Endocrine (2011) 40:462–471
DOI 10.1007/s12020-011-9483-0

ORIGINAL ARTICLE

Biochemical and histological liver changes occurred after iron supplementation and possible remediation by garlic consumption

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Received: 12 March 2011 / Accepted: 22 April 2011 / Published online: 8 May 2011

Abstract Iron liver excess is associated to biochemical and histological liver perturbations. Our aim was to know even if fresh garlic consumption can remediate these problems. Three groups of rats were utilized: control group A, iron overload group B and garlic and iron overload group C. Important morphological and biochemical modifications were obtained in group B rats comparatively to control group A. Indeed, body and liver weights and liver iron contents increased, respectively, by 12.5 ± 0.06%; 17 ± 0.25% and 35 ± 0.11% comparatively to controls. Radical cation scavenging ability in liver cytosol of group B rats was significantly low (54 ± 0,1%) in comparison to group A. Garlic consumption allowed the group C to achieve an increase by 46 ± 0,11 and 75 ± 0,14% of total antioxidant capacity comparatively to group A and B rats. For the serum ALAT, ASAT, triglyceride and LDH levels, they increased in iron-treated rats, respectively, by 25 ± 0.21; 15 ± 0.12; 30 ± 0.14 and 22 ± 0.16% comparatively to controls. These perturbations were accompanied by deep histological changes. After food fresh garlic supplementation, we had found a deep regulation of all modified parameters showing a hepatoprotective effect of garlic against iron liver excess. Garlic chemical compounds have curative effects on iron liver excess.

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Keywords Liver · Iron · Garlic · Rat · Histopathology · ASAT · ALAT · Triglyceride · Total antioxidant capacity

Introduction

Iron is an essential element for microbes, plants and higher animals. It is a component of heme and iron-sulphur centres in many key redox enzymes, and is an essential component of oxygen storage and transporting proteins such as haemoglobin and myoglobin [1]. It is also an integral part of several classes of enzymes, including cytochromes, enzymes involved in the synthesis of steroid hormones, detoxification of foreign substances in the liver, synthesis of neurotransmitters and DNA synthesis and breakdown. In all species, the concentration of iron in biological fluids is tightly regulated to provide iron as needed and to ovoid toxicity, because iron excess can lead to the generation of reactive oxygen species (ROS) [2]. Iron homeostasis must be maintained so that cells have sufficient iron for cell growth, but not excess because of its toxicity. This is achieved principally by controlling the activity of iron importers, exporters and storage proteins in a coordinated fashion [3].

In most circumstances, diets contain more iron than required to replace the small daily losses. Once iron has been ingested, humans and other mammals lack mechanisms to excrete excess iron. Therefore, tightly regulated absorption of intestinal iron is necessary [4]. After the intestinal absorption, the liver, or more specifically, the hepatocyte, plays a central role in iron homeostasis and is the major site of iron storage [5]. Other organs like spleen and bone marrow also store iron [6]. In all tissues, iron, as a transition metal, can promote Fenton reaction which leads to high amounts of ROS [7]. If not quenched by antioxidants, like polyphenols or enzymes, ROS can damage cells at different sites [8]. One

of the most important sources of antioxidant substances is vegetables. In fact, oxidative stress induced by excess iron exposition can be reduced by consumption of plants containing antioxidants [9].

In these last decades, growing interest is given to the association of specific components of human nutrition and oxidative stress [10]. The use of herbs of medicinal benefit has played an important role in nearly every culture on earth. In several countries, the use of medicinal plants is still the mainstay of health care [11]. Indeed, epidemiological data continue to lend support to the premise that dietary intake of vegetables, including garlic (*Allium sativum*), may be protective against the risk of various types of malignancies [12]. In fact, garlic is a bulbous plant, which contains some powerful antioxidant components, such as diallyl sulfide, diallyl trisulfide, 3-vinyl-1,2-dithiin and E,2-ajœne [12–14]. Therefore, one of the most beneficial effects of this plant is its hepatoprotective role [15].

Many studies had demonstrated that iron intoxication induced severe liver damage which progress to failure or HCC [16, 17]. Other studies have improved that the consumption of garlic gives liver protection [15]. However, there are no previous investigations dealing with the hepatoprotective role exerted by garlic consumption and iron exposition. Furthermore, many investigators used large doses of garlic or garlic oil in a relatively short experimental period [18, 19]. Therefore, little is known about the nutritional effects of garlic at ordinary levels of dietary intake.

The aim of this study was to demonstrate whether iron overload associated to uncooked garlic consumption promotes liver protection of growing young rats.

Materials and methods

Animals

According to the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Council of Europe No. 123, Strasbourg, 1985) and the review committee of our institution, rats rearing and experiments of this study were approved. In the present study, Suisse strain male rats, 2 months old, were purchased from the Central Pharmacy (SIPHAT, Tunisia). They were housed at 22 ± 3°C with light/dark periods of 12/12 h and a minimum relative humidity of 40%. They had free access to water and commercial diet (SICO, Sfax, Tunisia). The standard diet contained 73.44 mg of iron per gram of diet.

Experimental protocol

The initial number of young rats was 30, equally divided into three groups of ten individuals: controls (group A), overload with $FeCl_2$ at a dose of 150 mg/100 ml of drinking water (group B), overload with $FeCl_2$ and garlic, respectively, at doses of 150 mg/100 ml of drinking water and 5 g/100 g of dampen standard diet (group C). Sacrifice of all the group's rats was done 45 days after beginning treatments. The daily consumed food and water was precisely measured during all the treatment periods (Table 1).

Samples extraction

After anaesthesia with chloral hydrate by intraabdominal way, body weights of young rats were measured. The livers (10 per group) were carefully dissected out for weight, biochemical, mineral and histological analyses. Blood samples were collected from the brachial artery of young rats, and serum samples were withdrawn after centrifugation at 2,200×*g* for 15 min. All the liver and serum samples were kept at −80°C until analysis.

Biochemical and histological studies

Serum biochemical analysis

Serum samples were collected for iron, aspartate-amino-transferase (ASAT), alanine-amino-transferase (ALAT), triglyceride and lactate dehydrogenase (LDH) analysis. In these samples, mineral and enzymatic analyses were done according to Roche laboratories protocols using Hitachi

Table 1 Daily food, water, iron and garlic intake by 2-month-old rats: controls (group A), treated either by $FeCl_2$ (group B) or by both $FeCl_2$ and garlic (group C) for 45 days

Parameters and treatments	A	B	C
Food consumption (g/day/rat)	8.37 ± 0.65	3.78 ± 0.38***	4.46 ± 0.50***,+++
Water consumption (ml/day/rat)	2.83 ± 0.44	1.73 ± 0.53***	2.17 ± 0.65***,++
Ingested iron (g/day/rat)	0.59 ± 0.085	3.18 ± 0.78***	3.27 ± 0.46***
Ingested garlic (g/day/rat)	–	–	0.223 ± 0.025

Number of determinations ($n = 30$)

Treated B and treated C versus controls A: *** $P \leq 0.001$

Treated C versus treated B: ++ $P \leq 0.01$, +++ $P \leq 0.001$

912 analyser on which kits can be used. Commercial kits from Roche laboratories were used for iron (ref: 11970747 216), ASAT (ref: 10851124 216), ALAT (ref: 10851132 216), triglyceride (ref: 11488899 216) and LDH (ref: 11489305 216) analysis.

Iron liver contents determination

Liver iron content (seven livers per group) was realized after mineralization with nitric acid 25 ml/5 g liver. This process was done at 200°C until total nitric acid evaporation. Then, 50 ml of deionised water were, respectively, added to the samples before being analysed by spectrophotometric atomic absorption (Hitachi Z 6100 model) at 228.5 nm wave length.

Liver total antioxidant capacity determination

Liver cytosol extraction Liver rest parts (seven per group) were utilized for cytosol extraction. Cells fraction was realized after adding 10 ml of KCl (1.15%) to 1g of liver by using ultra-turrax at 4°C temperature.

ABTS assay in liver cytosol samples The Trolox equivalent antioxidant capacity (TEAC) assay is measuring the reduction of the ABTS radical cation by antioxidants. ABTS radical cation (ABTS+) was produced by reacting 7 mM ABTS stock solution with 140 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. For the study, ABTS+ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm. After addition of 1 ml of diluted ABTS+ solution to 50 µl of liver cytosol, or Trolox standard, the reaction mixture was incubated for 2 min in a glass cuvette at 30°C. The decrease in absorbance was recorded at 734 nm. All the measurements were performed in triplicate. The free radical scavenging capacity of the biological sample, calculated as inhibition percentage of ABTS+, was equated against a Trolox standard curve prepared with different concentrations (40–200 µmol/l). The results are expressed as mM of Trolox equivalents.

Liver histopathological analysis

Three livers were randomly selected from each group for light microscopy. They were taken and immediately fixed in a Bouin solution, embedded in paraffin and serially sectioned at 5 µm. Then, the sections were stained with haematoxylin eosin (HE) for routine histological examination, with Masson trichrome for collagen and with Perl's Prussian blue for iron accumulation.

Statistical analysis

Comparisons of mean values between rats treated groups (B and C) and control group (A) or between treated rats (group C) and (group B). Statistical differences were calculated using a one-way analysis of variance (ANOVA), followed by Student's *t*-test. Statistical significance was defined as a P value of less than 0.05. Values were expressed as the means followed by ecartype.

Results

Growth and feeding

Body growth rate variation

Animals used for all the three groups have, at the beginning of the experience, no body weight significant differences (Table 2). During the studied period, a regular increase in body growth rate was noted. However, the body growth rates of groups B and C were more important than that of controls (Table 2). Indeed, on the euthanizing day, we obtained for the utilized doses of iron and garlic (Table 1),

Table 2 Body weight (g), serum iron (µmol/l), liver iron (mg/l), serum triglyceride (mmol/l) and serum LDH (U/l) levels of young rats: controls (group A) and treated either by $FeCl_2$ (group B) or by both $FeCl_2$ and garlic (group C) for a period of 45 days

Parameters and treatments	A	B	C
Initial body weight (10)	181 ± 6	183 ± 4	185 ± 8
Final body weight (10)	230 ± 10	263 ± 16***	254 ± 6*, +++
Serum iron (10)	1.71 ± 0.08	1.93 ± 0.17*	1.65 ± 0.13+
Liver iron (7)	0,63 ± 0,14	0,97 ± 0,05***	0,37 ± 0,17***, +++
Serum triglyceride (10)	1.10 ± 0.08	1.58 ± 0.58*	0.63 ± 0.13***, ++
Serum LDH (10)	1296 ± 335	1657 ± 151**	764 ± 100*, +++

Number of determinations ()

Treated B and treated C versus controls A:* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$

Treated C versus treated B: + $P \leq 0.05$, ++ $P \leq 0.01$, +++ $P \leq 0.001$

respectively a significant increase by 12.5 ± 0.06% and 6 ± 0.6% in group B and C rat's body weights comparatively to controls (Table 2), respectively. In group C, a decrease in body weights by 7 ± 0.6% comparatively to the rats of group B was noted (Table 2).

Liver weights

We have obtained an increase in liver weights by 17 ± 0.25% of rats treated only by iron in drinking water (group B). For rats treated by both iron and garlic, liver weights have not shown any differences with those of controls, and they were 18 ± 0.17% less than those of group B rats (Fig. 1).

Food and water consumption

Food and water consumptions were decreased in group B and C rats. In fact, we have obtained a decrease in food consumption by 55 ± 0.2% and 48 ± 0.12% and in water by 39 ± 0.22% and 23 ± 0.14% (Table 1), respectively.

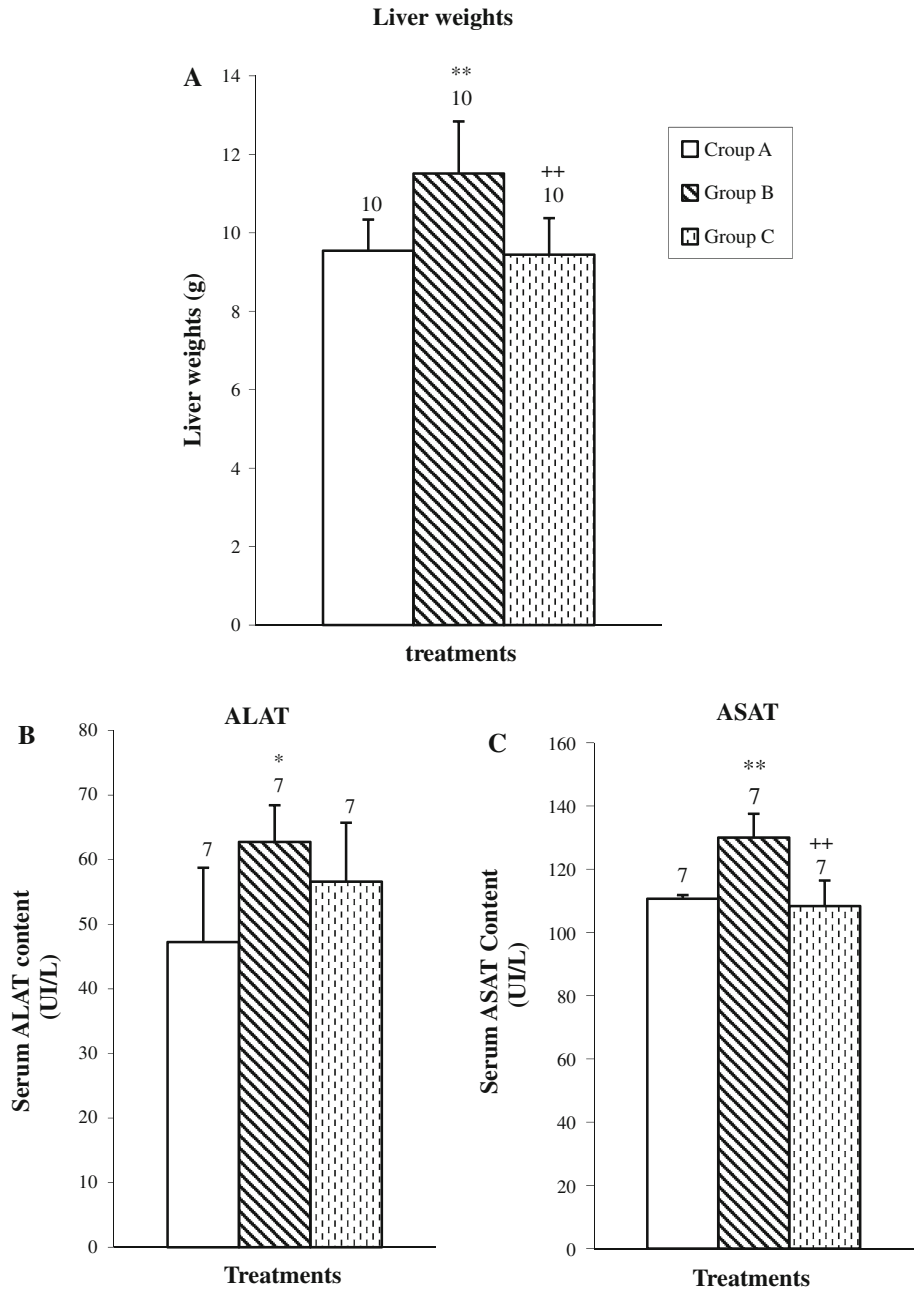

Fig. 1 Liver weights, ASAT and ALAT serum contents of young rats: controls (group A) and treated for a period of 45 days either by $FeCl_2$ (group B) or by both $FeCl_2$ and garlic (group C). Treated B and treated C versus controls A: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. Treated C versus treated B: $^{++}P \leq 0.01$

Serum biochemical markers

Group B-treated rats showed an increase in iron serum content by 11 ± 0.12% comparatively to controls. The administration of garlic to iron-treated animals induced a decrease by 15 ± 0.14% in iron serum content comparatively to the group B rats (Table 2).

The serum ALAT, ASAT, triglyceride and LDH levels increased in iron-treated rats, respectively, by 25 ± 0.21; 15 ± 0.12; 30 ± 0.14 and 22 ± 0.16% compared with controls (Fig. 1; Table 2). The supplementation of garlic induced a decrease in ALAT, triglyceride and LDH levels, respectively, by 17 ± 0.13, 42 ± 0.19 and 41 ± 0.22% compared with iron-treated animals and by 10 ± 0.16, 60 ± 0.25 and 53 ± 0.31% compared with control group (Fig. 1; Table 2). On the other hand, serum ASAT levels of group C rats showed no significant differences with control rats and a decrease by 17 ± 0.16% compared with iron-treated group B (Fig. 1).

Liver parameters

Liver iron contents

The animal treatment with $FeCl_2$ of group B induced an increase in liver iron contents (+35 ± 0.11%) compared with controls. On the other hand, in group C, rats treated with both iron and garlic provoked a decrease in liver iron contents by 41 ± 0.12% and 61 ± 0.10%, respectively, in comparison with control and group B-treated rats (Table 2).

Liver total antioxidant capacity

ABTS radical cation-scavenging ability in liver cytosol of group B rats was significantly low (54 ± 0, 1%) in comparison with rats fed normal diet (group A). Garlic consumption allowed for group C an increase by 46 ± 0,11 and 75 ± 0,14% of total antioxidant capacity compared with group A and B rats (Fig. 2).

Liver histological studies

After the HE staining liver sections of group B, it showed the presence of congested sinusoids. This induced disorders in hepatocytes arrangement. Moreover, some triglyceride vacuoles in hepatocytes cytoplasm had been observed, suggesting the presence of steatosis (Fig. 3b). In contrast, in control liver sections, the aspect of sinusoids and hepatocytes were normal (Fig. 3a). After garlic food supplementation, we had observed the absence of all the lesions obtained in group B liver sections (Fig. 3c).

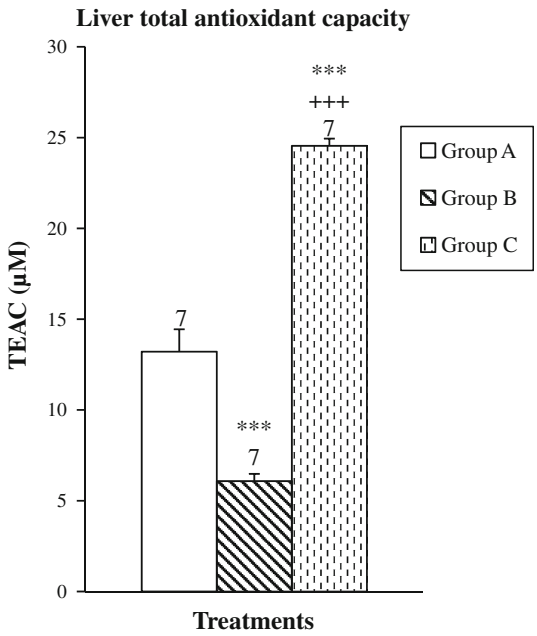

Fig. 2 Total antioxidant capacity in control and treated young rat's liver: controls (group A) and treated for a period of 45 days either by $FeCl_2$ (group B) or by both $FeCl_2$ and garlic (group C). Treated B and treated C versus controls A: $^{***}P \leq 0.001$. Treated C versus treated B: $^{+++}P \leq 0.001$

In group B liver sections, we had observed in the portal systems, after Masson trichrome coloration, an increase in collagen biosynthesis with important inflammatory cells infiltration compared with controls (Fig. 4a, b). However, for rats treated with garlic (group C), portal systems showed normal collagen content with the absence of inflammation (Fig. 4c).

With the objective being to better understand these histological changes, Perl's Prussian blue coloration was realized to show iron deposition. Therefore, we had observed blue kuppfer cells stained in group B liver sections indicating iron accumulation in comparison with controls (group A) and to garlic and iron-treated rats (group C) liver sections (Fig. 4d, e, f).

Discussion

Iron is a vital requirement for normal cellular physiology, with excessive intestinal absorption of iron as seen in hemochromatosis leading to its deposition in parenchymal cells of various organs, such as liver, heart and pancreas, resulting in cellular toxicity [20]. The potential of iron damage is particularly high in liver, the primary organ for storage of excess iron [5]. As there is no significant excretion of iron, excess uptake may be accompanied by

sever liver damage that progresses to liver failure or HCC [16].

In this study, we noted important growth impairment, blood biochemical modifications and liver histological changes. Indeed, rats of group B, treated only with iron, showed a significant increase in their body weights compared with controls. This result could not be explained by food consumption because we had found a significant decrease in daily food and water intakes. However, it may be explained by the fact that iron overload is associated to excess body weight, type 2 diabetes mellitus and hyperlipidaemia, this phenomenon being called as the insulin resistance-associated hepatic iron overload syndrome (IR-HIO) [21]. This syndrome is recently described in mild and moderate hepatic iron overload and is associated with features of insulin resistance [21]. In fact, results of many investigations support the existence of a pathogenetic link between insulin resistance and non-alcoholic fatty liver disease (NAFLD) [22, 23], and recent studies have underlined the role of iron overload as a possible determinant of NAFLD [21, 24]. Indeed, a link between iron and the progression of NAFLD has been supported by the observation that iron removal by phlebotomy can improve the insulin resistance and liver function in patients with NAFLD [25, 26]. At the beginning of our experiments, no significant body weight differences between control and treated group B rats were observed. However, after 45 days of iron treatment, we had obtained a significant increase in body weights compared with controls. The increase in body weights of iron overload group could be a result of the disturbance of endogenous insulin glucose and lipid metabolism. Another possible explanation of group B rat weights' increase is a general stimulation of collagen production. In fact, as it was demonstrated by Gardi and his collaborators [27], the net effect of iron excess on matrix remodelling is in favour of collagen accumulation.

After garlic food adjunction, we have obtained in group C rats a decrease in body weights compared with group B. This result was obtained despite the relative increase in daily food and water consumptions. The improvement of dietary intake may be due to the fact that garlic gives the food a special flavour and fragrance which can increase dietary consumption [28]. The decrease in body weights of rats of group C confirmed previous data of Baluchnejadmojarad and his collaborators [29] who had demonstrated that garlic oil treatment significantly decreased the body weights of extract-treated control and extract-treated diabetic rats. Moreover, Liu and his collaborators [30] showed that both garlic oil and diallyl trisulfide are able to ameliorate plasma insulin levels of diabetic rats. These data suggest that garlic can correct disorders obtained after the IR-HIO and induces more responsiveness to insulin effects which regulates glucose and lipid metabolic reactions and reduces body weights.

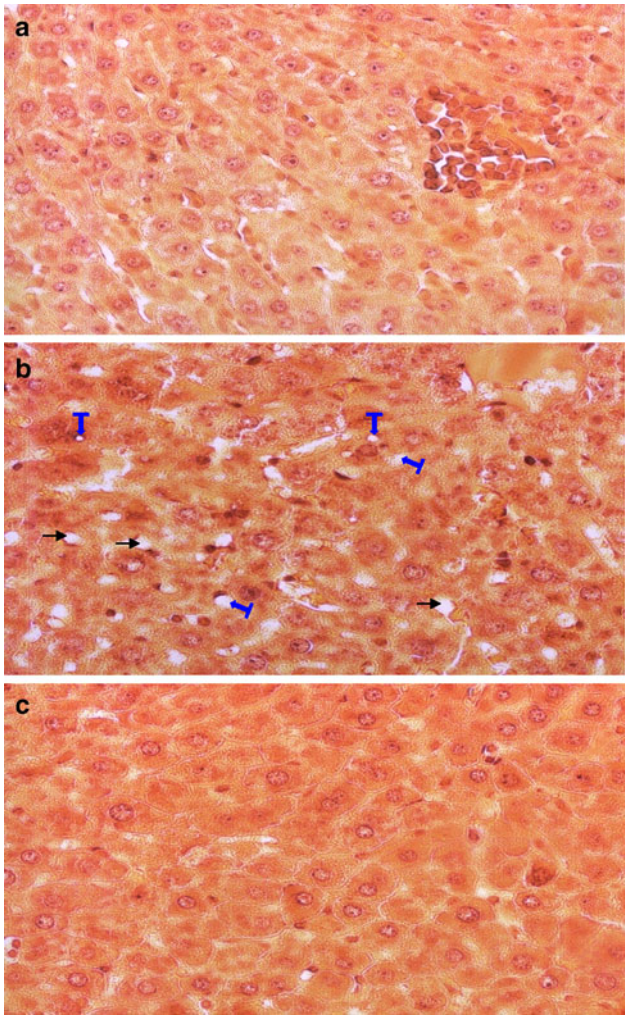

Fig. 3 Haematoxyline eosin liver-stained sections of young rats: controls (**a**) and treated either by $FeCl_2$ (**b**) or by both $FeCl_2$ and garlic (**c**) for a period of 45 days. ⟶ indicates sinusoid congestion, T indicates steatosis

Furthermore, we had found in group B rats an increase in blood iron levels suggesting an increase in intestinal iron absorption. In fact, iron was added in drinking water and to reach blood, iron must get over intestinal barrier. Intestinal iron absorption is regulated by several distinct signals [6]. In normal conditions, the regulatory mechanism involves sensing of iron stores, oxygen levels, plasma iron and/or putative erythroid factors by liver, which in turn, produces varying amounts of the peptide hepcidin, a negative regulator of duodenal iron absorption [31]. In fact, in case of iron blood excess hepcidin liver secretion is negatively regulated inducing an increase of iron intestinal absorption. Indeed, during iron overload, a miss of the regulation of hepatic genes occurs, some of them including hepcidin [32]. Moreover, it had been approved by many previous reports that the addition of Fe salts to the apical compartment of Caco-2 intestinal cells cultures increased cellular content of

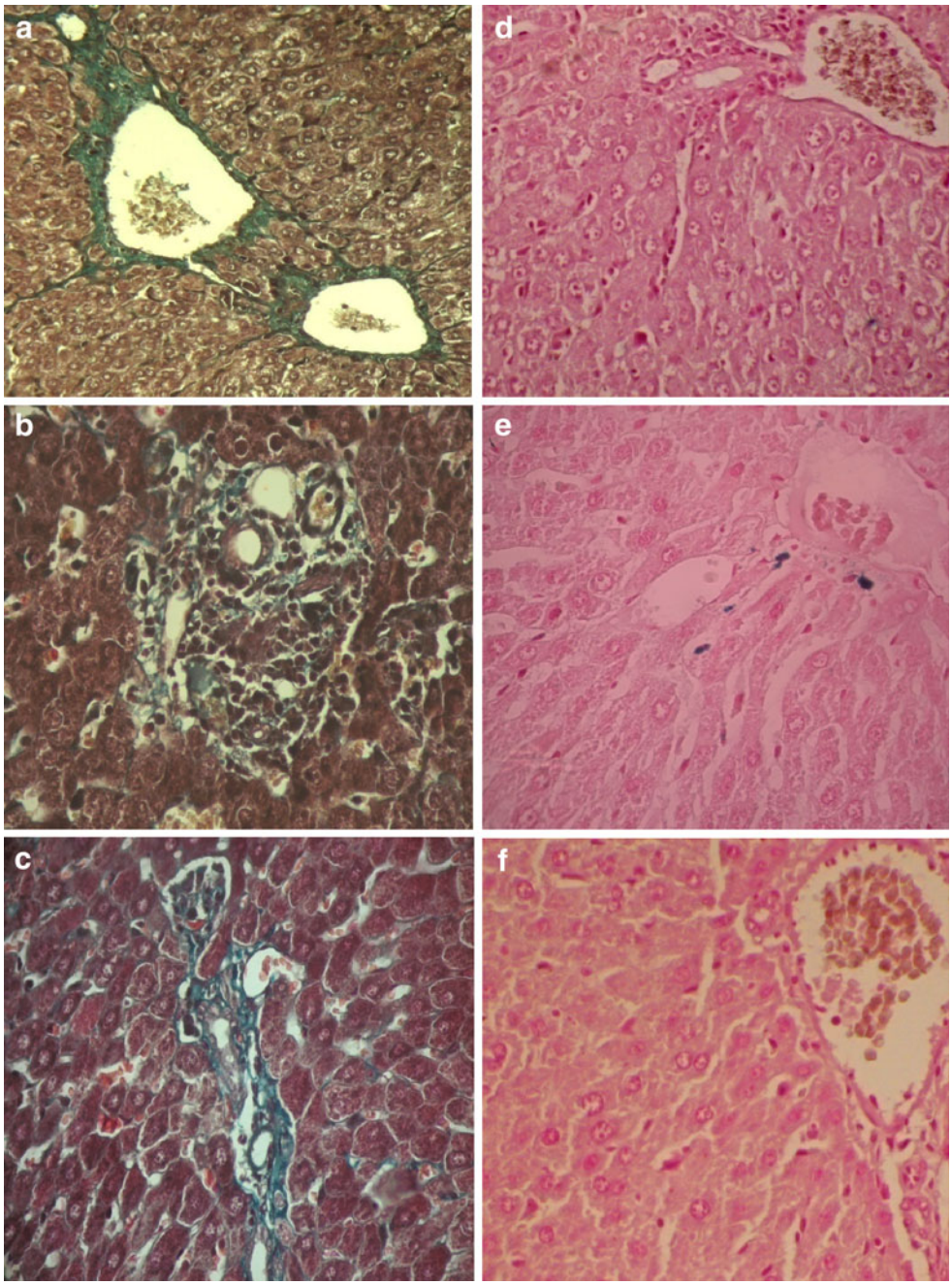

Fig. 4 Masson trichrome (**a**, **b**, **c**) and Perl's Prussian blue (**d**, **e**, **f**) stained liver sections of young rats: controls (**a**, **d**) and treated either by $FeCl_2$ (**b**, **e**) or by both $FeCl_2$ and garlic (**c**, **f**) for a period of 45 days

ferritin [33] and induced increase in iron intestinal absorption.

For group C rats, garlic started by exerting its regulatory effects on iron excess from intestine. In fact, we had obtained a decrease in serum iron levels. This may be explained by the fact that garlic is rich in polyphenolic compounds that are expected to inhibit iron absorption by forming iron complexes in the intestine, making dietary iron less available for absorption [34].

In the blood, iron is transported to the various organs especially those that use iron for heme production and that acquire and store iron [35]. The potential for iron damage is particularly high in liver, the primary organ for storage of excess iron [5]. In fact, we had found an increase of liver damage biomarkers as ALAT, ASAT and in LDH serum levels of group B-treated rats, which could be correlated to hepatocytes suffering and injuries. Moreover, we had found a decrease in liver total antioxidant capacity of iron-treated group rats. This result could be explained by important ROS production [36, 37]. Indeed, ferrous (Fe^{2+}) irons react through the Fenton reaction with hydrogen peroxide to produce ferric (Fe^{3+}) iron and highly reactive hydroxyl

radicals. This reaction is of particular importance in the liver because this organ has high steady-state production of O_2 and H_2O_2 from abundant mitochondrial activity [38]. As a result, the cellular oxidative stress induced by iron excess could cause alterations on membrane lipids, proteins and DNA favouring mitochondrial impairment, ATP disorders and genetic mutation, such as the mutation in p53 gene, a key gene of cell cycle control [39].

In group C rats, important increase in liver total antioxidant capacity and notable regulation of ASAT, ALAT and LDH were obtained indicating a hepato-protective effect exerted by garlic chemical components on liver cells. Indeed, it had been demonstrated by Thomas and his collaborators [15] that the organosulphur compound, diallyl disulphide (DADS), derived from garlic increased H-ferritin, L-ferritin and transferrin receptor genes in vitro in hepatic cells and in vivo in rat liver. Ferritin plays a key role in iron storage and detoxification by sequestering intracellular iron, and transferrin is involved in iron transport via endocytosis in hepatocytes [15]. In addition to its regulator effect on iron hepatocyte internalization and use, one of the most important biochemical properties of garlic is its antioxidant potential [40]. In cells, garlic plays direct and indirect effects on oxidative stress. It directly abolishes oxidative stress by increasing intracellular antioxidant compounds' content such as allicin, s-allylcysteine, diallylsulfide (DAS), DADS and diallyltrisulfide (DATS) [41, 42] and, indirectly, increases the levels of cellular antioxidant enzymes, such as superoxide dismutase, catalase, GSH-peroxidase and scavenging ROS [43]. As a result, better protection against liver oxidative damage and suffering was obtained in group C rats.

Liver weights' increase of group B rats may be explained by an increased mitotic activity. In fact, a number of agreements suggest that cycle control is disturbed during iron overload. Modifications of hepatocyte ploidy as well as an increase in liver volume have been frequently reported in vivo during iron overload [44, 45]. Furthermore, the hypertrophy of group B liver rats may be due to an increase in liver tissue water content. In fact, after HE-staining liver sections, we had observed, in contrast to control and group C rats, congested sinusoids suggesting an increase in water content. This result confirmed the data of Silva and his collaborators [46] who had obtained intense hydropic degeneration of hepatocytes after iron dextran rat treatments. Moreover, liver weights' increase may be explained by the improvement of collagen biosynthesis. Indeed, we had observed in the liver sections after trichromic coloration, portal system enlargement with increase in collagen mass and inflammatory cells invasion. This aspect confirmed previous data of Gardi and his collaborators [27] who had demonstrated that iron can enhance collagen synthesis. In fact, iron is essential for normal collagen synthesis since it is required as cofactor for prolyl-hydroxylase [47], and increased activity of this enzyme has been reported in various models of iron overload [48].

All these morphological and biochemical liver perturbations are due to important iron liver storage. Indeed, we had found, after mineral analysis, an increase in iron liver content of group B rats. This result confirmed liver histological results that show, after Perl's Prussian blue coloration, blue kuppfer cells staining which evidenced excess iron accumulation. The same result was obtained by Silva and his collaborators [46] who had observed blue kuppfer cells' coloration after Perl's Prussian blue coloration of liver sections of iron dextran-treated rats.

Another important histological aspect that was noted after the HE coloration of group B rats liver sections is the beginning of steatosis. This histological aspect was associated to increase in triglyceride serum levels. According to these findings, our experimental model with a period of 45 days $FeCl_2$ treatment is sufficient to increase lipid biosynthesis and to begin steatosis, which is characterized by accumulation of lipid droplets and triglyceride in liver cells [49]. This result confirmed the one obtained by Sorrentino and collaborators [50]who had found an association between increased hepatic iron sotres in non-alcoholic steato-hepatits (NASH) and HCC.

For group C-treated rats, liver steatosis and triglyceride blood levels were corrected compared with group B indicating that garlic chemical compounds prevent liver lipid and triglyceride accumulation. Indeed, garlic is endowed with hypocholesterolemic, hypoglycemic and hypolipidemic properties, and ingestion of garlic inhibit hepatic fatty acid synthesis by lowering the key enzymes [51]. The decrease in serum triglyceride levels obtained in group C-treated rats may be explained by a regulation of glucose metabolic reactions.

In conclusion, the association between the curative effects exerted by garlic chemical compounds and iron excess is a new approach in which we had tried to achieve better understanding and to get solutions for some physiological liver disorders not yet well known.

Acknowledgments The authors are indebted to Mrs Ines RGAIG, Miss Najet MEZGHANNI, Mr Slim LOUKIL and Mr Adel GARGOUBI for their skilful technical assistance. This research was supported by the Laboratory of Physiology in Superior Institute of Biotechnology of Sfax Tunisia and the Laboratory of Environnemental Bioproceeds in Center of Biotechnology by EEC contract ICA3-CT2002-10033, and the 'Contracts Programmes SERST', Tunisia.

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