Influence of garlic or its main active component diallyl disulfide on iron bioavailability and toxicity

Afef Nahdia,a,b Imen Hammamia,b Carole Brasse-Lagnelc, Nathalie Pilard,b Mohamed Hedi Hamdaouic, Carole Beaumontb,⁎, Michèle El Maya

aUnité de recherche n° 01/UR/08-07, Laboratoire d'histologie-embryologie et biologie cellulaire, Faculté de Médecine de Tunis, 1007 Tunis, Tunisie
bINSERM U773, Université Paris Diderot Paris 7, UFR de médecine site Bichat, 75018, Paris, France
cUnité de Recherche sur l'Anémie Nutritionnelle et la Biodisponibilité des Oligoéléments, Ecole Supérieure des Sciences et Techniques de la Santé de Tunis, BP 176 Bab-Souika, Tunisie

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Abstract

Garlic is regularly consumed and is known to have diverse biologic activities, particularly due to its antioxidant properties. In this study, we hypothesized that crude garlic can prevent iron-mediated oxidative stress in a rat model of nutritional iron overload, and we used an in vitro model to confirm the results. For the in vivo studies, rats received a basal diet supplemented with or without carbonyl iron (3%) and were fed distilled water or garlic solution (1g/kg body weight) by gavage for 3 weeks. The presence of both garlic and iron led to a 2-fold increase in plasma iron and a 50% increase in liver iron as compared with iron alone. However, garlic did not offer any protection against iron-induced oxidative stress. Duodenal divalent metal transporter-1 mRNA expression was fully repressed by iron and by the combined treatments but was also reduced by garlic alone. To confirm these data, we tested the effect of diallyl disulfide, one of the active components in garlic, in vitro on polarized Caco-2 cells. A 24-hour treatment decreased iron uptake at the apical side of Caco-2 cells but increased the percentage of iron transfer at the basolateral side. This probably resulted from a modest induction of ferroportin mRNA and protein expression. These results suggest that garlic, when given in the presence of iron, enhances iron absorption by increasing ferroportin expression. The presence of garlic in the diet at the dose studied does not seem to protect against iron-mediated oxidative stress.

Keywords: Iron absorption; Ferroportin; Garlic; Oxidative stress; Rats; Caco-2 cells

Abbreviations: AOPP, advanced oxidation protein products; DADS, diallyl disulfide; DMSO, dimethyl sulfoxide; DMT1, divalent metal transporter-1; Fe-NTA, iron-nitrilotriacetate; FPN1, ferroportin-1; MDA, malondialdehyde; PBS, phosphate-buffered saline; PIs, protease inhibitors; RT-qPCR, real-time quantitative polymerase chain reaction.

1. Introduction

Iron, the most abundant transition metal in the body, is required by all mammalian cells for growth and survival. However, excess iron in the body is potentially toxic, and iron-mediated oxidative stress is thought to be a significant factor in the pathogenesis of iron-overload disease. Iron-catalyzed lipid peroxidation promotes the formation of highly reactive aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal [1]. These molecules form covalent links to proteins, phospholipids, and DNA and cause considerable tissue damage.

In humans, iron homeostasis is based on the tight control of intestinal iron absorption and recycling of heme iron by tissue macrophages. There is no physiologic system to
eliminate the iron absorbed in excess of body requirements and iron losses only result from sloughing of epithelial cells in addition to menstrual blood loss in women [2]. Two crucial proteins are involved in intestinal iron absorption: divalent metal transporter-1 (DMT1) present at the apical membrane of duodenal enterocytes, exerting iron uptake into the cell and ferroportin-1 (FPN1) present in the basolateral membrane, exporting iron to the circulation. The amount of iron absorbed from the diet is influenced by multiple factors including iron content of the diet, the level of iron stores and the rate of erythropoiesis, inflammation, and pregnancy [3].

Iron homeostasis is mainly regulated by hepcidin, a peptide secreted by the liver in response to iron loading [4,5] and inflammation [6]. On the reverse, iron-deficiency anemia, stimulation of erythropoiesis, hypoxia, and dyserythropoietic disorders, all these conditions have been shown to fully repress hepcidin gene expression (see Darshan and Anderson [7] for review). This protein regulates plasma iron availability by limiting duodenal iron absorption and release of iron by macrophages through binding to ferroportin, inducing its internalization and degradation [8,9].

Garlic (Allium sativum) is a frequently used plant in Mediterranean cooking. In Tunisia, it is regularly consumed at various doses both crude and cooked, and its potential medical properties have been recognized for thousands of years [10,11]. In addition, garlic plays therapeutic effects such as in treatment of hypercholesterolemia [12,13], prevention of atherosclerosis [14,15], and of some cancers [16,17]. It also presents anticoagulant [18,19] and antihypertensive [20] properties. In contrast, excess consumption of garlic has been reported to cause toxicity, including anemia and gastrointestinal problems [21]. Some laboratories have reported that powder [22] or crude [23,24] garlic preparations altered male reproductive tract function.

Its impact on iron metabolism has only been poorly investigated. Some studies have reported that garlic oil suppresses iron-nitrilotriacetate (Fe-NTA)–mediated hepatic oxidative stress, tumor promotion, and toxicity [25]. Others also observed that pretreatment of animals with garlic oil attenuates oxidative stress, toxicity, and hyperproliferative response mediated by Fe-NTA in rat kidney [26].

In the present study, we hypothesized that garlic can prevent iron-induced oxidative damage. We investigated the effects of chronic administration of crude garlic on intestinal iron absorption in rats fed a normal diet or an iron-supplemented diet and on the parameters of oxidative stress. In addition, to get a better insight into the effects of garlic at the intestinal level, we studied iron absorption and transfer in Caco-2 cells grown in normal condition (ie, without iron supplementation) using diallyl disulfide (DADS), one of the main active [17] and the most studied components of garlic. Caco-2 cells were selected because they have an enterocyte-like phenotype, expressing numerous small intestinal brush border enzymes and nutrient transporters [27,28]. In addition, this cell line expresses the key iron transport proteins, including DMT1 [29] and FPN1 [30], and is the most commonly used model to study iron transport. Therefore, because garlic is largely used in Mediterranean cooking, it is important to evaluate its impact on iron absorption and toxicity.

2. Methods and materials

2.1. Plant extract

The variety of garlic (Allium sativum) used in the present study has pink bulbs and was purchased from a local market. This type of garlic contains 2.1% of proteins, 30% of carbohydrates, 1.5% of fibers, 0.2% of fat, 0.015% of vitamins, and 0.7% minerals. Everyday, cloves were peeled, sliced, ground to a paste, and then suspended in distilled water to 0.3 mg/mL to minimize volatile compound loss. The homogenate was centrifuged at 3000 × g for 10 minutes to remove particulate matter, and the supernatant fraction was used for the experiment.

2.2. Animals and diets

Twenty male Wistar rats weighing between 180 and 200 g were purchased from Charles River Laboratories (L’Arbresle, France), housed in stainless steel cages, and maintained in a controlled environment under standard conditions of temperature (25 ± 2°C), humidity, and light (12-hour light/dark cycle, lights on 0700-1900). The rats were allowed to acclimatize in the animal facility for a period of 1 week before the beginning of the study. During that period of time, they received a commercial pellet diet (SAFE, Augy, France) and water ad libitum. Animals were cared in accordance with criteria outlined in the European Convention for the Protection of Laboratory Animal, and the study had obtained institutional approval.

The diet [31] used during the experiment was prepared every 3 days by thoroughly mixing the ingredients and chemical compounds (Table 1) to obtain a homogeneous paste that was dried at 45°C, sliced, and stored at 4°C for a short time. The basal diet contained 50 mg Fe/kg diet as FeSO₄ 7H₂O. This iron supply corresponds to recommended supply in growing rats and is slightly lower than recommended supplies in humans [32]. For the iron-treated group, this diet was supplemented with 3% carbonyl iron (Sigma Chemical Co, St Louis, Mo). A fixed amount of distilled water was given daily to the animals by gavage, containing or not the garlic extract (1 g/kg body weight).

2.3. Experimental protocol

To investigate the effects of chronic administration of crude garlic on iron metabolism and oxidative stress, rats were weighed and randomly assigned into 4 groups of 5 animals each and treated for 3 weeks as follows:

Control group received the basal diet and was fed distilled water by gavage once a day.
Table 1

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Content (g/kg diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Powder skim milk&lt;sup&gt;a&lt;/sup&gt;</td>
<td>400</td>
</tr>
<tr>
<td>Corn oil</td>
<td>55</td>
</tr>
<tr>
<td>Maize starch</td>
<td>300</td>
</tr>
<tr>
<td>Sucrose</td>
<td>180</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>1</td>
</tr>
<tr>
<td>Choline</td>
<td>1.5</td>
</tr>
<tr>
<td>CaCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>20</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;P&lt;sub&gt;4&lt;/sub&gt;</td>
<td>20</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
</tr>
<tr>
<td>KCl</td>
<td>5</td>
</tr>
<tr>
<td>Vitamin mixture&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>Mineral mixture&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.3</td>
</tr>
<tr>
<td>FeSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> As source of protein.

<sup>b</sup> Vitamin mixture (unit/kg of mixture): synthetic vitamin A concentrate, 250 000 UI; cholecalciferol, 50 000 UI; α-tocopherol acetate, 0.100 g; thiamin hydrochloride, 0.100 g; riboflavin sodium phosphate, 0.075 g; pyridoxine hydrochloride, 0.100 g; ascorbic acid, 2.500 g; nicotinamide, 0.500 g; dexpanthenol, 0.200 g.

<sup>c</sup> Mineral mixture without iron (g/100 g mineral salt): MgSO<sub>4</sub>7H<sub>2</sub>O, 73.82; ZnSO<sub>4</sub>7H<sub>2</sub>O, 19.66; MnSO<sub>4</sub>H<sub>2</sub>O, 4.22; CuSO<sub>4</sub>5H<sub>2</sub>O, 0.73; KIO<sub>3</sub>, 0.06; Na<sub>2</sub>SeO<sub>3</sub>, 0.9.

Iron group received the basal diet supplemented with carbonyl iron (3%) and was fed distilled water by gavage.

Garlic group received the basal diet and was given garlic extract (1g/kg body weight) by gavage once a day.

Garlic + iron group received the basal diet supplemented with carbonyl iron (3%) and was orally treated with garlic solution (1g/kg body weight).

The animals received a fixed amount of food everyday, and the remaining food was weighed to calculate the daily food intake per group.

At the end of the experimental period, the rats were weighed, anesthetized by isofluorane (intrapertoneally), and killed. Blood was immediately collected in heparinized vials and centrifuged at 3,000 × g for 15 minutes. Plasma was stored frozen at −80°C until use. Liver, heart, spleen, and kidneys were dissected out, weighed, and sectioned, and then tissue fragments were kept at −80°C. Duodenum was removed, rinsed with cold 0.9% NaCl, and opened lengthwise, and mucosa was scraped from the organ with a clean glass microscope slide. This manipulation was performed on ice. Mucosa was immediately suspended in RNA extraction solution and frozen at −80°C.

2.4. Iron metabolism

Plasma iron and transferrin concentrations were quantified using an Olympus AU400 automate (Beckman-Coulter, High Wycombe, UK). Transferrin saturation was determined using the following formula: serum iron (μmol/L)/transferrin concentration (g/L) × 4.

Tissue iron content was determined by acid digestion of tissue samples as described previously [33], followed by iron quantification using an IL test (Instrumentation Laboratory, Lexington, Mass) on an Olympus AU400 automate.

2.5. Markers of oxidative stress

Plasma advanced oxidation protein products (AOPPs) were assayed as described [34]. Briefly, plasma diluted in phosphate-buffered saline (PBS) and chloramne standard solutions were mixed with 1.16 M potassium iodide solution. After incubation for 6 minutes, pure acetic acid was added and optical density was read at 340 nm (with a reference filter at 490 nm). Advanced oxidation protein product content in samples was determined by comparison with the predetermined chloramine standard curve.

The levels of lipid peroxidation products thiobarbituric acid–reactive substances, mainly MDA, were assessed in tissues according to the method of Buege and Aust [35], and protein content in supernatants was determined using Bradford method [36].

2.6. Histology

Liver tissue was fixed in formalin, routinely processed, and embedded in paraffin. Three-micrometer-thick paraffin sections were stained with Perls’ Prussian Blue method for iron visualization.

2.7. Caco-2 Cell cultures

To confirm the effect of garlic on intestinal iron absorption, we used an in vitro model of polarized cultured Caco-2 cells treated with one of the most active compounds of garlic: DADS.

Caco-2/TC7 cell line was obtained from Dr M. Rousset (INSERM U505, Paris, France) and was grown at 37°C in 5% CO<sub>2</sub>, 95% air incubator. Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen-Gibco, Cergy-Pontoise, France) supplemented with 10% fetal calf serum, 1% nonessential amino acids, 2 mmol glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin was used. Cells were seeded at a density of 12 × 10<sup>3</sup> cells/cm<sup>2</sup> onto 100-mm dishes. After reaching 80% to 90% confluence, cells were trypsinized and seeded onto permeable membrane filter supports (Transwell, 24- or 12-mm diameter, 0.4-μm pore size; Corning, Life Sciences, NY) and fed every 2 days with supplemented DMEM. The cells were grown for 11 days after confluence, and serum-free DMEM was added 24 hours before experiments. They were then cultured for 24 hours in the absence or in the presence of dimethyl sulfoxide (DMSO; 0.1%) or DADS (100 μmol in DMSO). Diallyl disulfide (CH<sub>2</sub> = CHCH<sub>2</sub>SSCH<sub>2</sub>CH = CH<sub>2</sub>; purity 80%) and DMSO were purchased from Sigma-Aldrich (Lyon, France).

2.8. Iron uptake studies

Caco-2 cells were grown on 12-mm permeable membrane filter, and formation of a monolayer of confluent cells was checked by measuring the transepithelial electrical resistance with an epithelial volt-ohmmeter (Millicell-ERS,
Millipore, France). At confluence, normal transepithelial electrical resistance values were greater than 310 Ω/cm². Twenty-four hours before iron absorption studies, cells were incubated with DMSO 0.1% or DADS 100 μmol in DMSO added on their apical side. Then the medium was removed, and cells were rinsed 3 times with Hank’s Balanced Salt Solution (HBSS) pH 6.5 on the apical side and with HBSS pH 7.5 on the basal side. A 10-μmol solution of iron was added at the apical side, consisting in a mixture of $^{55}$FeCl₃ (10 μCi/mL; PerkinElmer, Boston, Mass) and cold Fe-NTA (FeCl₃ mixed with a 4-fold molar excess of nitrilotriacetic acid) dissolved in HBSS adjusted to pH 6.5. After 120-min incubation, aliquots were removed from the basal and apical chambers, media were discarded, and the cell monolayer was rinsed twice with radio immuno precipitation assay (RIPA) buffer (50 mmol Tris pH 8, 150 mmol NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate) and scrapped into 500 μL of RIPA buffer. Aliquots of medium and the whole cell lysates were then subjected to scintillation counting to determine iron uptake (liquid scintillation analyzer TMI-CARB 2300 TR, PACKARD). Total iron absorption was calculated as the total counts in both the cell layer and the basal chamber. The percentage of iron transfer was calculated as the ratio of the total amount of iron present in the basal chamber at the end of the incubation period to the total iron absorption.

2.9. Western blotting

Caco-2 cells were washed once with PBS and scraped gently in a cold fresh solution of PBS/EDTA 2 mmol. After centrifugation for 5 minutes at 1400 rpm in the cold, the supernatant was removed and the cell pellet was resuspended in lysis buffer (10 mmol Tris-HCl pH 7, 1 mmol MgCl₂) containing protease inhibitors (Pis; Sigma P 8340 PIs cocktail) and phenylmethanesulphonylfluoride (PMSF) (10 μg/mL). After 3 freeze-thaw cycles, cell suspension was centrifuged at 3500 rpm for 10 minutes at 4°C; the supernatant containing microsomal and cytosolic fractions was again centrifuged at 75 000 revolutions/min (Beckman TLA100 rotor) for 45 minutes to separate the crude membrane fractions from the cytosolic proteins. Membrane pellets were resuspended in tris NaCl EDTA (TNE) buffer (100 mmol NaCl, 10 mmol Tris-HCl pH 7, and 10 mmol EDTA) containing PIs and PMSF. Fifteen micrograms of proteins of microsomal fraction was used for immunoblotting. Proteins were diluted in Laemmli buffer and loaded onto a 12% sodium dodecyl sulfate–polyacrylamide gel and electroblotted onto polyvinylidene fluoride (PVDF) membrane. The blots were then incubated with rabbit FPN antibody (dilution 1:3000), a kind gift from D. Haile, San Antonio, Tex, followed by incubation with peroxidase conjugated AffiniPure goat antirat immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA; dilution 1:30 000). Ferroportin was revealed using an enhanced chemiluminescence detection system (Immobilon Western kit; Millipore). The protein band was assessed according to its apparent molecular weight compared with standard proteins (Protein Ladder Plus; Fermentas Life Sciences, Saint-Rémy-lès-Chevreux, France). As an internal control, blots were also incubated with an anti β-actin antibody (Sigma-Aldrich).

2.10. RNA extraction and quantitative real-time polymerase chain reaction

Total RNAs were extracted from tissue or cultured cells using RNA-PLUS reagent (Q-Biogene, Europe) according to the manufacturer’s recommendations. The cDNAs were obtained by reverse transcription of 1 μg of total RNAs in a final volume of 100 μL. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed in duplicate with the CHROMO IV Detector (MJ Research, Boston, Mass) using SYBR Green RCR according to the manufacturer’s protocol. Amplification of specific transcripts was confirmed by melting curves profiles generated at the end of the PCR program. Primers used for amplification are indicated in Table 2. From tissue, each target gene (R) was normalized arbitrarily on Ct value for β-actin (ΔCt = CtR – Ctβactin) or ribosomal S14 mRNA. The relative amount of R mRNA levels between treated groups is given by $2^{-ΔΔCt}$, where $ΔΔCt = [ΔCt(R) of treated group] – [mean of ΔCt(R) of control group]. Relative gene expression is normalized to 1.0 (100%) of controls.

2.11. Statistical analyses

Results are expressed as the means ± SD. Statistical analyses were performed using SPSS 10.0 for Windows (SPSS, Chicago, Ill). For the in vivo study, to determine whether there were differences between all groups, a Kruskal-Wallis test was performed and was followed by a nonparametric Mann-Whitney U test to determine significant ($P < .05$) differences between pairs of groups (n = 5). For the in vitro study, a 1-way analysis of variance test was performed to determine whether there were differences between all groups, and it was followed by Bonferroni post hoc test if $P < .05$ to determine the significant differences between the 2 groups.

**Table 2**

<table>
<thead>
<tr>
<th>mRNA name</th>
<th>Primer names</th>
<th>Primer sequences (forward and reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>Rat actin</td>
<td>5′ ATCGTGCGCCGCCTAGGCACCA 3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′ TTGGCGCTTACGGTTAGGAGGG 3′</td>
</tr>
<tr>
<td>DMT1</td>
<td>Rat</td>
<td>5′ GCCTCGTGTCTTCGGACT 3′</td>
</tr>
<tr>
<td></td>
<td>IRE-DMT1</td>
<td>5′ GCTGTATCTCCGCTCAAGCAG 3′</td>
</tr>
<tr>
<td></td>
<td>Hum</td>
<td>5′ CTTCATATCCTGCTCTTCCCC</td>
</tr>
<tr>
<td></td>
<td>IRE-DMT1</td>
<td>5′ AAATCCTGAGACTGACTGGACC</td>
</tr>
<tr>
<td>Ferroportin</td>
<td>Hum FPNI</td>
<td>5′ GGAAGTCACAACCGCCAGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′ AAGGAAATTTTGGAGGCTCAGG</td>
</tr>
<tr>
<td>S14 ribosomal protein</td>
<td>Hum S14</td>
<td>5′ CAGGACCAAGACCCCCTGGA 3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′ ATCTTCATCCCCAGACGCAGACG 3′</td>
</tr>
<tr>
<td>Hepcidin</td>
<td>Rat Hepe</td>
<td>5′ TCTTCTTTCTTGCCAGG 3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′ TCTGCCCTGGTCTTCCC 3′</td>
</tr>
</tbody>
</table>
3. Results

3.1. Effect of garlic and/or iron administration on body weight

We induced nutritional iron overload in rats using carbonyl iron in the diet. The animals were fed an iron supplemented diet for 3 weeks, with or without garlic supplementation. All the body weights were increased at the end of the treatment. The presence of iron in the diet significantly reduced the food intake during the 3-week period from 637 ± 15 g in the controls to 511 ± 32 g (P = .008) in the iron group and 425 ± 54 g (P = .008) in the garlic + iron group. Therefore, the body weight gain of the rats was lower with iron alone (73 ± 25 g; P < .01) and with garlic + iron (21.2 ± 14 g; P = .01) than in control group (153 ± 10 g). The presence of garlic alone barely reduced the food intake (611 ± 20 g versus 637 ± 15 g), and consequently, the weight gain was only moderately attenuated with garlic alone compared with control (133 ± 7.5 versus 153 ± 10 g; P = .01).

3.2. Effect of garlic administration on serum and tissues iron concentrations

Iron alone induced a significant increase in serum iron and transferrin saturation as well as a more than 10-fold increase in liver iron. There was also a 3-fold increase in spleen iron and only a moderate increase in heart or kidney iron content (Table 3). Interestingly, the presence of both iron and garlic induced a 2-fold increase in serum iron and a transferrin saturation above 100%. There was also a noticeable, although not significant, increase in transferrin saturation in the presence of garlic alone, suggesting that garlic increases intestinal iron absorption. Consequently, the presence of both iron and garlic induced a significant increase (1.5-fold) in the extent of iron overload in liver as compared with the iron group but did not aggravate the iron burden in the other organs. In the presence of garlic alone, there was a slight significant increase in heart iron level (Table 3).

Observation of Perls-stained liver sections from rats receiving iron alone or garlic + iron showed that iron was predominantly deposited in the periportal hepatocytes with a more pronounced accumulation in the combined group (Fig. 1C, D) than in the iron supplemented group (Fig. 1A, B). Rare iron-overloaded Kupffer cells (hepatic macrophages) were also present (Fig. 1B, D; arrow heads) but their contribution to liver iron load was very low.

3.3. Effect of garlic administration on markers of oxidative stress

Iron in excess is known for inducing oxidative stress and garlic for exerting either antioxidant or oxidant properties depending on the ingested doses. Hence, we tested their respective effects using AOPP as markers of protein oxidative damage mediated by excess plasma iron. We found that only the combination of garlic and iron increased plasma AOPP, probably as a result of the important increase in serum iron (Table 3). Tissue iron overload was accompanied by enhancement in lipid peroxidation as shown by a significant increase in tissue MDA concentrations in iron supplemented group (Fig. 2, left panels), in all tissues except the spleen. In the liver, there was a perfect correlation between MDA level and tissue iron content ($R^2 = 0.67$), when the results of the 4 groups were combined (Fig. 2, on the right). This suggests that the presence of garlic in the diet does not offer protection against iron-mediated oxidative damage. In the heart, there was a 2-fold increase in MDA content (Fig. 2, on the left), despite a very modest increase in tissue iron (Table 3), suggesting that the heart is a more sensitive organ to iron-mediated toxicity than the liver. In the kidney, there was only a moderate increase in MDA in the treated groups (Fig. 2, on the left) and a significant correlation ($R^2 = 0.27$) between MDA level and kidney iron content (Fig. 2, on the right).

3.4. Effect of garlic and/or iron administration on liver hepcidin and duodenal DMT1 expressions

Nutritional iron overload is known to induce hepcidin gene expression [5]. Indeed, we found a robust increase in hepcidin mRNA level after iron ingestion (Fig. 3A), and

<table>
<thead>
<tr>
<th>Groups (n = 5)</th>
<th>Serum iron (μmol)</th>
<th>Transferrin saturation * (%)</th>
<th>Plasma AOPP (μmol)</th>
<th>Liver iron (μg Fe/g tissue)</th>
<th>Kidney iron (μg Fe/g tissue)</th>
<th>Spleen iron (μg Fe/g tissue)</th>
<th>Heart iron (μg Fe/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26.7 ± 3.2</td>
<td>39.0 ± 10.4</td>
<td>210.5 ± 67</td>
<td>63.6 ± 7.6</td>
<td>59.4 ± 3.3</td>
<td>296.2 ± 21.1</td>
<td>72.4 ± 5.1</td>
</tr>
<tr>
<td>Iron</td>
<td>41.3 ± 8.2 *</td>
<td>80.0 ± 24.3 A</td>
<td>149.4 ± 52</td>
<td>998.8 ± 45.4 A</td>
<td>84.8 ± 7.7 A</td>
<td>1040.7 ± 84.5 A</td>
<td>105.7 ± 13.6 *</td>
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<tr>
<td>Garlic</td>
<td>33.8 ± 7.8</td>
<td>63.8 ± 19.6</td>
<td>162.4 ± 19</td>
<td>70.6 ± 3.1</td>
<td>59.7 ± 1.6</td>
<td>184.0 ± 16.3</td>
<td>88.4 ± 2.7 A</td>
</tr>
<tr>
<td>Garlic +Iron</td>
<td>79.8 ± 4.9*A,B,C</td>
<td>136.8 ± 14.4 A,B,C</td>
<td>350.6 ± 177</td>
<td>1535.2 ± 105.4 A,B,C</td>
<td>116.0 ± 15.1 A</td>
<td>1122.0 ± 106.2 A,B,C</td>
<td>101.1 ± 10.4*A,B</td>
</tr>
</tbody>
</table>

Control group and garlic group received the basal diet. Both iron groups (with and without garlic) received the basal diet supplemented with carbonyl iron (3%). All animals were fed distilled water by gavage, with or without garlic extract (1g/kg body weight). Treatments lasted for 3 weeks. Results correspond to the means ± SD of 5 animals. The different pairs of groups were compared using the Mann-Whitney U test.

* Transferrin saturation was calculated using the formula serum iron (μmol/L)/Tf (g/L) × 4.
* P = .01, A: .01 < P < .05 versus control.
† P = .02, B: .02 < P < .05 versus iron-overloaded rats.
‡ P = .01, C: .01 < P < .05 versus garlic-treated rats.
there was no impairment when garlic was present in addition to iron.

To try to elucidate the origin of the increased iron overload observed in the presence of both iron and garlic, we measured isoform I DMT1 mRNA expression in the duodenum. There are 4 DMT1 isoforms, resulting from alternative splicing and the use of 2 alternative upstream promoters [37]. We limited our analysis to the mRNA encoding isoform I, the predominant isoform in the duodenum. Iron, with or without garlic supplementation, fully repressed DMT1 expression (Fig. 3B). In addition, we found a significant reduction ($P = .01$, Mann-Whitney U test) in DMT1 expression in garlic-treated group as compared with control.

3.5. Effect of DADS on iron absorption and protein expression in Caco-2 cells

Considering that duodenum DMT1 expression was repressed in garlic-treated rats, we wanted to confirm the effect of garlic (or one of its constituents) on iron absorption using an in vitro model of cultured Caco-2 cells. Diallyl disulfide was used instead of garlic because it is one of the most biologically active compounds [17] found in crude garlic. A monolayer of confluent Caco-2 was challenged with DADS (100 μmol) for 24 hours. Iron absorption and transfer were studied using $^{55}$Fe-NTA. Treatment with DADS resulted in a significant reduction in the total amount of iron absorbed at the apical side during a 120-minute incubation period ($P = .02$, Bonferroni post hoc test) as compared with the control group (DMSO-treated cells), as shown in Fig. 4A. However, there was an increase in the percentage of iron transferred to the basolateral side of the cells after incubation with DADS for 24 hours ($P < .01$, Bonferroni post hoc test), indicating that more iron was released by cells toward the basal chamber (Fig. 4B).

To investigate whether DADS-induced changes in iron absorption or transfer were mediated by changes in
expression of iron transporter proteins, isoform 1 DMT1 and FPN1 expression were determined in Caco-2 cells grown with normal iron concentrations.

Stimulating Caco-2 cells with DADS induced no change in DMT1 mRNA level (Fig. 4C) but resulted in a significant increase in ferroportin expression, both at the
mRNA (P < .01, Bonferroni post hoc test, Fig. 4D) and protein level (Fig. 4E).

4. Discussion

In the present study, we investigated the influence of garlic (or its main active component, DADS) on iron bioavailability and toxicity using in vivo (rats fed iron adequate diet or 3% iron carbonyl supplemented diet) and in vitro (the human intestinal Caco-2 cells) models. We chose carbonyl iron as a source of iron because it is a frequently used compound in nutritional iron-overload models. We found that garlic increased iron absorption as shown by increased plasma iron levels and higher degree of liver iron overload when iron was given in the presence of garlic. Under our conditions, the presence of garlic in the diet did not bring about any obvious protection against iron-mediated oxidative stress.

There are several possible mechanisms by which garlic can increase intestinal iron absorption. First, it can contribute to iron mobilization from the diet acting either as a chelator or as a reducing agent [38]. When a garlic clove is crushed, the odorless precursor alliin is rapidly converted into allicin, which decomposes instantly into other oil-soluble compounds, such as diallyl sulfide, DADS, diallyl trisulfide, and ajoene [39]. At the same time, S-allylcysteine is formed via a pathway other than alliin/allicin pathway [39]. All these compounds of garlic are biologically active [17]. Any of these components can theoretically increase iron bioavailability form carbonyl iron. In addition to allicin, a single clove of garlic contains several other compounds that can interfere with iron bioavailability such as polyphenols, a potent inhibitor of iron absorption [40], or vitamin C, a reducing agent that enhances iron absorption [41]. Our experiments in Caco-2 cells suggest that DADS tend to prevent iron uptake at the apical side of the enterocytes without altering DMT1 expression. These results suggest that DADS could reduce iron availability, although this effect may only be seen with Fe-NTA, the chemical form of iron used in the cell experiments.

The second mechanism by which garlic can interfere with intestinal iron absorption is by inducing changes in gene expression. Interestingly, we found that garlic alone diminished DMT1 mRNA expression in rat duodenal enterocytes, as compared with rat fed a normal diet without garlic supplementation. This did not lead to reduced iron stores, at least not on the time scale of the experiments (3 weeks). This reduction in DMT1 duodenal mRNA is probably not attributable to DADS because this component alone does not change DMT1 expression in Caco-2 cells. This result is at odds with the observation that garlic enhances intestinal iron absorption, as shown by a modest increase in transferrin saturation in the presence of garlic alone, or by a 50% increase when both iron and garlic are given to the animals. Based on the observation made in Caco-2 cells that DADS induces ferroportin expression at both the mRNA and protein levels, it is possible to speculate that garlic increases duodenal ferroportin expression, leading to increased export of iron, with a net increase in intestinal iron absorption. However, this would have to be confirmed by measuring ferroportin expression in the duodenum.

There was a huge increase in hepcidin mRNA expression in iron-overloaded rat livers, irrespective of the presence of garlic in the diet. This is in agreement with previous studies showing that nutritional iron overload increases hepcidin expression [42]. However, this increase was not sufficient to prevent increased intestinal iron absorption and onset of iron overload.

High-iron diet is known to enhance lipid peroxidation in vivo, as measured by thiobarbituric acid–reactive substances [43]. The production of reactive oxygen species by iron is mainly through the Fenton reaction, which eventually catalyzes the formation of hydroxyl radicals from superoxide or hydrogen peroxide [44]. These observations are consistent with immunohistochemical detection of increased levels of MDA and 4-hydroxyxnonenal adducts in liver and plasma.
proteins of rats maintained on diets containing iron carbonyl [45-47]. In the present work, we only observed increased plasma AOPP in rats receiving both iron and garlic. This is compatible with the observation that serum iron is very high in this condition, and transferrin saturation is more than 100%. This most certainly results in the presence of nontransferrin-bound iron, although we were not able to assess this directly. This form of iron is particularly toxic and prone to favor the formation of reactive oxygen species [48]. In the other conditions tested in this study, the plasma antioxidant defenses are probably sufficient to prevent iron-mediated oxidation.

In tissues, we observed marked differences in terms of oxidative responses. In the spleen, neither iron nor garlic affected MDA content. In the liver, there was a highly significant correlation between tissue iron content and MDA levels when combining the results from the 4 groups of animals. This result suggests that MDA levels reflect iron-mediated toxicity and that garlic does not offer any protection. In the heart, there was a greater dispersion of

Fig. 4. Effect of DADS (main active component of garlic) on iron uptake and protein expression in Caco-2 cells. Caco-2 cells were grown on filters, and confluent cells were incubated in control medium (control), with 0.1% DMSO alone (DMSO) or with 100 μmol DADS in DMSO (DADS). For the iron uptake studies, 55Fe-NTA with a final concentration of 10 μmol of iron was added at the apical side and the cells were incubated for 120 minutes. The total iron uptake (A) represents the sum of the iron transferred in the basal chamber and of the iron remaining in the cells after the 120-minute incubation period. Results are expressed as dpm/well. The percentage of iron transfer (B) is the ratio of the total amount of iron present in the basal chamber at the end of the incubation period to the total iron uptake. For gene expression studies, the cells were incubated for 24 hours in control medium (control), in the presence of DMSO alone (DMSO) or in the presence of DADS (DADS, 100 μmol dissolved in DMSO). Divalent metal transporter-1 (C) and ferroportin (D) mRNAs were measured by RT-qPCR and normalized to S14 mRNA. Ferroportin protein (E) was measured by Western blotting. Actin was used as an internal standard. Results correspond to the means ± SD (Bonferroni post hoc test). aP < .01, bP = .02 versus DMSO-treated cells.
the results, probably because of the increased sensitivity of the heart to iron-mediated toxicity [49,50]. Finally, in the kidney, iron with and without garlic, but also garlic alone, increased MDA contents. This is contradictory with results from the literature showing that pretreatment of animals with garlic oil attenuates oxidative stress and toxicity mediated by Fe-NTA in rat kidney [26]. However, one limitation of our study is the high dose of garlic that was used. It has been reported that the beneficial effects obtained by low doses of garlic (up to 500 mg/kg) on oxidative stress might be lost in the presence of higher doses of 1 g/kg and above [51].

Association between organosulfur compounds and iron metabolism [52,53] is seldom studied. Our data suggest that consumption of crude garlic could modify iron metabolism through changes in iron export via ferroportin. The nutritional relevance of such data to humans remains to be determined. Even if our data were somehow conflicting, due to the different models and more likely to the different iron status in the in vivo and in vitro models used, they highlight the need for additional studies testing different doses of garlic and possible interactions between iron therapy and garlic intake. These studies would be of interest for countries where there is a large consumption of garlic and where nutritional anemia is still a public health concern.

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References


