Heparins from porcine and bovine intestinal mucosa: Are they similar drugs?

Rafael S. Aquino*; Mariana S. Pereira*; Bruno C. Vairo; Leonardo P. Cinelli; Gustavo R. C. Santos; Roberto J. C. Fonseca; Paulo A. S. Mourão
Laboratório de Tecido Conjuntivo, Hospital Universitário Clementino Fraga Filho, Programa de Glicobiologia, Instituto de Bioquímica Médica, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Summary
Increasing reports of bleeding and peri- or post-operative blood dyscrasias in Brazil were possibly associated with the use of heparin from bovine instead of porcine intestine. These two pharmaceutical grade heparins were analysed for potential differences. NMR analyses confirmed that porcine heparin is composed of mainly trisulfated disaccharides →4-α-IdoA2S-1→4-α-GlcNS6S-1→. Heparin from bovine intestine is also composed of highly 2-sulfated α-iduronic acid residues, but the sulfation of the α-glucosamine units vary significantly: ~50% are 6- and N-desulfated, as in porcine heparin, while ~36% are 6-desulfated and ~14% N-acetylated. These heparins differ significantly in their effects on coagulation, thrombosis and bleeding. Bovine heparin acts mostly through factor Xa. Compared to porcine heparin on a weight basis, bovine heparin exhibited approximately half of the anticoagulant and antithrombosis effects, but similar effect on bleeding. These two heparins also differ in their protamine neutralisation curves. The doses of heparin from bovine intestine required for effective antithrombotic protection and the production of adverse bleeding effects are closer than those for porcine heparin. This observation may explain the increasing bleeding observed among Brazilian patients. Our results suggest that these two types of heparin are not equivalent drugs.

Keywords
Venous thrombosis, glycosaminoglycans, heparins, serpins, thrombosis

Introduction
Heparin has been used for more than 50 years for the treatment and prevention of thrombosis (1). This glycosaminoglycan is also required for extracorporeal circulation during cardiovascular surgery and renal dialysis (2). The development of low-molecular-weight heparin has extended and enhanced the use of this class of drug. Heparin consumption worldwide increased to 100 tons per year, requiring increased production of this compound. However, heparin production is limited since its sources are animal tissues.

The primary tissue source for the preparation of pharmaceutical grade heparin has changed over time from dog liver, where it was originally described, to bovine lung and finally porcine intestinal mucosa (3). Due to the higher anticoagulant activity of porcine mucosa heparin (4) and the advent of bovine spongiform encephalopathy, the production of bovine heparin has nearly ceased.

Heparin is a linear polysaccharide mainly composed of repeating trisulfated disaccharide units: a 2-sulfated, α-L-iduronic acid 4-linked to 6- and N-desulfated α-D-glucosamine (→4-α-IdoA2S-1→4-α-GlcNS6S-1→), with additional minor disaccharide structures corresponding to its variable sequences. Minor differences in the disaccharide composition have been observed among heparins from different sources. For example, heparin from bovine lung has a higher proportion of N-sulfated glucosamine units and lower proportion of N-acetylated residues than porcine mucosa heparin (4).

In the beginning of 2008, there were serious adverse events associated with heparin therapy caused by contamination with oversulfated chondroitin sulfate (5). Batches of the contaminated heparin were withdrawn from the American market. A similar event was observed in the European Union and Asia. These events demonstrated the need for stricter quality control of heparin preparations. Furthermore, they suggest that sources of heparin other than porcine intestinal mucosa should be developed.

Around the time of the worldwide crisis of heparin batches contaminated with oversulfated chondroitin sulfate, Roche Laboratory removed a well known and referenced unfractionated heparin (UFH) preparation from the Brazilian market. Subsequently, there...
were increasing reports of bleeding and peri- or post-operative blood dyscrasias associated with the use of heparin, mostly during cardiovascular surgeries (6). High rates of reoperations due to bleeding and increased numbers of consumption coagulopathies were observed. These events led the Brazilian National Health Surveillance Agency to publish a warning note about the use of heparin (7). Unfortunately, it was not possible to correlate clinical events with specific heparin batches due to the absence of appropriate records.

The first hypothesis to explain these events was the contamination of heparin preparations used in Brazil with oversulfated chondroitin sulfate, although the clinical symptoms were clearly distinct. The Brazilian patients showed a preponderance of bleeding and blood dyscrasias, while heparin preparations contaminated with oversulfated chondroitin sulfate produced mostly cardiovascular and anaphylactic manifestations (8). Furthermore, NMR (nuclear magnetic resonance) analyses of the heparin preparations used in Brazil excluded contamination with oversulfated chondroitin sulfate.

Subsequently, we observed that most of the heparins available in Brazil after withdrawal of the Roche product were from bovine intestinal mucosa. This is an unusual source of heparin. We now undertook a careful investigation of the heparins available in the Brazilian market. We also compared heparins from porcine and bovine intestinal mucosa concerning their structures and effects on coagulation, thrombosis and bleeding using in vitro and in vivo assays. We suggest that these two heparins are not equivalent drugs.

**Materials and methods**

**Heparin samples**

Forty batches of pharmaceutical grade heparin were obtained from different suppliers. Of these samples, 14 were from bovine and 23 from porcine intestinal mucosa. Three other batches contained mixtures of heparins from the two sources. All batches of heparin used in this study were sodium salts. Three batches showed broad 11 and 15 signals in the 1H NMR spectra, indicative of binding to divalent cations, and were omitted from the study. All batches of heparin conform to Brazilian regulations, which require a minimum anticoagulant activity of 140 UI mg⁻¹, checked exclusively by the coagulation assay with ovine plasma (recalcification time assay) using a single concentration of heparin. This is an imprecise method, nearly abolished from other pharmacopoeias. In the assays used in our laboratory, based on APTT, anti-IIa and anti-Xa activities, we always observed ~30% lower activity for bovine intestinal heparins than reported by the manufacturers based on the recalcification time assay. Names of suppliers were omitted for ethical reasons.

**Analysis of heparins by NMR**

1H and 13C one-dimensional (1D) and two-dimensional (2D) spectra of porcine and bovine heparins were recorded using a Bruker DRX 800 MHz apparatus with a triple resonance probe, as described previously (9). About 20 mg of each sample was dissolved in 0.5 ml of 99.9% deuterium oxide (Cambridge Isotope Laboratory, Cambridge, MA, USA). All spectra were recorded at 25°C with HOD (deuterated water exhibiting a peak due to exchange with residual H2O) suppression by presaturation. In some experiments, 1D 1H spectra were run at different temperatures (10°C to 40°C) to shift the HOD signal. For 1D 1H-NMR spectra, 16 scans were recorded. For the 2D 1H/1H COSY (correlation spectroscopy), TOCSY (total correlated spectroscopy), NOESY (nuclear Overhauser effect spectroscopy) and 1H/13C HSQC (heteronuclear single quantum coherence) experiments, spectra were recorded using states-TPPI (time proportion phase incrementation) for quadrature detection in the indirect dimension. TOCSY spectra were run with 4046 x 400 points with a spin-lock field of 10 kHz and a mixing time of 80 milliseconds (ms). NOESY spectra were recorded with a mixing time of 100 ms. The 2D 1H-13C Multiplicity-Edited HSQC spectra were recorded at 25°C with HOD suppression by presaturation, with 256 scans and increment number setup as 64, using states-TPPI for quadrature detection in the indirect dimension and run with 1024 x 256 points with globally optimised alternating phase rectangular pulses (GARP) for decoupling. Chemical shifts are displayed relative to external trimethylsilylpropionic acid at 0 ppm for 1H and relative to methanol for 13C.

**Gel filtration of heparins**

Porcine or bovine heparin (5 mg of each) was applied to a Sephadex-75 (HR10/30) column linked to an FPLC (fast performance liquid chromatography) system and equilibrated with 0.02 M Tris/HCl (pH 7.4). The column was eluted with the same solution at a flow rate of 0.5 ml min⁻¹, and fractions of 0.5 ml were collected and assayed for metachromasy using 1,9-dimethylmethane blue (10). The column was calibrated using blue dextran (average Mr=2000 kDa for VVo), cresol red (Mr=108 Da for Vt), chondroitin 6-sulfate from shark cartilage (average Mr=40 kDa) and the International Heparin Standard (average Mr=12 kDa).

**Agarose gel electrophoresis**

Porcine and bovine heparins were analysed by agarose gel electrophoresis, as described previously (11). About 15 μg of heparin was applied to a 0.5% agarose gel and run for 1 hour (h) at 110 V in 0.05 M 1.3-diaminopropane/acetae (pH 9.0). Heparin in the gel was fixed with 0.1% N-cetyl-N,N,N-trimethylammonium bromide solution. After 12 h, the gel was dried and stained with 0.1% toluidine blue in acetic acid:ethanol:water (0.1:5:5, v/v).
Activated partial thromboplastin time assay (APTT)

A mixture of 100 μl of human plasma and various concentrations of heparin were incubated with 100 μl of APTT reagent (kaolin phospholipid reagent from Biolab-Merieux AS, Rio de Janeiro, Brazil). After 2 minutes (min) of incubation at 37°C, 100 μl of 25 mM CaCl₂ was added to the mixtures, and the clotting time was recorded in a coagulometer (Amelung KC4A). The results were expressed as the ratio of clotting time in the presence (T₁) or absence (T₀) of different heparin concentrations. The anticoagulant activity was estimated as IU mg⁻¹ using a parallel standard curve based on the 5th International Heparin Standard (229 IU mg⁻¹), obtained from the National Institute for Biological Standards and Control (Potters Bar, UK). Solutions of bovine and porcine heparin were prepared on a weight basis and showed similar hexuronic acid contents when checked by the carbazole reaction.

Anti-IIa and anti-Xa activities of porcine and bovine heparins

Incubations were performed in 96-well plates. The final concentrations of the reactants were 10 nM antithrombin, 2 nM thrombin or factor Xa and 0 – 10 μg ml⁻¹ heparin in 40 μl of TS/PEG buffer (0.02 M Tris/HCl, 0.15 M NaCl, and 1.0 mg ml⁻¹ polyethylene glycol 8,000, pH 7.4). Thrombin or factor Xa was added last to initiate the reaction. After incubation for 60 seconds (sec) at 37°C, 25 μl of chromogenic substrate S-2238 for thrombin or S-2222 for factor Xa (Chromogenix AB, Mondal, Sweden) was added, and the absorbance at 405 nm recorded for 300 sec (Thermomax Microplate Reader, America Devices). The rate of change of the absorbance was proportional to the thrombin or factor Xa activity remaining in the solution. No inhibition occurred in control experiments in which thrombin or factor Xa was incubated with antithrombin in the absence of heparin. In addition, no inhibition was observed when thrombin or factor Xa was incubated with heparin alone over the range of concentrations tested. Protease activity was defined as 100% in control samples lacking heparin. The IC₅₀ is the concentration of heparin required for 50% inhibition of thrombin.

Figure 1: 1D ¹H NMR spectra at 800 MHz of porcine (A and C) and bovine (B and D) heparins. About 20 mg of each sample were dissolved in 0.5 ml of 99.9% D₂O, and the NMR spectra were recorded at 25°C. ¹H chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm. The residual water was suppressed by presaturation. The signals designated A1–6 correspond to H1–6 of 6- and N-disulfated α-glucosamine units; I1–5 to H1–5 of 2-sulfated α-iduronic acid residues; I1-deS to H1 of desulfated α-iduronic acid; B1 and C1 to H1 of N-acetylated and 6-desulfated α-glucosamine units, respectively. I1-A, I1-B and I1-C correspond to H1 of 2-sulfated α-iduronic acid units neighboring 6- and N-disulfated, 6-sulfated and N-acetylated or N-sulfated α-glucosamine units, respectively; I5-A, I5-B and I5-C are H5 of the same residues. C6 is H6 of the 6-desulfated α-glucosamine units. G1 and G2 correspond to H1 and H2 of β-glucuronic acid residues, respectively.
or factor Xa activity. Anti-IIa and anti-Xa activities were reported as unit mg⁻¹, using a parallel standard curve based on the International Heparin Standard (229 unit mg⁻¹).

### Assessment of venous antithrombotic activity

Antithrombotic activity was measured in rats with rabbit brain thromboplastin as the thrombogenic stimulus. We followed the institutional guidelines for animal care and experimentation. Rats (both sexes, ~200 g body weight, five animals per dose) were anaesthetised with an intramuscular injection of 100 mg kg⁻¹ body weight of ketamine (Cristália, São Paulo, Brazil) and 16 mg kg⁻¹ body weight of xylazine (Bayer AS, São Paulo, Brazil). Different doses of heparin were infused into the right carotid artery and allowed to circulate for 5 min. The inferior vena cava was isolated, and brain thromboplastin (5 mg kg⁻¹ body weight) from Biolab-Merieux AS (Rio de Janeiro, Brazil) was slowly injected intravenously; after 1 min, 0.7 cm of isolated vena cava was clamped off using distal and proximal sutures. After 20 min stasis, the thrombus formed inside the occluded segment was carefully pulled out, washed with phosphate-buffered saline, dried for 1 h at 60°C and weighted. Mean thrombus weight was obtained by the average weight from each group and then expressed as percentages of the weight, 100% representing absence of any inhibition of thrombus formation (thrombus weight in the absence of heparin administration).

### Table 1: ¹H and ¹³C chemical shifts (ppm) for residues of α-iduronic acid and α-glucosamine in bovine and porcine heparins.

<table>
<thead>
<tr>
<th>Residue</th>
<th>IdoA2S → GlcNS6S</th>
<th>IdoA2S → GlcNAc6S</th>
<th>IdoA2S → GlcNS</th>
<th>IdoA → GlcNS6S</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) ¹H chemical shifts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>A1</td>
<td>5.40</td>
<td>5.39</td>
<td>B1</td>
<td>5.38</td>
</tr>
<tr>
<td>A2</td>
<td>3.28</td>
<td>3.29</td>
<td>B2</td>
<td>3.86</td>
</tr>
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<td>A3</td>
<td>3.68</td>
<td>3.67</td>
<td>B3</td>
<td>ND</td>
</tr>
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</tr>
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<td>4.03</td>
<td>B5</td>
<td>ND</td>
</tr>
<tr>
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<td>4.39</td>
<td>B6a</td>
<td>ND</td>
</tr>
<tr>
<td>A6b</td>
<td>4.27</td>
<td>4.27</td>
<td>B6b</td>
<td>ND</td>
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<td>5.23</td>
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<td>I5-B</td>
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<td><strong>B) ¹³C chemical shifts</strong></td>
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<td>B2</td>
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<td>B5</td>
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<td>A6a</td>
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<tr>
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<td>B1-B</td>
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<td>I2-B</td>
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<td>71.8</td>
<td>I3-B</td>
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<tr>
<td>I4-A</td>
<td>78.0</td>
<td>78.5</td>
<td>I4-B</td>
<td>ND</td>
</tr>
<tr>
<td>I5-A</td>
<td>71.4</td>
<td>72.2</td>
<td>I5-B</td>
<td>70.4</td>
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*See the legends of Figs. 1 and S1 for the nomenclature of the NMR signals. Units of β-glucuronic acid were not included in the table. Data from Cavalcante et al., 2000 (18). Data from Yates et al., 1996 (19). Data from Chuang et al., 2001 (20). Sulfation and acetylation sites are indicated in bold and italic types, respectively. ND, not determined. Data from Guerrine et al., 2005 (21).
Citrated blood samples were collected (nine parts blood: one part 3.8% sodium citrate, v/v) from the carotid artery before infusion (control) and 5 min after intravascular administration of heparin.

**Ex vivo APTT**

The plasma was then separated by centrifugation (1,600 x g for 10 min) and assayed for ex vivo APTT, as described above. The results were expressed as ratios of clotting time in the presence (T₁) and in the absence (T₀) of different doses of heparin.
Bleeding effect

Wistar rats (both sexes, ~200 g body weight) were anesthetised with a combination of xylazine and ketamine, as described above. A cannula was inserted into the right carotid artery for administration of different doses of porcine or bovine heparin. After heparin had circulated for 5 min, the tail was cut 3 mm from the tip and carefully immersed in 40 ml distilled water at room temperature. Blood loss was determined 60 min later by measuring the haemoglobin dissolved in the water using a spectrophotometric method (12). The volume of blood was deduced from a standard curve based on absorbance at 540 nm.

Reversal of heparin effects by protamine

Incubations were performed in 96-well plates. The final concentrations of the reactants were 10 nM antithrombin, 2 nM factor Xa in 40 μl of TS/PEG buffer, porcine or bovine heparin (0.1 IU ml⁻¹ of each) and increasing concentrations of protamine chloride (Roche, São Paulo, Brazil) (0 – 0.25 units). Factor Xa was added last to initiate the reaction. After incubation for 60 sec at 37°C, 25 μl of chromogenic substrate S-2222 was added, and the absorbance at 405 nm recorded for 300 sec. Protease activity was defined as 100% in control samples lacking heparin.

Table 2: Integrals of ¹H signals as a percentage of the total area (mean ± SD) obtained from the ¹H NMR spectra of porcine and bovine heparins.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Porcine</th>
<th>Bovine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supplier I</td>
<td>II</td>
</tr>
<tr>
<td>Number of batches</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>A1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95.1 ± 0.8</td>
<td>94.5 ± 0.7</td>
</tr>
<tr>
<td>B1</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>C1</td>
<td>4.9 ± 0.8</td>
<td>5.5 ± 0.7</td>
</tr>
<tr>
<td>I1-B+C</td>
<td>5.0 ± 0.9</td>
<td>6.5 ± 0.7</td>
</tr>
<tr>
<td>I1-A</td>
<td>92.5 ± 1.1</td>
<td>91.5 ± 0.7</td>
</tr>
<tr>
<td>I1-deS</td>
<td>2.5 ± 0.5</td>
<td>2.0 ± 0.0</td>
</tr>
<tr>
<td>I5-C</td>
<td>3.6 ± 0.5</td>
<td>3.0 ± 0.0</td>
</tr>
<tr>
<td>I5-A+B</td>
<td>96.4 ± 0.5</td>
<td>97.0 ± 0.0</td>
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</table>

<sup>a</sup>See legends of Figs 1 and S1 and Table I for the nomenclature of the ¹H signals.

Figure 3: Gel filtration (A) and agarose gel electrophoresis (B) of heparins from porcine and bovine intestinal mucosa. A) Heparin from porcine (●) and bovine (❍) intestinal mucosa (5 mg of each) were applied to a Superdex 75 gel filtration column equilibrated with 20 mM Tris-HCl (pH 8.0). The column was eluted at a flow rate of 0.5 ml min⁻¹, and fractions of 0.5 ml were collected and assayed for their metachromasy. The column was calibrated using blue dextran (average Mr=2000 kDa for Vo), cresol red (Mr=108 Da for Vt), chondroitin 6-sulfate from shark cartilage (average Mr=40 kDa) and the International Heparin Standard (average Mr=12 kDa). B) Heparin and a mixture of standard glycosaminoglycans (15 μg of each) were applied to a 0.5% agarose gel, and electrophoresis was carried out for 1 h at 110 V in 0.05 M 1,3-diaminopropane:acetate (pH 9.0). Gels were fixed with 0.1% N-cetyl-N,N,N-trimethylammonium bromide solution. After 12 h, the gel was dried and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:1:5, v/v). CS, chondroitin 4-sulfate, DS, dermatan sulfate and H, heparin.
Results

Heparin disaccharide composition

The structures of pharmaceutical grade batches of heparin, prepared from either porcine or bovine intestinal mucosa, were analysed by NMR to determine their preponderant disaccharide substitution pattern. For a detailed structural analysis, we performed 1D and 2D NMR spectroscopy with both heparins. The \(^1\)H 1D spectra (Fig. 1) and the 2D \(^1\)H/\(^1\)H COSY, TOCSY and \(^1\)H/\(^13\)C HSQC experiments (see Supplementary Fig. S1 available online at www.thrombosis-online.com) were used to trace the spin systems for both heparins (Table 1A and B).

The complete \(^1\)H 1D spectrum is presented in Figure 1A and B, while the 5.48 – 4.70 ppm regions are shown in panels C and D. The porcine heparin spectrum (Fig. 1A and C) is consistent with a glycosaminoglycan composed of mainly trisulfated disaccharide-repeating units, \(\rightarrow\alpha\)-IdoA2S-1\(\rightarrow\rightarrow\rightarrow\alpha\)-GlcNS6S-1\(\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarr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Figure 3A. Both heparins were highly purified as a side effect of HOD suppression by presaturation. To overcome this problem, 1D 1H NMR spectra of mixtures containing increasing amounts of heparin from bovine intestine added to a fixed amount of porcine heparin confirmed the proposed assignments (see Supplementary Figs. S2 and S3 available online at www.thrombosis-online.com).

Heparin contains small amounts of β-glucuronic acid units (5). We investigated the occurrence of these residues in bovine and porcine heparins. The 1H anomeric signal of β-glucuronic acid resonates at 4.65 ppm (G1 in Fig. 1A and B), which may be underestimated as a side effect of HOD suppression by presaturation. To overcome this problem, 1D 1H NMR spectra were run at different temperatures (10°C to 40°C). At 10°C the HOD signal is shifted downfield, making it possible to visualise clearly the anomeric signal of β-glucuronic acid, which occurs in both bovine and porcine heparins. Furthermore, the H2 signal of this residue (G2 in Fig. 1A and B) resonates at 3.42 ppm (5). Again, bovine and porcine heparins do not differ in their G2 signal.

Molecular mass

High molecular mass is a requirement for high activity in UFH (4). We compared the molecular masses of heparins from bovine and porcine intestinal mucosa using gel filtration chromatography. A typical result is shown in Figure 3A. Both heparins were highly purified as a side effect of HOD suppression by presaturation. To overcome this problem, 1D 1H NMR spectra of mixtures containing increasing amounts of heparin from bovine intestine added to a fixed amount of porcine heparin confirmed the proposed assignments (see Supplementary Figs. S2 and S3 available online at www.thrombosis-online.com).

In conclusion, porcine intestinal heparin is composed mostly by the trisulfated disaccharide →4-α-IdoA2S-1→4-α-GlcNS6S-α-1→, while the α-glucosamine units of bovine heparin vary significantly: ~50% are 6- and N-disulfated, as in porcine heparin, while ~36% are 6-desulfated and ~14% are N-acetylated instead of N-sulfated (Fig. 2).

The 37 batches of pharmaceutical grade heparin, 14 from bovine and 23 from porcine intestinal mucosa and obtained from distinct suppliers, showed highly reproducible NMR spectra, and the variations among the signal areas in Table 2 are always <5%. Some integrals are derived from poorly resolved signals and must be interpreted carefully, especially in the cases of A1 and B1. Three batches of heparin had NMR spectra with signal areas that were intermediate between those observed for heparins from bovine and porcine intestinal mucosa (see Supplementary Fig. S4 available online at www.thrombosis-online.com). They correspond to batches containing mixtures of heparin from the two sources, as shown in the Supplementary Material (available online at www.thrombosis-online.com).

Figure 5: Effects of porcine (●) and bovine (○) heparins on venous thrombosis (A), on ex vivo anticoagulant activity (B) and on bleeding (C). A) Venous antithrombotic activity was investigated using a stasis + hypercoagulability thrombosis model in vena cava of rats. Different doses of bovine or porcine heparin were administered and allowed to circulate for 5 min. Then, thromboplastin (5 mg kg⁻¹ body weight) was slowly injected intravenously and 0.7 cm of the isolated vena cava segment was tied off. After 20 min stasis, the thrombus formed was dried and weighted. The results were expressed as % of thrombus weight, 100% representing absence of any inhibition of thrombosis (thrombus weight in the absence of heparin administration). B) Citrated blood samples were collected from the carotid artery before and 5 min after intravascular administration of heparin. Then, the plasma was assayed for ex vivo APTT. The results were expressed as ratios of clotting time after administration of different heparin doses (T₁) and of the control, saline treated animals (T₀). Values above 10 are not detected in the assay. C) Different doses of heparin were infused into rats. After 5 min, the rat’s tail was cut 3 mm from the tip and immersed in 40 ml of distilled water at room temperature. Blood loss was determined 60 min later by measurement of the haemoglobin in the water. The results were expressed as µl of blood loss. The insets in panels A and C express the dose-versus-response curves on the basis of anticoagulant activity (IU kg⁻¹). For clarity, only one standard error (SE) bar is shown in the panels. The curves in the panels were traced using the OriginPro 7.5 program. All the results are expressed as mean ± SE, n=5, *p < 0.01 and **p < 0.05 for bovine vs. porcine heparin using the Mann-Whitney Rank Sum test. The arrows in the insets of panels A and C indicate the dose of heparin required to achieve total inhibition of thrombosis (100 IU kg⁻¹ body weight).
polydisperse, but showed approximately similar average molecular masses. During agarose gel electrophoresis (Fig. 3B) these heparins run as a single band, excluding contamination with other glycosaminoglycans.

Anticoagulant activity

Bovine heparin has an anticoagulant activity of 104 ± 22 IU mg⁻¹ (mean ± SD, n = 7) by APTT, compared with a value of 197 ± 15 IU mg⁻¹ for porcine heparin (Fig. 4A). Assays using purified antithrombin and coagulation proteases also showed a significant difference between their anti-Xa activities (197 vs. 78 IU mg⁻¹, Fig. 4B) and an even more pronounced difference when thrombin replaced factor Xa (197 vs. 48 IU mg⁻¹ as anti-IIa activity, Fig. 4C). The three batches containing mixtures of bovine and porcine heparins had intermediate anticoagulant activity based on the APTT assay (see Supplementary Table S1 available online at www.thrombosis-online.com).

Antithrombotic activity

Anticoagulant activity does not always correlate with antithrombotic effect (13). To compare the antithrombotic activity of bovine and porcine heparins, we used a classical venous model of experimental thrombosis after intravascular administration of these two glycosaminoglycans. Clearly, bovine heparin requires twice the dose of porcine heparin, on a weight basis, to achieve the same antithrombogenic effect (Fig. 5A). The complete inhibition of thrombus formation was achieved with ~0.5 and ~1.0 mg kg⁻¹ body weight of porcine and bovine heparins, respectively. However, both heparins have similar dose-versus-response curves based on their anticoagulant activity (Fig. 5A, inset). Plasma collected from animals during the experimental thrombosis showed that bovine heparin requires approximately twice the dose of porcine heparin to achieve a similar anticoagulant effect (Fig. 5B). This indicates similar in vitro and ex vivo effects of these two heparins on blood coagulation.

Bleeding tendency

To compare bleeding tendency after intravascular administration of bovine or porcine heparins, rats were subjected to a blood loss assay. The results showed that both heparins have the same dose-dependent induction of bleeding on a weight basis (Fig. 5C). However, curves based on anticoagulant activity (Fig. 5C, inset) clearly showed that bovine heparin is twice as potent in inducing bleeding as porcine heparin.

Heparin neutralisation by protamine

Neutralisation of heparin at the end of extracorporeal circulation or when overdose of the drug is detected requires proper doses of protamine. Heparins with different chemical and biological properties, such as bovine and porcine heparins, may exhibit distinct protamine neutralisation curves. We investigated this feature by adding protamine at similar doses to bovine or porcine heparin (on an IU basis), and heparin neutralisation was assessed based on anti-Xa activity (Fig. 6). Clearly, bovine heparin requires significantly higher doses of protamine than porcine heparin to achieve neutralisation.

Discussion

Almost concurrently with the removal from the Brazilian market of a well known and referenced preparation of UFH, the number of reports from surgeons of bleeding and peri- or post-operative blood dyscrasias associated with the use of heparin started to increase. This event led us to perform a detailed survey of the pharmaceutical grade heparin batches available in the Brazilian market. Thirty-seven batches were carefully checked for heparin structure and anticoagulant activity, revealing that two types of heparins co-existed and were used interchangeably (6, 7). Of these samples, 23 batches of heparin were obtained from porcine intestinal mucosa and 14 others from bovine mucosa. The proportions of batch sources used in this study do not reflect the proportions of heparins available on the market, as bovine heparin still dominates the Brazilian market. Unfortunately, it was not possible to correlate clinical events with specific heparin batches due to the absence of appropriate records.

Our results indicate that these two types of heparins differ markedly in their preponderant disaccharide composition. Por
Heparin from bovine and porcine intestine also differ significantly in their anticoagulant activities. Porcine heparins have almost twice the activity of bovine heparins in the APTT assay (Fig. 4A). The difference is even more significant for the anti-IIa activities (Fig. 4C). Formation of the ternary complex between antithrombin, thrombin and heparin requires interaction of heparin and the protease, which depends mostly on the charge of the glycosaminoglycan (14). The lower charge density of bovine heparin may have led to a lower affinity for thrombin. Thus, instead of the balanced effects on thrombin and factor Xa observed for porcine heparin, heparin from bovine intestine acts mostly through factor Xa. Anti-Xa activity requires the formation of a heparin-antithrombin complex, without binding to the protease.

Heparin from bovine lung, which was commonly used until the 1980s, was extensively studied. Bovine lung heparin is composed mostly of trisulfated disaccharides, as is porcine heparin. Only minor pharmacological differences were observed between these two heparins (4). This past observation led to the misconception that bovine and porcine heparins are similar compounds, without considering that bovine heparins are extracted from two types of organs – lung and intestine. Furthermore, the initial analysis of heparin by NMR employed mostly 1D 13C spectra (16), which clearly cannot distinguish the complexity of disaccharide composition found in heparins from bovine intestine (see the 13C axis in Supplementary Fig. S1F available online at www.thrombosis-online.com). Curiously, the heparin used as the first British standard was obtained from bovine intestinal mucosa. Signals associated with undersulfation in this standard were attributable to either the presence of heparan sulfate or unusual heparin structures (15).

From a strictly academic point of view, heparin from bovine intestine is at the borderline between a “typical heparin” and a “highly sulfated heparan sulfate”. More important, however, are the practical implications of the differences observed between pharmaceutical grade heparins from these two sources. First, several pharmacopoeias have been or are in the process of being revised to establish more rigorous criteria to assess the purity of heparin (17). In these pharmacopoeias, porcine and bovine heparins are treated as similar compounds. Our results suggest these heparins should be described as different drugs. Second, health professionals use these two heparins interchangeably because they are usually unaware of the differences in structure, specific anticoagulant activity, side effects, and doses of protamine required for their neutralisation. Clear identification of heparins prepared from these two sources should aid in their medical use.

Bovine heparin requires twice the dose of porcine heparin on a weight basis to achieve similar effects on experimental thrombosis in rats (Fig. 5A), while both heparins induce similar levels of bleeding (Fig. 5C). Comparable bleeding is obtained at a dose of bovine heparin that has reduced antithrombotic effects. Thus, the doses of heparin from bovine intestine required for antithrombotic protection and production of adverse bleeding effects are more similar than for porcine heparin. This observation may explain the increasing reports of bleeding and peri- or post-operative blood dyscrasias observed in Brazilian patients. Reports of side effects were restricted mostly to cardiovascular surgeries, possibly due to the
high risk for bleeding in these patients and to the high doses of heparin used during the procedure. Approximately four- to eightfold higher doses of heparin are used during cardiovascular surgeries than in the treatment / prevention of thromboembolism, renal dialyses and vascular surgeries. We predict that heparins from bovine and porcine intestinal mucosa will differ in their doses required to achieve effective antithrombotic effect and in the frequency of bleeding side effect when tested in appropriate clinical trials for pathological conditions other than cardiovascular surgery.

Our results help clarify the increased frequency of undesirable effects associated with the use of this particular type of heparin. More significantly, this study indicates the information required before heparin preparations from new sources become available for medical use. During the present shortage of heparin, there is increased pressure to find new sources of this compound, but careful evaluation of the structure and biological effects of heparin from novel sources is required to assure therapeutic efficiency and minimal side effects.

In conclusion, our results suggest that pharmaceutical grade heparins obtained from porcine and bovine intestinal mucosa differ significantly in their preponderant disaccharide compositions, anticoagulant activities, bleeding tendencies and doses required to inhibit experimental thrombosis. We suggest that these two heparins are not equivalent drugs.

Acknowledgements

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento do Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ). We would like to thank Dr. Mauro S.G. Pavão and Ana M.F. Tovar for critical reading of the manuscript and Adriana A. Piquet for technical assistance.

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