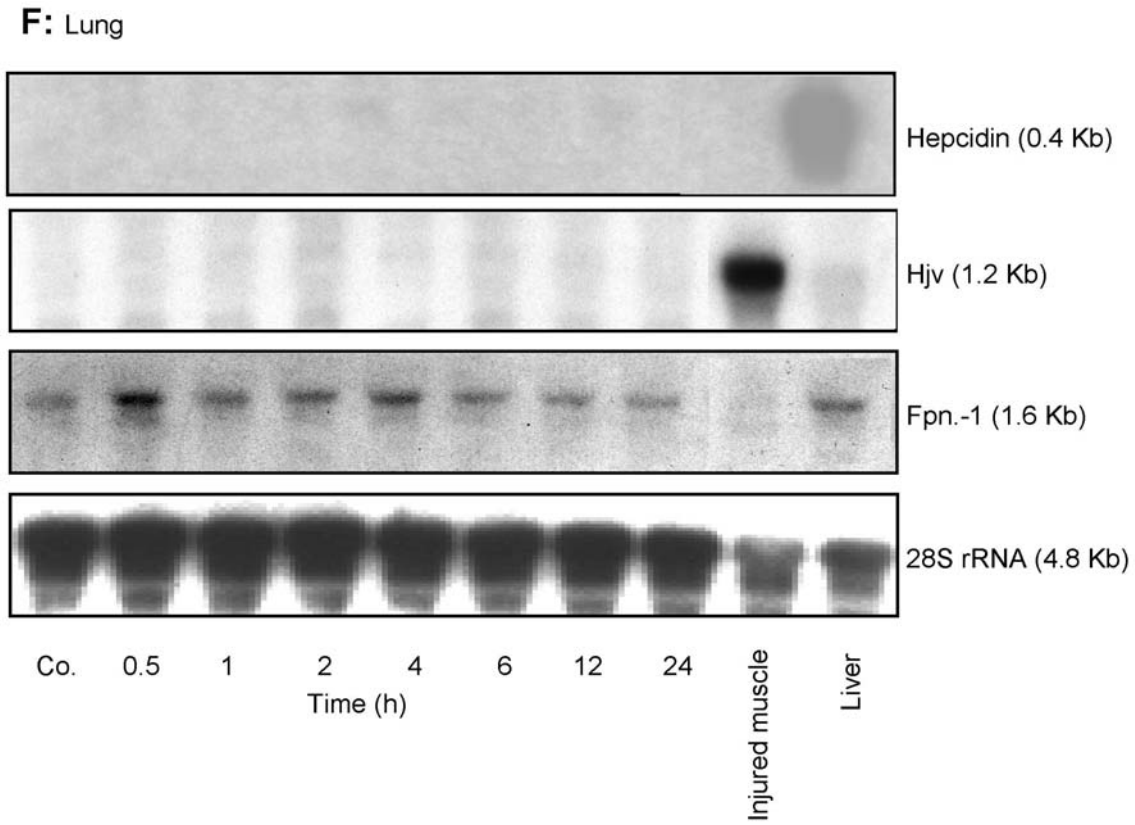

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Figure 23: Northern blot analysis of total RNA extracted from different organs of the TO injected rats taken at different time points (Materials and methods). Hepc, Hjv and Fpn.-1 specific cDNA probes radiolabel with [α-$_{32}$P] labeled deoxy-cytidin triphosphate (specific activity 3000ci/mmol) were used for hybridization. Hepc gene expression was not demonstrable by northern blotting in extrahepatic organs. Hjv could only be detected in the cardiac muscles (in the heart) but not in other extrahepatic organs. This could be due to the low relative abundance of the specific mRNA of Hepc and Hjv in extrahepatic organs. However, Fpn.-1 mRNA expression could be demonstrated in all the organs with a similar pattern of regulation with an exception of lung and injured muscle where an increased expression could be seen as compared to the other organs studied where Fpn.-1 mRNA expression was downregulated.

4.2 Partial-Hepatectomy-induced acute liver injury

4.2.1 Serum Analysis

4.2.1.I Serum iron levels

In case of acute liver injury induced by PH, statistically significant abridged serum iron levels were found early after the injury ($P < 10^{-4}$). Iron levels were declined to the lowest level (16.8 ± 1.03 µmol/l compared to controls 61.9 ± 2.5 µmol/l) by 8 hours of the liver injury (Figure 24).

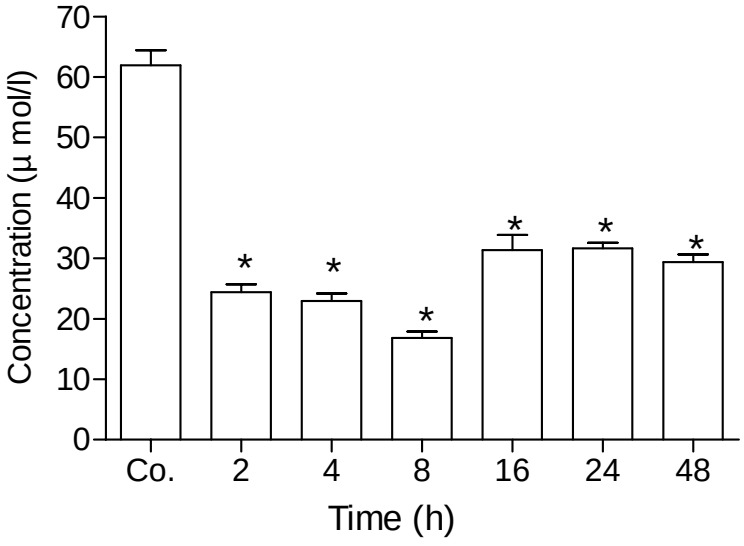


Figure 24: *In vivo* serum iron levels. The serum iron concentrations were measured as described in materials and methods. A quick and sharp decline of serum iron concentration was found (24.4 µmol/l compared to the control rats). Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=3).

4.2.1.II Serum transaminases

Transaminases (ALT and AST) catalyze the conversion of α-keto acids into amino acids by transfer of amino groups. AST and ALT levels significantly increase during liver disease. ALT is normally elevated in hepatobiliary disease and AST during liver parenchymal damage. Statistically significant increase in the serum level of AST and ALT were found in the PH rats compared to the control rats ($P < 10^{-4}$; Figure 25).

4.2.1.III Serum hepcidin pro-hormone

The acute liver injury after the resection of 70% liver induced changes in the serum hepcidin pro-hormone concentration. An increase in the serum hepcidin pro-hormone concentration was found at different time points; however, this increase was not statistically significant (Figure 26).

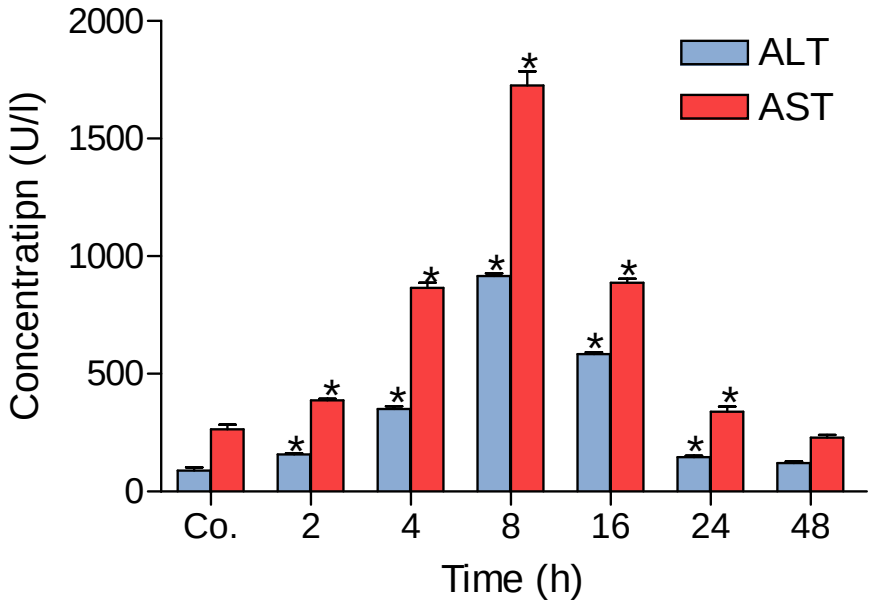


Figure 25: *In vivo* serum transaminase levels. The concentration was measured by routine clinical laboratory test as described in materials and methods. A strong increase in the AST and ALT concentrations after PH indicates the severe liver injury. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=3).

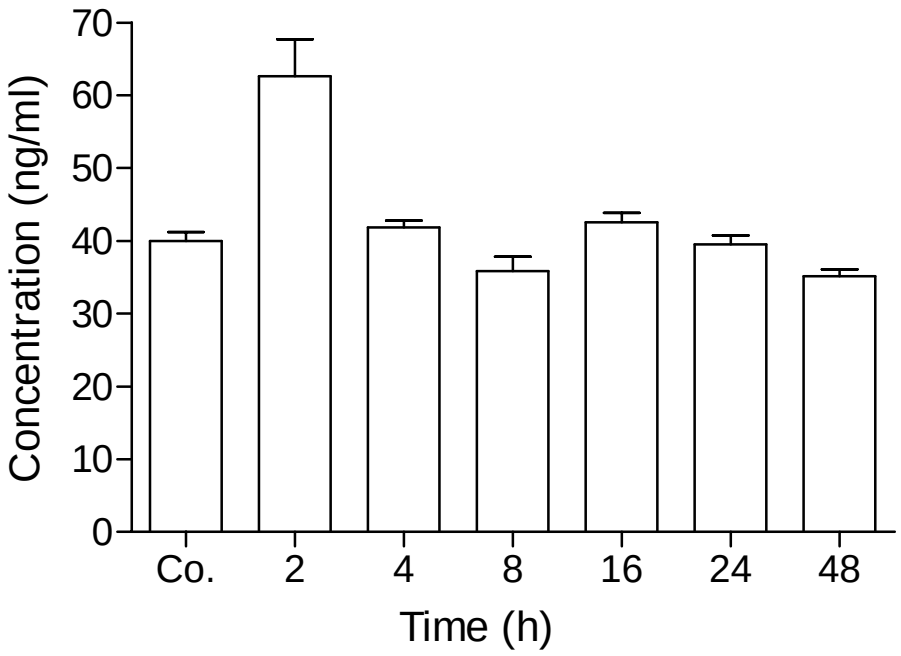


Figure 26: ELISA to study the serum level of hepcidin pro-hormone. Serum concentrations of hepcidin pro-hormone were not significantly changed after PH. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=3).

4.2.1.IV Serum levels of acute-phase cytokines

Serum analyses have shown significant changes in the acute-phase cytokines concentration during acute liver injury. After PH-induced acute liver injury, serum IL-6 and IL-1ß concentrations were significantly increased to 278 pg/ml of IL-6 compared to 126 pg/ml in control sera *($P = 0.0002$)* and 320 pg/ml of IL-1ß compared to 111pg/ml in control sera. However, IFN-γ concentration was significantly decreased from 4 to 24 hours after the injury ($P < 10^{-4}$; Figure 27).

4.2.2 Acute-phase cytokines gene expression in the livers of PH rats

PH-induced acute liver injury caused the upregulation of the acute-phase cytokine gene expression. A strong upregulation of IL-6 and IL-1ß gene expression was (15 ±0.84 and 2.06 ± 0.36 fold, respectively) statistically significant as a result of live injury. However, compared to IL-6 upregulation of IL-1ß gene expression was weaker. Changes in the IFN-γ and TNF-α gene expression were non-significant (Figure 28 A, B).

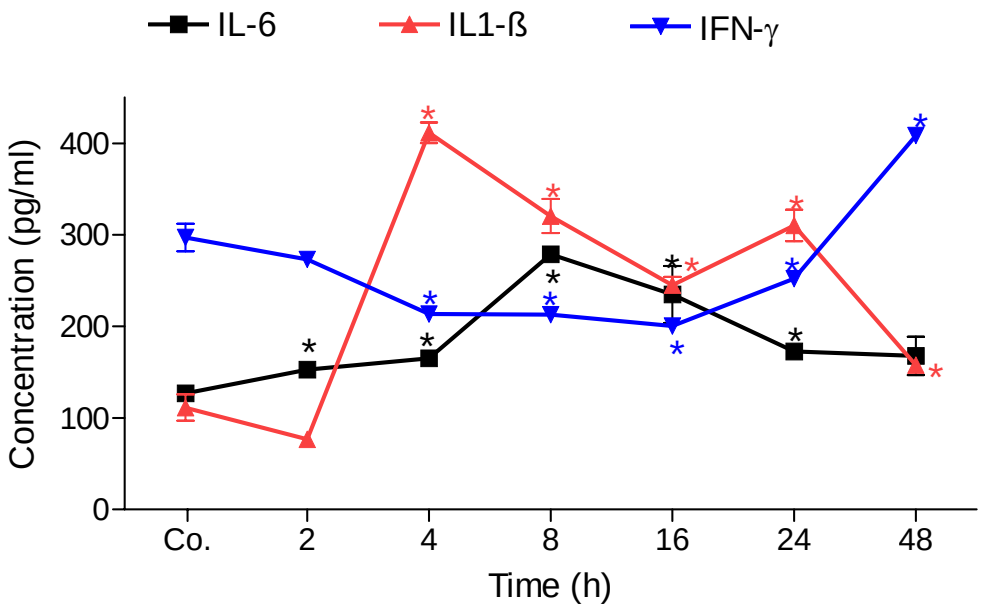


Figure 27: ELISA to study the serum level of cytokines during the liver injury. Serum levels of IL-6 were significantly elevated with a maximum concentration of (278.90 ± 3.7 pg/ml) 8 hours after PH. However, IFN-γ levels were significantly declined after PH. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=3)

4.2.3 Expression of Iron regulatory genes during PH-induced acute liver injury

Real-time PCR analysis revealed a significant time-dependent upregulation of Hepc and TfR1 gene expression (2.6 ± 0.8 fold by 8 hours and 4 ± 0.81 fold by 4 hours, respectively, $P < 0.05$, Figure 29 A, C). Tf and TfR2 gene expression was downregulated early after PH. The downregulation of both the genes was sustained throughout the course of experimental study (0.41 ± 0.10, 0.55 ± 0.04 fold); however, the downregulation of TfR2 gene expression was significant but not of the Tf ($P < 0.005$; Figure 29 A, C). DMT1, Dcytb, HFE, Heph and IREBP-1 gene expression was downregulated (0.74 ± 0.01, 0.49 ± 0.39, 0.42 ± 0.21, 0.21 ± 0.09 and 0.24 ± 0.06 by 16 hours; $P < 0.05$); however, only the downregulation of Heph and IRE-BP1 was significant (Figure 28 D-F). Hjv gene expression was significantly downregulated to a maximum of 0.2 ± 0.08 fold after the PH ($P = 2.2 \times 10^{-4}$; Figure 29 E). A time-dependent significant downregulation of ferritin-H and Fpn.-1 gene expression (0.45.± 0.09 and 0.5 ± 0.01 fold, respectively) was found as a result of liver injury (Figure 29 B). IRE-BP2 gene expression was significantly upregulated earlier after the liver injury;

however, at later time points the gene expression was downregulated significantly *(P < 0.01;* Figure 29 F).

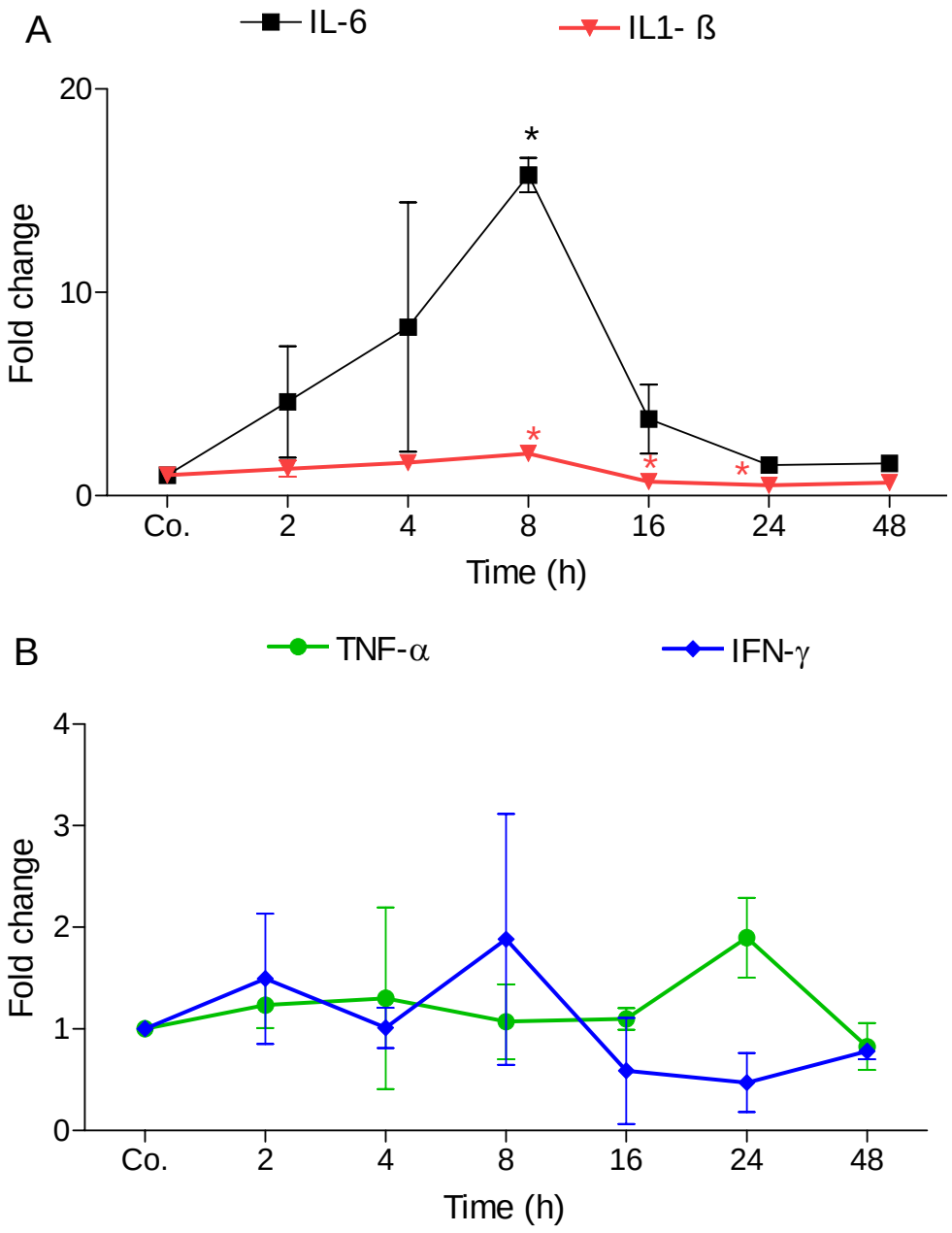
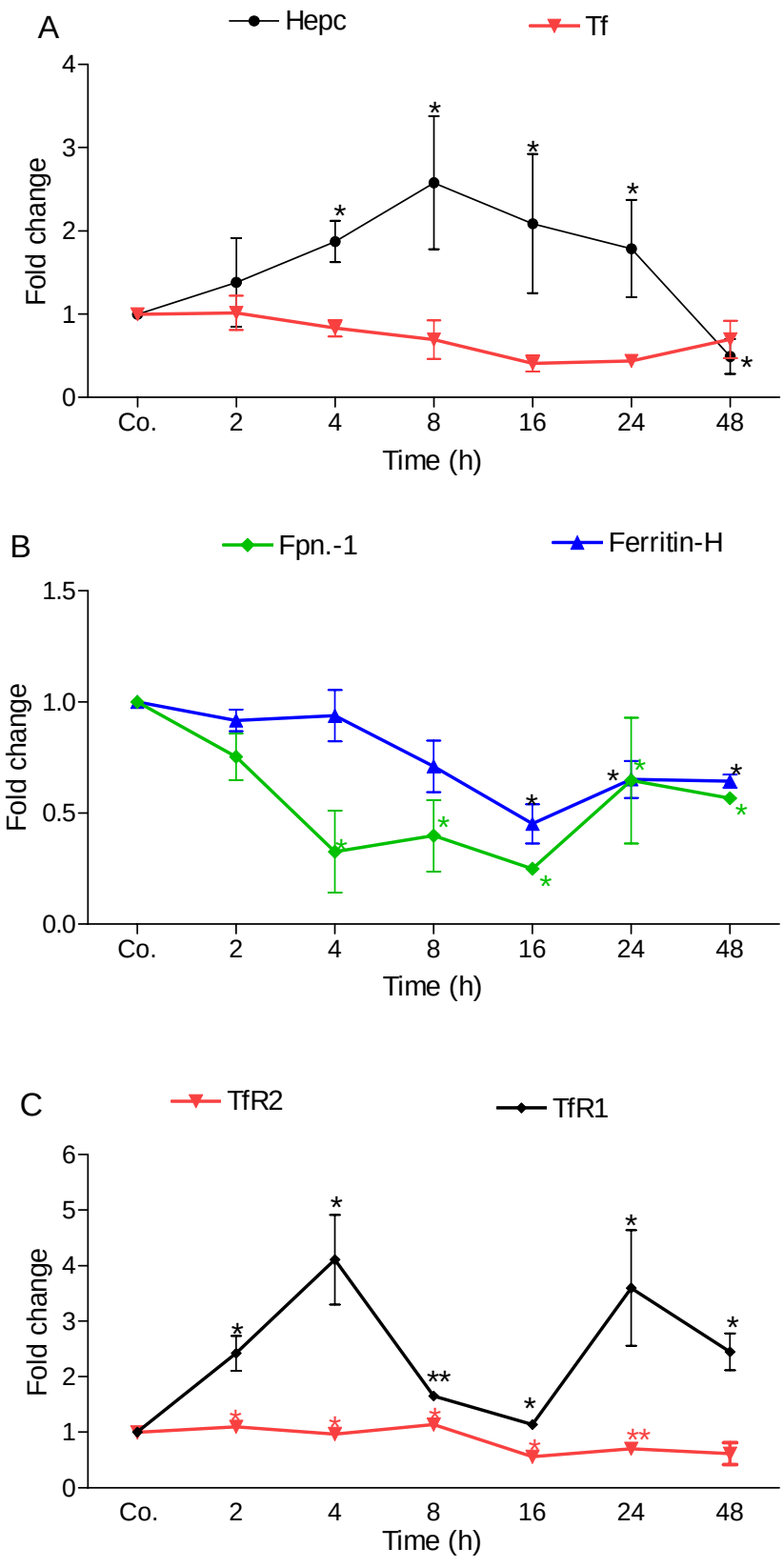


Figure 28: Real-time PCR analysis of rat liver RNA. Livers were obtained at different time points after the surgery (PH). Fold changes of IL-6, IL-1ß *(A)*, TNF-α and IFN-γ *(B)* gene expression after the injury shows significant upregulation of IL-6 and IL-1ß mRNA. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=3).



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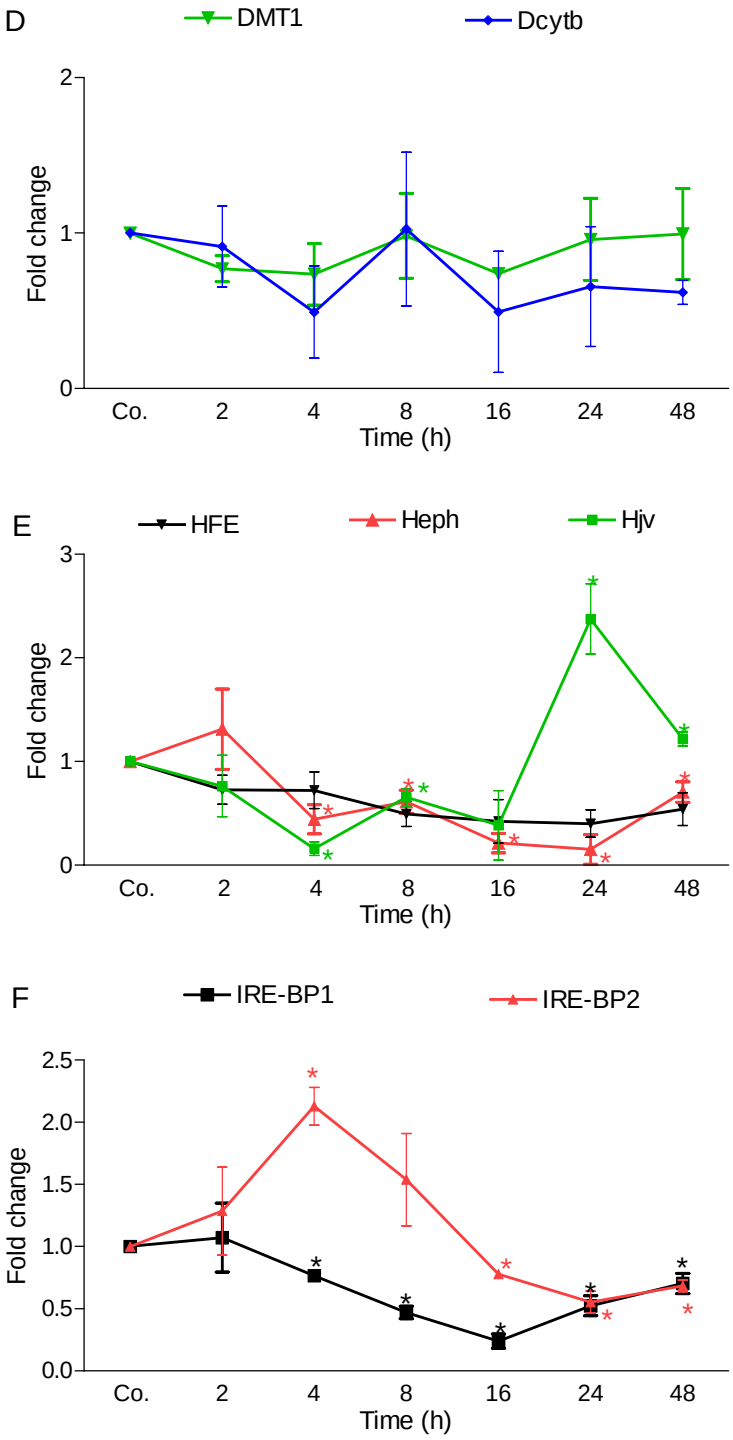


Figure 29: Real-time PCR -analysis of rat liver RNA. Livers were obtained at different time after operation (PH). Fold change of Hepc, transferrin (Tf), *(A)*, ferritin-H, Fpn.-1 *(B)*, TfR1, TfR2 *(C)*, DMT, Dcytb *(D)*, HFE, Hephaestin (Heph), Hjv *(E)* and IRE-BP1, IRE-BP2 after PH-induced liver injury. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=3).

4.2.4 Northern blot

The real-time PCR results were verified and supported by northern blot analysis. After the hybridization with specific cDNA probes we found increase of the Hepc mRNA expression to a maximum between 8 to 16 h after PH which is in agreement with our quantitative real-time PCR results. Similarly, as found by real-time PCR analysis Hjv and Fpn.-1 gene expression was decreased from 4–16 hours (Figure 30).

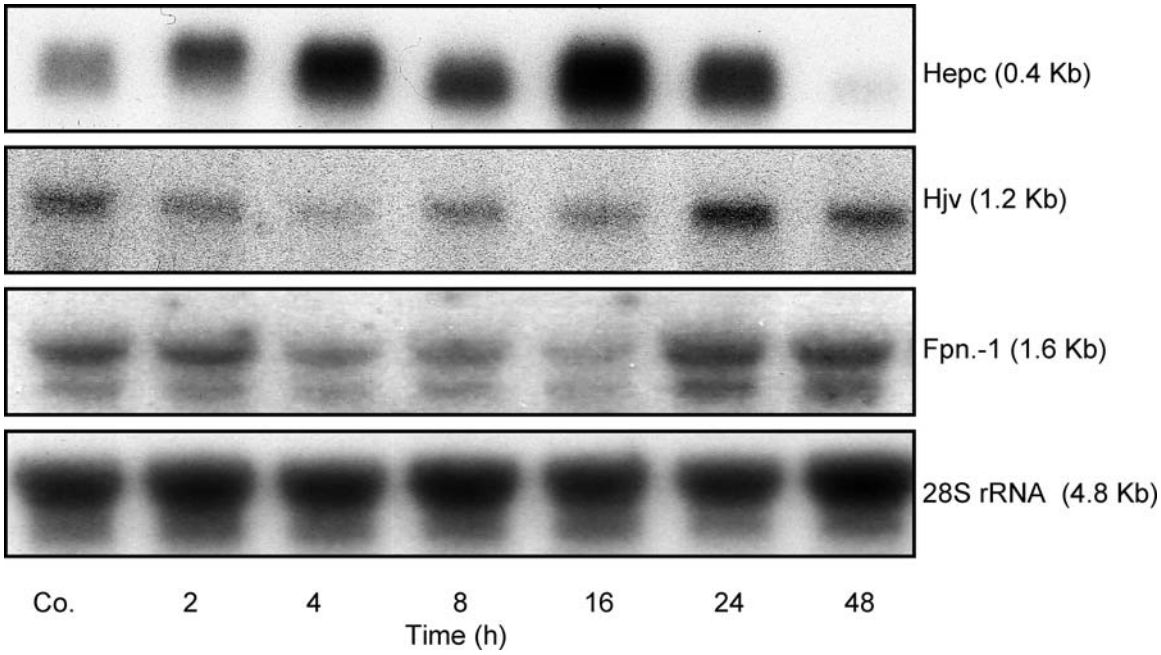


Figure 30: Northern blot analysis of Hepc, Hjv and Fpn.-1 mRNA levels in the livers of PH rats. A time course increase of Hepc gene expression was observed with a maximum expression between 8-16 hours in PH; however, Hjv and Fpn.-1 mRNA expression was downregulated at that time. Filters hybridized with Hepc, Hjv or Fpn.-1 specific cDNA probes were exposed to the autoradiographic film for different time periods at –80°C before developing.

4.3 CCl_4-induced acute liver injury

4.3.1 Serum analysis

4.3.1.1 Serum iron levels

In case of acute liver injury induced by oral administration of CCl_4, significantly declined serum iron levels were found early after the injury. Serum iron concentrations

were declined to 24.1 µmol/l ($P = 6 \times 10^{-4}$) compared to controls 66 µmol/l in CCl_4-treated animals (Figure 31).

4.3.1.II Serum transaminases

CCl_4-administration caused severe damage in the liver parenchyma. Consequently, AST and ALT levels were strongly increased in the sera at different time points compared to the control animals. This increase was statistically highly significant *($P < 10^{-4}$*; Figure 32).

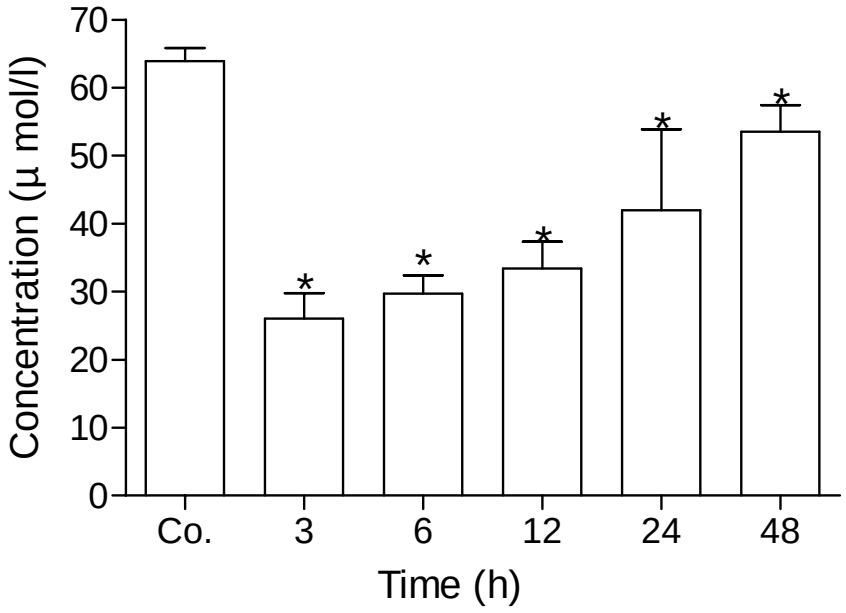


Figure 31: *In vivo* serum iron concentrations. A quick and sharp decline of serum iron level up to 26 µmol/l compared to 64 µmol/l in the control animals. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).

4.3.1.III Serum hepcidin pro-hormone

Serum hepcidin pro-hormone concentrations were increased as a result of hepatic injury induced by CCl_4-administration; however, this increase was statistically significant neither by Student's *t*-test nor by one way ANOVA (Figure 33).

4.3.1.IV Serum levels of acute-phase cytokines

Serum analyses have shown significant changes in the acute-phase cytokines concentration during acute liver injury. During CCl_4-induced liver injury, TNF-α and IL-1ß concentrations in the sera were significantly increased ($P < 0.05$). However, the

changes of IL-6 and IFN-γ serum concentrations were not statistically significant (Figure 34 A, B).

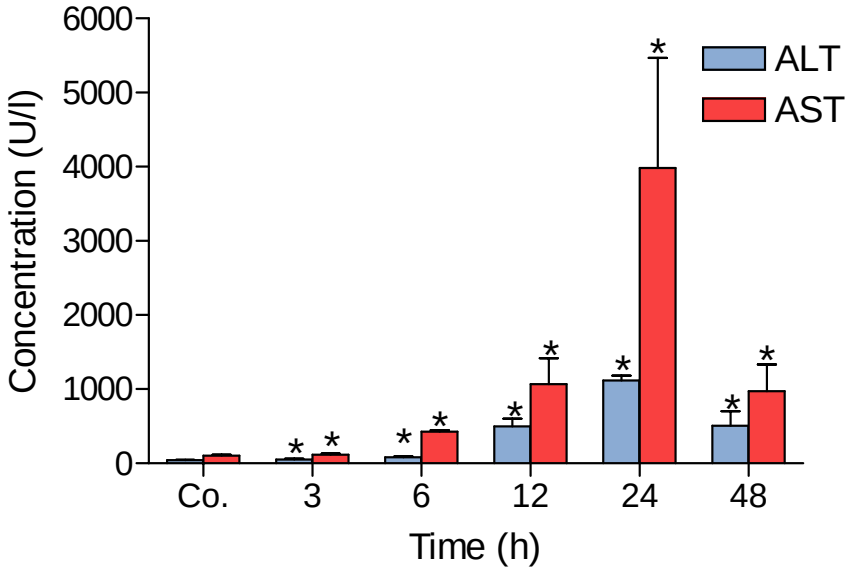


Figure 32: *In vivo* serum transaminase levels. A strong increase in the ALT and AST concentrations indicates the severe liver injury. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).

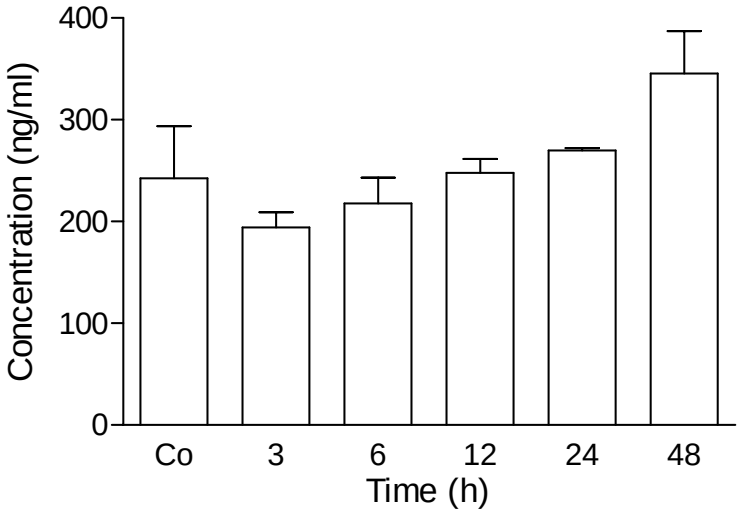


Figure 33: Serum concentration of hepcidin pro-hormone was not significantly affected by CCl_4-administration. A time dependent increase in serum hepcidin pro-hormone concentration was found in the rats with CCl_4-induced liver injury compared to control values (345 compared to 242 ng/ml). Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).

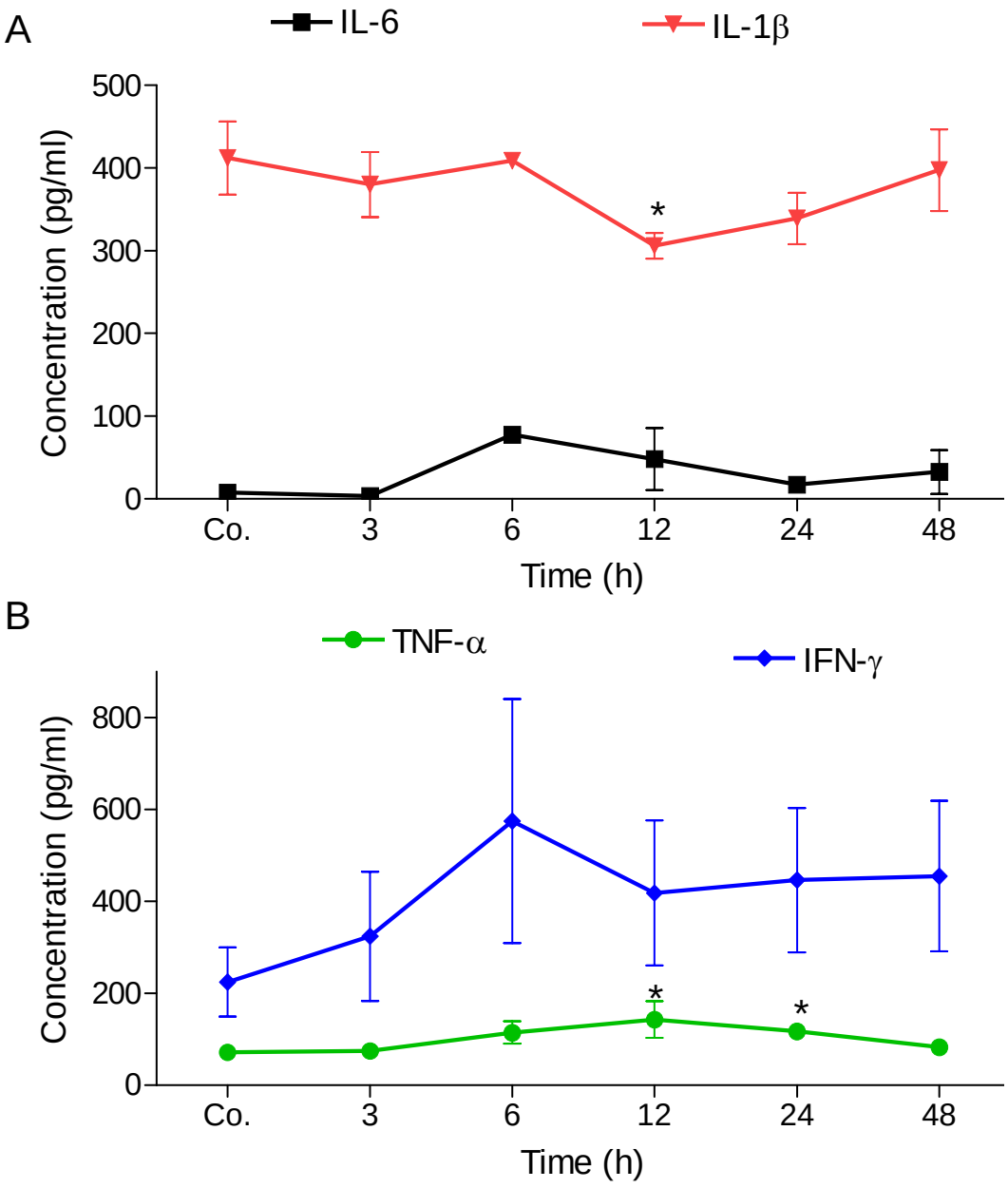


Figure 34: Serum acute-phase cytokines were studied by rat specific ELISA. In case of CCl_4-induced liver injury serum levels of IL-6 and IFN-γ were not significantly raised; however, TNF-α and IL-1ß concentrations were significantly elevated (143 ± 39 and 305 ± 15 pg/ml, respectively) 12 hours after the injury compared to control animals. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).

4.3.2 Acute-phase cytokines gene expression in the livers of CCl_4-administered rats

To study the role of specific acute-phase cytokines mediating the inflammation and regulating the Hepc gene expression along with the expression of other genes of iron metabolism, the expression of acute-phase cytokines at different time points after CCl_4-administration was studied by real-time PCR. CCl_4-administration caused local damage and strong and significant upregulation of IL-6 gene expression in the liver 6 hours after the injury with a highest peak at 12 hours (42 ± 18.1 fold, $P < 0.05$). Other acute-phase cytokines like IL-1ß, TNF-α and IFN-γ gene expression also attained the significant peaks 12 hours after the onset of the acute liver injury (6± 0.28, 17 ± 2.22 and 10 ± 0.378 fold, respectively; $P < 0.0002$; Figure 35).

4.3.3 Changes of the specific mRNA of the iron regulatory proteins

Oral administration of CCl_4 to the rats induced acute liver injury and significantly affected the expression of iron regulatory genes. Hepc gene expression was significantly upregulated (4.3 ± 0.73 fold; $P = 0.002$). The upregulation of ferritin-H and DMT1 gene expression (1.33 ± 0.15 and 2 ± 0.43 fold, respectively) during the course of study was not significant. TfR1 gene expression was significantly upregulated (9 ± 2 fold) earlier at 3 hours after CCl_4-administration and this expression remained above the basal level during the course of study ($P = 0.008$). On the other hand, Tf, Fpn.-1, Dcytb, HFE, Heph, Hjv and IRE-BP1 gene expression was (0.45 ± 0.06, 0.53 ± 0.11, 0.543 ± 0.08, 0.33 ± 0.05, 0.33 ± 0.12, 0.14 ± 0.08 and 0.40 ± 0.05 fold, respectively) significantly downregulated (Figure 36). Downregulation of TfR2 gene expression was significant by Student's *t*-test at different time points during the study; however, it was non-significant when analysed by one way ANOVA ($P = 0.13$; Figure 36C).

4.3.4 Northern blot analysis

Hepc mRNA levels were found to rise early reaching to the maximum expression level by 12 hours after the CCl_4-administration. On the other hand, Fpn.-1 and Hjv mRNA expression was downregulated at the same time (Figure 37). To ensure the equal loading of the samples an oligonucleotide complementary to 28S rRNA was used for hybridization.

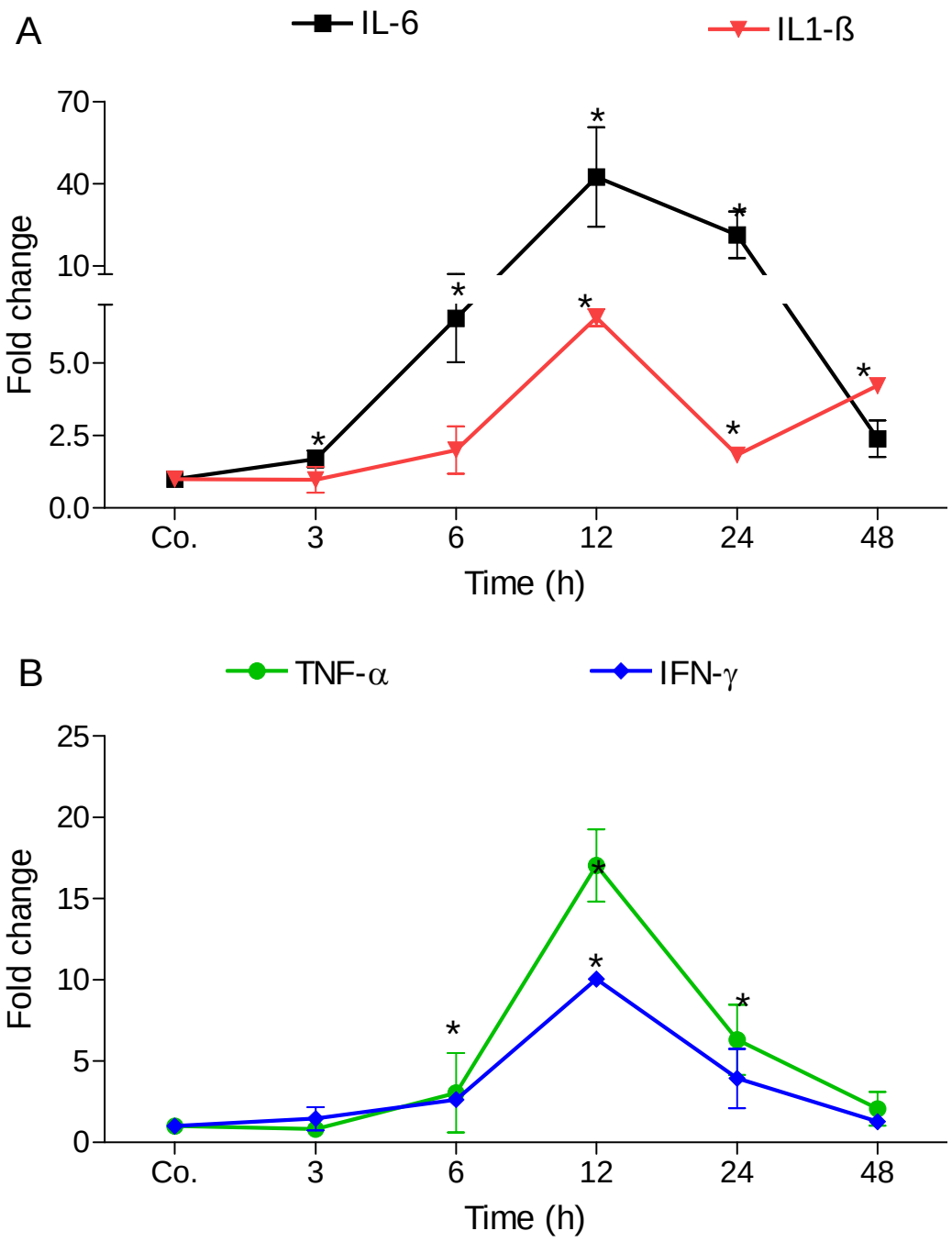
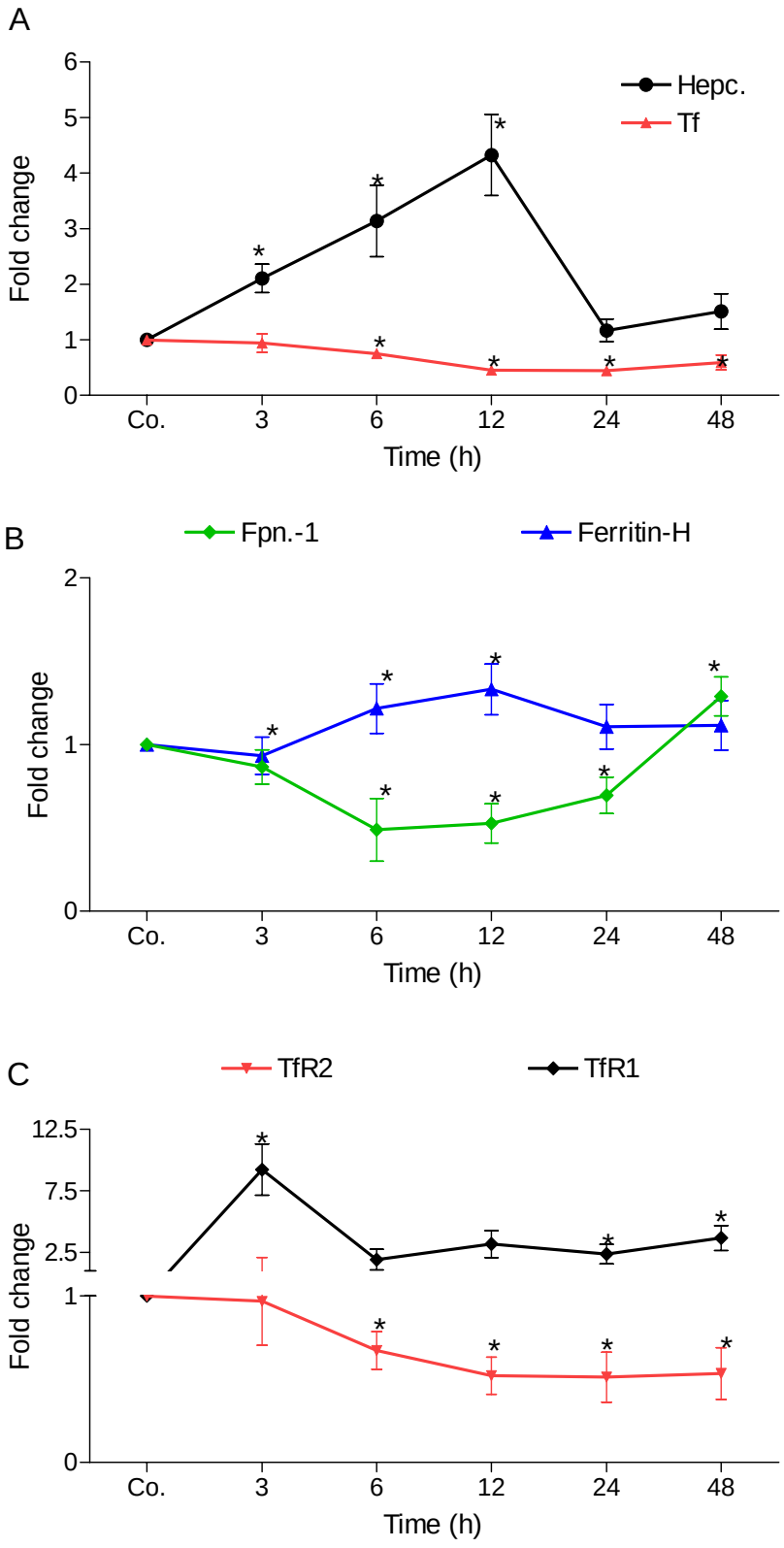


Figure 35: Real-time PCR analysis of total RNA from rat livers after CCl_4-induced acute liver injury for acute-phase cytokines. Livers were obtained at different time after intragastral administration of CCl_4. Significant changes in the IL-6, IL-1ß *(A)*, TNF-α and IFN- γ *(B)* gene expression was found during the liver injury. Values represent the amount of target mRNA compared to GAPDH mRNA. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).



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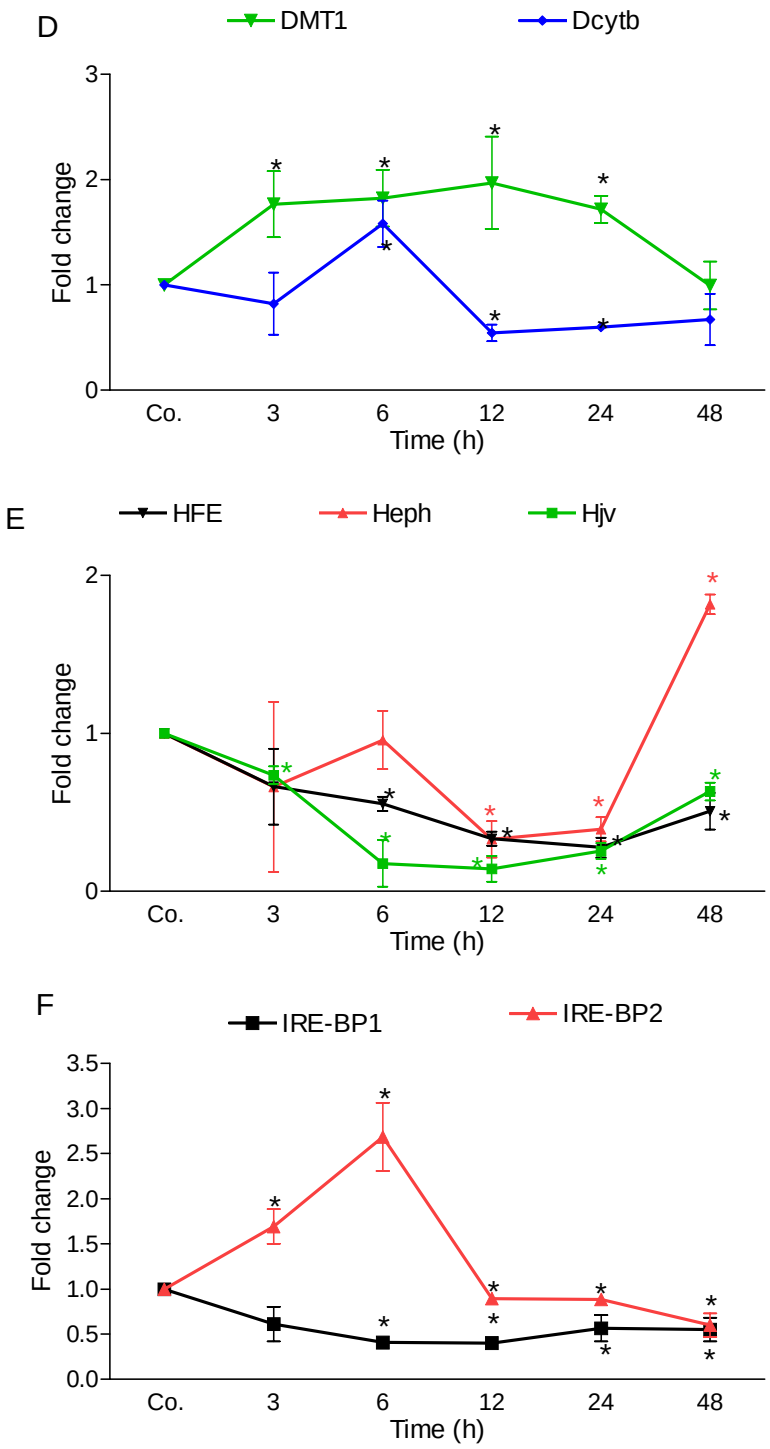


Figure 36: Real-time PCR analysis of total RNA isolated from rat liver at different time points after oral administration of CCl_4. mRNA expression of Hepc, Tf *(A),* Fpn.-1 and Ferritin-H *(B),* TfR2 and TfR1 *(C),* DMT1, Dcytb *(D)*, HFE, Heph and Hjv *(E),* IRE-BP1 and IRE-BP2 *(F).* Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=4).

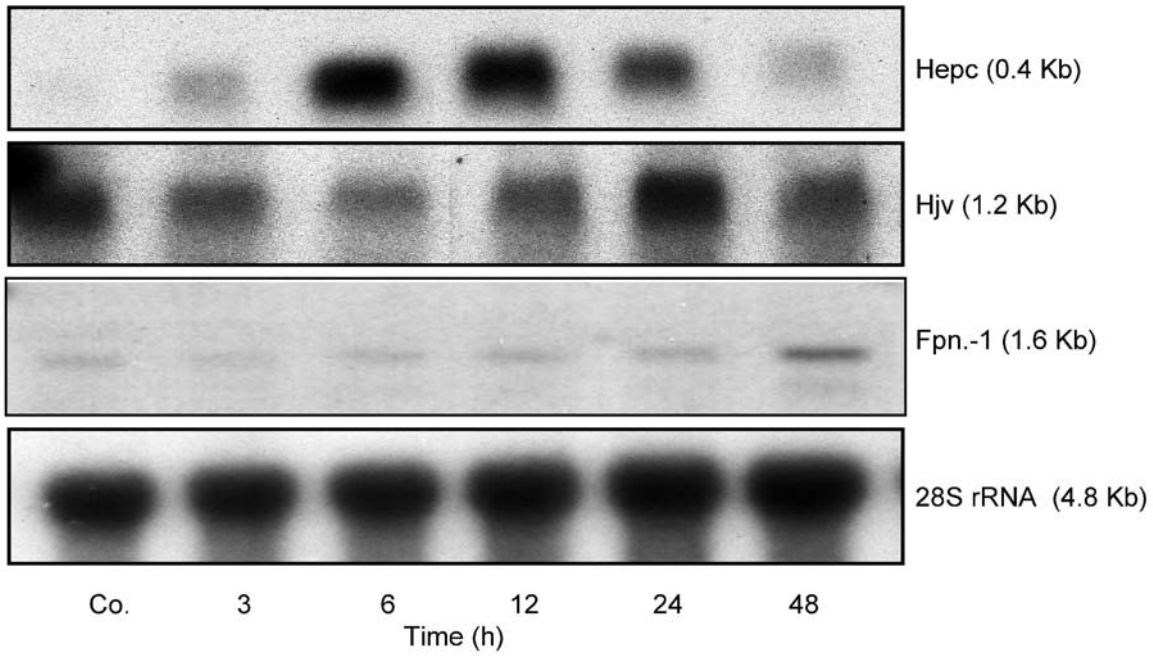


Figure 37: Northern blot analysis of Hepc, Hjv and Fpn.-1 specific mRNA in the livers, isolated from rats at different time points after oral administration of CCl_4. Filters carrying size fractionized RNA were hybridized with specific cDNA probes of Hepc, Hjv and Fpn.-1 and were exposed to the autoradiographic film for 5 days at -80°C before developing.

4.4 Changes of the specific mRNA of the Iron pathway proteins in vitro

4.4.1 Real-time PCR analysis

To study the role of hepatocytes in the expression and regulation of iron metabolism genes, hepatocytes were isolated, put into culture and stimulated with different acute-phase cytokines.

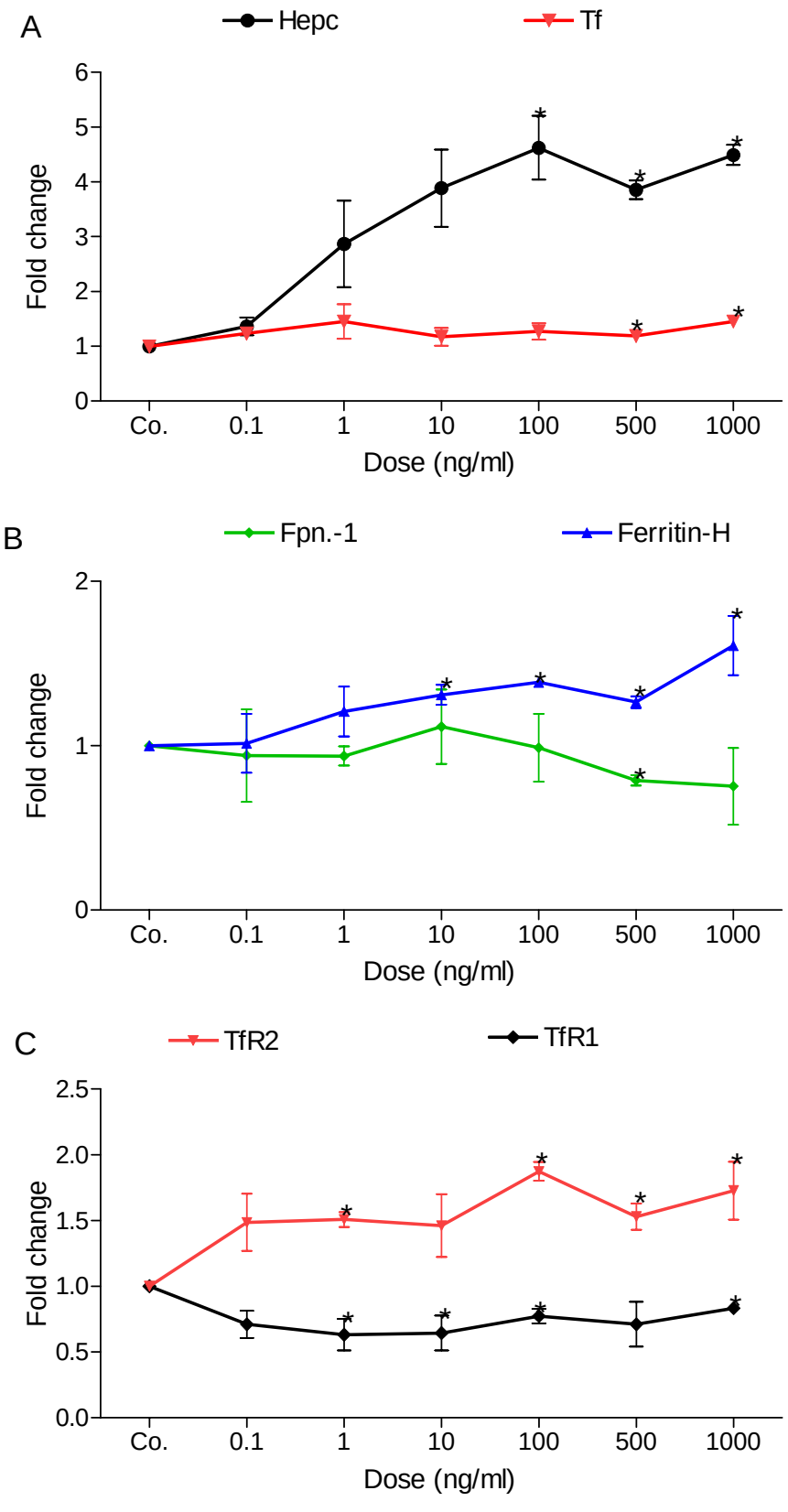
A dose-dependent significant upregulation of Hepc, Tf, Ferritin-H, TfR2, DMT1, Dcytb, HFE IRE-BP1 and IRE-BP2 gene expression was observed in the hepatocytes stimulated with IL-6 (4.33 ± 0.87, 1.45 ± 0.05, 1.38 ± 0.01, 1.87 ± 0.07, 6.32 ± 0.62, 1.35 ± 0.07, 1.48 ± 0.15, 1.12 ± 0.02 and 1.50 ± 0.09 fold, respectively; $P < 0.05$) at 100-500 ng/ml. TfR1, Hjv and Fpn.-1 gene expression was significantly downregulated (0.7 ±

0.05, 0.38 ± 0.03 and 0.8 ± 0.03 fold, respectively; $P < 0.05$) at 100-500 ng/ml dose (Figure 38 A-F).

Besides IL-6, hepatocytes in culture were stimulated with IL-1ß, TNF-α or IFN-γ Stimulation of the hepatocytes with IL-1ß caused non significant upregulation of Hepc gene expression (1.52 ± 0.55 fold, $P > 0.05$) at 1000 ng/ml dose. Similarly changes in the TfR2 and Fpn.-1 gene expression were not significant. However, IL-1ß caused significant upregulation of TfR1, DMT1, and HFE gene expression (2 ± 0.2, 6.08 ± 0.64 and 1.92 ± 0.05 fold, respectively; $P < 0.05$) at 500 ng/ml dose. Tf, ferritin-H, Dcytb, Hjv, IRE-BP1 and IRE-BP2 gene expression was significantly downregulated (0.82 ± 0.03, 0.78 ± 0.05, 0.67 ± 0.10, 0.5 ± 0.005, 0.60 ± 0.03 and 0.66 ± 0.02 fold respectively, $P < 0.05$) whereas the downregulation of Fpn.-1 gene expression was not significant (0.6 ±0.2 fold, $P > 0.05$) at 500 ng/ml dose (Figure 39 A-F).

TNF-α did not show significant effects on the Hepc and ferritin-H gene expression. However, Tf, TfR2, DMT1 and IRE-BP1 gene expression was significantly upregulated to (1.21 ± 0.08, 1.35 ± 0.06, 2.79 ± 0.09, 1.26 ± 0.07 fold, respectively; $P < 0.05$). Fpn.-1 and TfR1, Dcytb, HFE, Hjv, and IRE-BP2 gene expression was significantly downregulated (0.6 ± 0.02, 0.4 ± 0.02, 0.84 ± 0.00, 0.64 ± 0.02, 0.73 ± 0.01, 0.85 ± 0.02 fold, respectively; $P < 0.05$; Figure 40 A-F).

Hepatocytes stimulated with IFN-γ have shown no significant changes in Hepc, TfR1, HFE, and Hjv gene expression. However, significant upregulation of Tf, Fpn.-1, Ferritin-H, Dcytb, IRE-BP1 and IRE-BP2 gene expression was observed, on the other hand, TfR2 gene expression was downregulated in the hepatocytes stimulated with IFN-γ. (Figure 41 A-F)



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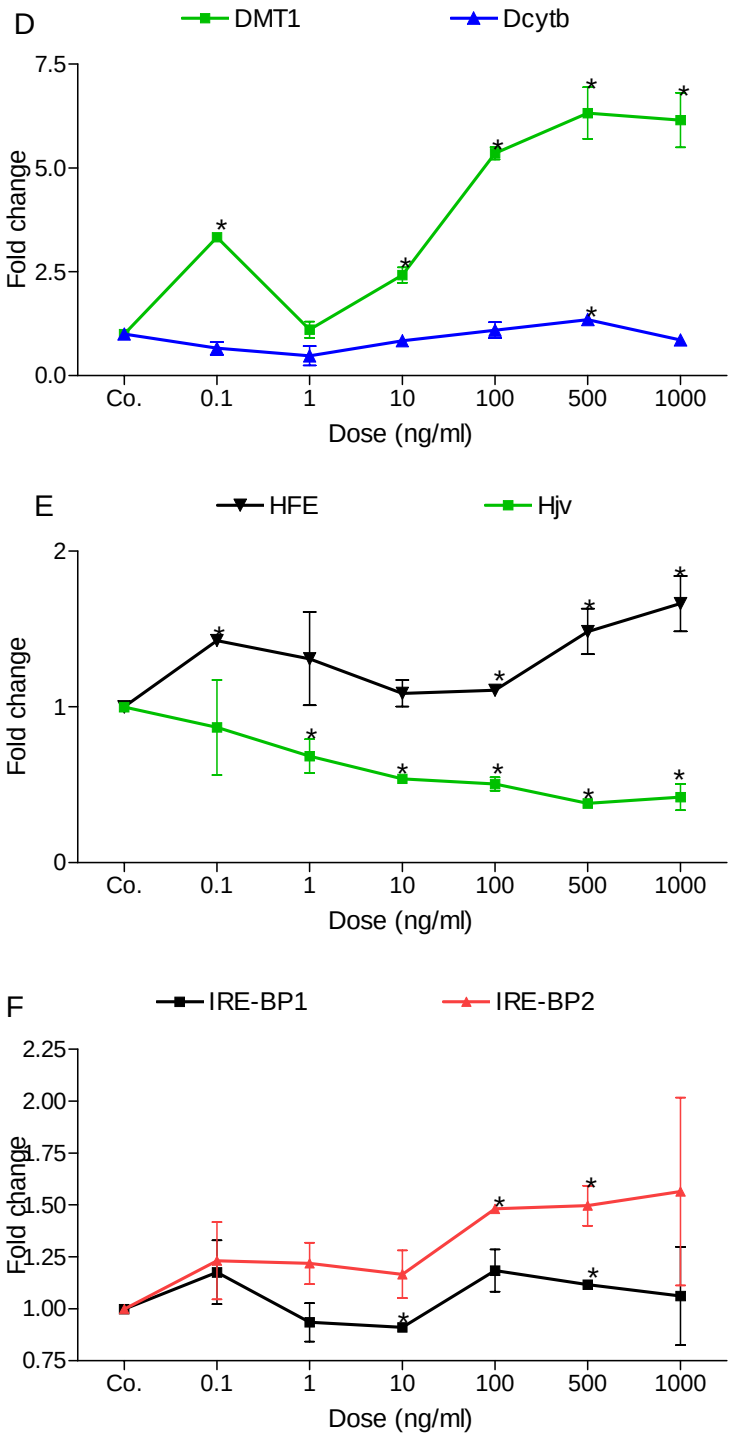
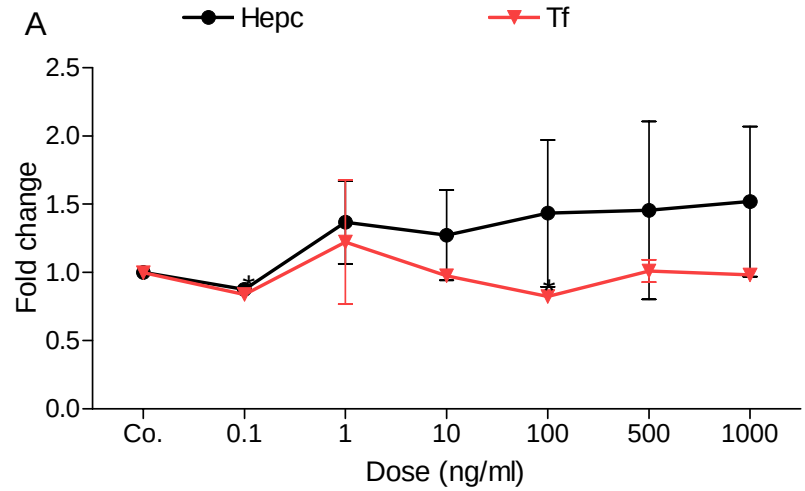
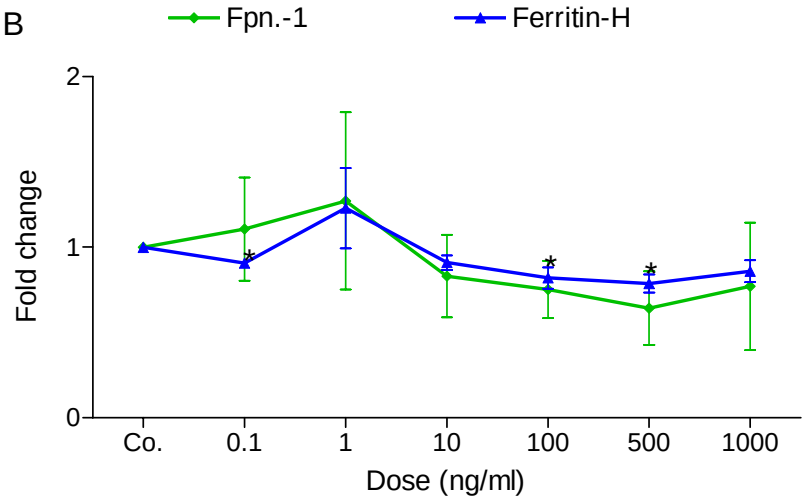
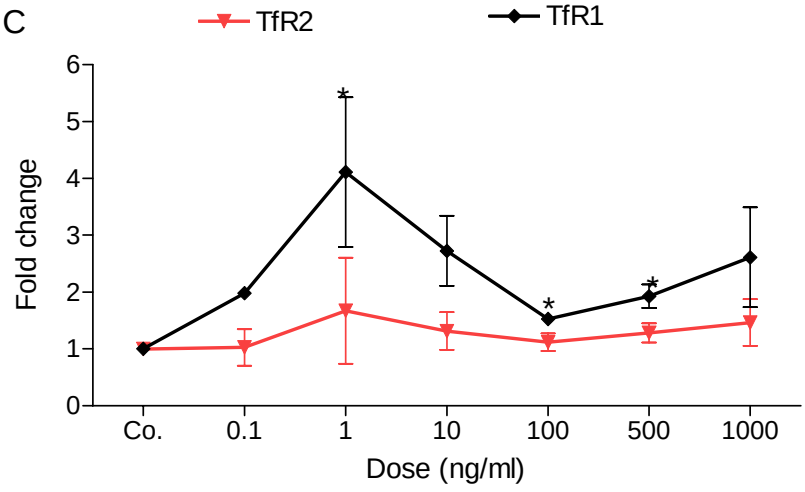


Figure 38: Real-time PCR analysis of rat hepatocytes RNA (*in vitro*). Rat hepatocytes were stimulated with different doses of IL-6 for 3 hours. Experiment was started 24 hours after plating. Fold change of Hepc, Tf *(A),* ferritin-H, Fpn.-1 *(B),* TfR1, TfR2 *(C)* DMT1, Dcytb *(D)*, HFE, Hjv *(E)* and IRE-BP1, IRE-BP2 *(F).* Values represent the amount of target mRNA compared to ß-actin mRNA. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=3).







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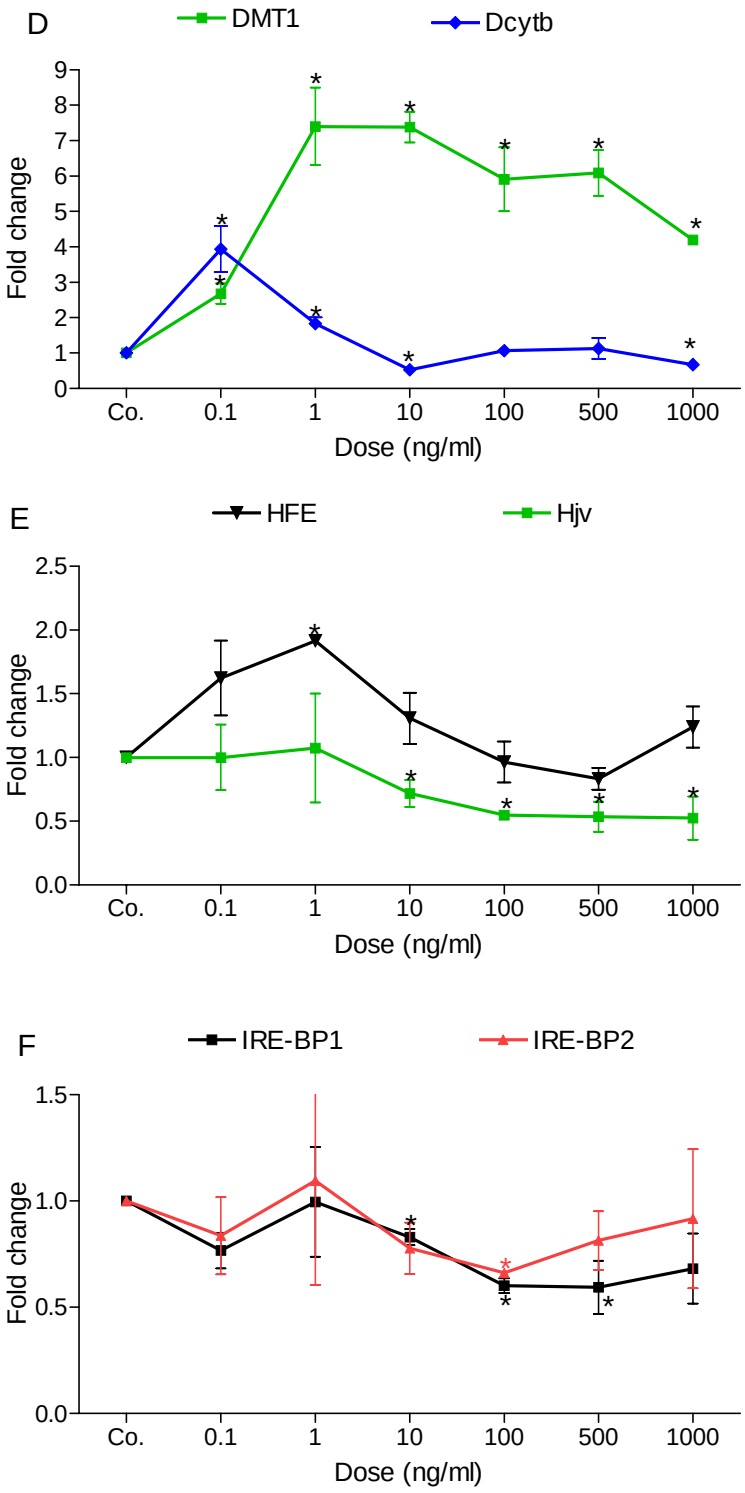
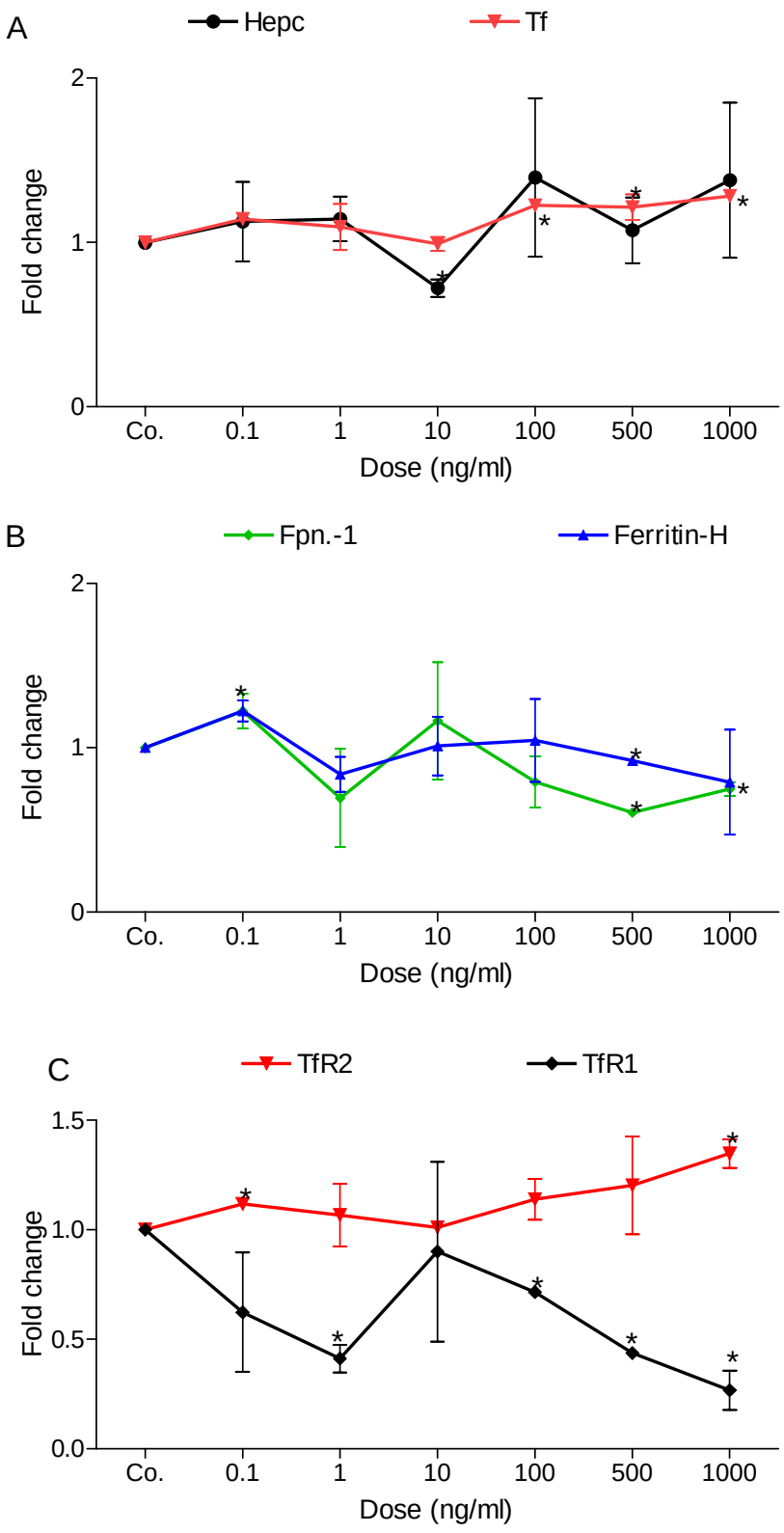


Figure 39: Fold change of Hepc, Tf *(A),* Ferritin-H, Fpn.-1 *(B),* TfR1, TfR2 *(C)* DMT1, Dcytb *(D)*, HFE, Hjv *(E)* and IRE-BP1, IRE-BP2 *(F)* in the rat hepatocytes stimulated with different doses of IL-1ß for 3 hours by real-time PCR. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=3).



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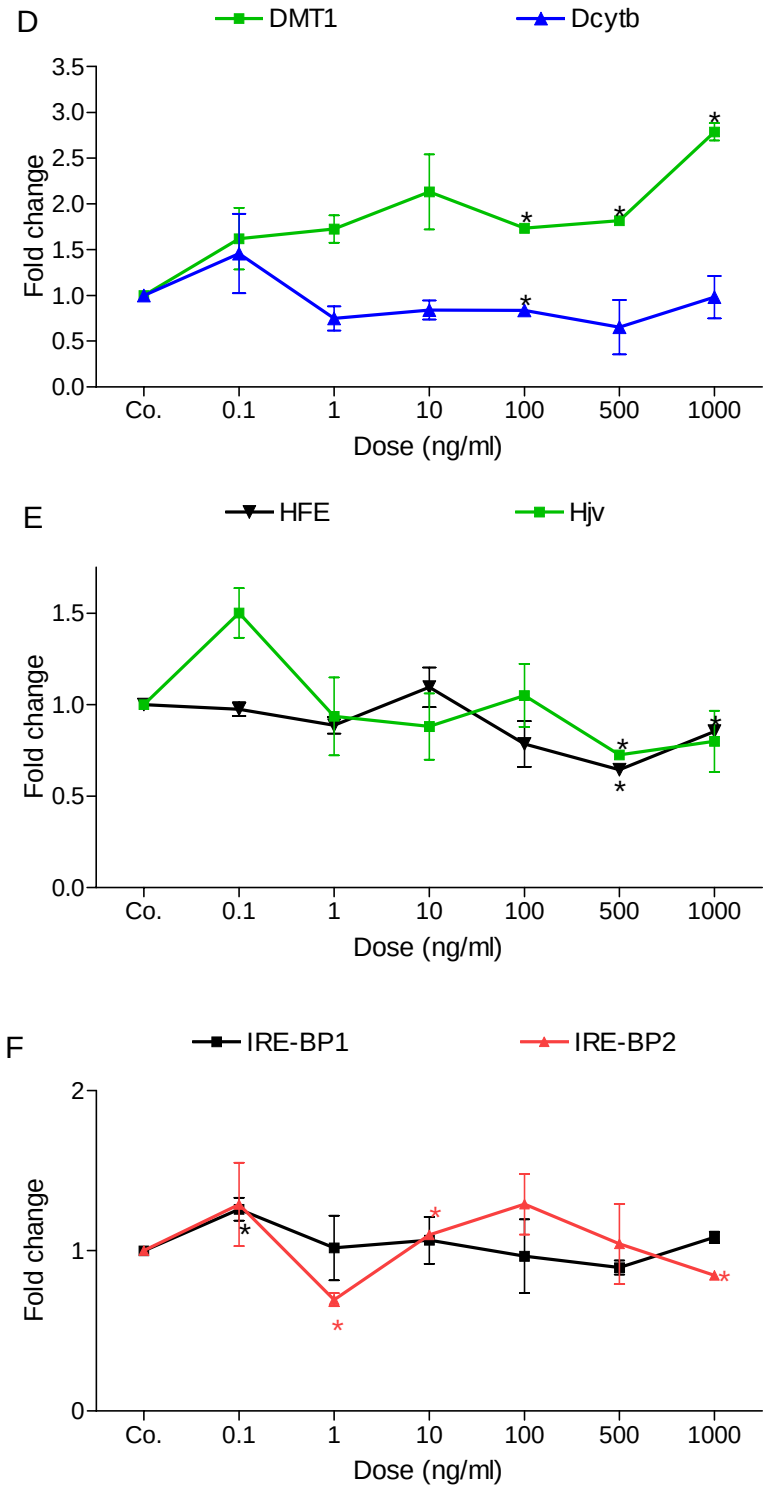
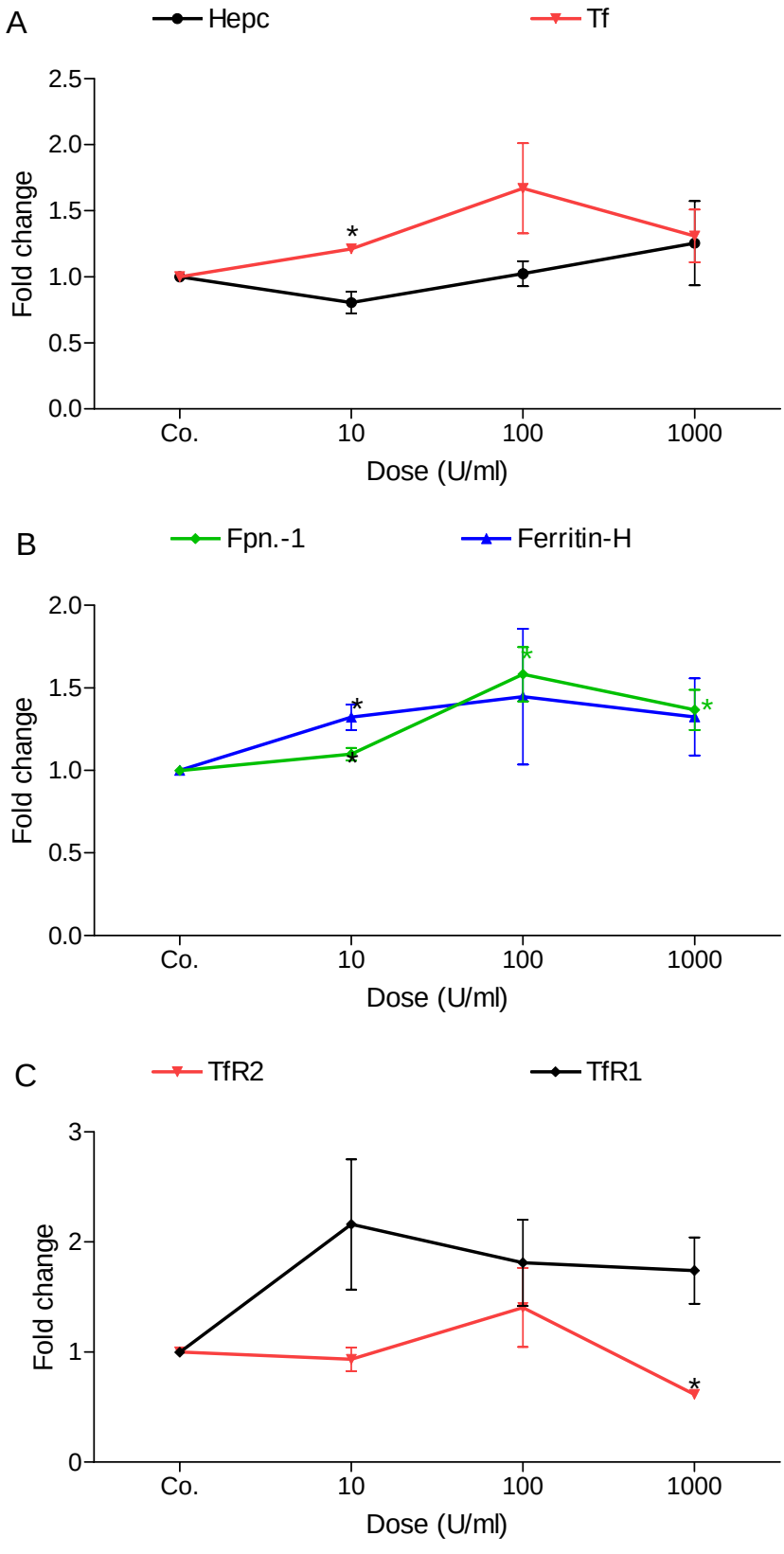


Figure 40: *In vitro* real-time PCR analysis of total RNA isolated from rat hepatocytes stimulated with TNF-α. Fold change of Hepc, Tf *(A),* Ferritin-H, Fpn.-1 *(B),* TfR1, TfR2 *(C)* DMT1, Dcytb *(D)*, HFE, Hjv *(E)* and IRE-BP1, IRE-BP2 *(F)*. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=3).



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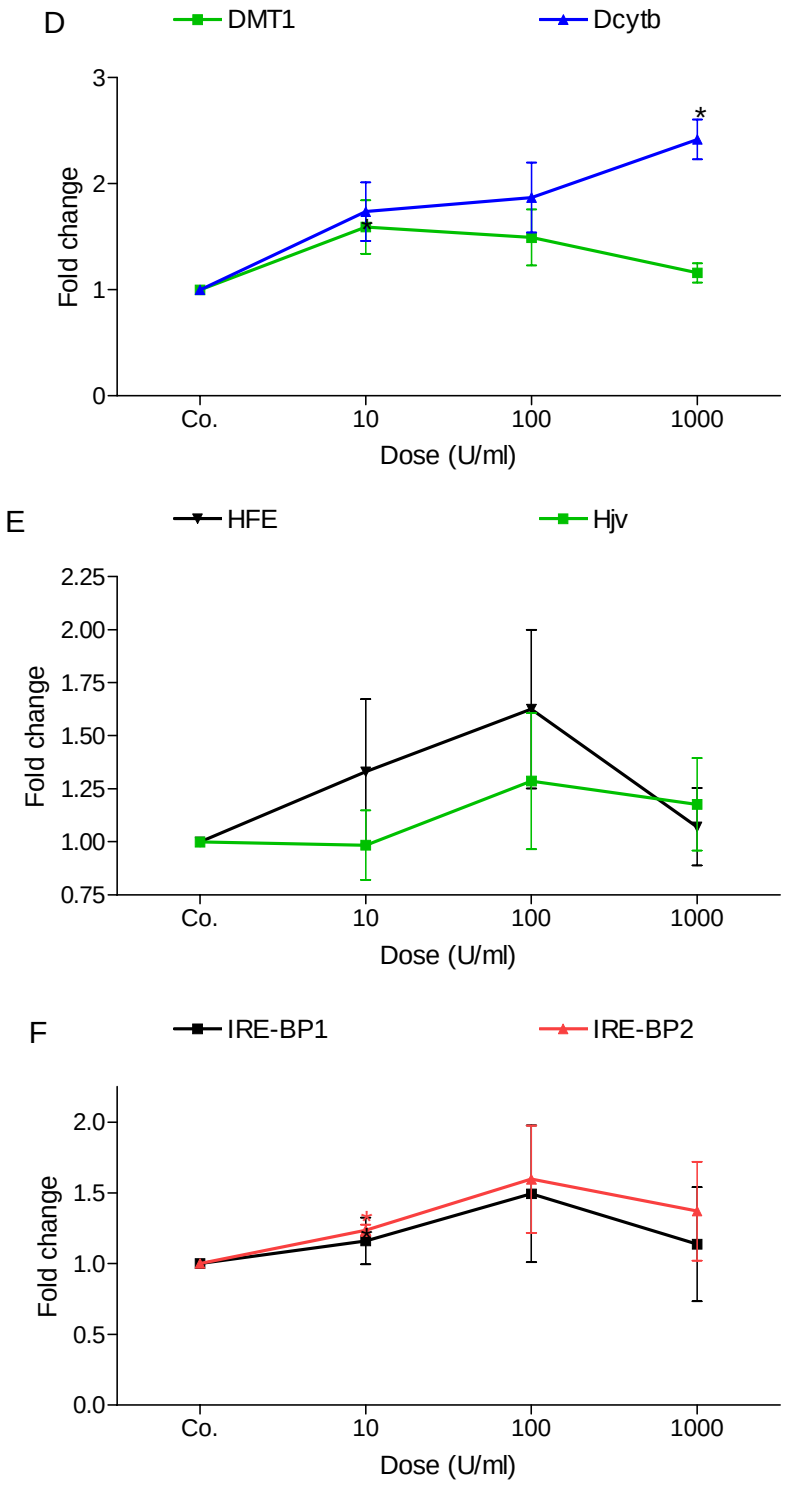

Figure 41: Effects of IFN-γ on the expression of iron regulatory proteins. Graphs show fold changes of iron regulatory genes in rat hepatocytes treated with different doses of IFN-γ for 3 hours. Results represent the mean value ± SEM (*$P < 0.05$, analysed by Student's *t*-test; n=3).

4.4.2 Northern blot analysis (in vitro)

In vitro studies with hepatocytes stimulated with IL-6 showed dose-dependent upregulation of Hepc to a maximum level at 100 ng/ml dose. A dose-dependent downregulation of Hjv and Fpn.-1 mRNA was clearly demonstrated by northern blot (Figure 41). Specific cDNA probes were developed as described in section 3.2.6. 28S rRNA was used to show the equal loading of RNA (Barbu and Dautry, 1989). By this technique we confirmed that Hepc and Fpn.-1 are two oppositely regulated but timely related genes (Figure 42) as was Hjv.

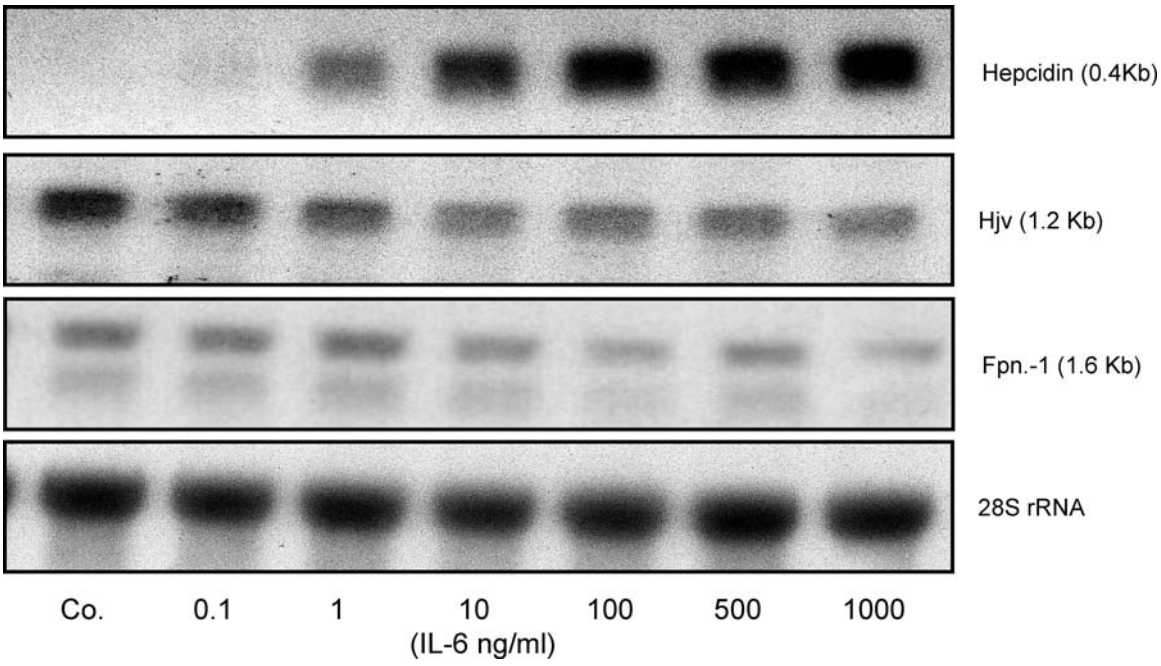

Figure 42: Northern blot analysis of RNA extracted from isolated rat hepatocytes. Hepatocytes were stimulated with different doses of IL-6. Total RNA was used for analysis as described in materials and methods. Filters were exposed to autoradiographic film at -80°C before developing.

5. DISCUSSION

In the present work, we studied the changes in the expression of the Hepc and other genes, recently observed to be involved in the regulation of iron metabolism, induced in the liver; which is considered to be the central organ for body iron economy, in different other organs in response to intramuscular injection of TO. At the same time the local expression of the genes of acute-phase cytokines known to be involved in the regulation of iron-metabolism genes especially IL-6 and IL-1ß were studied. For this study, *in vivo* model of TO-induced APR in the rat was chosen, because it is considered to better appreciate the role of acute-phase mediators in the changes observed in the liver and eventually in the organs. Partial-hepatectomy- (PH) and CCl_4-induced liver injury models were selected to study the regulation of iron metabolism genes during acute liver injury and the possible mediators for this regulation.

5.1 Acute-phase-response

Intramuscular TO-injection induced significant changes of the serum iron-concentration without a measurable change in the serum hepcidin pro-hormone concentration; however, hepcidin gene expression in the liver was significantly upregulated. The possible reason for this contradiction could be; firstly, the quantitative upregulation of the hepcidin at transcriptional level was not so strong which could induce the measurable and significant changes in the serum levels of the protein as we recently shown that serum level of CINC-1 increases to 15 fold when the hepatic expression of CINC-1 was upregulated to 260 folds in response to the TO induced localized inflammation (Sheikh *et al*, 2006a). Similarly, significant increase of the serum levels of the hormone have been reported associated with strong upregulation of hepatic hepcidin gene expression in response to γ-irradiation (Christiansen *et al*, 2006). Secondly, the ELISA used to study the serum hepcidin concentration was not specific to detect the biologically active hepcidin-25 as it was developed with the specific N-terminal hepcidin antibody EG(2)-HepN, instead, it is very specific to detect the hepcidin pro-hormone (25-84 amino acids) in the serum (Kulaksiz *et al*, 2004; Sheikh *et al*, 2006b). Furthermore, the depressed levels of the hepcidin pro-hormone could be because of the increased demand of bioactive hepcidin for iron regulation during the APR. So far no practical

assay has been reported for active hepcidin-25 though attempts have been made to develop an assay system by using SELDI-TOF MS (Tomosugi *et al*, 2006). The kinetics of the hepatic Hepc upregulation and decline in the serum iron levels also supports that hepcidin is involved in the iron regulation as the significant maximum upregulation of hepatic hepcidin was at 6 hours whereas significant decline of iron level was delayed to 12 hours.

Serum levels of IL-6 and IL-1ß were strongly upregulated whereby the level of IL-6 was (at its maximum) 9 times higher than that of IL-1ß. IL-6 gene expression was dramatically upregulated in the injured muscle whereas it was downregulated in the liver considered to be major target organ for the cytokine. On the contrary, in the model of LPS induced inflammation the IL-6 gene expression was upregulated in the liver (Lee *et al*, 2004) that could be due to the reason that injected LPS distributes throughout the body and local presence of the LPS might be responsible for the upregulation of hepatic IL-6. On the other hand, TO induced local irritation is a model of sterile inflammation in which the inflammatory agent is restricted to a specific area i.e. the muscle and the local inflammation results in the recruitment of the inflammatory cells as well the upregulation of the IL-6 in the injured muscle (Sheikh *et al*, 2006a). As the inflammatory stimulus is localized which could not approach the liver where it could cause the IL-6 upregulation. Hepatic IL-1ß and TNF-α gene expression was significantly upregulated during APR.

Hepc gene expression was observed not only in the liver but also in the extrahepatic organs studied. Furthermore, the APR-induced upregulation of Hepc found in the liver could also be detected in the extrahepatic sites with similar kinetics. An exception was represented by the lung where Hepc gene expression was downregulated. In that organ Hepc gene expression seems to be qualitatively relevant when compared the Ct values (Table 4) with those of the liver on one side and with those of spleen or kidney on the other side and it seems to be of the same order of magnitude as in the heart. The first part of these results is confirmatory of previously published data (Canonne-Hergaux *et al*, 2005; Frazer *et al*, 2004; Lee *et al*, 2004; Montosi *et al*, 2005; Nicolas *et al*, 2002); however, the second part has not yet been described in this comprehensive form.

The changes of gene expression of the different proteins in the injured muscle are worth to be observed in more details. In fact, Hepc gene expression decreased during

development of inflammation whereas Fpn,-1, ferritin-H, IRE-BP1, IRE-BP2, DMT1, and Heph gene expression were upregulated. This pattern of expression may be in part due to the mRNA originating from the inflammatory cells more than muscle itself. Further investigations are needed to better understand these findings.

Hjv gene expression could be confirmed in the skeletal muscle, in the heart and in the liver (Roy and Andrews, 2005). However, Hjv gene expression was clearly detectable in the kidney, lung and in smaller amounts in other organs. Previous studies by our group and other groups have established that the Hepc and Hjv are conversely regulated genes (Niederkofler *et al*, 2005; Nisbet-Brown, 2004; Sheikh *et al*, 2006b). In non-HFE-HH, Hepc gene expression is reduced (Bondi *et al*, 2005; Roy and Andrews, 2005) which indicates that Hjv could be involved in Hepc regulation but by which mechanism is still not clear.

In mammals, iron bioavailability for erythropoiesis and other vital functions is regulated at three principal sites: duodenal uptake, release from hepatic stores and recycling of scavenged iron from senescent red blood cells via reticuloendothelial macrophages (Hentze *et al*, 2004). During APR iron disappeared from the circulation to a significantly low level by 24 hours of the TO injection. Fpn.-1 gene expression was downregulated in the liver and in the extrahepatic organs studied except in the lung where an upregulation was seen. These changes of the gene expression were parallel to the significant decline in the serum iron level with the lowest concentration by 24 hours. Hepc interacts with Fpn.-1 leading to its internalization and degradation (Fleming and Bacon, 2005; Nemeth *et al*, 2004a) and the signal for downregulation of Fpn.-1 may be initiated as a consequence of Hepc and Fpn.-1 interaction (Frazer and Anderson, 2005). Heph, a ferroxidase (Vulpe *et al*, 1999) works in association with Fpn.-1 and takes part in the iron transfer across the basolateral membrane in the circulation. Hepatic Heph gene expression was significantly downregulated at 4-12 hours of APR supporting the hypothesis that Heph could also be involved in the export of iron across the basolateral membrane along with Fpn.-1. In the extrahepatic organs Heph gene expression was variable and only significant changes were in the spleen.

Ferritin-H, an intracellular iron associated protein that keeps the iron in bioavailable and nontoxic form (Ferreira *et al*, 2000), gene expression was upregulated in

the liver, kidney and lung while in the heart, small intestine and colon significant downregulation was observed. Hepatic DMT1 gene expression was upregulated whereas Dcytb gene expression was significantly downregulated in the same organ. As the brush border iron uptake is regulated by the interaction of DMT1 and Dcytb; however, how these two components of the iron uptake machinery are regulated is still a question. Previous studies have shown a strong effect of iron concentration on expression of these two proteins. In case of blood loss decrease in serum iron concentration is a signal to increase the uptake of dietary iron in the intestine, consequently, the DMT1 and Dcytb gene expression was upregulated (Chen *et al*, 2003; Frazer and Anderson, 2005). DMT1 gene expression was downregulated in the small intestine which could be due to the local Hepc expression; however, Dcytb gene expression was found significantly upregulated in small intestine as expected (Mena *et al*, 2006). Besides small intestine DMT1 gene expression significantly downregulated in the spleen and kidney; however, in the lung DMT1 gene expression upregulated which is in agreement with the previously published work (Nguyen *et al*, 2006; Wang *et al*, 2005), where DMT1 upregulation was observed in response to LPS treatment in mouse and epithelial cells; however, this upregulation was not significant. Dcytb gene expression was downregulated in the lung but upregulated in the kidney and in the small intestine.

An early upregulation of transferrin “an iron carrier protein in the blood” was found with a subsequent downregulation in the liver when the iron was depleted from the circulation. TfR1 and TfR2 which can take up the transferrin-bound iron via receptor mediated endocytosis (Cairo and Pietrangelo, 1994) were upregulated. Hepatic TfR1 gene expression was upregulated earlier while TfR2 gene upregulation was delayed to 6 hours. Tf gene expression was significantly downregulated in the heart and kidney but not in the lung and small intestine where it significantly upregulated. TfR2 gene expression was significantly upregulated in the kidney and in the lung whereas, an opposite regulation was observed in the small intestine and in the spleen. TfR1 gene expression was changes significantly only in the spleen. As reported before Hepc gene expression is regulated during inflammation or altered iron conditions through more than one signalling pathways like IL-6, HFE, Hjv and TfR2 (Lee *et al*, 2004). An increase in TfR2 in this study was accompanied by an increase in Hepc gene expression however,

Hepc gene expression was increasing already after 1 hour of the TO injection on the other hand, TfR2 gene expression was delayed, which indicates regulation of the Hepc gene expression via some other pathways most possibly by IL-6.

Hepatic-HFE gene expression was significantly downregulated during acute-phase. HFE forms a complex with TfR1 (Waheed *et al*, 1997) and the binding sites of HFE and diferric-Tf overlap (Fleming and Britton, 2006). HFE undergoes endocytosis with TfR1 (Lebron *et al*, 1998). Despite of iron overload, patients with HFE mutations and HFE deficient mice have Hepc levels below the normal control mice which suggest that HFE is required for Hepc gene expression (Bridle *et al*, 2003; Muckenthaler *et al*, 2003). However, Hepc gene expression is not completely abolished in HFE–deficient mice which indicate the existence of intact Hepc regulatory setup (Jacolot *et al*, 2004). The question still to be answered is how Hepc and HFE are regulated; our findings suggest an inverse relationship of Hepc and HFE gene expression in liver during acute-phase. HFE gene expression was downregulated in the other organs as well.

Iron responsive elements play crucial role in the transcription regulation of ferritin-H, TfR1 and DMT1 gene expression. In iron-depleted state, IRE-BP binds to single IREs located in the 5' untranslated regions of ferritin and represses translation initiation (Gray and Hentze, 1994) or 3' region of TfR1 and DMT1 mRNAs and enhance the translation. IRE-BP1 and 2 gene expression was upregulated 6 hours after the TO injections. IRE-BPs are critical determinants of the post-transcriptional regulation of TfR expression. IRE-BPs have a major role in determining the iron storage capacity of cells by regulating translation of both H- and L-ferritin mRNA. In addition, the mRNA-encoding DMT1 and Fpn.-1 contain iron-responsive element (IRE) like sequences, suggesting that IRE-BP might possibly affect the use of these mRNAs (Gunshin *et al*, 1997; McKie *et al*, 2000; Donovan *et al*, 2000). DMT1 expression is iron regulated in some but not all situations (Fleming *et al*, 1999; Han *et al*, 1999; Wardrop and Richardson, 1999), whereas other studies indicate that Fpn.-1 mRNA abundance responds to alterations in iron status (McKie *et al*, 2000). Iron regulates the synthesis of ferritin and TfRs largely through the regulated interaction of cytosolic RNA-binding proteins, IRE-BPs, with the 28-nucleotide IRE in their mRNA. The IRE is a stem-loop structure composed of a 6-nucleotide CAGUGX loop at the end of an RNA helix. The

RNA helix or stem contains a bulged-nucleotide region that is five base pairs 5` of the first nucleotide of the loop, which is a critical determinant in the recognition of IREs by IRE-BPs. H- and L-ferritin mRNAs have a single IRE in their 5`-UTR, very close to the 5` cap structure, whereas TfR mRNAs contain five IREs in their 3` UTRs (Eisenstein, 2000).

When IRE-BPs are active for RNA binding, as is the case under iron-limiting conditions, IRE-BP1 or IRE-BP2 binds to the IRE in ferritin mRNA and blocks its translation. When IRE-BPs are bound to a 5` IRE, the cap-binding complex (eIF4F) binds to the mRNA but cannot make functional contact with the 43S pre-initiation complex (Muckenthaler *et al*, 1998). Hence, in the repressed state, ferritin mRNAs appear to have entered the initiation pathway but are blocked at an early step. The presence of the cap-binding complex on the repressed mRNA may provide a mechanism that promotes efficient competition for translation after IRE-BP inactivation (Muckenthaler *et al*, 1998). In a similar manner, evidence suggesting that IREs may be stimulators of translation, in the absence of IRE-BP binding, also may explain the efficiency with which ferritin mRNAs compete with other cellular mRNAs for translation when the iron level is high (Dix *et al*, 1992). The efficiency with which the IRE-BPs and IREs modulate mRNA translation is reflected in the 50- to 100-fold range of iron regulation of ferritin expression (Chen *et al*, 1997; Eisenstein, 2000; Shull and Theil, 1982).

In contrast to ferritin mRNAs, IRE-BPs indirectly modulates TfR mRNA translation by influencing its rate of degradation. Two regions within the 3` UTR of TfR mRNA are required for iron regulated stability of mRNA. These include the IRE, of which only three appear to be required, and a region referred to as the rapid turnover determinant. When IRE-BPs are bound to TfR mRNA, they retard its degradation, and the message has a half life of 6 hours. When IRE-BPs are not bound to the mRNA, it has a much shorter half life (< 1h). Unlike most eukaryotic mRNAs examined to date, the degradation of TfR mRNA does not appear to be initiated by deadenylation (Binder *et al*, 1994). Much remains to be understood about the control of TfR mRNA stability, including the role of other post-transcriptional steps (Seiser *et al*, 1993) and the identification of a nuclease system that degrades the mRNA when iron levels are high. Finally, the newly discovered protein TfR2 is encoded by an mRNA that lacks IREs,

indicating that in some circumstances regulation of the number of Tf-binding sites in the cell would not be controlled by IRE-BP (Kawabata *et al*, 1999). In contrast, expression of TfR2 appears to be refractory to alterations in iron status, at least in liver (Fleming *et al*, 2000). At the site of injury the expression of iron regulatory genes Hepc, HFE, Hjv and TfR2 was downregulated while the genes responsible for iron trafficking like Fpn.-1, Heph, DMT1 and Dcytb were upregulated.

Despite the injury in the hind limb muscle of the rat the expression of mRNA of acute-phase cytokines in the liver was not so strongly induced as it was found in the injured muscle. Significantly high expression of IL-6 mRNA was observed 2 hours after the TO injection with a maximum (3427 fold) at 6 hours in the injured muscle. Besides IL-6, IL-1β gene expression was also upregulated to 400 fold at the same time. Other acute-phase cytokines studied were TNF-α and IFN-γ. Upregulation of TNF-α gene expression was slightly delayed. IFN-γ gene expression was upregulated significantly at 2 hours after the TO injections in the injured muscle; however, this upregulation was not quantitatively similar to the IL-6 gene expression when compared the Ct values (Table 4). In the liver, IL-6 and IFN-γ gene expression was downregulated however, the IL-1β and TNF-α gene expression was weakly upregulated.

The elevated expression of these acute-phase cytokines at protein level was demonstrated in the serum using specific ELISAs. A similar pattern of increase in serum IL-6, and IL-1β concentration was found to a maximum 2018 pg/ml at 6 hours and 150 pg/ml at 12 hours whereas the delayed increase in the serum concentration of IFN-γ was found to a maximum 495 pg/ml after 12 hours of the commencement of APR. Serum TNF-α concentration was unchanged in response to TO induced APR. Previous studies have shown IL-6 as the principle mediator of TO induced APR (Tron *et al*, 2005); however, recent studies by our group and Lee *et al* (Lee *et al*, 2005) suggest that IL-1β along with IL-6 could be involved to mediate the consequences of the inflammation (Dinarello, 2006; Sheikh *et al*, 2006a) and induce hepcidin gene-expression *in vivo*.

Northern blot analysis supported our real-time PCR findings for hepatic Hepc, Hjv and Fpn.-1 gene expression. An upregulation of Hepc and downregulation of Hjv and Fpn.-1 gene expression was clearly visible in the blots exposed to autoradiography. These changes of the Hepc and Hjv gene expression could be detectable by real-time PCR but

not by northern blot analysis in the extrahepatic organs. IL-6 was differently upregulated in several organs (up to 20 fold in the heart) which indicate that besides the acute-phase cytokines coming through the blood (e.g. into the liver as it may be the case for IL-6) local changes in the acute-phase cytokine gene expression may also play some role in local iron regulation. In a previous report (Tron *et al*, 2005) this was not fully appreciable by northern blot analysis.

5.2 Acute liver injury

Second part of this work deals with the regulation of iron metabolism proteins during PH- or CCl_4-induced direct liver injury. In this case the liver not only has to respond to the damaging noxae but also have to protect itself and carry on the synthesis of vital acute-phase reactants in order to protect the body.

Serum levels of transaminases, iron and hepcidin pro-hormone and of IL-6, IL-1ß, TNF-α and IFN-γ were determined. Elevated concentrations of AST and ALT in the serum were found after the PH and CCl_4 administration. AST and ALT are liver injury marker enzymes. Elevated serum levels of ALT reflect the hepatobiliary disease; however, AST levels increase during liver parenchymal damage. As a result of direct liver injury both ALT and AST levels were elevated significantly. In PH rats; however, the serum concentration of hepcidin pro-hormone was slightly increased and serum iron levels were sharply declined early after the onset of injury. The obvious explanation for why the serum hepcidin pro-hormone levels were unchanged but the serum iron levels were sharply declined is that the hepcidin pro-hormone assay does not reflect the level of active hepcidin.

Serum concentrations of IL-6 and IL-1ß were significantly increased but the level was much lower than those observed in the rat under TO induced acute-phase conditions. IFN-γ serum concentration was significantly reduced in the partially-hepatectomized rats. On the other hand, no significant increase of IL-6 or IFN-γ was found in the serum of CCl_4-treated rats, whereas IL-1ß and TNF-α concentrations were significantly elevated 12 hours after the administration of the toxin. IL-6 specific mRNA was increased in the liver but this increase was not as strong as were in the classical acute-phase models (Kemna *et al*, 2005; Tron *et al*, 2005). This could be one of the reasons why serum levels

of IL-6 were not significantly elevated in CCl_4-induced liver injury. Its local action; however, could be sufficient to regulate the expression of acute-phase genes.

A similar pattern of changes in the expression of iron metabolism genes was observed during acute liver injury induced by PH or CCl_4 administration; however, the expression differs quantitatively. The changes of the hepatic Hepc, Hjv gene expression together with the changes of other genes involved in iron metabolism in response to direct liver injury showed a similar pattern. Hepatic Hepc, TfR1 and IRE-BP2 gene expression was upregulated after resection of 70 % of the liver. An early upregulation of these genes was observed in the liver of CCl_4-treated animals. Tf, TfR2, Dcytb, HFE, Heph, and IRE-BP1 gene expression was downregulated in both the models of acute liver injury.

In both models, Hjv gene expression was downregulated together with Hepc upregulation. At the same time the expression of the ferritin-H and Fpn.-1 genes was downregulated. In the liver of CCl_4-treated animals expression of ferritin-H gene was slightly upregulated whereas Fpn.-1 gene expression was downregulated 0.5 fold after 6 hours of liver injury. DMT1 gene expression behaved oppositely in two animal models. It was variably regulated after PH; however, a significant upregulation was observed in CCl_4-induced liver injury.

The gene expression of acute-phase cytokines IL-6, IL-1ß, TNF-α and IFN-γ was found to be upregulated in the damaged livers. In partially-hepatectomized livers upregulation of IL-6, IL-1ß and IFN-γ started by 2 hours was at peak by 8 hours of the PH; however, upregulation of TNF-α gene expression was weak and slightly delayed. In CCl_4-treated animals upregulation of Hepc gene expression was observed earlier than the upregulation of IL-6 gene expression. In contrast, the time kinetics for IFN-γ, IL-1ß and TNF-α gene expression was similar to that of Hepc gene expression though all these cytokines attained their peaks by 12 hours of the liver injury. Upregulation of IL-6 gene expression was seen at 6 hours, whereas significant upregulation of Hepc gene expression was detectable already at 3 hours after CCl_4-administration; this may suggest the presence of some other regulatory mechanisms for Hepc and Hjv gene expression. The possible reason for downregulation of the Hepc gene expression at later time could mark the beginning of necrosis of hepatocytes in response to the toxin.

5.3 In vitro studies

To study the effect of single cytokines on the Hepc and other genes expression isolated rat hepatocytes were treated with the single cytokines at different doses at 24 hours after isolation and plating. The data obtained from *in vitro* studies supports the assumption that IL-6 may be sufficient to induce the changes of Hepc gene expression observed in different models. The kinetics of the changes of Hepc, Hjv and Fpn.-1 gene expression on one side and those of IL-6 on the other side suggests that in CCl_4-induced liver injury besides IL-6 some other factors could be involved in Hepc, Hjv and Fpn.-1 gene regulation. IFN-γ could be one of such factors, as IFN-γ gene expression was upregulated after CCl_4-treatment along with IL-1ß and TNF-α. Therefore, we studied the effect of IFN-γ treatment on isolated rat hepatocytes. No significant changes of Hepc, Hjv or Fpn.-1 gene expression were found in hepatocytes stimulated with IFN-γ. Several converging lines of evidence from recent work have established that TNF-α and IL-6 are important components of the early signalling pathways after local injury (Michalopoulos and DeFrances, 2005) induced by PH. Previous studies suggested that endotoxin, one of the key stimulants leading to TNF-α production by Kupffer cells, may be involved in PH (Cornell, 1981; Cornell *et al*, 1990). During recovery after PH-induced liver injury the role of TNF-α is to regulate secretion of IL-6. IL-6 is secreted by Kupffer cells, and this secretion is stimulated by TNF-α (Gauldie *et al*, 1992). Our data supports this hypothesis; in fact an upregulation of IL-6 starting 2 hours after the PH may have induced the expression of the Hepc gene after resection of 70% of the liver. Interestingly upregulation of Hepc specific transcripts of a similar order of magnitude as observed in our study have been found in the liver of mice 6 hours after the PH (Kelley-Loughnane *et al*, 2002).

Northern blot analysis confirmed the findings of real-time PCR analyses that Hjv gene expression was downregulated during hepatic injury at the same time when Hepc gene expression was upregulated. Similar data have been shown at 6 hours after treating the mice with LPS (Krijt *et al*, 2004; Niederkofler *et al*, 2005). Furthermore, we could reproduce the changes observed *in vivo* by IL-6 treatment of isolated hepatocytes. This could mean that IL-6, on one hand inhibits Hjv gene expression and on the other hand, it replaces the stimulatory effect of Hjv by directly upregulating the Hepc gene expression in hepatocytes. In fact the *in vitro* studies seem to suggest that IL-6 is major acute-phase

mediator which induces Hepc gene expression (Ganz and Nemeth, 2006; Nicolas *et al*, 2002); however. IL-1ß can also play some role in this expression as reported earlier (Lee *et al*, 2005). Our results also confirm, at least in part, that IL-6 may be sufficient to induce the Hepc gene expression in acute inflammation (Nemeth *et al*, 2004b).

From these findings we can conclude that Hepc gene expression is upregulated during TO induced APR not only in the liver but also in the other organs and this hormone can regulate the expression of different proteins like Fpn.-1, DMT1, HFE, Hjv and other iron regulatory proteins studied during the APR. IL-6 produced at the site of injury coming to the liver could be the main mediator of the APR; however, local expression of IL-6 in different organs could contribute to regulate iron metabolism locally. Furthermore, most of the so far known genes of the iron-metabolism expressed in the liver and in the other organs suggest that besides the liver the skeletal muscle and other organs may participate in the regulation of serum iron concentration and in systemic iron metabolism. The key difference in the TO induced APR and acute liver injury induced by PH or CCl_4 is the source of IL-6. In the former model, the IL-6 is produced in the injured muscle and act on the liver in endocrine manner to induce the expression of acute-phase-proteins whereas in the later case IL-6 is produced in the liver most probably by resident macrophages and act in a paracrine manner to stimulate the Hepc (acute-phase-proteins) expression by the hepatocytes. Acute liver injury in the animal models results in hypoferraemia with significant increase in the concentration of liver injury markers. Hepc gene expression is regulated conversely with Hjv and Fpn.-1gene expression. Increased hepatic Hepc gene expression during acute liver injury could be the result of more than one inflammatory mediator; however, IL-6 could be designated as one of the principle mediators. In parallel, IL-6 also modulates the expression of Hjv and Fpn.-1 genes which are known to act in concert with ferritin-H and transferrin directly on the hepatocytes.

6. REFERENCES

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7. LIST OF PUBLICATIONS AND MEETINGS

Diese Arbeit wurde durch die Deutschen Forschungsgemeinschaft im Rahmen des Sonderforschungsbereich SFB402 und das Graduiertenkollegs GRK335 gefördert

Diese Arbeit wurde in der Abteilung Gastroenterologie und Endocrinologie am Zentrum Innere Medizin der Georg-August-Universität Göttingen angefertig.

Publications

1. **Sheikh N**, Batusic DS, Dudas J, Tron K, Neubauer K, Saile B and Ramadori G. Hepcidin and hemojuvelin gene expression in rat liver damage: in vivo and in vitro studies. *Am J Physiol Gastrointest Liver Physiol* 291: G482-G490, 2006.
2. **Sheikh N**, Tron K, Dudas J, and Ramadori G (2006). Cytokine-induced neutrophil chemoattractant-1 is released by the noninjured liver in a rat acute-phase model. *Lab Invest* 86: 800-814.
3. **Sheikh N.,** Christiansen H., Saile B, Reuter F., Rave-Fränk M., Hermann R. M., Dudas J., Hille A., Hess C. F., and Ramadori G., γ-radiation leads to up-regulation of hepcidin and to down-regulation of hemojuvelin and ferroportin-1 gene expression in rat liver. *Radiology* 2006, 10.1148/radiol.2421060083
4. **Sheikh N**, Dudas J, Tron K, and Ramadori G (2006). Changes of gene-expression of iron regulatory proteins during turpentine oil induced acute-phase-response (APR) in the rat.

Abstracts published

1. **Sheikh N.,** Tron K., Dudas. and Ramadori G.: Cytokine-induced neutrophil chemoattractant-1 (CINC-1) is released by the non-injured liver in a rat acute-phase model.. Z. Gastroenterol. Dev. 44, 792 (2006).
2. **Sheikh N.,** Tron k., Dudas J., Saile B., Batusic D. and Ramadori G.: "Hepcidin and hemojuvelin gene expression in rat liver damage: *in vivo* and *in vitro* studies" J. Hepatol. Dev. 44 (2), S81-82 (2006).
3. **Sheikh N.,** Tron K. and Ramadori G.: Heat Shock proteins: come to rescue during acute phase response (APR). Z. Gastroenterol. Dev. 44, (2006).

4. **Sheikh N.,** Tron K., Dudas J. and Ramadori G.: HO-1 gene expression in extrahepatic organs of rats in response to turpentine oil induced APR. Poster presented in HO conference 2005, the fourth international congress from October 6-9, 2005, Boston, USA.
5. **Sheikh N.,** Christiansen H., Tron K., Dudas J., Saile B., Hess C. F. and Ramadori G.: Hepcidin: Regulation in different animal models. Z. Gastroenterol. Dev. 43, 860 (2005).

Meetings

1. 61 Jahrestagung der Deutschen Gesellschaft für Verdauungs- und Stoffwechselkrankheiten from September 13-16, 2006, Hannover, Germany (Poster presentation).
2. 41st Annual meeting of "The European association for the study of the liver" April 26-30, 2006, Vienna, Austria (Poster presentation).
3. HO conference 2005, the fourth international congress from October 6-9, 2005, Boston, USA (Poster presentation).
4. 60 Jahrestagung der Deutschen Gesellschaft für Verdauungs- und Stoffwechselkrankheiten from September 14-17, 2005, Köln Germany (Poster presentation).

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NADEEM SHEIKH

9. DEDICATION

To my family

10. ZUSAMMENFASSUNG

[Einleitung] Eisen ist ein essentielles Element welches für viele Redoxprozesse in allen Eukarionten und den meisten Prokarionten gebraucht wird. Serumeisenspiegel fallen bei bakterieler Infektion, Entzündung und bei Verletzungen ab. Der exakte Mechanismus und die Änderung der Genexpression der bei der Eisenregulation beteilgten Proteine ist in der Leber, aber auch in anderen Organen, noch nicht gänzlich geklärt.

[Methoden] In der präsentierten Arbeit haben wir Terpentinöl (TO) in die hintere Extremität von Ratten injiziert um eine akut Phase Antwort (APR) zu povozieren. weiterhin haben wir mit partieller Hepatektomie (PH) und Tetrachlorkohlenstoff (CCl_4) Leberschäden induziert, um Änderungen der Genexpression der akut-Phasecytokine und der Proteine zu untersuchen, von denen man weiß, dass sie an der Eisenregulation beteiligt sind.

[Ergebnisse] Die Serumkonzentration von Pro-Hepcidin hat sich nicht signifikant verändert. Es konnte jedoch ein signifikanter Abfall des Serumeisenspiegels beobachtet werden. Ebenfalls wurden die Serumkonzentrationen der akut-Phasencytokine IL-6, IL-1ß, TNF-α and IFN-γ signifikant verändert. Aufgrund der erhöhten Konzentration von Il-6 im Serum könnte man diesen als Hauptmediator bezeichnen, welcher für die Regulation der Geneexpression von Hepcidin und der anderen Eisen-regulatorischen Gene während der APR durch TO zuständig ist. Dieser Anstieg der Serumkonzentration war mit einer vermehrten Expression von IL-6 mRNA im geschädigten Muskel der TO und in den Lebern von PH behandelten Ratten assoziert. Im CCl_4-Modell sind Serumspiegel von IL-6 nicht signifikant gestiegen. Jedoch war in der Leber eine deutliche Hochregulation auf Transkriptionsebene zu beobachten.

Als Folge der Entzündung durch TO hat sich die Genexression von Hepcidin gesteigert sowie von Transferrin (Tf), Transferrin-Rezeptor TfR1 and TfR2, ferritin-H, Eisen-responsives Element-Bindungsprotein IRE-BP1 und IRE-BP2. Die Genexpression von Hämojuvelin (Hjv), Ferroportin-1 (Fpn.-1), duodenales Cytochrom-B (Dcytb), Hämochromatose-Gen HFE and Hephaestin (Heph) wurde herrunterreguliert. Außerhalb der Leber, wurde die Genexpression von Eisen-metabolisierenden Proteinen im verletzten Muskel und extrahepatischen Organen untersucht. Es wurde beobachtet, dass alle

Organe, die auch eine lokale Steigerung der IL-6 Genexression zeigten, mit Ausnahme vom verletzten Muskel und der Lunge, eine verstärkte Hepcidin-Genexpession aufwiesen. Ähnliche Expressionschemata zeigten sich für Fpn.-1 in verschieden Organen: in Lunge und Muskel ist Hepcidin herunterreguliert und Fpn.-1 erhöht. In der Leber und den anderen untersuchten Organen, ist Hepcidin gesteigert und Fpn.-1 herunterreguliert.

Bei akuten Leberschäden die durch PH oder CCl_4 hervorgerufen werden, verhalten sich die untersuchten Gene mehr oder weniger gleich. Die Genexpression von Hepcidin sowie TfR1 und IRE-BP1 wurde signifikant durch die Schädigung gesteigert, während die anderen Gene, als Ergebniss des Traumas, herunterreguliert wurden. Als Antwort auf die PH-Operation war die Serumkonzentration von IL-6 erhöht. Nicht so bei CCl_4 behandelten Ratten. Hier war keine signifikante Erhöhung der Werte zu finden. Eine mögliche Erklärung hierfür könnte die regionale Wichtigkeit und Verwertung von IL-6 in der Leber sein. Es sei erwähnt, dass neben IL-6 auch andere Mediatoren wie zum Beispiel IL-1β an der Regulation des Eisenstoffwechsels beteiligt sein könnten.

[Zusammenfassung und Kommentar] Aus den Ergebnissen können wir zusammenfassen, dass im Rattenmodell der sterilen abszess-induzierten systematischen APR, die Veränderungen der Genexpression von denen im Eisenstoffwechsel teilnehmenden Proteinen in allen Organen, mit Ausnahme der Lunge, qualitativ ähnlich ausfallen. Im verletzten muskel jedoch könnte die Veränderung der Exression von Hepcidin, Hjv und Fpn.-1-Genen eine spezifisch entzündliche Veränderung darstellen. Diese Veränderungen könnten in der Leber, sowie in anderen Organen durch die Haupt-akut-Phasemediatoren induziert werden. Wenn die Leber selber Ziel der Noxe ist, könnten die akut-Phasecytokine, die lokal gebildet werden, eine entscheidende Rolle bei der Eisenregulation spielen und für die Veränderungen der am Eisenstoffwechsel beteiligten Proteine verantwortlich sein

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