Nonheme Iron Absorption in Young Women Is Not Influenced by Purified Sulfated and Unsulfated Glycosaminoglycans¹,²

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Abstract
Meat is a well-known enhancer of iron absorption, yet the molecular entity mediating the effect remains obscure. Recently published data indicate that highly acidic sulfated glycosaminoglycans (GAG) from fish and chicken muscle are effective stimulants of iron uptake in Caco-2 cells. Two fully randomized stable isotope studies with crossover design were performed in a group of 16 apparently healthy young women to assess the effect of purified sulfated and unsulfated GAG on human iron absorption. Iron absorption was measured on the basis of erythrocyte incorporation of $^57$Fe or $^{58}$Fe 14 d after the administration of labeled semisynthetic meals (SSM) based on egg albumin, corn oil, maltodextrin, and water. The meals were consumed with or without added sodium hyaluronate (NaH, 300 mg) or chondroitin sulfate (CS, 360 mg) as representative unsulfated and sulfated GAG, respectively. The level of GAG added was 3 times (NaH) to about 10 times (CS), the amount expected to be present in 150 g beef muscle. Geometric mean iron absorption from SSM containing NaH (21.2%) or CS (19.4%) did not differ from that of SSM without GAG (19.5 and 20.3%, respectively). NaH and CS at those levels do not affect human nonheme iron absorption. J. Nutr. 137: 1161–1164, 2007.

Introduction
Meat is a well-known enhancer of nonheme iron absorption (1–3), yet the molecular entity in meat responsible for this effect remains elusive. Until now, the meat factor was assumed to be a protein component (4–6) of cellular origin; however, recent in vitro data suggest that glycosaminoglycans (GAG)³ may also play a role as part of the meat factor. Huh et al. (7) showed enhanced radioiron uptake by Caco-2 cells in the presence of a purified dilute acid-soluble fraction from haddock or chicken. Notably, the purified acid-soluble fraction was very low in protein, peptides, and amino acids (<2.3 g/100 g extract after HPLC), but contained mainly acidic carbohydrate structures most probably derived from GAG in the extracellular matrix (7). Caco-2 cells exhibit a human small intestinal phenotype upon differentiation (8) and when compared with in vitro digestion and dialysis, usually give the same direction of response to enhancers and inhibitors of iron absorption as do human subjects (9–12).

GAG are part of the connective tissue between the muscle fibers and represent ~0.1% of muscle wet weight (13). Seven different types of GAG have been described (Table 1), the differences being related to disaccharide subunit composition and degree of sulfation. Contributions of the subclasses to the total GAG content of muscle tissue vary, depending on factors like species, age, and tissue source (14,15). In mouse muscle tissue, hyaluronic acid (HA) accounted for ~70% of the total GAG, whereas dermatan sulfate (chondroitin sulfate B), the major fraction among the sulfated GAG, contributed 20% (14). The remainder was made up by chondroitin sulfate (CS) and heparan sulfate. Likewise, chondroitin sulfate B was reported to be the major sulfated GAG in rabbit muscle (16) and porcine skeletal muscle epimysium (17), namely, 80–90% of total sulfated GAG. However, others reported chondroitin sulfate A/C (i.e., 4- or 6-sulfated CS) to constitute 44% of the total hexuronic acid content in rabbit muscle (18). HA consists of repeating disaccharide units of N-acetyl-glucosamine and D-glucuronic acid. CS differs from HA in that N-acetyl-glucosamine is replaced by N-acetyl-galactosamine and the C4 or C6 of the galactosamine is sulfated. Whereas HA is the only unsulfated GAG, CS can be viewed as representative of sulfated GAG.

We designed the present absorption studies to evaluate the possibility that GAG are part of the meat factor, and assessed the influence on iron absorption of purified NaH (the sodium salt of HA) and CS as model GAG. Interestingly, iron-chondroitin sulfate complexes (Condrofer, Blutal) have been used in the treatment of iron deficiency anemia (19,20) and hyaluronic acid has been shown, in vitro, to bind iron (21). Iron absorption from a semisynthetic meal (SSM; hydrolyzed corn starch, chicken egg white, corn oil) served with NaH or CS was compared with iron absorption from the SSM alone. Absorption was measured based on the incorporation of the stable iron isotope labels into erythrocytes (22,23).

¹ Supported by ETH Zurich.  
² Author disclosures: S. Storcksdieck genannt Bonsmann, no conflicts of interest; T. Walczyk, no conflicts of interest; S. Renggli, no conflicts of interest; and R. F. Hurrell, no conflicts of interest.  
³ Abbreviations used: CS, chondroitin sulfate; GAG, glycosaminoglycan(s); HA, hyaluronic acid; SSM, semisynthetic meal.  
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Subjects and Methods

Subjects. Sixteen apparently healthy women (aged 19–34 y; maximum body weight 60 kg) were recruited from the staff and student populations of ETH Zurich, University of Zurich and the University Hospital Zurich. The subjects participated in 2 studies, which were performed in a randomized, crossover design.

Exclusion criteria included pregnancy or lactation and known gastrointestinal or metabolic disorders. No medication (except oral contraceptives) or vitamin or mineral supplements were allowed during the study. Women regularly taking vitamin or mineral supplements were allowed during the study period.

Study design. Iron absorption was determined with the use of a double stable-isotope technique and based on erythrocyte incorporation of $^{57}$Fe or $^{58}$Fe 14 d after test meal administration. Each subject received a total of 4 test meals (meal A twice, and meals B and C once each; see below). All test meals were consumed between 0700 and 0900, after the subjects had fasted overnight, under standardized conditions. Each subject acted as her own control and received the purified GAG in a fully randomized crossover design.

A few days prior to the first test meal administration (day 0), a baseline venous blood sample was drawn after an overnight fast to determine iron status (plasma ferritin, hemoglobin), and subjects’ height and weight were recorded.

The subjects consumed the first 2 labeled test meals on 2 consecutive days (d 1 and 2). Fourteen days later (d 16), a second blood sample was drawn, followed by the administration of test meals 3 (d 17) and 4 (d 18). Another 14 d later (d 32), a final blood sample was drawn.

Test meals. The control meal (meal A) was a semisynthetic meal (SSM) consisting of 67 g hydrolyzed corn starch (CStar MD 01910, Cargill Cerestar, Blattmann AG), 35 g corn oil (Mazola, Unilever), 35.2 g egg white (Monark Egg) corresponding to 30 g protein and 200 mL high-purity (18 MIU) water. Pineapple flavor (74185–33, Givaudan), which is devoid of carboxylic and phenolic groups likely to interact with iron, was added at 0.05% (v:w). SSM shakes were prepared freshly the day before consumption. All test meals were consumed between 0700 and 0900, after the subjects had fasted overnight, under standardized conditions. Each subject acted as her own control and received the purified GAG in a fully randomized crossover design.

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The amount of NaH was estimated to equal 2 to 3 times the daily consumption with a meat intake of ~150 g/d (13,24). For CS, the same amount on a molar basis, 360 mg, was added to allow for better comparison, although CS intake would most probably be closer to 20–30 mg/d. NaH had a molecular weight of 1000–1200 kDa and contained 97.05% hyaluronic acid according to the certificate of analysis. CS was of ~20 kDa molecular weight and contained 92.3% chondroitin sulfate (10% C4-sulfated and 90% C6-sulfated), the remainder being protein (~6%), ash, and water. High-purity water (230 g) was served as a drink.

With each control and test meal, a separate high-purity water drink (70 g) containing 4 mg of iron, added as isotopically labeled ferrous sulfate ($^{57}$FeSO$_4$ or $^{58}$FeSO$_4$) on the morning of consumption, was served. Subjects were asked to start with the SSM and to intermittently drink the water. No time restriction was imposed but all subjects finished their SSM within 5–25 min. Control and test meals were fed once per study. To alleviate any unpleasant aftertaste, the subjects received a peppermint-flavored Wrigley’s Eclipse Flash strip after each meal.

Iron status measurements. Venous blood samples (7 mL) were drawn into EDTA-treated vacutainers (368453, Becton Dickinson) a few days before the first test meal administration and again 14 d after the first and second set of test meals on d 16 and d 32 of the study, respectively. Samples were analyzed for iron status indexes (plasma ferritin, hemoglobin; d 0) and for the incorporation of $^{57}$Fe and $^{58}$Fe into erythrocytes (d 16, d 32). Hemoglobin was measured in fresh whole blood using the cyanmethemoglobin method (DS941, Sigma). Plasma was separated and stored frozen for later ferritin analysis with the use of an enzymatic immunoassay (Immulite, DPC Buhlmann GmbH). Commercial quality control materials for hemoglobin (Digitana AG) and ferritin (Immulite) were run with each analysis.

Statistics. Student’s paired $t$ test was used to evaluate study data. Values were logarithmically transformed before statistical analysis (EXCEL 2002 SP3, Microsoft ). Results are presented as geometric means (±1 SD) and were considered statistically significant at $P < 0.05$. Post-hoc power calculations for paired $t$ tests were performed using GraphPad StatMate for Windows, version 2.00 (GraphPad ) with $n = 14$ in study 1 and $n = 15$ in study 2. The same 14 subjects in study 1 and study 2 were used for paired comparison between the NaH-containing SSM and the CS-containing SSM after normalization for mean isotope absorption from the GAG-free reference SSM in both studies.

Results

Subjects. Of 16 subjects recruited into the 2 studies, 15 completed the CS arm and 14 completed the NaH arm. Reasons for dropout were taste aversion and accidental spilling of isotope

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TABLE 1 Composition of repeating disaccharide units of GAG

<table>
<thead>
<tr>
<th>GAG</th>
<th>Disaccharide, Sulfates per disaccharide</th>
<th>Repeating disaccharide (A–B)$_n$</th>
<th>Monosaccharide A</th>
<th>Monosaccharide B</th>
<th>n, μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronic acid</td>
<td>50–10000</td>
<td>β-D-GlcUA</td>
<td>β-D-GlcNAc</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Chondroitin 4-sulfate</td>
<td>20–60</td>
<td>β-D-GlcUA</td>
<td>β-D-GalNAc</td>
<td>0.2–1.0</td>
<td></td>
</tr>
<tr>
<td>Chondroitin 6-sulfate</td>
<td>20–60</td>
<td>β-D-GlcUA</td>
<td>β-D-GalNAc</td>
<td>0.2–2.3</td>
<td></td>
</tr>
<tr>
<td>Dermatan sulfate$^2$</td>
<td>30–80</td>
<td>α-L-IdUA</td>
<td>β-D-GalNAc</td>
<td>1.0–2.0</td>
<td></td>
</tr>
<tr>
<td>Heparan sulfate and heparins$^1$</td>
<td>10–60</td>
<td>α-D-GlcUA</td>
<td>β-D-GalNAc</td>
<td>0.2–3.0</td>
<td></td>
</tr>
<tr>
<td>Keratan sulfate</td>
<td>5–40</td>
<td>β-D-GlcNAc</td>
<td>β-D-Gal</td>
<td>0.9–1.8</td>
<td></td>
</tr>
</tbody>
</table>

$^1$ These GAG may contain 2 different types of disaccharide units [modified from (32)].

$^2$ GlcNAc, N-acetylglucosamine; GaINAc N-acetylgalactosamine; IdUA, iduronic acid; GlcUA, glucuronic acid; Gal, galactose.
Iron absorption. The geometric mean absorption from the control meal was 19.5% in the NaH study and 20.3% in the CS study (Table 2). Iron absorption from the SSM containing GAG was 21.2% for NaH and 19.4% for CS. Absorption from test meals did not differ from control (NaH: $P = 0.42$; CS: $P = 0.64$). After normalization for mean iron absorption from the GAG-free SSM (20.5%) in the 14 subjects who participated in both studies, absorption from the GAG-containing meals did not differ ($P = 0.50$). The study design allowed detecting a change in iron absorption of ~30% at 80% power.

**Discussion**

We studied the effect of the purified glycosaminoglycans sodium hyaluronate and chondroitin sulfate as a possible part of the meat factor on nonheme iron absorption from a semisynthetic meal. This study was prompted by recent reports of acidic carbohydrate structures of the GAG type as enhancers of iron uptake in Caco-2 cells (7). In contrast to the increased iron uptake in vitro (300–500% of the control), we observed no enhancement of iron absorption in humans.

Data on meat consumption from the European Prospective Investigation into Cancer and Nutrition (EPIC) study showed that total meat intake ranged between 46–127 g/d in women and 79–242 g/d in men (24). Assuming that the total glycosaminoglycan content in meat of different species does not vary greatly, the estimated GAG intake would thus amount to 46–127 mg/d in women and 79–242 mg/d/men. Unfortunately, data on GAG composition in meat used for human consumption is scarce. In mouse muscle tissue, the unsulfated GAG hyaluronic acid (HA) constitutes ~70% of total GAG (14), the remainder being sulfated GAG like chondroitin sulfate B (dermatan sulfate) or heparan sulfate. Thus, daily HA and sulfated GAG intakes probably range between 32–169 mg/d and 14–73 mg/d, respectively. The rationale for using 300 mg of NaH was to simulate an intake 2–3 times the daily consumption of nonvegetarians. To allow for direct comparison between NaH and CS, we fed the same molar amounts of CS (calculated on a disaccharide subunit basis), although dietary CS intake would be only about one-fifth of NaH intake. It is still possible but unlikely that it could be enhancing at lower levels.

Soluble HA-iron complexes form at a molar ratio of 3:1 (HA disaccharide:Fe) and iron interacts specifically with the acetylated nitrogen of N-acetyl-glucosamine in aqueous solution (21). In the present study, a GAG disaccharide:Fe molar ratio of ~10:1 was employed. Molar ratio is a key issue in the study of enhancers and inhibitors of iron absorption. Usually, dietary factors influencing iron absorption show a more or less pronounced dose-response relation (26–29). The only known exception is EDTA, which, when added to meals of low bioavailability at a molar ratio of ≤1:1, acts as an enhancer (30,31), whereas higher EDTA:Fe ratios lead to decreased iron absorption (30). Thus, the lack of any effect at the rather high molar GAG:Fe ratio used in the present study makes it unlikely but not impossible that a lower molar GAG:Fe ratio would change dietary iron bioavailability. If larger amounts of GAG had a beneficial effect, however, they would have to be provided by dietary supplements.

During synthesis, GAG can undergo several chemical modifications such as (de-)sulfation, (de-)acetylation or epimerization. Huh et al. (7) report that preliminary characterization of their chicken and fish extracts suggested highly acidic oversulfated heparan sulfate moieties as major constituents of the fractions enhancing Caco-2 cell iron uptake and virtual absence of proteins, peptides, and amino acids. It is still possible that this small specific fraction of muscle GAG could be an iron absorption enhancer. However, it should be stressed that these Caco-2 studies were made by adding the acid-soluble fractions onto Caco-2 cells directly without the addition of a meal. When other positive or negative ligands are present from partially digested food, the influence of the acid extract could be very different.

The high cost of commercial heparan sulfate prevented its use in the present study. Large-scale production for human feeding trials of the fish extract used in the Caco-2 experiments was attempted but was unsuccessful. Whether the chemical modification of GAG substantially modulates their influence on iron bioavailability remains to be determined. Generally, differences among the different types of GAG largely relate to their sequence of disaccharide subunits so that fragments of low molecular weight become increasingly similar to one another. To what extent GAG are modified during digestion, i.e., gastric acid treatment as simulated by HCl incubation in the study by Huh et al. (7), is difficult to tell. However, gastric digestion in humans would probably release low-molecular-weight oligosaccharides from larger GAG molecules in a similar fashion to that observed in the Caco-2 study.

In conclusion, the purified glycosaminoglycans sodium hyaluronate and chondroitin sulfate had no detectable effect on iron absorption from a semisynthetic meal. The GAG doses administered represented intake levels achievable with very high meat consumption or low-dose supplement use. It is unlikely that these compounds constitute a major part of the meat factor. However, the potential role of chemical modifications to the GAG structure and the GAG:Fe molar ratio warrant further investigation.

**Acknowledgments**

The help of Eberhard Denk, Ralf Biebinger, and Rita Wegmüller in performing the human studies is greatly appreciated. Special thanks go to Christophe Zeder for preparing the isotope solutions and helping with data evaluation, and to Charlotte Züllig and Marlies Krähenbühl for taking blood samples.

**Table 2** Iron absorption in 19–34 y-old women from a semisynthetic meal with and without added sodium hyaluronate (study 1) or chondroitin sulfate (study 2)

<table>
<thead>
<tr>
<th>Meal</th>
<th>Plasma ferritin (μg/d)</th>
<th>Iron absorption (mean, 95% CI)</th>
<th>Absorption ratios (μg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (SSM)</td>
<td>19.49 (10.53; 36.05)</td>
<td>0.74–1.61</td>
<td>B: A</td>
</tr>
<tr>
<td>B (+ NaH)</td>
<td>21.24 (12.18; 37.01)</td>
<td>1.09</td>
<td>C: A</td>
</tr>
<tr>
<td>A (SSM)</td>
<td>20.33 (9.59; 43.09)</td>
<td>0.66–1.39</td>
<td>0.95</td>
</tr>
<tr>
<td>C (+ CS)</td>
<td>19.42 (8.73; 41.18)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Values are geometric means ± SD, n = 13 (study 1) or 15 (study 2) [modified from (32)].

2. SSM, semisynthetic meal; NaH, sodium hyaluronate; CS, chondroitin sulfate.

3. SSM plus 300 mg sodium hyaluronate.

4. SSM plus 500 mg chondroitin sulfate.

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