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Abstract Free iron leads to the formation of pro-oxidant reactive oxygen species (ROS). Humic acids (HAs) enhance permeability of cellular wall and act as a chelator through electron transferring. This study was designed to test chelator effect of HA on iron as well as its anti-oxidant effect against the iron-induced hepatotoxicity and cardiotoxicity. The rats used were randomly divided into four groups (n = 8/group): group I (the control group); group II (the HA group), humic acid (562 mg/kg) was given over 10 days by oral gavage; group III (the iron group), iron III hydroxide polymaltose (250 mg/kg) was given over 10 days by intraperitoneal route; and group IV (the HA plus iron group), received the iron (similar to group II) plus humic acid (similar to those in groups II and III) group. Blood and two tissue samples both from liver and heart were obtained for biochemical and histopathological evaluations. Iron deposition, the iron-induced hepatotoxicity, and cardiotoxicity were demonstrated by histopathological and biochemical manner. However, no significant differences were observed in the serum biochemical values and the

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histopathological results among the iron and the HA plus iron groups in the liver tissue but not in the heart tissue. The protective effects of humic acid against iron-induced cardiotoxicity were shown but not against hepatotoxicity in our study.

Keywords Iron · Hepatotoxicity · Humic acid · Chelation · Cardiotoxicity

Introduction

Iron is an essential trace element of the body, being found in functional form in hemoglobin, myoglobin, and cytochrome enzymes with iron sulfur complexes [1]. Elementary iron plays principal role in oxygen transport, energy production, and mitochondrial respiration as it can serve as both electron donor and recipient [2]. Besides these principal functions, it also serves as a cofactor for many enzymes including respiratory enzymes and those involved in RNA and DNA synthesis [3]. Both haem and ionic (non-haem) iron are absorbed by duodenal enterocytes. The oxidized (Fe³⁺) form in dietary non-haem iron reduces to ferrous iron (Fe^{2+} form) by ferrireductase. Then, the ferrous iron is transported into the cell by heme carrier proteins, the divalent metal transporter or the integrin-mobil ferrin pathway [4]. In here, the absorbed iron is either stored or transported from out of the enterocyte to the blood by ferroportin. Then, the ferrous iron is oxidized by a multi-copper oxidase protein called hephaestin before being bound by plasma transferrin. Iron bound by plasma transferrin is transported to storage organs for iron such as erythrocyte, the liver, muscle, and macrophages of the reticuloendothelial system. Iron is sequestrated in these storage organs in the form of hemoglobin, ferritin, myoglobin, or hemosiderin. The uptake of transferrin-bound iron by the liver is mediated by



transferrin receptor 1 (TfR1) and TfR2. The absorption of iron is dependent on the body's iron stores, hypoxia, and rate of erythropoiesis [5]. Usually, iron is exported via ferroportin (Fpn) which is controlled by hepatocyte-reproduced hepcidin [6]. Fpn-based iron release from cells is an essential and important limiting step for systemic iron homeostasis. Hepcidin especially reduces the activites of ferroportin. Especially, hepcidin acts as a negative regulator of Fpn in the systemic iron overload. Hepcidin production in the hepatocyte may be controlled via TfR1/HFE, TfR2 genes, and hemojuvelin [7].

The body regulates iron uptake and also prevents excess. Normally, in therapeutic dosing, 10 to 35 % of iron is absorbed. It is transported by transferrin in plasma and stored in reticuloendothelial cells by ferritin in attempt to maintain free concentration. In addition, transport and storage proteins become saturated and caused the increase of free iron in the circulation for cellular toxicity. However, these protective mechanisms are inadequate in acute intoxication [8]. The amount of iron that is absorbed in an overdose situation is unknown. The iron toxicity is due to the amount of elemental iron ingested [9]. Ingestions of more than 60 mg/kg can be associated with serious toxicity. But, elemental iron content of each form of iron preparation is different. This is because in several previous studies, application time, form of preparation, and dosage of iron are administered differently [1, 10-13].

The iron-induced hepatotoxicity/hepatic necrosis occurs 12 to 96 h after ingestion, and the absorption of iron rate is less in oral intake than in parenteral intake because of the preffered parenteral route in the experimental study. Peak serum iron concentrations occur 2 to 3 h after therapeutic dosing and 4 to 6 h after ingestion of an overdose [9]. The 10-day administration, form of preparation, and dosage of iron were determined based on all of these reasons, previous different studies, and our preliminary study [1, 10–12].

If iron-binding capacity of transferrin is excessed, nontransferrin bound iron (NTBI) occurs. Despite positive effects, if NTBI from the bloodstream occurs, iron can accumulate in the liver, pancreas, other endocrine organs, and heart in case of its overload. Hepatotoxicity is the most common finding in patients with iron overload. There may be abnormal liver function tests as well as serum ferritin, iron level, ironbinding capacity, and transferrin saturation [14]. The best estimate of the severity of the iron toxicity can be determined by measuring the serum iron concentration within acute phase [15]. Total iron-binding capacity (TIBC) reflects the level of iron in the blood that can be bound to transferrin. TIBC is not used for measurement of the severity of acute iron toxicity [16]. Serum ferritin is one of the most important markers to estimate iron toxicity. The level of ferritin in the blood indirectly indicates the level of iron present in the liver. In a previous study, it was used and shown to enhance its level [10]. However; acute iron toxicity was assessed with liver enzymes such as aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT). These tests are also very useful. They are often used to indicate iron toxicity [11, 12]. In case of iron overload, free iron, a pro-oxidant, causes formation of reactive oxygen species (ROS) as a result of many reactions required for energy production. The iron failed to be detoxified by anti-oxidant system and causes oxidative stress through hydroxyl radicals in tissues where it accumulates [17, 18]. It leads to several injuries, including fibrosis and necrosis due to the pro-inflammatory mediators being induced by tissue injury [19]. Several chelators are used to prevent iron deposition in tissues, which regulate iron cycle. Desferrioxamine is the most widely used iron chelator despite many disadvantages [20]. Thus, several studies have been conducted on many alternative chelators [20, 21].

Humic acids (HAs) are organic compounds and a main component of humic substances that are formed as a result of decomposition of plant and animal residues in soils and aqueous systems [22–24]. HAs have been shown to exhibit various effects due to their varied chemical structures, the content of heterogenous functional groups, their adsoprtion capacity, and their capability to form complexes [25]. As examples, in animals, they have shown a strong tendency to bind different materials, such as heavy metals [26, 27] and also anti-oxidant [28], immunostimulatory, and anti-goitrogenic effects [29].

This study was designed to test chelator effect of HA on iron as well as anti-oxidant effect against iron-induced hepatotoxicity and cardiotoxicity in search of alternative chelators that can be used in hemochromatosis, thalassemia, and other disorders characterized by iron deposition.

Materials and Methods

Animals

The study was conducted at Inonu University Experimental Animal Research Center (IUEARC). It was approved by the Inonu University Ethics Committee on Experimental Animal Research (reference number: 2014/A-33). The study protocol was designed in accordance to the International Medical Board of Animal Experiments guidelines accepted by the Inonu University Ethics Committee on Experimental Animal Research. Thirty-two female Wistar Albino rats (weighing 210–230 g; mean, 220 g) were used in the study. The rats were supplied by IUEARC. The rats were randomly assigned into four groups (eight rats in each). All animals were fed with standard commercial pellet diet and water ad libitum. Rats were starved a day with 12-h fasting before experiment. All rats were housed under standard conditions with temperature of 21 ± 2 °C and humidity of 60 ± 5 % by maintaining a 12-h dark-light cycle.

Preparation of the HA

Forty-five percent of HA was obtained from Farmavet Internationals Pharmaceuticals Company. Initially, humic acid was dissolved by deionized water and followed by filtering the solution through a membrane. Then, the stock solution was prepared by dissolving 1 g humic acid in 8.5 mL of 0.85 M NaOH, then it was centrifuged at 3000 rpm for 30 min and exactly dissolved. The prepared solutions were stored at $4^{\circ}C$ [30].

Administration of Iron

As oral iron intake in our preliminary studies failed to provide adequate tissue accumulation, we gave iron intraperitoneally since it is easier. Iron III hydroxide polymaltose at a dose of 250 mg/kg was given over 10 days by intraperitoneal route. We chose 10 days as acute exposing time [31].

Experimental Design

Thirty-two rats were randomly assigned into four groups with eight rats each.

Group I (control): control rats were fed by standard commercial pellet diet and water ad libitum.

Group II (HA): humic acid at a dose of 562 mg/kg (HA, 45 %; Farmavet Internationals Pharmaceuticals Company) was given over 10 days by oral gavage.

Group III (iron): iron III hydroxide polymaltose at a dose of 250 mg/kg (Ferrum Hausman Vial, Abdi Ibrahim Pharmaceuticals) was given over 10 days by intraperitoneal route.

Group IV (HA plus iron): iron III hydroxide polymaltose (250 mg/kg; i.p.) plus humic acid (562 mg/kg; PO) were given over 10 days.

Health status was monitored and recorded throughout the experiments in all groups. In all rats, laparotomy was performed under anesthesia (xylazine, 70 mg/kg and ketamine, 10 mg/kg) via 3-cm median incision 24 h after last dose given on day 11 [32]. Blood sample (5 mL) was drawn by intracardiac route for measurement of ALT, AST, iron-binding capacity (TIBC), and transferrin saturation (TS) by Olympus autoanalyzer (Olympus Instruments, Tokyo, Japan). For determination of iron in blood samples, an Architect 1600 atomic absorption spectrometer was used to determine the iron concentration. In the same time, Abbott trademark commercial iron analysis kit was used for this determination. Then, rats were killed. In addition, the liver and the heart specimens were obtained for biochemical and histopathological evaluations. Tissue samples were stored at -70 °C for biochemical assays and in 10 % formalin for histopathological evaluations.

Oxidative stress and anti-oxidant system markers were analyzed after thawing tissue samples at room temperature.

The doses of iron and humic acid were determined based on previous dose–response studies [27, 31].

Biochemical Analysis

Two hundred milligrams of frozen liver and heart tissue was cut into pieces on dry ice and homogenized in 10 vol of icecold Tris–HCl buffer with respect to tissue weight (50 mmol/ L, pH 7.4) using a homogenizer (Ultra Turrax IKAT18 basic homogenization; Werke, Staufen, Germany) for 3 min at 6000 rpm. The supernatant solution was extracted with an equal volume of an ethanol/chloroform mixture (3/5, volume per volume (v/v)). After centrifugation at 3000×g for 30 min, the upper layer was used in the analysis of total tissue protein levels.

Determination of Malondialdehyde

The malondialdehyde (MDA) contents of the homogenates were determined spectrophotometrically by measuring the presence of thiobarbituric acid reactive substances (TBARS) [33]. Three milliliters of 1 % phosphoric acid and 1 mL 0.6 % thiobarbituric acid solution were added to 0.5 mL of homogenate pipetted into a tube. The mixture was heated in boiling water for 45 min. After the mixture cooled, the colored part was extracted into 4 mL of *n*-butanol. The absorbance was measured by spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan) at 532 and 520 nm. The amount of lipid peroxides was calculated as TBARS of lipid peroxidation. The results were given in nanomoles per gram of tissue, according to a prepared standard graph.

Determination of Protein Content

Protein content of the samples was determined by the method of Lowry et al. [34] using bovine serum albumin as a standard.

Determination of Superoxide Dismutase Activity

Total superoxide dismutase (SOD) activity was determined according to the method of Sun et al. [35]. The principle of the method is the inhibition of nitrobluetetrazolium (NBT) reduction using the xanthine–xanthine oxidase system as a superoxide generator. One unit of SOD was defined as the enzyme amount causing 50 % inhibition in the NBT reduction rate. SOD activity was expressed units per milligram protein.

Determination of Catalase Activity

Catalase (CAT) activity was determined according to Aebi's method [36]. The principle of the assay is based on the

determination of the rate constant $(k; s^{-1})$ or the H₂O₂ decomposition rate at 240 nm. Results are given as *k* per gram of protein.

Determination of Glutathione Peroxidase Activity

Determination of glutathione peroxidase (GPX) activity was measured by the method of Paglia and Valentine [37]. An enzymatic reaction in a tube containing NADPH, glutathione (GSH), sodium azide, and glutathione reductase was initiated by addition of H_2O_2 , and the change in absorbance at 340 nm was observed by a spectrophotometer. Activity was calculated in units per gram of protein.

Determination of Glutathione Content

GSH content in liver and heart tissue as non-protein sulfhydryl was analyzed following a previously described method [38]. Aliquots of tissue homogenate were mixed with distilled water and 50 % trichloroacetic acid in glass tubes and centrifuged at 3000 rpm for 15 min. The supernatants were mixed with Tris buffer (0.4 mol, pH 8.9) and 5,5'-dithiobis (2-nitrobenzoic acid (DTNB), 0.01 mol) was added. After shaking the reaction mixture, its absorbance was measured at 412 nm within 5 min of the addition of DTNB against blank with no homogenate. The absorbance values were extrapolated from a glutathione standard curve and given as GSH (µmol/g tissue).

Determination of Total Anti-oxidant Capacity

Total anti-oxidant capacity (TAC) levels were determined using a novel automated colorimetric measurement method developed by Erel [39]. In this method, the hydroxyl radical, the most potent biological radical, is produced by the Fenton reaction and reacts with the colorless substrate *O*-dianisidine to produce the dianisyl radical, which is bright yellowishbrown in color. Upon the addition of the samples, the oxidative reactions initiated by the hydroxyl radicals present in the reaction mix are suppressed by the anti-oxidant components of the sample, preventing the color change and thereby providing an effective measure of the total anti-oxidant capacity of the sample. The assay has excellent precision values, which are lower than 3 %. The results were given as millimoles of trolox equivalent per liter.

Determination of Total Oxidant Status

Total oxidant status (TOS) was determined using a novel automated measurement method, developed by Erel [39]. The oxidants present in the sample oxidize the ferrous ion–*O*-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric iron makes a colored

complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the samples. The assay was calibrated with hydrogen peroxide, and the results were calculated in terms of micromoles of H_2O_2 equivalent per liter.

Measurement of Oxidative Stress Index

The ratio of the TOS to TAC was accepted as the oxidative stress index (OSI), an indicator of the degree of oxidative stress [39]. OSI value was calculated from the formula: OSI (arbitrary unit) = TOS/TAC. The OSI value of the liver and heart samples was also given as an OSI (arbitrary unit).

Histological Analyses

Liver and heart tissue specimens were fixed in 10 % buffered formalin. After routine tissue processing, including fixation, dehydration, and paraffin, 5- μ m thickness sections were obtained and stained by hematoxylin and eosine and Prussian blue. All preparations were evaluated by a pathologist blinded to groups.

Statistical Analysis

The data were expressed as median (min–max) values. The variables were compared by Kruskal–Wallis *H* test among the groups. When significant differences were determined, multiple comparisons were carried out by using the Mann–Whitney *U* test with Bonfernoni correction. P < 0.05 was considered as significant. IBM SPSS statistics version 22.0 for Windows was used for statistical analyses. The present study has four groups having a total of 32 subjects. This information is provided in "Animals" and "Materials and Methods": "The rats were randomly assigned into four groups (eight rats in each)." The descriptive statistics for the groups were given in Tables 1, 2, and 3.

Results

Body and Liver Weights

None of the animals died during the experimental period. There was no difference between the body and liver and heart weights before and after the experiments among the groups (data not shown).

Biochemical Results

The results of the biochemical parameters of the pro-oxidant and anti-oxidant systems belonging to the liver tissue are

Table 1 Chai	nges in the activities of ox	idant and anti-oxidant p	parameters of liver tiss	ue in control and experi	mental rats			
Groups $(n = 8)$	MDA (nmol/g tissue) Median (min-max)	SOD (U/mg protein) Median (min-max)	CAT (k/g protein) Median (min-max)	GPX (U/g protein) Median (min-max)	GSH (µmol/g tissue) Median (min-max)	TOS (µmol/g tissue) Median (min-max)	TAC (trolox Eq/L) Median (min-max)	OSİ (arbitrary unit) Median (min-max)
Control HA Iron HA + iron <i>P</i> value	8.64 (6.24–10.75) 8.52 (6.69–21.36) 21.94 (14.53–32.19)*· ** 17.50 (10.75–19.53)* <0.0001	1.71 (1.54–2.02) 1.27 (1.13–2.01) 0.90 (0.84–1.215)* 1.27 (1.21–1.75)**** <0.0001	16.48 (10.85–18.58) 15.10 (7.74–23.20) 12.98 (9.15–20.52) 14.21 (10.85–27.45) >0.05	241.56 (208.22–293.15) 167.46 (127.94–254.30) 115.60 (89.25–165.53)* 145.95 (121.54–270.28) <0.0001	12.49 (8.07−15.69) 12.29 (8.07−14.27) 6.97 (6.27−11.43)* 9.28 (4.97−13.84) <0.01	31.16 (21.45–35.22) 40.47 (21.45–51.72) 53.49 (29.74–65.41)* 37.42 (30.27–49.61) <0.01	0.88 (0.85–1.30) 0.85 (0.63–1.16) 0.81 (0.60–1.30) 0.88 (0.55–1.07) >0.05	33.77 (16.47–40.29) 44.31 (34.40–69.23) 65.90 (33.77–80.97)* 50.77 (16.47–70.36) <0.05
Results are exp <i>HA</i> humic acid *P < 0.05 versu	pressed as median (min-ma las control; $**P < 0.05$ vers	ux), <i>n</i> = 8 sus HA; *** <i>P</i> < versus	iron					
Table 2 Char	nges in the activities of ox	idant and anti-oxidant p	barameters of heart tiss	sue in control and experi-	imental rats			
Groups $(n = 8)$	MDA (nmol/g tissue) Median (min-max)	SOD (U/mg protein) Median (min-max)	CAT (k/g protein) Median (min-max)	GPX (U/g protein) 6 Median (min-max) 1	GSH (µmol/g tissue) Median (min-max)	TOS (μmol/g tissue) Median (min-max)	TAC (trolox Eq/L) Median (min- max)	OSİ (arbitrary unit) Median (min–max)
Control	5.48 (2.96–7.54)	0.88 (0.66–1.10)	3.40 (2.63–4.12)	56.14 (47.32–65.82)	13.12 (11.37–18.97)	4.58 (3.45–5.87)	0.90 (0.72–1.06)	5.25 (4.35–6.45)
HA	6.76 (3.57–8.01)	0.87 (0.71–0.90)	3.11 (1.97–4.28)	40.65 (36.33–45.07)	12.42 (10.22–14.73)	5.28 (4.50–6.29)	0.89 (0.79–1.01)	6.01 (4.82–8.40)
Iron	11.99 (8.39–15.84)* ^{, **}	0.69 (0.55–0.86)*	2.09 (1.34–3.15)*	28.09 (22.63– 8 33.58)*	3.99 (6.27–13.18) *	6.68 (5.69–8.27)*	0.87 (0.72–0.96)	7.87 (6.60–10.09)*
HA + iron	6.43 (3.57–9.62)***	0.90 (0.74–0.93)***	3.23 (2.58– 5.27)***	37.94 (30.27–54.55) 1	13.88(12.10-16.13)***	5.06 (4.38–5.55)***	0.85 (0.80–1.08)	5.98 (4.51– 6.46)***
P value	<0.001	<0.01	<0.05	<0.0001	<0.01	<0.001	>0.05	<0.001
Results are expr	ressed as median (min-ma	(x), n = 8						

H4 humic acid*P < 0.05 versus control; **P < 0.05 versus HA; ***P < 0.05 versus iron

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Groups $(n = 8)$	ALT (U/L) Median (min–max)	AST (U/L) Median (min–max)	Iron (mg/dL) Median (min–max)	TIBC (mg/dL) Median (min-max)	TS (%) Median (min–max)
Control	25.00 (20.00-32.00)	50.00 (21.00-72.00)	157.500 (132.00–184.00)	110.35 (101.80–131.20)	132.60 (122.79–150.00)
HA	28.00 (14.00-52.00)	67.50 (54.00-87.00)	159.50 (103.00-182.00)*	122.00 (97.60–167.70)	135.01 (81.74–150.83)
Iron	212.00 (138.00-436.00)*, **	278.50 (101.00-502.00)*, **	414.50 (394.0-428.0)*, **	41.00 (40.00-41.10)** **	1009.74 (960.97–1043.90)*, **
HA + iron	63.50 (26.00–155.00)	162.50 (159.00-227.00)*	413.00 (230.00–516.00)*' **	49.05 (40.00-73.00)*' **	874.63 (323.94–1230.00)*' **
P value	< 0.001	< 0.001	< 0.0001	< 0.0001	< 0.0001

 Table 3
 Comparison of the serum biochemical parameters among the study groups

HA humic acid, *ALT* alanine transaminase, *AST* aspartate transaminase, *TIBC* total iron-binding capacity, *TS* transferrin saturation (iron/TIBC \times 100) **P* < 0.05 versus group I; *P* < 0.05 versus group II

presented in Table 1. Briefly, the MDA levels were found to be significantly higher in the iron group when compared with the control group (P < 0.0001). However, no significant difference was found in the MDA levels between the iron and the HA plus iron groups (P > 0.05). When SOD, CAT, GPX, and GSH levels were compared between the iron and the control groups, it was observed that there were significant differences in SOD, GPX, and GSH levels (P < 0.0001, P < 0.0001, and P < 0.05, respectively) (Table 1). When the same parameters were compared between the iron and the HA plus iron groups, only significant difference was observed in SOD levels (P < 0.005) (Table 1). When TOS, TAC, and OSI levels were compared between the iron and the control groups, it was observed that there were significant differences in both TOS and OSI levels (P < 0.001 and P < 0.005) (Table 1). When the same parameters were compared between the iron and the HA plus iron groups, no significant difference was found.

In heart tissue, the MDA levels were found to be significantly higher in the iron group when compared with the control group (P < 0.0001). Also, a significant difference was found in the MDA levels between the iron and the HA plus iron groups (P < 0.05). There were significant differences in SOD, CAT, GPX, and GSH levels between the iron and the control groups (*P* < 0.05, *P* < 0.05, *P* < 0.0001, and *P* < 0.05, respectively) (Table 2). When the same parameters were compared between the iron and the HA plus iron groups, there were differences proved by statistical analyses in SOD and GSH levels (P < 0.01 and P < 0.05, respectively) (Table 2). Significant differences in TOS and OSI levels were observed when comparing between the iron and the control groups (P < 0.05 and P < 0.001) (Table 2). The similar differences were observed when the iron and the HA plus iron groups were also compared in the same parameters (P < 0.05 and *P* < 0.05) (Table 2).

When ALT, AST, iron, TIBC, and TS levels were compared between the iron and the control groups, significant differences in all parameters evaluated were found. However, when the same parameters were compared between the iron and the HA plus iron groups, no significant statistical difference was observed (P > 0.05) (Table 3).

Histopathological Results

In the control and the HA groups, all organs were observed to be in normal morphology without iron deposition (Figs. 1 and 2).

In the assessment of iron deposition in liver, no iron deposition was observed in the control and the HA groups, while similar morphological findings were observed in the hematoxylin and eosine (H&E)-stained samples from the iron and the HA plus iron groups: brown pigments within tissue macrophages, hepatocytes cytoplasm, Kuppfer cells, and biliary duct epithelium at portal areas were observed as being more prominent at zone 2. In addition, binucleation was noted in the some nuclei of hepatocytes, while nuclear pyknosis in some others (Fig. 1).

In the H&E- and Prussian blue-stained heart samples, no iron deposition was observed in the HA group while degenerative nuclear changes, interstitial edema, and inflammatory cells were observed in the iron group. Diffuse iron deposition in myocytes was striking in all subjects (Fig. 2).

Hepatic iron deposition grading system proposed by LeSega was used to rate iron pigment [40] (Table 4). When Prussian blue-stained sections were evaluated, macroscopic blue coloration was noteworthy. As in H&E sections, iron deposition in more than 75 % of all hepatocytes (LeSega grade 4) was observed on light microscopy as globules being larger at zone 2. No significant difference was observed in the amount and distribution of iron between the iron and the HA plus iron groups.

Discussion

Iron causes disruption in several physiological functions of the tissue where it accumulates. There are many previous and ongoing studies on the mechanism in which iron deposition causes the pathology. In these studies, it has been reported that ROS elevation has a major role in the iron-induced hepatotoxicity [41, 42]. Increased ROS stimulates oxidative stress and release of pro-inflammatory mediators, causing tissue damage [43, 44]. Moreover, ROS elevation causes cell death by leading

Fig. 1 a, b No pigment was observed in the liver tissue of controls (H&E, ×400; Prussian blue, $\times 400$): c. d no vellow-brown pigment was observed in the liver tissues in the HA group (H&E, ×400; Prussian blue, ×400); e diffuse yellow-brown pigment was striking in the liver tissue in the iron group (H&E, \times 400); f diffuse iron deposition around central vein and all zones (Prussian blue, ×400); g presence of yellow-brown pigment in the HA plus iron group (H&E, ×400); h diffuse iron deposition in the HA plus iron group, as being more prominent at zone 2 (Prussian blue, ×400)



apoptosis [41]. Many anti-oxidants, anti-inflammatory agents, or chelators have been used in the studies, which aim to decrease ROS activity resulted from free iron [21, 45]. In the present study, we preferred to use HA as chelator and anti-oxidant since there is almost no study in the medical literature on HA. HA is a compound that naturally occurs in water and soil and has many functions due to its electron-transferring properties. Among these, anti-inflammatory, anti-oxidant, and chelation effects were tested in our study.

The liver and the heart are organs primarily affected by iron that is able to accumulate in several tissues. In the extracts of both tissues (liver and heart), significant increases were observed in the MDA production in the group given iron compared with controls in our study. The MDA is released as a result of lipid peroxidation in cellular lipid membrane caused by active ROS [12]. This finding confirmed lipid peroxidation and increased oxidative stress induced by iron. The finding of increased lipid peroxidation induced by iron was in agreement with previous studies [41]. Marked reduction was observed in lipid peroxidation induced by the iron in the group received the iron plus HA in the heart tissue but not in the liver tissue. Probably, this is because it is due to that heart muscle cell membranes and sarcoplasmic membranes are in direct contact with the extracellular fluids. Excessive exposure to iron toxicity of the membranes is particularly easy [46].

Fig. 2 a, b Heart tissue with normal morphology in the control group (H&E, ×400; Prussian blue, $\times 400$); c, d no iron deposition was observed in the heart tissue in the HA group (H&E, ×400; Prussian blue, ×400); e yellow-brown pigment was observed in the heart tissue in the iron group (H&E, $\times 400$); f blue-colored iron deposition was observed in the heart tissue in the iron group (Prussian blue, ×400); g yellow-brown pigment deposition was observed in the heart tissue the HA plus iron group (H&E, $\times 400$); h bluecolored iron deposition was observed in the HA plus iron group (Prussian blue, ×400)



These results demonstrated anti-oxidant activity of HA. The similar results were observed in the studies that aimed to reduce the lipid peroxidation induced by iron, thus, oxidative stress [20, 45].

Table 4 LeSega iron deposition grading

Grade 0: no iron deposition Grade 1: iron deposition in less than 25 % of hepatocytes Grade 2: iron deposition in 25–50 % of hepatocytes Grade 3: iron deposition in 50–75 % of hepatocytes Grade 4: iron deposition in more than 75 % of hepatocytes In the present study, iron treatment led to a significant decrease in SOD, CAT GPX activities, and GSH concentration when compared with the control group in the liver and heart tissue extracts. It is known that there are enzymatic and nonenzymatic anti-oxidant systems in several tissues in order to protect integrity of biological membranes against oxidative stress. Normally, there is equilibrium between oxidant and anti-oxidant systems. The imbalance favoring oxidant system plays an important role in the development of many disorders. The SOD, CAT, and GPX are endogenous enzymatic antioxidants, protecting cell membrane against ROS [10]. SOD plays a major role in the formation of hydrogen peroxide, whereas GPX and CAT play a role in the enzymatic catabolism of ROS. [12]. GPX is a crucial anti-oxidant enzyme involved in preventing the harmful accumulation of intracellular hydrogen peroxide. It has been found to be more effective than catalase at removing intracellular peroxides under many physiological conditions [47]. Our findings support the hypothesis that iron-induced tissue injury is associated with ROS, which depletes the anti-oxidant system. Iron is a well-known inducer of ROS. ROS which increases lipid peroxidation can lead to cell disfunction. Harmful effects of iron which has been associated with ROS-induced oxidative damage were shown in the previous studies [12]. HA treatment along with iron treatment led to a significant increase in the SOD and GSH levels when compared with the iron group in the rat heart but led to a significant increase in only SOD in the liver. Probably, this situation is due to the fact that heart muscle cell membranes and sarcoplasmic membranes are in direct contact with the extracellular fluids. However, they were shown that HA, used against adverse effects of iron, enhanced enzymatic and non-enzymatic anti-oxidant system. This suggests that HA is a promising compound that may be protective and therapeutic in iron-induced tissue injury. In a study by Naik et al., increased lipid peroxidation and decreased enzymatic anti-oxidant system in the iron-induced hepatotoxicity [48] were shown. This result was in agreement with our study. In addition, in the different previous studies, HA was shown to significantly increase SOD and decrease MDA levels in a focal cerebral ischemia model. This may be due to the reduction of oxidative stress [49]. These results were in agreement with the present study. In another previous study, a significant decrease in SOD after humic acid treatment [28] was also observed.

GSH cycle is the most important intracellular anti-oxidant defense mechanism. It is used as substrate for activity of several anti-oxidant enzymes. In particular, GPX is a glutathionedependent enzyme. It is known that, in case of decreased or depleted glutathione, decreased GPX activity causes accumulation of toxic products by enhancing oxidative stress [12]. In the present study, iron caused reduction in GPX and GSH levels in both the liver and the heart tissues. These results were in agreement with previous studies [48].

In the present study, TOS and OSI levels were increased while TAC level was decreased in the heart and the liver tissues in the group receiving iron alone. However, TOS and OSI levels were significantly decreased in the heart and but not the liver tissues, while TAC level did not increase significantly in the group receiving the HA plus iron when compared with the group receiving iron alone. Probably, this is due to the fact that the heart muscle cell membranes and sarcoplasmic membranes are in direct contact with the extracellular fluids. This indicated that iron increased while HA reduced oxidative stress [50]. In the present study, HA seems to reduce oxidative stress and increase anti-oxidative stress in the heart better than the liver. In the present study, it was found that there were significant differences in all parameters when ALT, AST, iron, TIBC, and TS levels were compared between groups receiving iron and controls. This finding confirmed that an experimental model was designed successfully. In a study by El-Baky et al., several biochemical parameters were shown to increase during iron-induced toxicity [45]. Again, in a study by Jensen et al., an association among iron and liver enzymes or TS [51] was observed. In our study, no significant differences among the groups receiving iron alone and HA plus iron were shown. Our results are in agreement with the abovementioned studies.

Histopathological examination is the gold standard in the assessment of tissue injury. When the group receiving iron was compared with controls in a histopathological manner, it was seen that there were apparent changes in the group receiving iron, which was indicated by demonstration of iron deposition in periportal area and relevant pathological changes, as being more prominent at zone 2 of the liver. Our results are in agreement with those found by Gao et al. [52]. It is seen in the diseases associated with iron deposition. However, iron was given over 10 days in our study, and acute changes were more prominent. It was seen that the changes persisted in the group receiving HA in addition to iron. In the present study, iron was found to cause degenerative changes, inflammation, edema, and iron deposition by histopathology and the major effect of iron deposition is tissue injury secondary to oxidative stress, which manifested as degenerative nuclear changes, interstitial edema, inflammation, as well as diffuse iron deposition in all cells. Tissue injury induced by iron is supported in previous studies on iron deposition.

Marked tissue injury was demonstrated in subjects receiving iron while no histopathological change was observed in the group receiving the HA plus iron. In the latter group, it was observed that HA had some effects on lipid peroxidation products and anti-oxidant system. By increasing MDA and decreasing SOD, GPX, CAT, and GSH, iron was shown to cause increased ROS and lipid peroxidation products by influencing nucleic acids, lipids, and proteins in cell membranes through oxidative stress.

Conclusion

Consequently, high doses of iron can accumulate in several tissues. This deposition alters physiological functions of the tissue. The present study was shown that HA was protective against iron-induced hepatotoxicity and cardiotoxicity via its anti-oxidant and free radical scavenger effects in both histopathological and biochemical levels. Presumably, it exerts this effect by reducing ROS activity through anti-oxidant features such as lipid peroxidation inhibition and enhanced enzymatic and non-enzymatic anti-oxidant systems. We think that HA can be used as a protective agent against iron-induced hepatic and heart injury. However, further clinical studies are needed to demonstrate the protective effects of HA against ironinduced hepatotoxicity and cardiotoxicity.

Conflict of Interest The authors declare that they have no conflict of interest regarding the publication of this paper.

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