LETTER TO THE EDITOR

ALTERATIONS IN HEPATIC TRACE ELEMENTS AND *FERRITIN* EXPRESSION IN HIGH ENERGY DIET FED RAT MODEL

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To the Editor

HED especially rich in fats has established itself as one of the major contributors to obesity and hyperglycemia. Immoderate use of lipid-rich food often triggers inflammatory responses and disturbs cellular metabolic homeostasis, thus serving to be a principal initiator of many metabolic syndromes (1).

Trace elements (TEs) are inevitable to the human body due to their multiple structural, cofactor and signaling functions (2). TEs, in particular iron abnormalities, are common in obese and overweight people (3). Several studies have shown the presence of close inter-relationship between TE homeostasis and obesity-linked disorders (2).

Ferritin protein plays a pivotal role in cellular inflammation, iron metabolism, cell proliferation, apoptosis and oxidative damage (4). In response to iron concentration in the cell, *ferritin* expression (*ferritin-L, ferritin-H*) increases at both transcriptional and translational level (5). Numerous factors, such as iron overload, chemokines production, cytokine release, prostaglandins, lipopolysaccharides, second messengers, growth factors, hypoxia and hyperoxia, and oxidative stress, are responsible for ferritin regulation either directly or indirectly. Complicated feed-back mechanism exists between cytokines and ferritin, hence controling pro-inflammatory as well as anti-inflammatory mediators (6).

The key objective of the current study was to develop a rat model by feeding male Wistar rats on HED, hepatic TEs profiling, and to measure the *ferritin* mRNA expression.

MATERIALS AND METHODS

High energy diet

To develop a fat-rich HED rat model, six-week-old rats were randomly, divided into two dietary groups (n=5): dietary group I (dry milk, sucrose, and rat chow) providing 18.5% of calories from fats, and dietary group II (dry milk and rat chow) providing 30% of calories from fats. A control group was fed on rat chow and water *ad libitum.* The trace elements of commercially available rat chow (Fe: 220µg/g, Cr: 0.70 µg/g, Mg: 2100 µg/g, Zn: 78 µg/g), supplemented diet I and diet II (Fe: 215 µg/g, Cr: 0.65 µg/g, Mg:0: 2100µg/g, Zn:80 µg/g) were also noted. The setup of rats was maintained in the animal house of the Department of Zoology, University of the Punjab, Lahore, Pakistan, under set standards (12 h light/ dark cycle, 24-25°C room temperature, and water *ad libitum*) on their assigned diets for the period of sixteen weeks. Wistar rats were then dissected. Excised livers were preserved at -80°C until further processed.

Key words: ferritin expression, hepatic trace elements, inflammation, iron metabolism.

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Wet digestion of liver samples

Wet digestion method was used for trace metal estimation through atomic absorption spectrophotometer (model number: AA 800, S/N800S8090101, Autosampler, technique: AA flame). For acid digestion, 10 ml of HNO_3 (1+1) was added to each of the liver samples (1 gram). Flasks were heated to the temperature of 90–95°C and were refluxed for 10–15 min without allowing them to boil. Deionized water (DI=2 ml) was added to each sample. Afterward, 3 ml of H_2O_2 (30%) was added to acid digested samples. Samples were warmed until effervescence stopped and then H_2O_2 (10 ml) was added in portions of 1 ml each time and heated again. Finally, the digestion flasks were leveled to 100 ml by adding DI water.

Expression of ferritin genes

Ferritin gene expression was assessed, adopting a previously reported method (7)

Total RNA isolation

For total RNA isolation, TRIzol reagent was added to liver tissues, followed by homogenization and centrifugation; 0.2 ml of chloroform was added to the homogenate thus obtained, that resulted in the phasic separation of RNA, which was then concentrated using isopropanol, was washed by ethanol and hence partially air-dried RNA pellets were re-suspended in RNAase-free water. RNA was quantified and qualified using Nanodrop (UV/VIS Spectrophotometer, model, Optizen Nano Q).

cDNA generation

cDNA was generated for further downstream PCR application, using Revert Aid first Stand cDNA kit (Cat #K1621, Fermentas) as per the manufacturer's guidelines.

Ferritin genes (Ferritin-H and Ferritin-L) amplification of cDNA

Already reported specific primers were used for polymerase chain reaction (PCR) (Table I). β-actin served as housekeeping gene during the process.

Polymerase chain reaction

The conditions for different stages of Polymerase Chain Reaction (PCR) cycle were optimized. The results of PCR products were analyzed by gel electrophoresis (2% agarose in Tris-acetate EDTA buffer), using ready-to-use gene ruler (Thermo Scientific Gene Ruler™ 100bp Plus DNA Ladder). Results were documented using Gel Doc-it™ imaging system.

Statistical analysis

Estimation of comparative fold change in mRNA expression from control and experimental rat groups was carried out using Image J software and Graph Pad Prism 5 (California, USA). One-way ANOVA test was applied for comparative statistical study. Columns thus obtained showed fold changes along with error bars that represent the Standard Error of Mean (SEM) (n=5, *P<0.05, **P<0.01 and ***P<0.001).

RESULTS

Hepatic trace elements profiling

Hepatic trace elements profiling showed substantial statistical alterations in both dietary groups fed on HED from the control group (Fig. 1).

Iron

The hepatic iron concentration in control groups was 53.17 mg/kg. Experimental groups fed on HED showed an iron overload. The concentration of iron was 84.25 mg/kg in DG I and 107.5 mg/kg in DG II (P=0.0021) (Fig. 1a).

Chromium

A positive rise of 6.050 mg/kg of chromium contents was observed in DG I (Fig. 1b). A negative trend of decreased metal contents 2.175 mg/kg was noticed in DG II in comparison to control where contents were 3.483mg/kg (P=0.0001).

Magnesium

Magnesium liver contents (Fig. 1c) of DG I 56.88 mg/kg showed no significant variance from control 60.42 mg/kg. However, magnesium contents were found significantly decreased in DG II 41.88 mg/kg.

Zinc

Zinc contents of DG I (4.765 mg/kg) were noticed to be decreased significantly from control (11.41 mg/kg). DG II was found to have 10.97 mg/ kg of zinc contents. Statistical comparison of DG I and DG II illustrated significant decrease between the two dietary groups (Fig. 1d).

Table I. *Specific primer sequences (forward and reverse) used during gene expression study.*

Primers	Forward 5'⟶3'	Reverse 5'⟶3'	Product length	Reference
Ferritin-L	AAC CAC CTG ACC AAC CTC CGT A	TCA GAG TGA GGC GCT CAA AGA G	103	(8)
Ferritin-H	GCC CTG AAG AAC TTT GCC AAA T	TGC AGG AAG ATT CGT CCA CCT	110	(9)
β-Actin	TGT CAC CAA CTG GGA CGA TA	AAC ACA GCC TGG ATG GCT AC	195	(9)

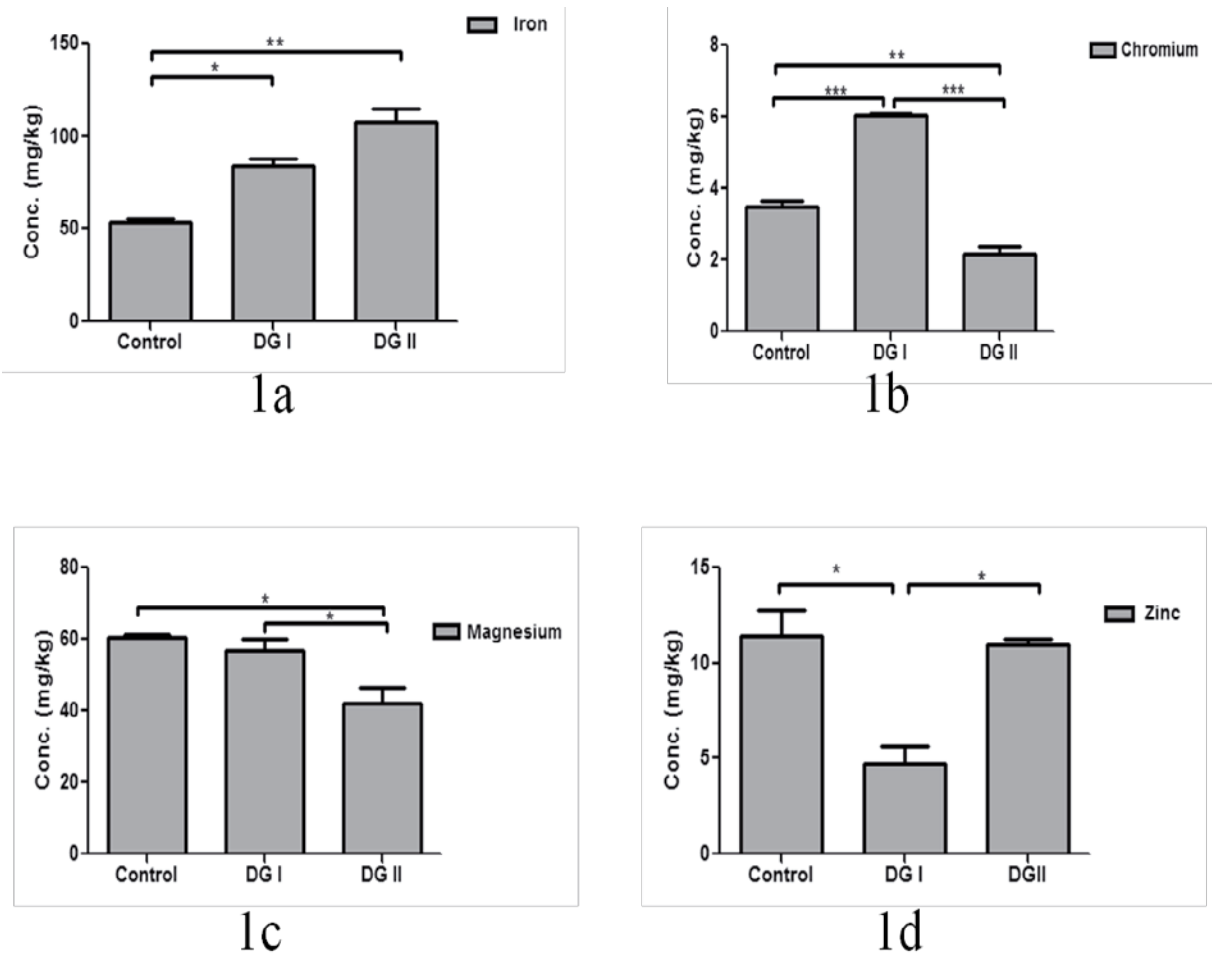

Fig. 1. *Statistical analysis of iron, chromium, magnesium and zinc contents in the liver of rats fed on HED against control.*

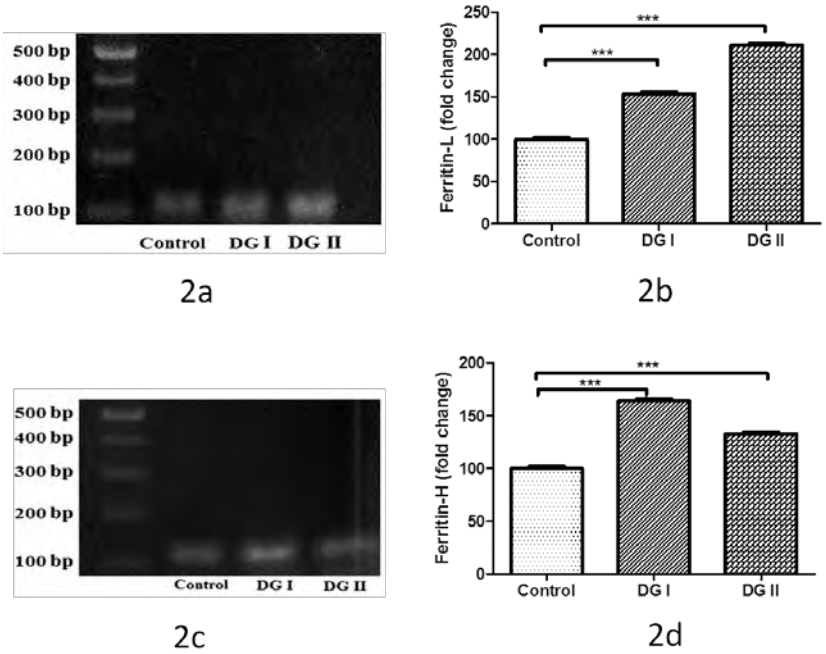

Fig. 2. *Fold change in mRNA expression of Ferritin-L (**a**, **b**) and Ferritin-H (**c**, **d**).*

Ferritin gene expression

qPCR analysis showed increased expression of *Ferritin-L* and *Ferritin-H* in experimental groups fed on HED. *Ferritin-L* gene expression was found to be up-regulated significantly in both dietary groups ($P < 0.0001$). The up-regulation of mRNA expression was more conspicuous in DG II (Fig. 2a, 2b). DG I had more positive induction of *ferritin-H* mRNA than DG II ($P < 0.0001$) (Fig. 2, c, d).

DISCUSSION

The present study exhibited significant variability of trace elements in dietary groups fed on a high energy diet rich in fats. We found total iron contents were altered when rats were fed on HED. Dietary fat affects hepatic iron levels as reported in a previous rat model (10). Chromium overload was observed in DG I in comparison to DG II. Chromium contents in conjunction with copper and zinc were determined in diabetic rats by Wu et al. Their results pointed to elevated chromium concentration in the liver (11). The difference in trends of the dietary groups may be the reflection of differential dietary intake. A reduced level of magnesium in dietary groups in our experiment, though not very significant, may be the result of decreased absorption due to dietary fat, or probably due to mild level of oxidative stress and inflammation that developed in some tissues (12). An analogous trend of zinc deficiency was observed in DG I that can be compared to the results of reduced metal contents by Tinkov et al., in Wistar rats (2).

Ferritin mRNA expression analysis through qPCR showed a significantly increased *ferritin* level in both dietary groups fed on HED. *Ferritin-L* expression was more unregulated in DG II in comparison to DG I. *Ferritin* induction due to altered iron status is a well-established phenomenon in rats. Expression of acute phase genes in relation to changes in hepatic iron regulatory proteins has already reported by Sheikh et al. (8-9). Our results are concurrent with the results by many previous studies reporting increased iron uptake by the liver in acute phase response, in many rat models, along with elevated mRNA. Changes in intracellular *ferritin* level could also be related to modifications

in iron status (8).

Our present findings conclude that high energy diet rich in fat alters hepatic trace elements. Moreover, a perturbed iron metabolism results in hepatic iron overload as confirmed through enhanced *ferritin* mRNA expression. It is therefore suggested to cut down high energy fatty diet intake in our routine life, so as to protect our body from its harmful effects.

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