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ORIGINAL ARTICLE

Mechanistic study of regulation of iron homeostasis by *N. sativa* seeds and *P. ovata* husks on high fat/high sucrose diet induced non-alcoholic fatty liver disease

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Abstract

Background In recent years, nonalcoholic fatty liver disease (NAFLD) has reached epidemic proportions. Characteristic findings in NAFLD patients are elevated iron stores, as iron plays an important role in the pathophysiology of chronic liver disease. The current study was aimed at investigating the possible protective effects of *N. sativa* seeds and *P. ovata* husks on the regulation of iron homeostasis in NAFLD.

Methods Two age groups of Wistar rats (four weeks and twelve weeks old), further subdivided into four groups were fed on high fat/high sucrose (HF/SF) diet for sixteen weeks to induce NAFLD and randomized into three groups (HF/SF diet control (Group I), HF/SF diet with *N. sativa* seeds (Group II) and HF/SF diet with *P. ovata* husks (Group III) and normal diet, serving as negative control (Group 0). At the end of the experiment, histochemical analysis of hepatic sections, biochemical evaluates of the blood, and gene expression analysis were conducted.

Results The results revealed that both *N. sativa* seeds and *P. ovata* husks possess the capacity to maintain iron homeostasis by regulating the level of blood hemoglobin, serum iron contents, expression of key genes involved in iron metabolism, and iron deposition in hepatic sections. While *N. sativa* seeds proved more effective.

Conclusions *N. sativa* seeds are a more potent iron regulator compared to *P. ovata* husks at reducing the iron overburden associated with NAFLD.

Keywords High fat high sucrose diet · Iron metabolism · *N. sativa* · Non-alcoholic fatty liver disease · *P. ovata*

Introduction

As indicated by epidemiological data, the rate of chronic liver disease is increasing with every passing year [1]. NAFLD is a metabolic disorder, it has attained epidemic proportions in the past couple of years. Because nowadays, conditions like diabetes mellitus, obesity, and metabolic syndrome are prevalent in the general population. Chronic liver disease is generally caused by NAFLD as per recent data [2]. However, treatment options for chronic liver disease are very limited and there is room for research in curative management to find better approaches. At present, there is no precise NAFLD therapy, and the only useful option is to bring lifestyle changes like weight loss and increasing physical activity [3].

Recent studies have highlighted the part of iron in the pathology of chronic liver disease. Patients with NAFLD were observed to have increased iron levels as indicated by hyperferritinemia, with transferrin saturation mildly increased and liver biopsies were observed to have mild iron deposits (hemosiderosis) [4]. Iron, important for the synthesis of hemoglobin, is also vital for cells for iron-sulfur and

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heme clusters production. These clusters are constituents of enzymes/proteins responsible for significant physiological processes like metabolic reactions, repair and replication of nucleic acid, respiration, and host defense [5].

Although iron has a biological significance, it is also potentially toxic; naturally it is strictly controlled at systemic and cell levels to avoid both its overload and deficiency [6]. Regardless of the cause, Iron becomes toxic when it goes beyond cell storage capacity resulting in the formation of reactive oxygen species (ROS). This leads to cellular damage through taking over anti-oxidant defense along with active lipid peroxidation. Eventually, clinical issues like diabetes, arthropathy, liver cirrhosis, hepatocellular carcinoma, hypogonadism, cardiomyopathy, and osteoporosis may arise [7].

An antimicrobial hepatic peptide hormone, known as hepcidin strictly regulates the iron level [8, 9]. Increased iron levels simulate its synthesis and up regulation, it suppresses the expression of ferroportin-1 (FPn-1) which is an iron expresser in intestinal cells and macrophages, and results in a drop in serum iron levels [10]. Transferrin and its receptors are key proteins responsible for the cellular uptake and transport of iron [11]. Plasma iron and transferrin are bound together. Transferrin is a widely available protein involved in iron binding. Through transferrin receptors, mediated process, iron requirement of most of the cells is fulfilled by taking iron bounded transferrin from extracellular fluids [12].

The medicinal utilization of plants has a history allied with man's evolution [13]. *Nigella sativa (N. sativa)* is a pharmacologically significant plant and reported to have therapeutic potential as an antioxidant, antimicrobial, anticancer, anti-diabetic, anti-inflammatory, gastroprotective, renal protective, and most importantly hepatoprotective agent [14, 15]. *Plantago ovata (P. ovata)* husk is indigestible in humans and is frequently consumed as a dietary fiber source. Recent research have confirmed that *P. ovata* husk shows promising potential in hypolipidemic and hypoglycemic effects in type 2 diabetics, hypercholesterolemia, and hypersensitive individuals and experimental models [16–18]. Despite of numerous pharmacological studies conducted throughout the world on *N. sativa* seeds and *P. ovata* husks still, there is much to investigate. Therefore, the current revision is designed to analyze the possible advantageous effects of these herbs on iron homeostasis against NAFLD in high fat diet fed (HFD) rats.

Methodology

Experimental design

Two age groups of Wistar rats (Four weeks weaning of 30 g and twelve weeks adult of 150 g) were taken. All groups were subdivided separately into four groups in which one was selected as negative control (Group 0) feed on regular rat chow diet throughoutthe investigational study. The remaining were designed as Group I, II, and III. Group I served as positive control and fed on a rich fat/high sucrose rich diet (HF/HS) [19] with minor modifications in the composition 34% Rat chow + 33% Sucrose + 20% commercially available tea whitener + 13% H_2O. The diet of group II was HF/HS + 50 g *N. sativa* seeds and group III was fed on a diet combination containing HF/HS + 50 g *P. ovata* husk [20]. Rats had *ad libitum* access to water and food. The same diet was regularly fed them for 16 weeks under standard temperature, light and dark cycle.

Blood and tissues collection

After 16 weeks, rats were fasted for 12 h and euthanized by Intraperitoneal (IP) inoculation of Narcuron (1.5 ml/kg body weight). 1ml of the blood was taken in ethylene diamine tetra acetic acid (EDTA) coated vials for hemoglobin (Hb) and red blood cells (RBCs) count and rest (4-6ml) in the gel coated tubes for serum separation. After dissection liver was excised and divided into two parts. The left liver lobe was stored in TRIzol reagent at -80 **°C** for gene expression analysis and the right lobe was stored in 10% formalin for histological analysis.

Hematological analysis

Blood samples were collected in vacationer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) covered by anticoagulant EDTA at the end of the experiment. RBCs count and Hb concentrations were calculated by using an automatic blood cell counter (Cell counter analyzer, MS9-5 V; Melet Schloesing Laboratoires, Osny, France).

Serum iron status estimation

For serum isolation, the blood was retained for two to three hours at about 25 **°C** and centrifuged at 5000 rpm for 20 min. Serum iron concentration and unsaturated iron-binding capacity (UIBC) were calculated using an automatic biochemical analyzer, Cobas 8000 c701 (*Roche*). Total iron binding capacity (TIBC) was calculated as the sum of serum iron and UIBC values. Concentrations of serum ferritin and serum transferrin levels were calculated

with a microplate reader (Rayto RT-6100, Shanghai, China) using commercially available Elisa assay kits (Ferritin rat ELISA kit, Transferrin rat ELISA kit, *Abnova*) according to the manufacturer's instructions.

Gene expression analysis (reverse transcription-polymerase chain reaction)

TRIzol reagent was used to extract out total RNA from 100 mg of the corresponding liver tissue. Total RNA was then quantified with a Nanodrop 2000 (Thermo Scientific). From each sample, 1.5 µg of total RNA was taken for first-strand cDNA synthesis using the First Strand cDNA Synthesis Kit (Thermo Scientific). Gene expression of hepcidin (forward: AGGACAGAAGGCAAGATGGCA and reverse: TGTTGAGAGGTCAGGACAAGGC), transferrin (forward: CCGTGACCACATGAAAACCG and reverse: GGAGAGCCGAACAGTTGGAA), and ferroportin-1 (Fpn-1) (forward: CAGACTTAAAGTGGCCCAGACG and reverse: ACAAGGCCACATTTTCGACG) was determined using reverse transcription-polymerase chain reaction and for normalization, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: GAAGGTCGGTGTGAACGGAT, and reverse: ATGAAGGGGTCGTTGATGGC) was used. Primers were designed by Primer-BLAST on NCBI.

Histochemical observations

Small pieces of liver of each experimental group, fixed in 10% formalin were dehydrated by various ascending concentrations of ethanol. Liver tissues were then cleared through xylene and fixed in paraffin wax to prepare blocks. Tissues embedded within the blocks were cut into Sect. (4 µm thick) using a rotary microtome. The sections were further processed for staining with Prussian blue iron staining for histochemical findings, mounted with DPX, and examined under the light microscope.

Statistical analysis

All the data were analyzed using GraphPad Prism 5. One-way *ANOVA* was applied to observe the difference among groups. *Post-Hoc* Tukey test was applied to see a comparison among groups. A P-value ≤ 0.05 was considered statistically significant.

Results

Hemoglobin

The blood Hb was significantly higher in weaning rats of groups W-I, W-II, and W-III in comparison with control

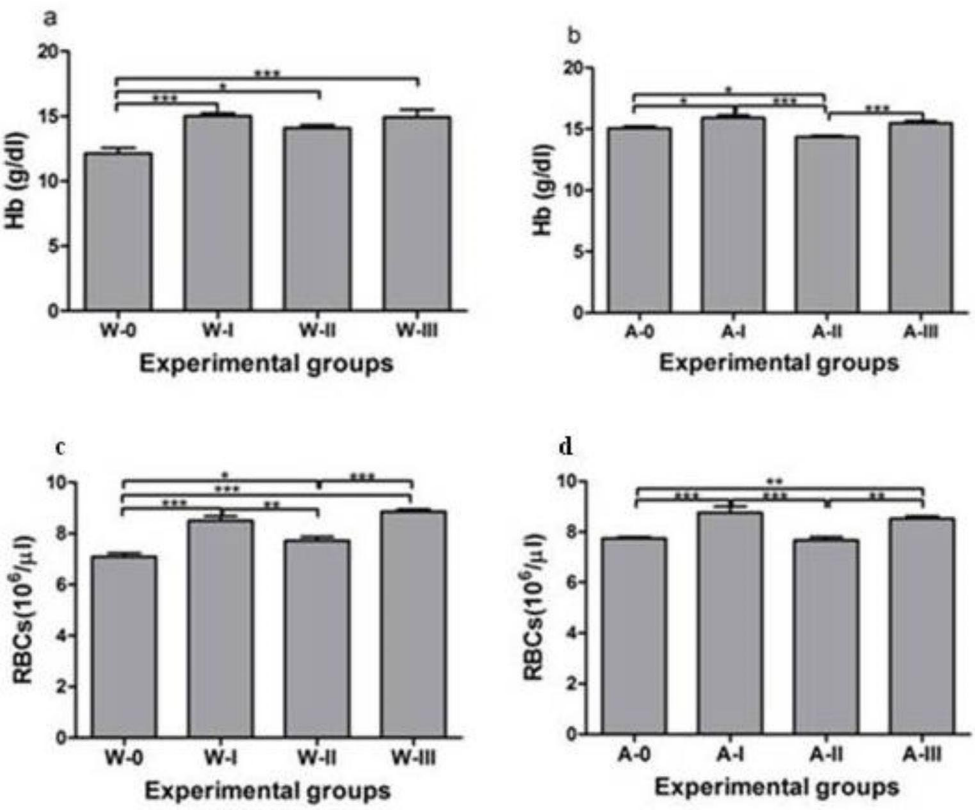

Fig. 1 Hemoglobin and red blood cells count (10^6/µl) level in the investigational rats in contrast to the control rats, A = Adult, W = weaning. Results are Mean ± SEM, analyzed by one-way ANOVA (post-hoc Tukey's test) with n = 10. Significance levels are *=P ≤ 0.05, **=P ≤ 0.01, ***=P ≤ 0.001

W-0. In adults significantly elevated Hb was found in group A-I, while significantly lower Hb was found in group A-II as compared to control A-0. The intergroup comparison

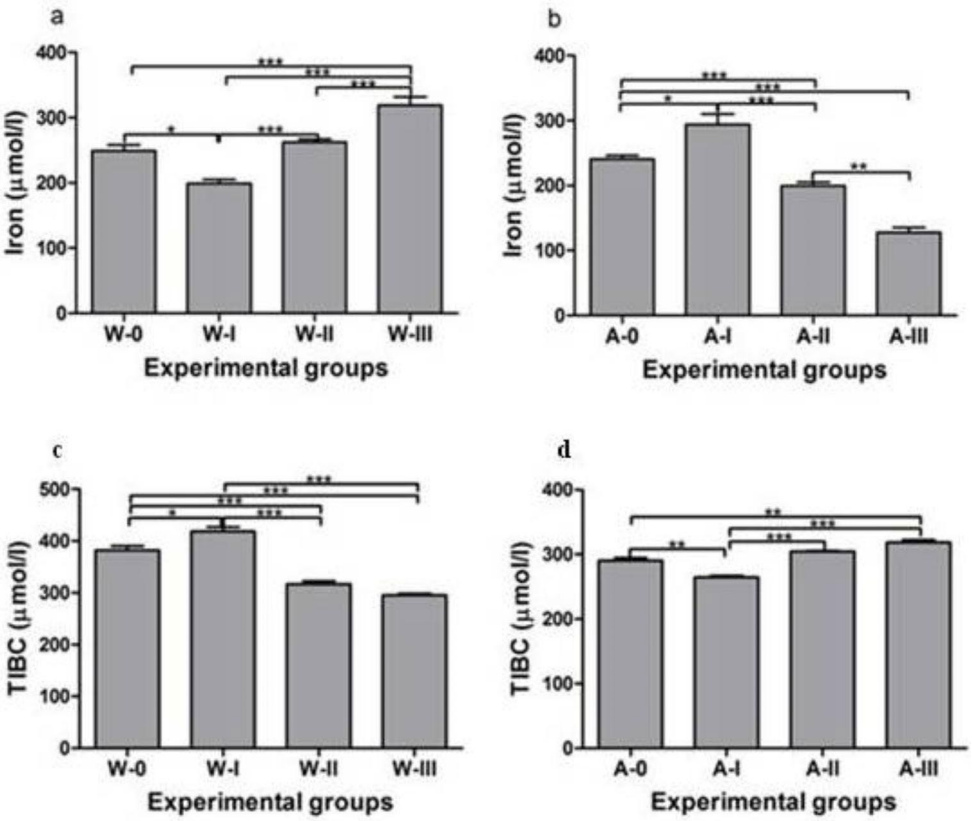

Fig. 2 Serum iron (µmol/l) and total iron binding capacity (µmol/l) in the experimental rats as compared to the control rats, A = Adult, W = weaning. Results are Mean ± SEM, analyzed by one-way ANOVA (post-hoc Tukey's test) with n = 10. Significance levels are *=P ≤ 0.05, **=P ≤ 0.01, ***=P ≤ 0.001

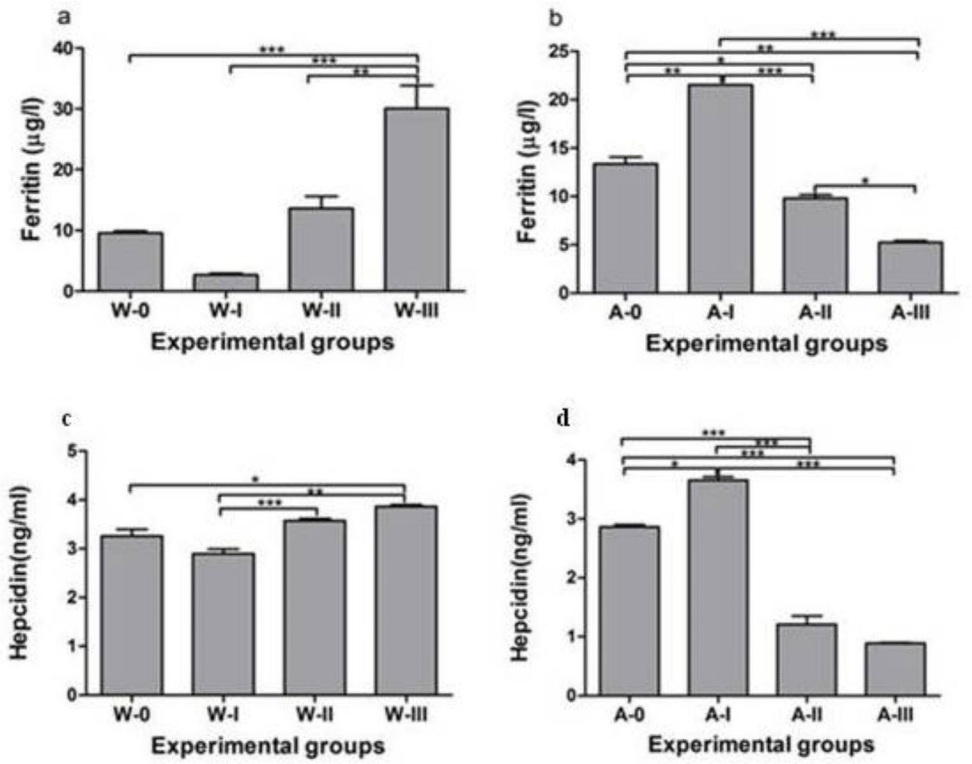

Fig. 3 Serum ferritin (µg/l) and Hepcidin (ng/ml) in the experimental rats as compared to the control rats, A = Adult, W = weaning. Results are Mean ± SEM, analyzed by one-way ANOVA (post-hoc Tukey's test) with n = 10. Significance levels are *=P ≤ 0.05, **=P ≤ 0.01, ***=P ≤ 0.001

revealed significantly lower Hb in rats of group A-II when compared with groups A-I and A-III (Fig. 1a and b).

Red blood cells (RBCs) count

The significant increase in RBCs count was analyzed in groups W-I and W-III as compared to the control group W-0. The RBCs count in group W-II was significantly lower than groups W-0, W-I, and W-III. The RBCs count of adult rats in groups A-I and A-III were significantly elevated than control group A-0 while, group A-II showed a significantly lower RBCs count than group A-I and A-III (Fig. 1c and d).

Serum iron

Serum iron level was considerably elevated in weaning rats group W-III in contrast to control group W-0. In an intergroup comparison, a significant decline in serum iron was examined in groups W-I and W-II as compared to W-III. Similarly, in adult rats, a considerable increase of serum iron in group A-I and a decline in A-II and A-III were found in comparison with the control group A-0. The intergroup comparison showed a considerably high serum iron levels in group A-I and lesser in group A-III when compared with group A-II (Fig. 2a and b).

Total iron binding capacity (TIBC)

The serum TIBC was significantly declined in weaning rat groups W-II and W-III in comparison with the W-0, while increased in group W-I. Intergroup comparison showed a significantly lower serum TIBC in groups W-II and W-III as compared to W-I. However, there was a significantly lower serum TIBC of adult group A-I, when compared with groups A-0 and A-II. The serum TIBC of group A-III was significantly elevated than groups A-I and A-0 (Fig. 2c and d).

Serum ferritin

There was a significant elevation in the serum ferritin levels of the weaning rats group W-III as compared to W-0, W-I, and W-II. However, significantly higher serum ferritin in adult group A-I while lower serum ferritin in A-II, A-III was present in comparison with the control group A-0. In an intergroup comparison significantly elevated serum ferritin levels were observed in groups A-II and A-I as compared to group A-III. However, group A-I had significantly higher serum ferritin levels than group A-II (Fig. 3a and b).

Serum hepcidin

Serum hepcidin was significantly higher in weaning group W-III in comparison with the control group W-0. In the

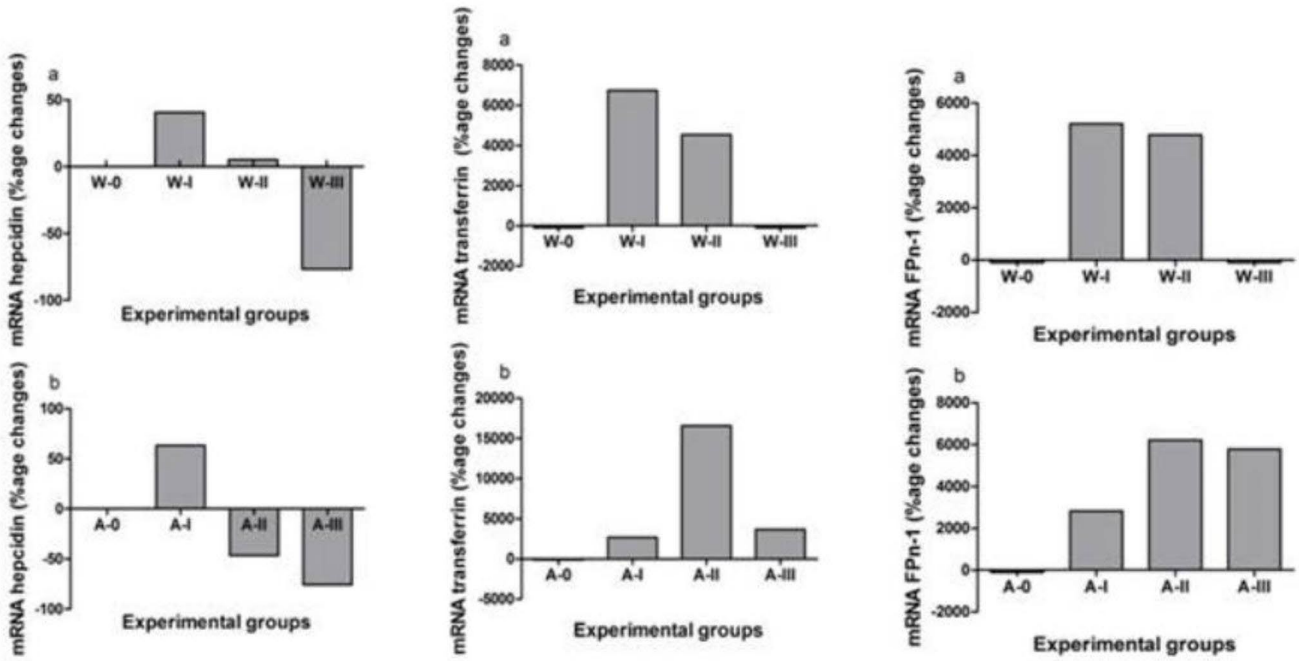

Fig. 4 Hepatic mRNA hepcidin, transferrin and Ferroportin-1 (FPn-1) expression in experimental groups in contrast to the control groups. Results are shown as percentage changes. A = Adult, W = weaning

intergroup comparison, a significant decline in serum hepcidin was examined in groups W-I and W-II as compared to W-III. Similarly, in adult rat groups a significant elevation of serum hepcidin in groups A-I and a decline in groups A-II and A-III was observed in comparison with A-0. The intergroup comparison revealed a significantly lower serum hepcidin level in group A-II and A-III when compared with group A-I (Fig. 3c and d).

mRNA expression

Variations in the hepatic mRNA expression of hepcidin were prominent in the weaning and adult rat experimental groups as compared to their respective control groups, W-0 and A-0. Up regulation of hepatic mRNA expression of hepcidin was observed in the positive control groups W-I and A-I, whereas it was down regulated in the groups W-II, W-III, A-II, and A-III when compared with the positive control groups W-I and A-I. Diet formulation with *P. ovata* husks had the maximum impact to reduce the iron deposits of hepatocytes. Hepatic mRNA expression of ferroportin-1 and transferrin showed an inverse relationship with hepcidin expression. Their maximum expressions were evident in A-II and A-III groups of adult rats while no changes were observed in the W-0, A-0, W-I, and W-III groups (Fig. 4).

Iron staining

Histopathological examination revealed that there were no considerable hemosiderin granules found in hepatocytes, kupffer cells, and sinusoidal spaces of hepatic sections of control rats. In the case of weaning rats, experimental groups I and II showed iron deposition in the form of blue stained hemosiderin granules within the hepatocytes and sinusoidal spaces as compared to control rats. While very less iron deposition in hepatic sections of the weaning rats of group III was observed as compared to other experimental groups (I and II). In adult rats, iron deposition was higher in HF/HS diet group in comparison with the control group and other experimental groups (Fig. 5).

Discussion

The current study was designed to analyze the effects of high fat diet as well as supplements of fat dipping herbs (*N. sativa* seeds and *P. ovata* husks) on iron homeostasis in the weaning and adult rats (*Rattus norvegicus)*.

The hematological results revealed a significant increase in RBCs counts, and Hb levels in the rats of experimental group W-I and A-I fed on HF/HS diet when compared to their respective controls. The effect of this diet was nullified to some extent in experimental groups W-II and A-II fed on *N. sativa* seeds supplemented diet which brought values of RBCs counts near to the baseline when compared to their respective controls. This normalization was more in adult rats fed on *P. ovata* husks where Hb levels were also close to the control values of group A-0. In the diagnosis of various diseases and pathological conditions induced by drugs, heavy metals, dyes, industrial toxins, pesticides, and several other components the hematological indices have widely been used [21]. It has been documented that many biochemical and hematological abnormalities occur in chronic liver diseases. A huge data on chronic “alcoholic liver disease” (ALD) has reported significant decreases in RBCs count and Hb levels [22]. Chronic alcoholism is associated with inflammation and haematotoxic effects, while NAFLD has a limited effect on hematological parameters [23]. Some

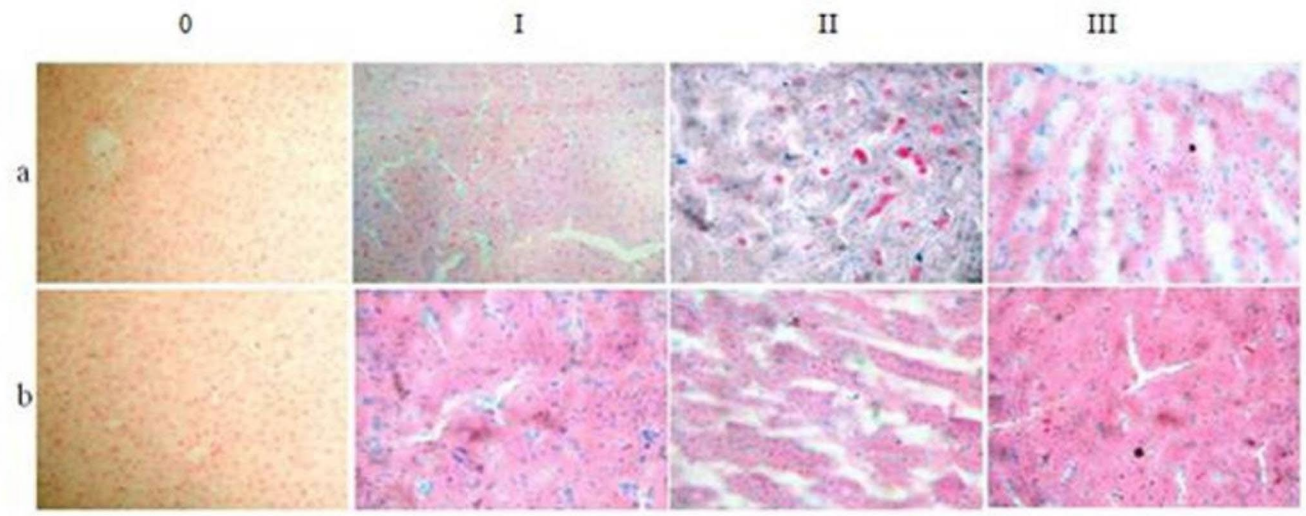

Fig. 5 Representative photomicrographs of liver sections of weaning rats (a) and adult rats (b) stained with Prussian blue

studies on NAFLD have reported its asymptomatic nature [24]. The increase in RBCs count can be due to the increase in erythropoietin (EPO), a positive hepatic acute phase protein synthesized during inflammation [25].

The most recurrent liver disease in the USA is NAFLD mainly reported in children as well as one-third of all USA adults [26]. Most patients with primary hepatic iron overload fit the criteria of NAFLD [27]. The results obtained by the present study revealed that HF/HS diet, affected the weaning and adult groups W-I and A-I differentially and significantly. Serum iron and hepcidin levels were enhanced in adult groups as compared to the weaning group. Similarly, different responses to supplemented diet were observed for serum iron and hepcidin where *N. sativa* seeds and *P. ovata* husks sharply reduced the serum iron and hepcidin levels in adult groups A-II and A-III while slightly elevated levels of serum iron and hepcidin were observed for weaning groups W-II and W-III as compared to control group. Almost similar trends were observed for serum ferritin levels for HF/HS, *N. sativa* seeds and *P.ovata* husks supplemented diets.

With the current advancement regarding iron metabolism in patients with hereditary hemochromatosis at the molecular levels, mass data advocates a connection between distorted iron metabolism and NAFLD [28]. It is reported that iron reduction therapy, such as with phlebotomy, in patients with NAFLD recovers metabolic problems and the level of hepcidin and ferritin in the present study were also observed to be raised significantly in group A-I as per previous studies [29].

Plasma concentration of transferrin is measured by the total iron binding capacity (TIBC) of plasma [30]. TIBC of group W-II and W-III of weaning rats was decreased significantly and a probable explanation from literature is that when the plasma iron concentration surpasses the iron binding capacity of transferrin, iron ascends in the body as non-transferrin bound iron and instigate the constitution of reactive oxygen species (ROS) resulting in cellular smash up [31]. However, an opposite trend was observed in adult rats fed on *N. sativa* seeds in group A-II and *P. ovata* husks in group A-III, where its levels were increased in comparison with adult HF/HS fed group A-I.

In the adult groupsupplemented with *N. sativa* (group A-II), there were found significantly lower concentrations of iron, hepcidin, and ferritin, and higher values of TIBC in comparison with group A-I. It may be due to the antioxidant property of the *N. sativa* as reported previously [32]. In adult group A-III the diet consisting of *P. ovata* seeds has an ameliorating effect on hepcidin and ferritin levels of serum because of the presence of antioxidants in these seeds. Also using it with other watersoluble fibrous plants has established the anti-inflammatory and antioxidant effects when used in a grouping with cocoa, hazelnuts and phytosterol [33, 34].

Elevated hepatic hepcidin gene expression and iron accumulation in our studies were exactly as per documented in NAFLD patients. In iron overload linked inflammation, the expression of hepcidin is raised [10]. According to Sheikh et al., 2007 and Malik et al., 2011 the expression of the hepcidin gene elevates by many folds in the liver and extrahepatic tissues during acute phase response [23, 28, 29]. An upregulated hepcidin expression in acute phase response induced by turpentine oil and *in vitro/in vivo* hepatic damage has also been reported in rats. In our experimental design, the transferrin and Fpn-1 genes expressions were down regulated and are strongly supported by the studies conducted previously on rats [11, 32]. Fpn-1 is an inflammatory negative acute phase protein and provides an inverse relationship between hepcidin and Fpn-1genes expression [10, 11]. In HF/HS weaning and adult (W-I and A-I) groups of the current study the down regulated Fpn-1expression is related to the iron overload reported in NAFLD [33].

Iron deposition in periportal hepatocytes and sinusoidal cells is mildly reported in some cases of NASH. Elevated iron deposits estimation histochemically/biochemically, are linked with increased portal fibrosis in non-alcoholic steatohepatitis (NASH). Paradoxical research concerning iron overload and HFE mutations along with the succession of liver fibrosis and NASH are reported. The mechanism behind NASH linked to iron overload is not very clear. Moreover, this high hepatic iron concentration in NASH is relative to portal hepatic fibrosis [35].

Conclusions

In the present study, *N. sativa* seeds and *P. ovata* husks ameliorated the iron overburden associated with NAFLD by regulating the levels of blood hemoglobin, serum iron, the regulation of key genes involved in iron metabolism, and iron deposition in hepatic sections. In addition, both *N. sativa* seeds and *P. ovata* husks were able to improve iron overload but *N. sativa* seeds proved more potent to reduce the iron overburden as compared to *P. ovata* husks.

Author contribution ASA carried out the experimental work, data analysis, interpretation of data and manuscript draft. TA participated in data analysis, interpretation of data and manuscript draft. NS and SA participated in experimental work. NS provided the concept, participated in the design of study, supervised the manuscript draft and approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Data availability The data used to support the findings of this study is

available from the corresponding author upon request.

Declarations

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethics approval The study was performed in accordance with the guidelines for the care and experimentation protocols of laboratory animals approved by local ethical and review committee of the Institute of Zoology, University of the Punjab, Lahore.

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