

NON-ALCOHOLIC FATTY LIVER DISEASE AND ASSOCIATED CHANGES IN SERUM HEPCIDIN, IRON, FERRITIN-R LEVELS AND TOTAL IRON BINDING CAPACITY IN WEANING WISTAR RATS (*RATTUS NORVEGICUS*)

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ABSTRACT

The purpose of the present experimental study was to analyze the impact of fat induced NAFLD and fat reducing agents on iron metabolism. Four groups (n=10) of weaning *Rattus norvegicus* (30g) were designated as Con (Control), I, II, and III. For 16 weeks Con group was fed on 100% rat chow, I, II and III were provided with diet "A" (20% Sucrose + 33% Nestle tea whitener + 34% ground pallet diet +13% water). The diets of group II and III were provided with 5% *Nigella sativa* and 5% *Plantago ovata* husk as supplements respectively. After keeping the animals under experimental conditions for 16 weeks, serum samples were taken to analyze iron metabolism related parameters. In comparison with Con group significant changes were observed in the serum hepcidin, iron, ferritin-R levels and Total iron binding capacity of experimental groups. Conclusively, we can say that serum hepcidin, iron and ferritin-R concentrations as well as total iron binding capacity are associated with NAFLD. *N. Sativa* and *P. ovata* have impact on iron regulation in diet induced NAFLD.

Key words: Ferritin-R, Hpcidin, NAFLD, TIBC. *N. sativa* seeds, *P. ovata* husk.

INTRODUCTION

Non alcoholic fatty liver disease (NAFLD) has a wide spectrum including Non-alcoholic steatohepatitis (NASH) (Ahmed *et al.*, 2009). This hepatic disease is common in children and adults (Schwimmer *et al.*, 2005). NAFLD is concerned with defective iron metabolism resulting in overload. A relationship between hepatic iron and NASH or its progressive stage has been reported in the last decade (Fargion *et al.*, 2001; Manousou *et al.*, 2011).

Iron is the vital bio element required by the body due to its flexible chemistry and redox activity but becomes potentially hazardous due to its catalytic generation of highly reactive hydroxyl radicals (Sheikh *et al.*, 2007; Wang and Pantopoulos, 2011). The reported distribution of iron in the body is; as a component of haemoglobin of red blood cells (2.1g in human), macrophages (up to 600mg), myoglobin of muscles (up to 300mg), whereas excess body iron is stored in liver (Andrews, 1999; Olsson and Norrby, 2008).

The strict regulation of serum iron is maintained by an antimicrobial hepatic peptide hormone, hepcidin (Sheikh *et al.*, 2006). Its synthesis is stimulated by an elevated iron levels, which down regulates the only known iron expresser ferroportin-1 (FP-1) in macrophages and intestinal cells and reduces serum iron (Fleming and Ponka, 2012). It is expressed in hepatocytes, primarily as a precursor, pro- peptide, the

proteolytic processing of which yields a bioactive molecule of 25 amino acids secreted into blood stream. Following iron intake and under inflammatory conditions, hepcidin accumulates and results into decreased dietary iron absorption and iron retention in macrophages. Pathological elevation of hepcidin level may lead to establishment of iron deficiency and as a result anemia is developed (Weiss and Goodnough, 2005). Further cofactors are required for iron dependent activation of hepcidin, yet its mechanism is still unclear. There is no mechanism for excretion of iron from mammalian body except sloughing of mucosal and skin cells or bleeding (Wang and Pantopoulos, 2011).

Excessive intracellular iron can be stored and detoxified when it binds to ferritin in cytosol. Ferritin is conserved protein that assembles into a shell like structure that provides the space for the storage of up to 4500 Fe⁺³ in form of ferric oxy- hydroxide phosphate (Shi *et al.*, 2008). Serum ferritin, a marker of iron accumulation, is related to iron overload and advanced fibrosis in patients with NAFLD (Shim, 2012). Hyperferritinemia has been frequently observed in patients with metabolic syndrome and NAFLD (Zelber-Sagi *et al.*, 2007).

Total iron binding capacity (TIBC) is indirect measurement of the primary iron transport protein, transferrin (TF) (Aisen *et al.*, 1966, Kasvosve and Delanghe, 2002). According to a theoretical study two molecules of iron can bind one molecule of TF at two

**The Data is part of Ph.D. work of first author.

high affinity binding sites for ferric ion (Baker and Lindley, 1992). TF concentration and total iron binding capacity (TIBC) are currently used to assess iron status (Kasvosve and Delanghe, 2002). The level of iron should be tightly regulated in the body as even mild overload might lead to numerous metabolic diseases including NAFLD (Jiang *et al.*, 2004; Kim *et al.*, 2011).

Being the vital organ for the excess iron storage, liver is more susceptible to iron damage (Andrews and Schmidt, 2007). Probably primary hepatic iron overload engages several steps mechanism, for the direct induction of lipid peroxidation (LPO) and activation of stellate cells and enhanced production of collagen (Bedossa *et al.*, 1994, Lee *et al.*, 1995). Iron status plays a tenacious role in LPO, the peroxidation is maximum when the ratio of the ferrous (Fe^{+2}) to ferric (Fe^{+3}) is 1 (Ryan and Aust, 1992).

N. sativa is a miraculous plant with countless pharmacological significance including uricosuric, anti-diabetic, anti-fertility, choleric, antihistaminic with hepato-protective, anti toxic and anti oxidant properties (Salem, 2005). *In vitro* studies demonstrate that extracts of *N. sativa* seeds due to its anti oxidant properties inhibit the hemolytic activation of scorpion and snake venom, it is protective against protein degradation, elevated osmotic fragility caused by H_2O_2 , LPO, laryngeal carcinoma cells, apoptosis induced by lipopolysaccharides and cortisol. *In vivo* studies on *N. sativa* seed oil confirms that it is hepato-protective against CCl_4 induced toxicity (Nagi *et al.*, 1999).

P. ovata husk is soluble in water, indigestible in human beings and are often used as a source of dietary fiber. The inert bulk of the husks help, provide a constant volume of solid material irrespective of other aspects of the diet or any disease condition of the gut. Some recent researches have also proved their promising role in lowering cholesterol and controlling diabetes (Anderson *et al.*, 2000; Galisteo *et al.*, 2005).

The current study is designed to find out the impact of *P. ovata* and *N. sativa* on the iron burden in fat induced inflammatory conditions in the body of the weaning rats.

MATERIALS AND METHODS

Diet Formulations: Four different diet formulations were prepared and designated as **A**, **B**, **C**, and **D**. Diet **A** was prepared according to Naderali *et al.* with modifications, it contained 20% Sucrose + 33% Nestle tea whitener + 34% ground pallet diet +13% water (Naderali *et al.*, 2001). The composition of diet **B** and **D** was 5% *N. sativa* or *P. ovata* husk /Kg of **A**, respectively. Diet **C** was consisting of 100 % rat chow.

Animals and experimental protocol: Four groups (n=10 for each group) of weaning rats (*R. norvegicus*) of 30g

were designated as one control (**Con.**) and three treated groups (I, II and III) and were fed on diet compositions **C**, **A**, **B**, and **D** respectively. Animals were housed for 16 weeks in the animal house of the Department of Zoology, University of the Punjab, Lahore, Pakistan.

Sample Collection and Storage: After overnight fasting animals were anaesthetized with Norcuron (150 μ l/100g of body weight) blood samples were collected in serum gel vacutainers directly by cardiac puncture. Kept for half an hour at room temperature, clotted blood was centrifuged at 5000rpm for 20 minutes to separate the serum and stored at -20° for further use.

Serological studies: Serum samples were used to assess the levels of iron, hepcidin, ferritin-R and TIBC by using the commercially available kits by Human (Germany).

Statistical analysis: The data are expressed as the Mean \pm SEM and percentage changes. One-way ANOVA with Post host Tukey's test was performed using GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com". The level of significance was set at $P < 0.05$.

RESULTS

Serum Iron: Serum iron levels were significantly elevated in experimental group I ($P < 0.01$). An intergroup comparison exhibited significantly lower levels of iron in group II ($P < 0.01$) and III ($P < 0.01$) in comparison with group I (**Figure 1a**). An increase in the percentage of serum iron level was observed in all the three experimental groups (**Table 1**).

Serum Hpcidin: The serum hepcidin levels were significantly augmented in experimental group I ($P < 0.01$) when compared with Con group with the maximum increase in the intergroup comparison ($P < 0.001$) (**Figure 1b**). There was a rise in percentage of serum hepcidin I and III while a decline in group II (**Table 1**).

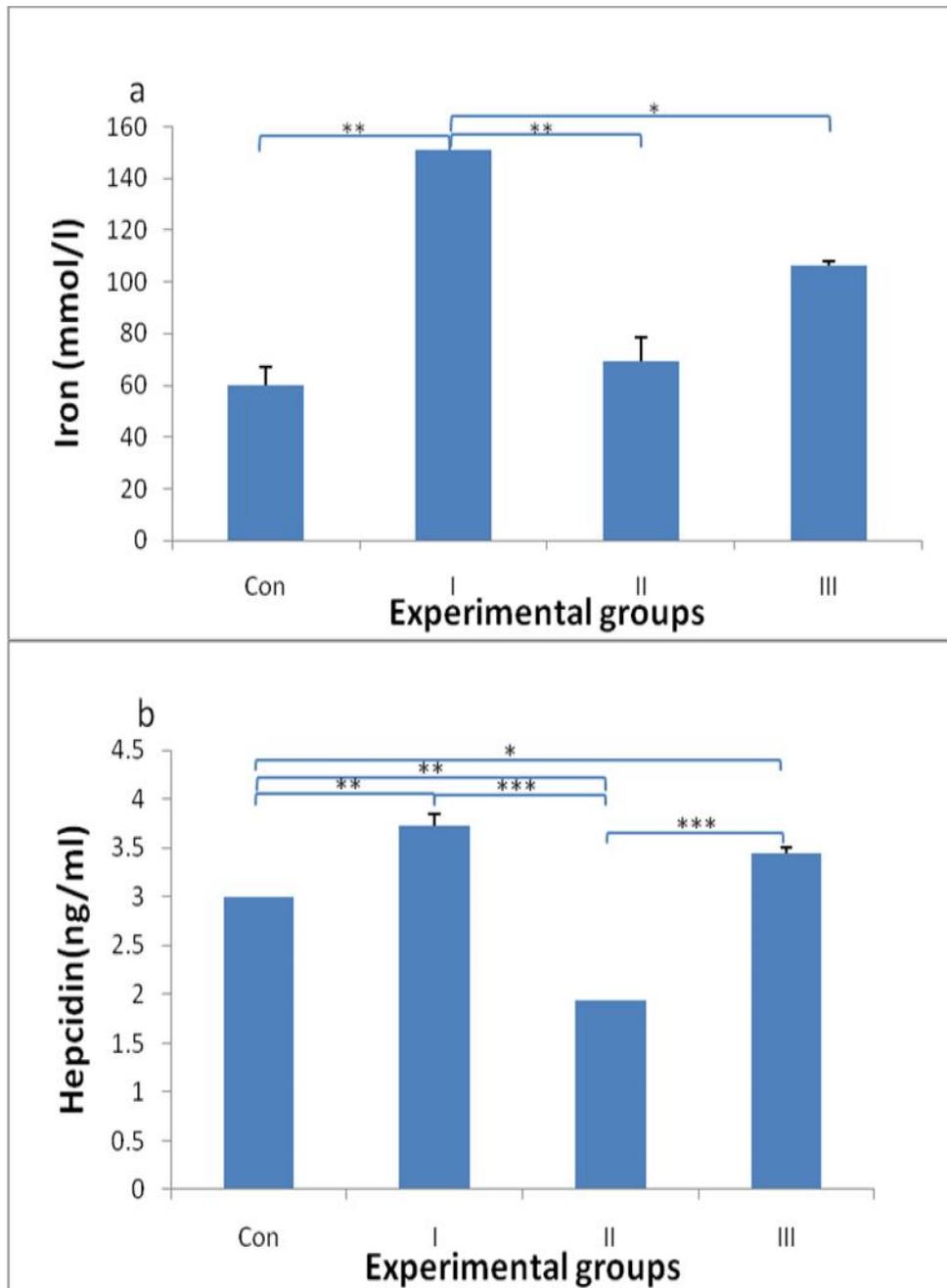
Serum Ferritin-R: In experimental group I significant increase in serum ferritin-R was observed in comparison with Con group. Intergroup comparison revealed significantly lower serum concentration of Ferritin-R in group II ($P < 0.001$) and III ($P < 0.05$) as compared to group I (Figure 1c). An elevation in the percentage of serum ferritin-R in group I while a decline in group II and III was observed (Table 1).

Total iron binding capacity (TIBC): TIBC was significantly higher in group II ($P < 0.05$) in comparison with group I and III (**Figure 1d**). A drop in the serum percentage of TIBC was examined in group I. However, a boost up was observed in group II and III (**Table 1**).

Table 1: Percentage changes in serum level of iron, hepcidin, ferritin-R and TIBC, in *R. norvegicus*.

	Control (%)	I	II	III
Hepcidin	100	24.52	35.39	15.14
Ferretin-R	100	169.14	57.00	21.58
Iron	100	151.67	15.83	77.50
TIBC	100	21.76	6.99	16.43

Con= Control group fed on diet C, I= Treated group fed on diet A, II= Treated group fed on diet B, III= Treated group fed on diet D,
 = Increase, = Decrease.



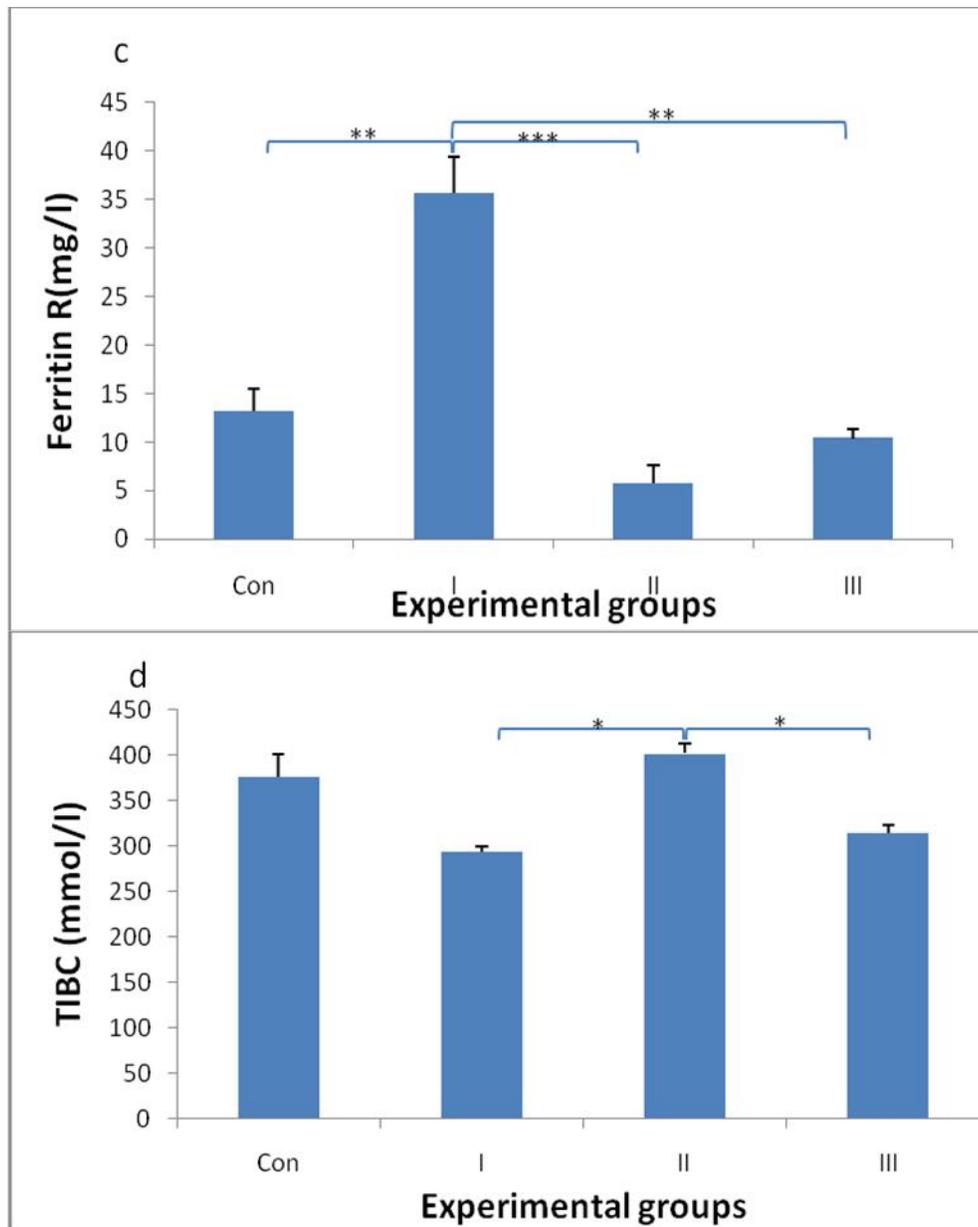


Figure 1: Serum iron (a), Hcpidin (b), Ferritin-R (c), and TIBC (d) of weaning rats: Con (group fed on diet C), I (group fed on diet A), II (group fed on diet B) and III (group fed on diet D). *= P 0.05, **= P 0.01, *=P 0.001.**

DISCUSSION

The most recurrent liver disease in much of the developed world, North America and US is NAFLD mainly reported in children as well as one third of all US adults (Cohen *et al.*, 2011). Most patients with primary hepatic iron overload fit the criteria of NAFLD (Mendler *et al.*, 1999). The present study was conducted to investigate the fat induced iron overload and the impact of *P. ovata* husk and *N. sativa* on iron burden in *R.*

norvegicus, as plant materials have been found efficient iron reduction therapeutic tools.

The results obtained by the present study revealed that the animals in group I had a significantly elevated serum iron. This elevation is strongly supported by the studies conducted in the past decades reporting a relationship between hepatic iron and NASH or its progress. With current advancement in iron metabolism in patients with hereditary hemochromatosis at molecular level, a mass data advocate a connection between distorted iron metabolism and NAFLD (Shim, 2012). Iron diminution therapy, such as with a phlebotomy, in

patients with NAFLD recovers metabolic snags and the level of hepcidin and Ferritin-R were also observed to be raised significantly in group I in accordance with the previous studies (Valenti *et al.*, 2011).

The association between hepcidin and iron metabolism was primarily introduced by Pigeon *et al.*, during studies of the hepatic response to iron burden (Pigeon *et al.*, 2001). Hepcidin secretion is well allied with serum ferritin levels, which is amplified by both iron overload and inflammation. It is indicated that hepcidin production by hepatocytes is regulated by both iron and infection. Lipopolysaccharides act on hepatic macrophages and Kupffer cells to encourage the synthesis of cytokines and IL-6 as a result of which the production of hepcidin mRNA in hepatocytes is induced (Lee and Beutler, 2009, Nemeth *et al.*, 2003). In the most recent study it is suggested that the major determinant of hepcidin regulation in NAFLD, apart from HFE genotype (Nelson *et al.*, 2012).

Iron is transported in plasma after binding with transferrin. Plasma concentration of transferrin is measured by TIBC of plasma (Morgan, 1961). TIBC of group I was decreased significantly due to the fact observed by the past literature that when the plasma iron concentration surpasses the iron binding capacity of transferrin, iron ascends up in the body as non- transferrin bond iron and instigate the constitution of reactive oxygen species (ROS) resulting in cellular smash up (Cabantchik *et al.*, 2005; Papanikolaou and Pantopoulos 2005).

In group supplemented with *N. sativa* (group II) there were expected significantly lower concentrations of iron, hepcidin and ferritin-R and higher values of TIBC in comparison with group I. It may be due to the antioxidant property of the *N. sativa* verified by the previous research (Salem, 2005). Garlic (*Allium sativum*), *Withania somnifera* (Ashwagandha) *Curcuma longa* (Turmeric) are the medicinal plants and have some antioxidant properties, they are reported to be promising in the natural treatment of the iron overload (Bhattacharya *et al.*, 2000; Ghorbel *et al.*, 2011; Reddy & Lokesh, 1996). In this regard, findings of our research are contributing towards iron control in NAFLD associated iron overload.

In group III the diet supplemented with *P. ovata* also had positive impact to decrease iron, hepcidin and ferritin-R concentrations of serum; this may be due to the presence of antioxidants in *P. ovata* husk. A current study with other water soluble fibrous plant has confirmed the anti-inflammatory and anti-oxidant effects when used in a combination with cocoa, hazelnuts, phytosterols (Sola *et al.*, 2012). There is need for further research in this regard.

Conclusively, it can be said that hepcidin, iron and ferritin-R concentrations as well as total iron binding capacity are related with NAFLD. *N. sativa* and *P. ovata* have an impact on iron regulation in diet induced

NAFLD. Therefore, we can say that the use of natural herbs like *P. ovata* and *N. sativa* can be significant tools for the treatment of the NAFLD associated iron overload.

Acknowledgments: The study was supported by the funds under Indigenous Ph.D. Scholarship Batch IV [PIN # 17-5-4 (Bm4-217)]. The authors wish to thank Higher Education Commission of Pakistan for providing funds for the current research project.

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