Composition and structure elucidation of human milk glycosaminoglycans

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To date, there is no complete structural characterization of human milk glycosaminoglycans (GAGs) available nor do any data exist on their composition in bovine milk. Total GAGs were determined on extracts from human and bovine milk. Samples were subjected to digestion with specific enzymes, treated with nitrous acid, and analyzed by agarose-gel electrophoresis and high-performance liquid chromatography for their structural characterization. Quantitative analyses yielded ~7 times more GAGs in human milk than in bovine milk. In particular, galactosaminoglycans, chondroitin sulfate (CS) and dermatan sulfate (DS), were found to differ considerably from one type of milk to the other. In fact, hardly any DS was observed in human milk, but a low-sulfated CS having a very low charge density of 0.36 was found. On the contrary, bovine milk galactosaminoglycans were demonstrated to be composed of ~66% DS and 34% CS for a total charge density of 0.94. Structural analysis performed by heparinas showed a prevalence of fast-moving heparin over heparan sulfate, accounting for ~30–40% of total GAGs in both milk samples and showing lower sulfation in human milk (2.03) compared with bovine (2.28). Hyaluronic acid was found in minor amounts. This study offers the first full characterization of the GAGs in human milk, providing useful data to gain a better understanding of their physiological role, as well as of their fundamental contribution to the health of the newborn.

Keywords: chondroitin sulfate/dermatan sulfate/glycosaminoglycans/heparin/human milk

Introduction

It is a well-known and established fact in pediatrics that infants that are not breastfed have a greater incidence of disease compared with those that are breastfed (Cunningham 1979; Kovar et al. 1984). In fact, human milk, apart from its nutritional value, contains several bioactive substances (secretory immunoglobulin A, lactoferrin, lysozyme etc.), which exert documented protective effects against infection (Hamosh 2001; Morrow et al. 2005). Moreover, in the last few years, evidence has been obtained that some human milk glycans (such as glycoproteins, glycolipids and especially oligosaccharides), behaving as cell-surface receptor homologs, inhibit pathogen bindings, thus protecting the newborn against several enteric infections (Hanson 2007; Newburg 2009). On the contrary, knowledge regarding the content, structure and function of milk glycosaminoglycans (GAGs) is very scanty. In fact, to date, there is only one study available on the evaluation of human milk GAGs (Newburg et al. 1995) and no data exist on bovine milk, which is commonly used for the preparation of infant formulas.

GAGs are highly sulfated, linear polysaccharides constituted by repeating disaccharidic units. They are generally grouped into four classes: (1) hyaluronan (HA); (2) keratan sulfate; (3) galactosaminoglycans, represented by chondroitin sulfate (CS) and dermatan sulfate (DS); (4) glucosaminoglycans with heparan sulfate (HS) and heparin (Hep). Each specific disaccharidic unit is made up of an N-acetyhexosamine [N-acetylgalactosamine (GalNAc) or N-acetylgalactosamine (GlcNAc)] alternated with a hexuronic acid (glucuronic or iduronic acid) with the exception of keratan sulfate that contains galactose instead. The capacity of GAGs to interact with other components strictly depends on their structure, such as charge density, the presence of nonsulfated regions and sulfatation on the specific carbohydrate backbone (Jackson et al. 1991). Moreover, it is important to remember that also minimal modifications within the chains may substantially modify the biological and pharmacological properties of the molecule.

Recent studies on GAGs have enabled us not only to obtain more detailed information on their molecular structure, but also to provide a sharper definition of their physiological role in several biological processes such as blood coagulation, cell growth and differentiation and cell–cell and cell–matrix interactions (Jackson et al. 1991; Raman et al. 2005). In particular, it has been shown that some cell-surface receptors are constituted by GAGs (Sasiskehrhan et al. 2006), which in this...
way directly participate in infective and inflammatory processes.

In human milk, Newburg et al. (1995) found the presence of various sulfated GAGs, in particular CS, DS, HS and Hep, and demonstrated that CS or a not further defined CS-like moiety was able to inhibit the binding of the human immunodeficiency virus (HIV) envelop glycoprotein gp120 to the cellular CD4 receptor. From this study, it emerges that human milk GAGs could play a role as soluble receptors and would therefore have the power to inhibit the binding of different pathogens to the intestinal mucosa, thus protecting the infant from infections as already reported for other human milk glucidic components (Hanson 2007; Newburg 2009). However, no characterization of these polysaccharides is available also considering that the capacity of these macromolecules to interact with other components strictly depends on their structure and properties, such as charge density, the presence of nonsulfated regions and sulfatation on the specific carbohydrate backbone (Jackson et al. 1991; Raman et al. 2005; Sasisekharan et al. 2006). Finally, no data are available on the backbone (Jackson et al. 1991; Raman et al. 2005; Sasisekharan et al. 2006). Furthermore, the products of the enzymatic digestions (unsaturated HS/Hep and HA/CS/DS disaccharides) were also qualitatively and quantitatively evaluated by reverse-phase-high-performance liquid chromatography-fluorescence-electrospray ionization-mass spectrometry [RP-HPLC-Fp-ESI-MS] (Deakin and Lyon 2008; Volpi 2009).

Figure 2 shows the agarose-gel electrophoresis of milk GAGs, both untreated and treated with enzymes. Four bands are evident in bovine milk having migration mobility, respectively, comparable with CS, DS, HS/FM-Hep and SM-Hep. On the contrary, two main bands corresponding to CS/DS and HS/SM-Hep were observed for the human sample (also confirmed by repeating electrophoresis at decreasing GAG amounts, not shown) both in pool and single donors milks.

The issue addressed in this study is to fill the gap in the knowledge of the fine structure (and chemical properties) of human milk GAGs in comparison with those of bovine milk to have a structure–function relationship related to these macromolecules.

Table I. Quantitative evaluation of the total GAGs in milk samples and structural characterization of their GAG components

<table>
<thead>
<tr>
<th></th>
<th>Human milk</th>
<th>Bovine milk</th>
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<tbody>
<tr>
<td>Total GAGs (as uronic acids; mg/L)</td>
<td>416.2 ± 85.6</td>
<td>60.2</td>
</tr>
<tr>
<td>% GAGs, mg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS</td>
<td>55.5 ± 7.8, 231.0</td>
<td>21.4, 12.9</td>
</tr>
<tr>
<td>DS</td>
<td>1.7 ± 0.6, 7.1</td>
<td>39.7, 23.9</td>
</tr>
<tr>
<td>FM-Hep</td>
<td>39.2 ± 6.2, 163.1</td>
<td>29.4, 17.7</td>
</tr>
<tr>
<td>SM-Hep</td>
<td>2.3 ± 1.0, 9.6</td>
<td>5.0, 3.0</td>
</tr>
<tr>
<td>HA</td>
<td>1.3 ± 0.5, 5.4</td>
<td>4.5, 2.7</td>
</tr>
<tr>
<td>% CS/DS/HA disaccharides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔDi-4s</td>
<td>22.4 ± 3.6</td>
<td>59.8</td>
</tr>
<tr>
<td>ΔDi-2s</td>
<td>0.7 ± 0.2</td>
<td>1.8</td>
</tr>
<tr>
<td>ΔDi-6s</td>
<td>13.2 ± 2.3</td>
<td>27.5</td>
</tr>
<tr>
<td>ΔDi-0s</td>
<td>62.4 ± 7.9</td>
<td>6.1</td>
</tr>
<tr>
<td>ΔDi-HA</td>
<td>1.3 ± 0.5</td>
<td>4.8</td>
</tr>
<tr>
<td>CS/DS properties</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4s/6s ratio</td>
<td>1.70</td>
<td>2.17</td>
</tr>
<tr>
<td>Charge density</td>
<td>0.36</td>
<td>0.94</td>
</tr>
<tr>
<td>GlcA (%)</td>
<td>96.5 (HPLC)/97.1 (Agarose)</td>
<td>38.1 (HPLC)/34.0 (Agarose)</td>
</tr>
<tr>
<td>IdoA (%)</td>
<td>3.5 (HPLC)/2.9 (Agarose)</td>
<td>61.9 (HPLC)/66.0(Agarose)</td>
</tr>
<tr>
<td>Hep/HS disaccharides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔDi-H-tris</td>
<td>52.7 ± 8.2</td>
<td>72.3</td>
</tr>
<tr>
<td>ΔDi-H,N,6dis</td>
<td>2.9 ± 0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>ΔDi-H,2,Ndis</td>
<td>8.1 ± 1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>ΔDi-H-Ns</td>
<td>13.8 ± 2.2</td>
<td>5.4</td>
</tr>
<tr>
<td>ΔDi-H-6s</td>
<td>8.5 ± 1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>ΔDi-H-0s</td>
<td>14.0 ± 1.3</td>
<td>19.1</td>
</tr>
<tr>
<td>Hep/HS properties</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Charge density</td>
<td>2.03</td>
<td>2.28</td>
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</table>

The results are the mean of three different analyses. The coefficient of variation % was always found to be lower than 20% for all analyses. Furthermore, for human milk, data were obtained from the analysis of samples from seven different healthy subjects and reported as the mean ± SD. CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; FM-Hep, fast-moving heparin; SM-Hep, slow-moving heparin; 4s/6s ratio, ratio between 4-sulfated disaccharide, ΔDi-4s and the sulfated disaccharide in position 6 of the N-acetylgalactosamine unit, ΔDi-6s; GlcA, glucuronic acid; IdoA, iduronic acid. The percentages of the two different uronic acids have been calculated by means of agarose gel (Agarose) and RP-HPLC-Fp (HPLC) after treatment with chondroitinases ABC and B.
Furthermore, in a specific treatment with chondroitinase ABC produced in human milk, the disappearance of the band having greater mobility and, in the bovine sample, the quite total disappearance of the two bands having the same mobility as CS and DS occurred, thus confirming the galactosaminoglycan nature of these species (Figure 2). Indeed, contrary to human, ~5% of the bovine milk band having the electrophoretic mobility of CS was found resistant to chondroitinase ABC probably due to the high percentage of iduronic acid in this polymer (see Table I). Digestion with chondroitinase B, which selectively cleaves DS, produced different results in the two milk GAG samples. In fact, no effect on the electrophoretic pattern of human milk GAGs was obtained, whereas the complete disappearance of the band corresponding to DS was observed in the bovine milk extract (Figure 2). These results confirm that almost the entirety of human milk galactosaminoglycans are constituted by CS, whereas DS is present in significant amounts in the bovine milk sample.

RP-HPLC-Fp (Volpi 2009) after chondroitinase ABC of human milk GAGs (Figure 3A, upper panel) shows the production of a large percentage of nonsulfated disaccharide: 62% of ΔDi-0s ([ΔUA-GalNAc]2), 22% of ΔDi-4s ([ΔUA-GalNAc][4s]) and ~13% of ΔDi-6s ([ΔUA-GalNAc][6s]) species also confirmed by RP-HPLC-Fp-ESI-MS (Figure 4; Volpi 2009). No disulfated disaccharides (or the trisulfated one) were detected. As a consequence, the galactosaminoglycans showed a very low charge density value of 0.36 and a 4/6-sulfated ratio of ~1.7 (Table I). On the contrary, the disaccharides obtained from bovine milk GAGs treated with chondroitinase ABC (Figure 3B, upper panel) mainly produced ΔDi-4s (60%) and ΔDi-6s (27%) with a low percentage of ΔDi-0s (6%) (and no disulfated disaccharides) accounting for a high charge density value of 0.94 and a 4/6-sulfated ratio of ~2.2 (Table I). Specific treatment with chondroitinase B of bovine GAGs generated almost exclusively the ΔDi-4s disaccharide, as expected (Trowbridge and Gallo 2002; Figure 3B, lower panel) confirming the presence of a DS polymer in a concentration ~62–66%, according to agarose-gel determination (66%). On the contrary, only very low amounts of disaccharides were observed after chondroitinase B digestion of human milk GAGs (Figure 3A, lower panel), showing the virtual absence of iduronic acid (Table I) and, obviously, the presence of a very high content of glucuronic acid (97%), according to agarose-gel electrophoresis. As a consequence, human milk galactosaminoglycans are almost exclusively constituted by a low-sulfated CS as opposed to the bovine sample formed by sulfated DS (~65%) and CS (~35%) (Table I). Finally, by means of RP-HPLC-Fp-ESI-MS, isomeric nonsulfated HA and CS disaccharides and isomeric monosulfated CS disaccharides were distinctly separated and unambiguously identified by their retention times and mass spectra in a negative ionization mode generally confirming the previous results and also showing the presence of low percentages of ΔDi-2s ([ΔUA(2s)-GalNAc], the CS disaccharide sulfated in position 2 of the glucuronic acid [ΔUA(2s)-GalNAc], the CS disaccharide sulfated in position 2 of the glucuronic acid [ΔUA(2s)-GalNAc], and of HA determined by its disaccharide ΔDi-HA (ΔUA-GlcNAc) after treatment with chondroitinase ABC (Table I).

Glucosaminoglycans (HS/FM-Hep and SM-Hep) were identified by agarose-gel electrophoresis before and after specific treatment with nitrous acid (Conrad 2001). A clear disappearance of the bands having the same migration mobility of the standard HS/FM-Hep and SM-Hep in bovine and human samples was observed (not shown), confirming the presence of HS and/or Hep with its two components (Table I). Quantitative evaluation performed on different donors showed the presence of ~41% glucosaminoglycans on the total of GAGs (Table I) and sensitive to nitrous acid. By agarose-gel electrophoresis (Figure 1), ~95% of total glucosaminoglycans was related to HS/FM-Hep and ~5% to SM-Hep (bovine milk showed ~13% SM-Hep of the total glucosaminoglycans). However, due to the very complex and heterogeneous structure of these polysaccharides, after elimination of CS/DS component, disaccharide composition was performed on milk samples, untreated and treated with chondroitinase ABC (ABC) or chondroitinase B (B). ST, standard; CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; FM, fast-moving heparin; SM, slow-moving heparin.

**Figure 1.** Agarose-gel electrophoresis of GAGs from human milk samples obtained from seven healthy mothers (from A to G). ST, standard; CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; FM, fast-moving heparin; SM, slow-moving heparin.

**Figure 2.** Agarose-gel electrophoresis of GAGs from bovine milk (BM) and human milk (HM) samples, untreated and treated with chondroitinase ABC (ABC) or chondroitinase B (B). ST, standard; CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; FM, fast-moving heparin; SM, slow-moving heparin.
extracts by using heparinases and separation of unsaturated disaccharides by RP-HPLC-Fp after their derivatization with 2-aminoacridinone (AMAC; Deakin and Lyon 2008). Figure 5 illustrates the HPLC separation and fluorimetric detection of the main six disaccharides forming HS/Hep (Deakin and Lyon 2008). As evident, the trisulfated disaccharide ΔUA(2s)-GlcNS(6s) typical of Hep was found the main species (≏53–73%) forming human and bovine milk glucosaminoglycans along with lower percentages of disulfated, monosulfated and nonsulfated disaccharides (Figure 5 and Table I). As a consequence, FM-Hep (and not HS) was characterized as the major glucosaminoglycan species present in milk even if minor differences were observed between human and bovine samples. In fact, as illustrated in Table I, human milk showed an FM-Hep having a lower percentage of trisulfated disaccharide and a overall greater content of disulfated and monosulfated species. This different disaccharide composition is related to a lower sulfated Hep in human milk (2.03) compared with bovine sample (2.28) (Table I) also according to the minor percentage of SM-Hep determined in human extract by electrophoresis. In fact, FM-Hep species is characterized by a lower sulfate group amount than SM-Hep component having a greater charge density (Volpi 1993).

In conclusion, the definitive quantitative GAG composition of human and bovine milk, obtained from the evaluation of the results derived from several analytical procedures, is reported in Table I. As appears evident, along with important structural modifications of CS, substantial differences may be observed between the two samples. Total GAGs from human milk have been found to be ~7-times more than those in the bovine sample. Furthermore, ~50–60% of human milk GAGs are constituted by undersulfated CS with the remaining formed of FM-Hep (~40) whose absolute amounts are, respectively, ~23 and 7–8 times higher than those in the bovine milk. On the other hand, the main bovine milk GAGs are DS (40%), FM-Hep (30%) and high charge density CS (21%).

Discussion

To date, the only data reported in the literature on milk GAGs are those described by Shimizu et al. (1981) and Newburg et al. (1995). Furthermore, Shimizu et al. (1981) exclusively analyzed milk fat globule membranes showing that isolated GAGs were 5–10 times higher in human than in bovine milk membranes. The major GAG in bovine membranes was HS (70%) and the remainder (30%) was represented by CS sulfated both on C4 and C6, whereas in human membranes, HS was found to be the main macromolecule along with a second unidentified polysaccharide. The second study (Newburg et al. 1995) was solely performed on GAGs from whole human milk obtained from a pool of 30 healthy mothers.

Fig. 3. RP-HPLC-Fp chromatograms of unsaturated disaccharides from human (A) and bovine (B) milk GAGs treated with chondroitinase ABC (+ lyase ABC) or chondroitinase B (+ lyase B) and fluorotagged with AMAC.
showing the presence of Hep, HS, CS, DS and a not-clearly identified CS-like molecule without any quantitative evaluation, structural characterization or comparison with bovine milk.

In our study, the total GAG amount in human milk was found to be 416 mg/L, with the bovine sample, used as a basic source for the preparation of infant formulas, having a much lower content. Furthermore, we confirm that the GAG fraction in milk samples is very complex, being formed of the unique glucosaminoglycan FM-Hep, glucosaminoglycans CS and DS and minor percentages of HA. Human milk CS was characterized as an undersulfated polymer having a low charge density value generally much lower than the mammalian polymers (Volpi 2009). On the contrary, bovine milk galactosaminoglycans were found to be composed of ~66% DS and ~34% CS for a total charge density of 0.94. Interestingly, we demonstrated in milk samples the presence (~30–40%) of a quite unique glucosaminoglycan species characterized as FM-Hep that is a lower sulfated component of Hep formed of iduronic acid and possessing a greater

![Figure 4](image-url)
percentage of trisulfated disaccharide and higher charge density than HS (Volpi and Maccari 2002; Deakin and Lyon 2008) and anticoagulant activity (Volpi 2003). Without any accurate and specific analytical evaluation, this polysaccharide may be easily confused with HS generally possessing variable but lower iduronic acid and trisulfated disaccharide percentages, and a charge density between ~0.5 and 1.5 (Vongchan et al. 2005) with lower or absent anticoagulant activity.

Furthermore, this macromolecule was found structurally quite different between human and bovine milks with the last one having a higher charge density. The significance of these variations between samples of different origins such as the physiological role of FM-Hep milk are at the moment unknown.

In this study, we used pooled milk to have a robust characterization of the general pattern of components. However, we also performed quantitative and qualitative characterization of GAGs extracted from milk samples of seven different healthy subjects to have information about possible intraindividual variation also considering that many other human milk components may have 4–5-fold variation within mothers (Hamosh 2001). No great variations were observed for various subjects, and the qualitative/quantitative composition of GAGs was confirmed quite similar in all human samples with very great diversity compared with bovine milk. However, we should consider that human milk samples were from healthy donors, with a full-term delivery and at the same time of milk withdrawal. We cannot exclude modifications in GAGs composition and structure of milk from donors having different conditions. Studies are in progress to clarify possible modifications of GAG structure depending on parturient conditions along with analyses to determine if milk GAGs are linked to a core protein during their biosynthetic processes to produce proteoglycans.

As concerns the possible physiological role of GAGs in the gastrointestinal tract of breastfed newborns, it is important to underline that undigested GAGs reach the small intestine, because there are no specific enzymes present on the intestinal wall able to degrade them. Therefore, these molecules could behave as soluble receptors having the power to interact with pathogens and to compete for their adhesion to the intestinal wall, as already demonstrated for other human milk (oligo)saccharides (Hanson 2007; Newburg 2009). In fact, accumulated evidence indicates that, during their evolution, many microorganisms (pathogenic bacteria, viruses and parasites) have adapted in order to exploit the GAG chains of proteoglycans of specific cells and tissues for efficient invasion, propagation and survival in the host (McClure et al. 1992; Rostand and Esko 1997; Wadstrom and Ljungh 1999).

Of the several GAG types present in animal cells and tissues, the majority of the GAG-recognizing microorganisms use HS chains of HS proteoglycans as receptors for infecting the hosts (McClure et al. 1992; Rostand and Esko 1997; Wadstrom and Ljungh 1999). In addition to HS chains as receptors for the adhesion to and invasion of host cells, several types of microorganisms have also been demonstrated to use CS. For example, Toxoplasma gondii has been reported to use the CS chains of proteoglycans as the host cell receptor (Carruthers et al. 2000), and in the efficient infection of Herpes simplex virus 1, glycoprotein C of the virus binds CS chains (Mårdberg et al. 2002). Furthermore, the spirochete Barretta burgdorferi, the causative agent of Lyme disease in humans, shows strain-specific differences in GAG binding with certain strains having the capacity to specifically recognize CS/DS (Parveen et al. 2003). Moreover, the human bacterial pathogen Streptococcus pyogenes expresses a number of surface adhesins which bind CS/DS as well as HS and Hep (Frick et al. 2003). Finally, it is worth mentioning that a

![RP-HPLC-Fp chromatograms](https://academic.oup.com/glycob/article-abstract/21/3/295/1988638/113.25196638)
uniquely undersulfated extracellular CS proteoglycan, rather similar in structure to that characterized in this study, localized in the intervillous space of the placenta, is the receptor for infected red blood cell adherence (Achur et al. 2003). This indicates that the molecule(s) responsible for blocking gp120 binding to CD4, as also demonstrated for human milk (Newburg et al. 1995), may also provide a wide range of biologically active specific anti-infectious properties.

We did not perform the inhibition of the binding of the HIV envelope glycoprotein gp120 to its host cell receptor CD4 by human milk GAGs as a previous study clearly showed that the inhibitory molecule was a CS or a chondroitin-like molecule (Newburg et al. 1995). In this further research, we demonstrated that CS (and may be also FM-Hep) from human milk possesses a very peculiar structure and properties different form that of bovine milk generally adopted for an alternative feeding of infants. Furthermore, this new knowledge will permit the screening of milk sources of various animal origins to have a possible more similar GAGs composition. Moreover, based on these new data, we will be able to evaluate possible differences in the human milk GAG pattern depending on various factors, such as the stage of lactation (Newburg 2009), ethnic group or the presence of pathological conditions.

In conclusion, for the first time, this study offers a full characterization of the complex GAGs in human milk, providing basic data which are useful to explore their metabolic fate and their physiological roles, as in the case of other human milk glycans, such as oligosaccharides (Coppa and Gabrielli 2008). Moreover, new knowledge on human milk composition will be a source of basic information for the preparation of improved infant formulas.

Materials and methods

Materials

Hep, CS, DS, HS and HA standards, papain from papaya latex (EC 3.4.22.2), chondroitinase ABC from Proteus vulgaris (EC 4.2.2.4) and chondroitinase B from Flavobacterium heparinum (EC 4.2.2.2) were from Sigma-Aldrich (St. Louis, MO). Heparinase I from F. heparinum (EC 4.2.2.7), heparinase II from F. heparinum (no EC number) and heparinase III from F. heparinum (EC 4.2.2.8) were supplied by Sigma-Aldrich. The SM-Hep and the FM-Hep components of Hep were purified as reported previously (Volpi 1993). All unsaturated HA, CS/DS and HS/Hep disaccharide standards used were from Seikagaku Corporation (Tokyo City, Japan). QAE Sephadex® A-25 anion-exchange resin was from Pharmacia Biotech (Uppsala, Sweden). Microcon YM-3 filters having a cut-off of 3000 were from Millipore (Billerica, MA). All other reagents were of the analytical grade.

The carbazole assay for uronic acids was performed according to Cesaretti et al. (2003), and nitrous acid treatment was carried out as reported by Conrad (2001).

Milk samples

Human milk samples were obtained, after written consent, from 30 healthy mothers having delivered term newborns at 30–45 days post partum. Samples were pooled and stored at −20°C until used. Milk samples from seven healthy mothers also having delivered term newborns at 30–45 days post partum were obtained and individually analyzed. Whole bovine milk was a pool from three commercially available products.

Purification of the milk GAGs

Five milliliters of milk was defatted with acetone. After centrifugation at 10,000 × g for 10 min and drying at 60°C for 24 h, the pellet was solubilized in 20 mL of 100 mM Na-acetate buffer, pH 5.5, containing 5 mM ethylenediaminetetraacetic acid and 5 mM cysteine. Two hundred micrograms of papain was added and the solution incubated for 24 h at 60°C in a stirrer. After boiling for 10 min, the mixture was centrifuged at 5000 × g for 15 min, and three volumes of ethanol saturated with sodium acetate were added to the supernatant. After storing at +4°C for 24 h, the precipitate was recovered by centrifugation at 5000 × g for 15 min and dried at 60°C for 6 h. The dried precipitate was dissolved in 20 mL of 50 mM NaCl and after centrifugation at 10,000 × g for 10 min, the supernatant was applied to a column (2 cm × 7 cm) packed with QAE Sephadex® A-25 anion-exchange resin equilibrated with the same NaCl solution. GAGs were eluted with a linear gradient of NaCl from 50 mM to 1.2 M for 0–150 min using low-pressure liquid chromatography (Biologic LP chromatography system from Bio-Rad, Milan, Italy) at a flow of 1 mL/min. Fractions positive to the uronic acid assay (Cesaretti et al. 2003) were collected. Three volumes of ethanol saturated with sodium acetate were added to the pooled fractions and stored at +4°C for 24 h. The precipitate was recovered by centrifugation and dried at 60°C for 12 h. The dried precipitate was dissolved in 100 μL of distilled water and further analyzed.

Agarose-gel electrophoresis

Agarose-gel electrophoresis in barium acetate/1,2-diaminopropane was performed essentially as reported elsewhere previously (Volpi and Maccari 2002), and quantitative analysis was carried out according to Dainese et al. (2007).

Quantitation of constituent galactosaminoglycans disaccharides

Twenty microliters of the purified milk GAGs was incubated with 60 μL (~40 mU) of chondroitinase ABC or chondroitinase B (~200 mU) for 20 h at 37°C in 50 mM Tris-Cl, pH 8.0 (Volpi 1993; Dainese et al. 2007; Volpi 2009). The unsaturated disaccharides produced were derivatized with AMAC as described previously (Volpi 2009). The fluorescent disaccharides were analyzed by on-line RP-HPLC-Fp-ESI-MS as reported in detail in a previous publication (Volpi 2009).

Quantitation of constituent glucosaminoglycans disaccharides

Twenty microliters of the purified milk GAGs was incubated with chondroitinase ABC as reported previously, and undigested glucosaminoglycans were precipitated by adding five volumes of 95% ethanol at −20°C overnight. After centrifugation at 5000 × g for 15 min, pellets were dried at 60°C for...
12 h. After reconstitution with 50 µL of 0.1 M sodium acetate and 0.1 M calcium acetate, pH 7.0, a mixture formed of 5 mU each of heparinas I, II and III was added and incubated at 38°C overnight. The unsaturated disaccharides produced were derivatized with AMAC as described previously. The fluorescent HS/Hep disaccharides were analyzed by RP-HPLC-Fp as reported in detail by Deakin and Lyon (2008).

**Determination of uronic acid percentages**

The percentages of glucuronic and iduronic acid in the galactosaminoglycans portion were determined by agarose-gel electrophoresis and by the RP-HPLC-Fp separation of disaccharides both after treatment with chondroitinase ABC, able to degrade glucuronic and/or iduronic acid composed polysaccharides, i.e. CS and DS, and chondroitinase B capable of acting solely on iduronic acid formed polymers (just DS).

**Charge density**

The sulfate-to-carboxyl ratio (charge density) was determined by enzymatic degradation after the RP-HPLC-Fp separation of unsaturated disaccharides. The ratio was calculated considering the presence and the percentages of carboxyl and sulfate groups for each disaccharide.

**Quantitation of GAG species**

CS/DS were quantitatively determined by agarose-gel electrophoresis and confirmed by the RP-HPLC-Fp-ESI-MS separation of constituent disaccharides (Volpi 2009) together with their structural characteristics. FM-Hep and SM-Hep were quantified by agarose-gel electrophoresis (Volpi 1993; Dainese et al. 2007), and HA percentage was derived from its unique constituent disaccharide separated and quantified by RP-HPLC-Fp-ESI-MS (Volpi 2009).

**Contributors**

NV developed the applied methodologies. LZ, TG, FM and DB performed the experimental procedures and analyses. EB contributed in milk sample collection, literature search and final data analysis. NV, GVC and OG designed and developed the experimental design, performed data analysis and wrote the manuscript. All authors reviewed and approved the study.

**Conflicts of interest**

None declared.

**Abbreviations**

AMAC, 2-aminoacridinone; CS, chondroitin sulfate; DS, dermatan sulfate; ESI, electrospray ionization; FM, fast-moving; Fp, fluorescence; GAG, glycosaminoglycan; GaINAc, N-acetylgalactosamine; GlcNAc, N-acetylgalactosamine; HA, hyaluronan; HIV, human immunodeficiency virus; HPLC, high-performance liquid chromatography; HS, heparan sulfate; Hep, heparin; MS, mass spectrometry; RP, reverse-phase; SM, slow-moving.

**References**


