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Biological and structural analyses of bovine heparin fractions of intermediate and high molecular weight

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ABSTRACT

Low molecular weight heparin, which is generally obtained by chemical and enzymatic depolymerization of unfractionated heparin, has high bioavailability and can be subcutaneously injected. The aim of the present investigation was to fractionate bovine heparin using a physical method (ultrafiltration through a 10 kDa cut-off membrane), avoiding structural modifications that can be caused by chemical or enzymatic treatments. Two fractions with different molecular weights were obtained: the first had an intermediate molecular weight (B-IMWH; *Mn* = 9587 Da) and the other had a high molecular weight (B-HMWH; 22,396 Da). B-IMWH and B-HMWH have anticoagulant activity of 103 and 154 IU/mg respectively, which could be inhibited by protamine. Both fractions inhibited α-thrombin and factor Xa *in vitro* and showed antithrombotic effect *in vivo*. Moreover, *ex vivo* aPTT assay demonstrated that B-IMWH is absorbed by subcutaneous route. The results showed that ultrafiltration can be used to obtain two bovine heparin fractions, which differ on their molecular weights, structural components, anticoagulant potency, and administration routes.

1. Introduction

Heparin is a linear polysaccharide consisting mainly of →4)-α-D-glucosamine-(1→4)-α-L-iduronic acid-(1→ repeating groups, with a complex pattern of substitution of *N*- and *O*-sulfates, and *N*-acetil groups (Casu, Naggi, & Torri, 2015). It acts as an anticoagulant mainly by binding to antithrombin (AT) and heparin cofactor II (HCII), enhancing the rate at which they inactivate enzymes involved in coagulation (Bourin & Lindahl, 1993; Casu, 1985). Although largely effective, the clinical use of heparin has limitations. Through its negative groups, it presents non-specific binding with many plasma proteins which affect its bioavailability. Thus, the anticoagulant effect of heparin is unpredictable, with high risk of bleeding, which requires a close clinical monitoring of the patient for a safe use (Mousa, 2007).

Low molecular weight heparin (LMWH), which has a molecular weight between 3800 and 6500 Da, is obtained by enzymatic or chemical depolymerization of unfractionated heparin (UFH), which has a molecular weight between 5000 and 40,000 Da (Johnson & Mulloy, 1976; Keire et al., 2015; Linhardt, 2003). LMWH presents less non-specific binding with plasma proteins, higher bioavailability, predictable anticoagulant response and a longer plasma half-life, when compared to UFH (Cohen, 2000; Hirsh, 1998). Moreover LMWH can also be subcutaneously injected, and its use does not require a close clinical monitoring (Bratt, Törnebohm, Widlund, & Lockne, 1986; Kakkar, 2004).

Heparin for pharmaceutical uses is obtained from porcine intestines or bovine lung. However, since the nineties bovine heparins are no longer used in Europe and in the United States of America because of the possibility of contamination with the prion agent of the bovine spongiform encephalopathy (BSE), related to variant Creutzfeldt–Jakob disease (vCJD) in humans (Kort, Buijsman, & Boeckel, 2005). However, there is no evidence that bovine heparin is related with BSE in humans. Bovine heparin has been continuously used in India, Brazil and Argentina, and no case of vCJD has been reported in these countries (Keire et al., 2015). Although there are many studies related to heparin, few researches have been performed with bovine heparin, probably due to the decrease of its use. Moreover, there is no commercialization of bovine low molecular weight heparin, and no studies on its possible application were found.

The aim of the present investigation was to fractionate bovine heparin using a physical method (ultrafiltration), avoiding structural modifications that can be caused by chemical or enzymatic

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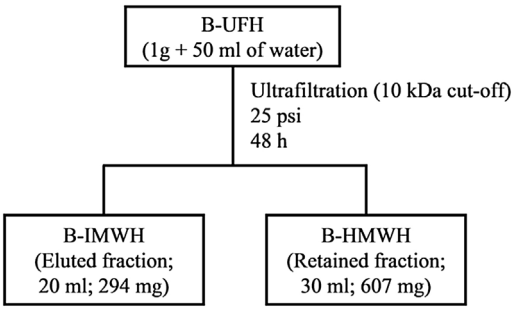

Fig. 1. Scheme of fractionation of bovine heparin using ultrafiltration. B-UFH (bovine unfractionated heparin); B-IMWH (Bovine Intermediate Molecular Weight Heparin); B-HMWH (Bovine High Molecular Weight Heparin).

treatments. Bovine heparin fractions with intermediate and high molecular weights were obtained, assayed for anticoagulant and antithrombotic activities, and structurally compared by nuclear magnetic resonance analysis.

2. Materials and methods

2.1. Materials

Bovine unfractionated heparin (Lot: 11101411), supplied by Extrasul (Jaguapitã – Paraná – Brazil), and international standard of porcine heparin with 200.47 IU/mg (6th International Standard 2009–unfractionated heparin).

2.2. Methods

2.2.1. Fractionation of the bovine heparin

Bovine unfractionated heparin (B-UFH; 1 g) was dissolved in distilled water (50 ml) and submitted to ultrafiltration in a Sartorius apparatus (model 16249) using a 10 kDa cut-off membrane (regenerated cellulose membrane, 47 mm, filter code: PLGC, Millipore) to obtain an eluted (B-IMWH; Bovine Intermediate Molecular Weight Heparin) and a retained (B-HMWH; Bovine High Molecular Weight Heparin) fraction (Fig. 1). Ultrafiltration was conducted at 25 psi, for 48 h, when 20 ml of the material were filtered. After this time the flow rate stopped. Both fractions were lyophilized in a Modulyo freeze drier (Edwards).

2.2.2. Analysis of nuclear magnetic resonance (NMR): determination of major components and molecular weight

The NMR analyses were performed in a 600 MHz spectrometer (Avance III, Bruker), equipped with a QXI inverse probe of 5 mm. The samples were dissolved in D_2O and then analyzed at 30 °C. The chemical shift of ^1H and ^{13}C were referenced in relation to 0.001% of TMSP-d_4 (2,2,3,3- tetradeuterium-3-trimethylilsilylpropionate) as internal standard ($\delta = 0$). 1D ^1H NMR were performed after 90° (p1) pulse calibration by evolution until 360° using a start p1 of 4 μs plus increment of 2 μs (p1 6.4–7.0 μs), calculation of offset (1885.0–1885.6 Hz) to obtain a spectrum width of 4795 Hz, using 16 scans to give a signal/noise ratio (S/N) of at least 1000:1 for the anomeric region (90° pulse, relaxation delay = 4.0 s, number of time domain points = 65,536 and acquisition time = 6.832 s). Integration of H-1 areas was performed without tube spinning and respecting a HDO signal with a medium half line varying from 1.0–1.2 Hz and TMSP 0.8–1.0 Hz. Presaturation of residual HDO was carried out with the pulse program zgpr, which included presaturation during relaxation delay, using a relaxation delay = 4.0 s, number of time domain points = 65,536 and acquisition time = 6.832 s. 2D-NMR HSQC, heteronuclear correlation via double inept transfer with decoupling during acquisition, using sensitivity improvement trim pulses in inept transfer and shaped pulses for all 180° pulses on the ^{13}C channel (hsqcetgpsisp 2.2 on Bruker spectrometers), was performed as described by Torri and Guerrini (2008). The spectral widths for Q-HSQC were 3595 Hz (^1H) and 5031 Hz (^{13}C), experiments being recorded for quadrature detection in the indirect dimension, using 24 scans per series of 1 K × 320 W data points with zero filling in F1 (2 K) prior to Fourier transformation. NMR signals were assigned based on literature data (Alekseeva et al., 2014; Bhaskar et al., 2015; Fu et al., 2013; Guerrini, Guglieri, Naggi, Sasisekharan, & Torri, 2007; Naggi et al., 2016).

Analysis of major components of heparins was performed taking into account the areas of the anomeric signals on 2D-NMR HSQC spectra.

The molecular weight (*Mn*) was determined as descripted by Desai and Linhardt (1995) method, using the formula below:

$$Mn = [\{S_{int}/(S_{red} \times 1.15) + 1\}/2] \times [\text{average disaccharide mass}]$$

S_{red} and S_{int} are obtained by integration of signals in the anomeric region of 2D-NMR HSQC, where S_{red} is the total volume of the $^1H/^{13}C$ correlation of the reducing ends and S_{int} refers to internal units.

2.2.3. Anticoagulant activity

The anticoagulant activity of the heparins was determined *in vitro* by comparing their ability to increase the aPTT (activated partial thromboplastin time) of recalcified citrated sheep plasma with the ability of a reference preparation of heparin calibrated in International Units. A standard curve (log of aPTT × IU of heparin) was obtained using varied concentrations of an international standard of porcine heparin with 200.47 IU/mg. The results were expressed as activity mean ± standard error of the mean (SEM) ($n = 2$).

The aPTT assays were determined with a Dade Actin kit (Dade Behring, Marburg, DE), in a COAG-A-MATE XM coagulometer (Organon Teknika Corporation), using a pool of citrated sheep plasma. Plasma (100 μl) was incubated at 37 °C with saline or heparin (100 μl) for 1 min. Then, rabbit cephalin (100 μl) was added. After 2.5 min, 0.025 M $CaCl_2$ (100 μl) was added and the clotting time measured.

2.2.4. Inhibition of the anticoagulant effect of the heparins by protamine sulfate

The effect of protamine sulfate on the anticoagulant activity of the heparins was determined by aPTT. 100 μl of saline solution, or heparin (0.25 IU in 50 μl) plus protamine sulfate (0.00–0.10–0.25–0.40–0.55–0.70–0.85–1.00–1.50–2.00–5.00 μg in 50 μl) were incubated at 37 °C with citrated sheep plasma (100 μl) for 1 min. Then, rabbit cephalin (100 μl) was added. After 2.5 min, 0.025 M $CaCl_2$ (100 μl) was added and the clotting time measured. aPTT was expressed as mean ± standard error of the mean (SEM) ($n = 2$).

2.2.5. Inhibition of α-thrombin and factor Xa

The assays were performed in 96-well plates. The final concentrations of the reactants included 100 nM antithrombin (AT) or 15 nM heparin cofactor II (HCII), 6 nM α-thrombin or 8 nM factor Xa (Haematologic Technologies) and 1×10^{-6} to 1 UI of heparin in 75 μl of TS/PEG buffer (0.02 M Tris/HCl, 0.15 M NaCl, and 1.0 mg/ml polyethylene glycol 8000, pH 7.4). The α-thrombin or factor Xa was added last to initiate the reaction. After 1 min of incubation at 37 °C, 25 μl of chromogenic substrate S-2238 for α-thrombin or S-2222 for factor Xa (Chromogenix AB) were added (100 μM final concentration), and absorbance at 405 nm recorded over 5 min (Multimode microplate reader, Infinite M200, Tecan). The change of absorbance was proportional to the α-thrombin or factor Xa activ-

ity. In the absence of heparin the α-thrombin or factor Xa activity was considered 100%.

2.2.6. Animals

Experiments were conducted on male or female Wistar rats (180–220 g) from the colony of Federal University of Paraná, Curitiba, Brazil. They were maintained under standard laboratory conditions (12 h light/dark cycle, temperature 22 ± 2 °C), with standard pellet food and water *ad libitum*. The animals were anesthetized with an intramuscular injection of a mixture of ketamine (100 mg/kg body weight) and xylazine (16 mg/kg). The Institutional Ethics Committee of Federal University of Paraná approved all the procedures adopted in this study (authorization number 428).

2.2.7. Venous thrombosis

Thrombus formation was induced by promoting a combination of stasis and hypercoagulability (Berry, Girard, Lochot, & Lecoffre, 1994; Vogel, Meleuman, Bourgondiën, & Hobbelen, 1989). Rats were anesthetized and their right carotid artery was cannulated for injection of vehicle (Phosphate buffered saline – PBS; 0.136 M NaCl, 0.0268 M KCl, 0.0081 M Na_2HPO_4, 0.00147 M KH_2PO_4, pH adjusted for 7.2 with 1 M HCl), heparin and thromboplastin. The abdominal vena cava was dissected, and loose sutures were placed between the right renal vena and femoral veins, and in the left renal vena. PBS or heparin was infused into the right carotid artery and allowed to circulate for 5 min. Thrombus formation was then induced by injection of thromboplastin (5 mg/kg body weight), and 20 s later by stasis of a 0.7 cm segment of the abdominal vena cava. After 20 min, the thrombus formed inside the occluded segment was then pulled out, washed with PBS, freeze dried for 24 h, and weighed. For each group ($n \geq 6$), the thrombus weight mean ± standard error of the mean (SEM) was determined and expressed as percentage of thrombosis, with 100% representing absence of any inhibition of thrombus formation (thrombus weight with PBS administration).

2.2.8. Ex vivo *aPTT*

Vehicle (PBS) or heparins (750 IU/kg) were injected in rats subcutaneously in the dorsal region (500 μl/kg). After 1.5 h the rats were anesthetized and their right carotid artery was cannulated to collect 0.5 ml of blood, which was immediately placed in a microtube with 50 μl of 3.8% sodium citrate solution. Blood samples were collected 2 and 3 h after injection of PBS or heparins. Then, the blood was centrifuged at 2000 rpm by 10 min to obtain the citrated plasma. In order to determine the aPTT, plasma (50 μl) was incubated at 37 °C by 1 min. Then rabbit cephalin (50 μl) was added. After 2.5 min, 0.02 M $CaCl_2$ (50 μl) was added and the clotting time measured. Results were expressed as *ex vivo* aPTT mean (s) ± standard error of the mean (SEM) ($n = 2$).

2.2.9. Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM) and the statistical significance of the results was determined using one-way analysis of variance (ANOVA), followed by Tukey's test. Data were considered different at a significance level of $p < 0.05$. The IC_{50} and ED_{50} values were determined by nonlinear regression using GraphPad Prism version 3.02 for Windows (GraphPad Software, Inc.).

3. Results

3.1. Fractionation of the bovine heparin and anticoagulant activity

The bovine unfractionated heparin (B-UFH; *Mn* = 16,824 Da) was submitted to ultrafiltration using a 10 kDa cut-off membrane to obtain an eluted (B-IMWH; Bovine Intermediate Molecular Weight Heparin; 33% yield; *Mn* = 9587 Da) and a retained (B-HMWH; Bovine High Molecular Weight Heparin; 67% yield; *Mn* = 22,396 Da) fraction (Fig. 1).

Table 1
Anticoagulant activity, molecular weight and yield of B-UFH, B-IMWH, and B-HMWH.

Heparins	Activity (IU/mg)[a]	*Mn* (Da)	Yield (%)[b]
B-UFH	142 ± 1.62	16,824	–
B-IMWH	103 ± 0.73***	9587	33
B-HMWH	154 ± 0.58**	22,396	67

[a] The anticoagulant activity was determined by aPTT, using an international standard of porcine heparin with 200.47 IU/mg as reference. The results were expressed as activity mean ± SEM (n = 2). $p < 0.01$** and $p < 0.001$*** when compared with B-UFH.
[b] The yield was based on the quantity of recovered material (901 mg).

The anticoagulant activity of B-UFH, B-IMWH, and B-HMWH was 142, 103 and 154 IU/mg respectively (Table 1). B-HMWH was 8.7% more potent than B-UFH, whereas B-IMWH was 27% less potent than B-UFH.

3.2. Effect of the heparins on inhibition of α-thrombin and factor Xa

B-IMWH and B-HMWH were incubated with α-thrombin in the presence of AT or HCII, and with factor Xa in the presence of AT. As expected, both bovine heparin fractions inhibited α-thrombin and factor Xa in these conditions. The inhibitory effect of B-IMWH on thrombin in the presence of AT was lower than that of B-HMWH (Fig. 2A). However, the inhibitory effects of the heparins on thrombin in the presence of HCII and on factor Xa in the presence of AT were very similar (Fig. 2B and C).

3.3. Effect of protamine sulfate on the anticoagulant activity of the heparins

Protamine sulfate was able to neutralize the anticoagulant effect of both B-IMWH and B-HMWH (Fig. 3), in a dose dependent way.

3.4. In vivo *antithrombotic activity of the heparins*

The antithrombotic activity of the heparins was investigated upon a venous thrombosis model in rats (Fig. 4). In the control group, which received PBS, the dried thrombus weight was 5.0 ± 0.8 mg (mean ± SEM), corresponding to 100% thrombosis. B-IMWH and B-HMWH inhibited thrombus formation with an ED_{50} of 0.17, and 0.06 IU/kg respectively. However, there was no statistically significant difference between the results of B-IMWH and B-HMWH.

3.5. Ex vivo *anticoagulant effect after subcutaneous injection of the heparins*

Ex vivo aPTT was evaluated after subcutaneous injection of 750 IU/kg of B-IMWH and B-HMWH in rats (Fig. 5). The aPTT mean for the negative control group (PBS) was 18.72 and 18.78 s for the blood collected in the second and third hour after administration of PBS, respectively. B-IMWH increased at 6.8 and 4.5 times the aPTT of the animals 2 and 3 h after its subcutaneous injection, respectively. Subcutaneous administration of B-HMWH increases slightly the aPTT when compared with B-IMWH.

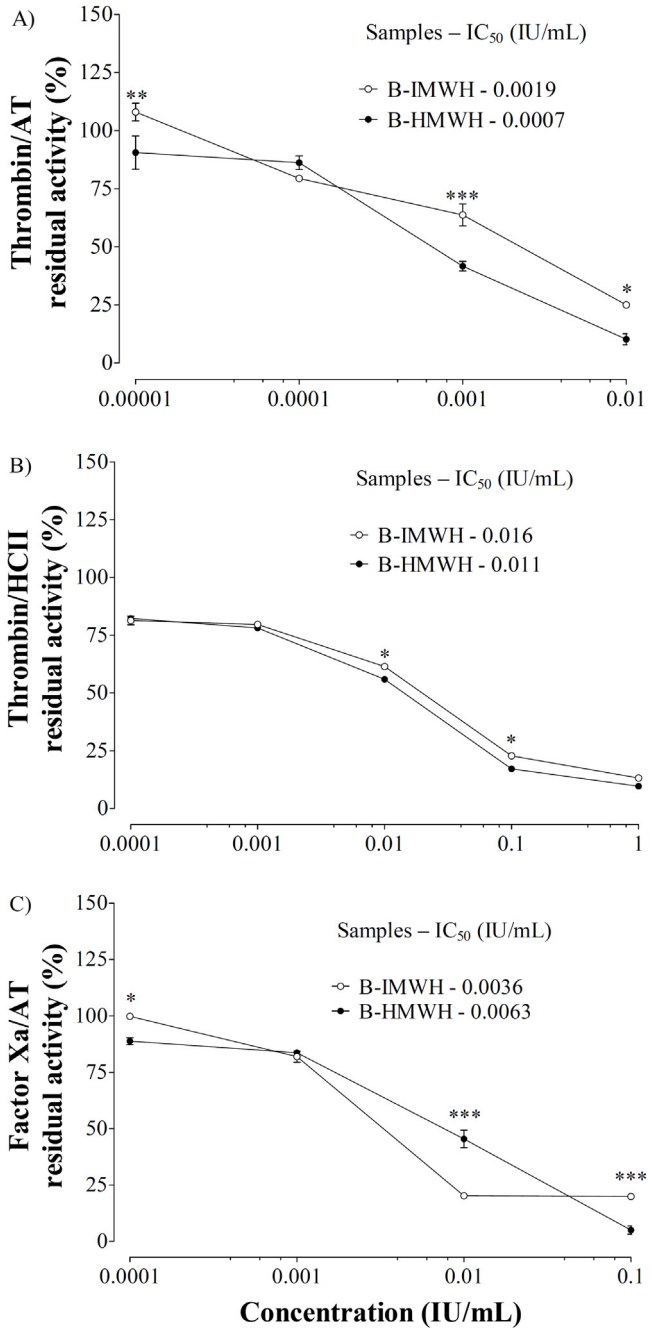

Fig. 2. Effect of the heparins on inhibition of α-thrombin by AT (A), α-thrombin by HCII (B), and factor Xa by AT (C). 6 nM of α-thrombin or 8 nM factor Xa and 100 nM AT or 15 nM HCII were incubated with different concentrations of B-IMWH (○), or B-HMWH (●). After 1 min at 37 °C, specific chromogenic substrate was added, and α-thrombin or factor Xa activity expressed as a proportion of the absorbance at 405 nm (means ± SEM, $n = 3$ with, with $p < 0.05^{*}$, $p < 0.01^{**}$ and $p < 0.001^{***}$), with 100% of activity considered as the absorbance achieved without the addition of heparin.

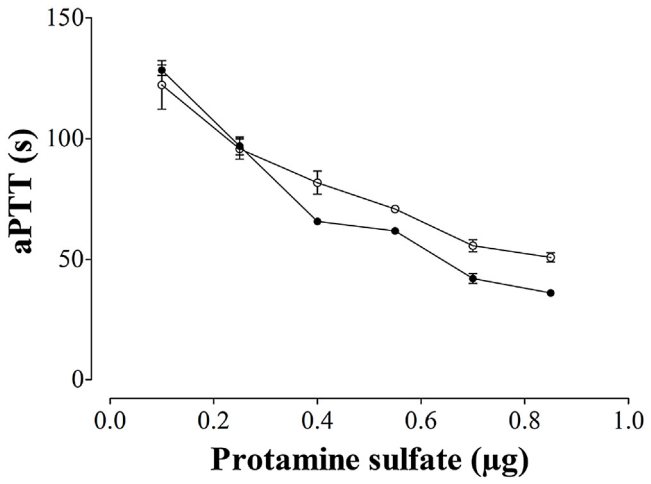

Fig. 3. Effect of protamine sulfate on the anticoagulant activity of B-IMWH (○) and B-HMWH (●). 100 µl of saline solution, or heparin (0.25 IU in 50 µl) plus protamine sulfate in different concentrations (50 µl), were incubated at 37 °C with citrated sheep plasma (100 µl) for 1 min. Then, rabbit cephalin (100 µl) was added. After 2.5 min, 0.025 M $CaCl_2$ (100 µl) was added and the clotting time measured. The results were expressed as aPTT mean ± SEM (n = 2). There was no statistically significant difference between B-IMWH and B-HMWH.

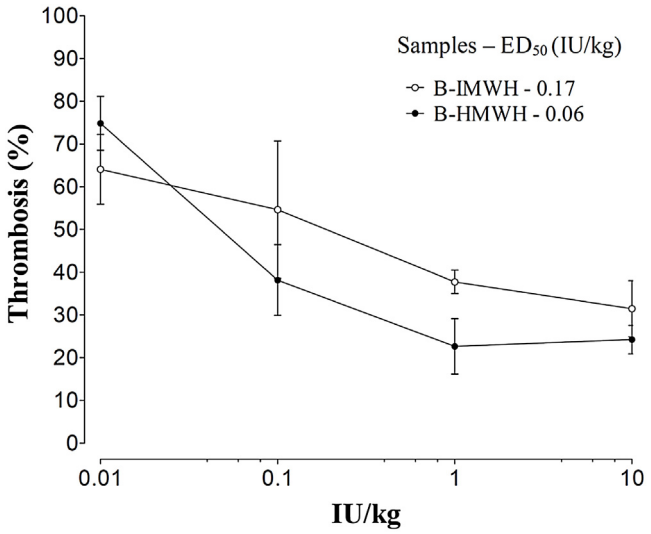

Fig. 4. Venous antithrombotic effect after intravascular administration of B-IMWH (○) or B-HMWH (●). Thrombus formation was induced by promoting a combination of stasis and hypercoagulability. Different doses of the heparins were administered in the right carotid artery and allowed to circulate for 5 min. Thromboplastin (5 mg/kg body weight) was then injected and 20 s later, 0.7 cm of an isolated segment of the abdominal vena cava was tied off. After stasis for 20 min, the thrombus formed on the interior was pulled out, dried and weighed. Results are expressed as % of thrombosis (mean ± SEM, $n \geq 6$), 100% representing absence of any thrombosis inhibition (thrombus weight in the absence of heparin administration). There was no statistically significant difference between B-IMWH and B-HMWH.

3.6. Compositional comparison of the heparins

The compositional comparison of the heparins was made by analysis of major components determined by 2D-NMR HSQC (Fig. 6).

B-IMWH presented difference in the proportion of almost all components when compared to B-UFH. Among the differences is the higher percentage of reducing end-units of α-$GlcNSO_3$ ($ANS_{\alpha\text{-red}}$) and α-GlcNAc ($ANAc_{\alpha\text{-red}}$), 2.9 and 1.2 times respectively, and the higher proportion of the disaccharide GlcA-$GlcNSO_3$3,$6SO_3$ (G-A*), which is present in the pentasaccharide considered the binding site to AT. In the other hand, B-HMWH was more similar to B-UFH (Table 2).

4. Discussion

Bovine heparins are no longer used in several countries because the theoretical risk of contamination with the prion of bovine spongiform encephalopathy. However, the procedures to purify heparin from animal tissues probably eliminate any possibility of contamination by pathogens. Another problem concerning the

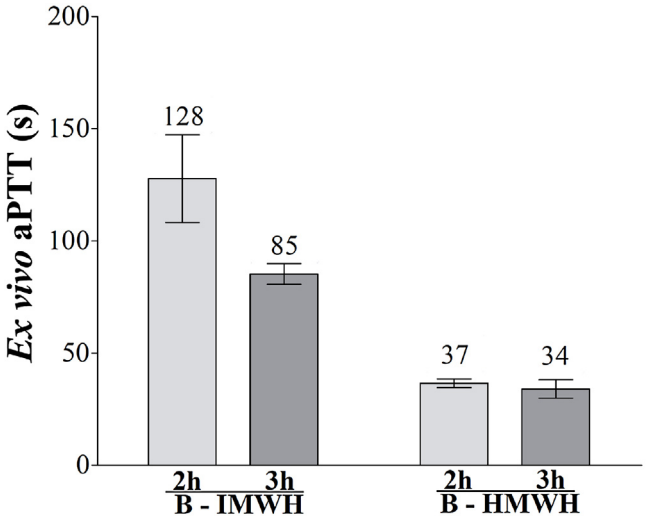

Fig. 5. *Ex vivo* anticoagulant effect after subcutaneous injection of the heparins. Blood was collected 2 or 3 h after subcutaneous injection of PBS or heparins in rats, and used to determine the aPTT. Results were expressed as ex vivo aPTT mean (s) ± SEM (n = 2). There was no statistically significant difference between 2 h and 3 h.

Table 2
Percentage of the major components of B-UFH, B-IMWH, and B-HMWH.

		%	Fraction ratio/B-UFH[b]	
		B-UFH[a]	B-IMWH	B-HMWH
Glucosamines [1]	$ANS_{\alpha\text{-red}}$	1.2	↑ 2.9	↓ 0.8
	$ANAc_{\alpha\text{-red}}$	0.7	↑ 1.2	↓ 0.8
	$ANS_{\beta\text{-red}}$	1.5	↓ 0.6	↓ 0.7
	ANS-I	4.6	=1.0	=1.0
	A*	1.2	↓ 0.6	=1.0
	ANS-G	14.5	↓ 0.8	↓ 0.9
	ANAc-G	76.3	=1.0	=1.0
	ANS-I2S			
Uronic acids [2]	I2S	79.3	=1.0	=1.0
	I2S-ANH2			
	I-A6S	1.7	↓ 0.9	↓ 0.6
	I-A	3.9	↓ 0.6	↓ 0.9
	G-A*	0.4	↑ 1.6	↓ 0.6
	G-ANS	5.1	↑ 1.1	↓ 0.8
	G-ANAc	7.7	↑ 1.3	↑ 1.2
	G2S	1.8	↑ 1.5	↓ 0.8
LR [3]	G-Gal	54.8	=1.0	=1.0
	Gal	22.7	↑ 1.1	↑ 1.2
	Xyl	22.5	↓ 0.8	↓ 0.8

$ANS_{\alpha\text{-red}}$ – reducing end-unit of α-$GlcNSO_3$; $ANAc_{\alpha\text{-red}}$ – reducing end-units of α-GlcNAc; $ANS_{\beta\text{-red}}$ – reducing end-unit of β-$GlcNSO_3$; ANS-I – $GlcNSO_3$-IdoA; A* – $GlcNSO_3$3,$6SO_3$; ANS-G – $GlcNSO_3$-GlcA; ANAc-G – GlcNAc-GlcA; ANS-I2S – $GlcNSO_3$-$IdoA2SO_3$; I2S – $IdoA2SO_3$; I2S-ANH2–$IdoA2SO_3$- $GlcNH_2$; I-A6S – IdoA-$GlcN6SO_3$; I-A – IdoA-GlcN; G-A* – GlcA-$GlcNSO_3$3,$6SO_3$; G-ANS – GlcA-$GlcNSO_3$; G-ANAc – GlcA-GlcNAc; G2S – $GlcA2SO_3$; G-Gal – GlcA-Gal; Gal – galactose; Xyl – xylose.

[a] Percentage of the major components in the B-UFH by 2D-NMR HSQC. 100% is equivalent to the total of: 1–Glucosamines; 2–uronic acids; and 3–Linkage Region (LR).

[b] Number of times of increase (↑) or decrease (↓) of the component in relation to B-UFH.

bovine heparin is its anticoagulant activity significantly lower than that of porcine. Porcine unfractionated heparin normally has anticoagulant activity of at least 180 IU/mg, whereas bovine unfractionated heparins normally have activity lower than 150 IU/mg (Keire et al., 2015; Kotoku, Yosizawa, & Yamauchi, 1967; Lasker & Stivala, 1966; Liberti & Stivala, 1967; Radoff & Danishefsky, 1981; Rosenfeld, Prior, & Girardi, 1991).

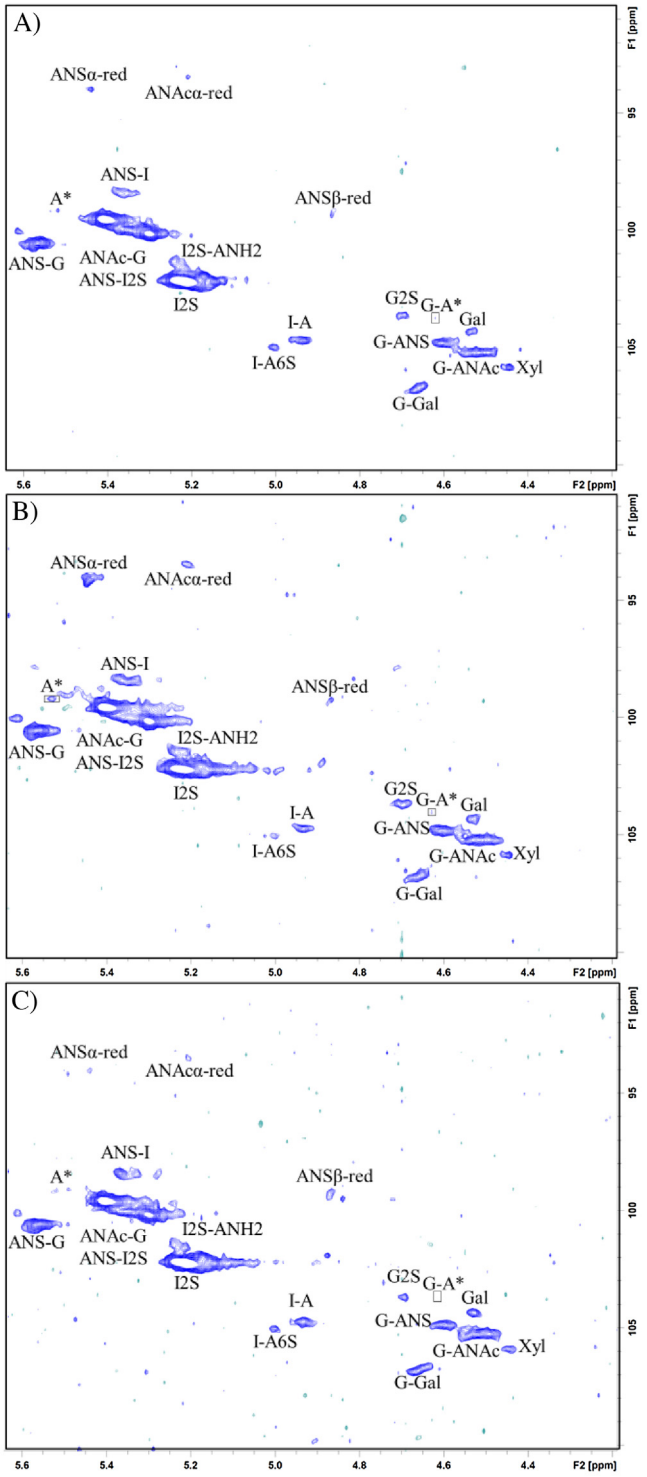

Fig. 6. 2D HSQC NMR. A) anomeric region of B-UFH; B) anomeric region of B-IMWH; C) anomeric region of B-HMWH. $ANS_{\alpha\text{-red}}$ – reducing end-unit of α-$GlcNSO_3$; $ANAc_{\alpha\text{-red}}$ – reducing end-units of α-GlcNAc; $ANS_{\beta\text{-red}}$ – reducing end-unit of β-$GlcNSO_3$; ANS-I – $GlcNSO_3$-IdoA; A* – $GlcNSO_3$3,$6SO_3$; ANS-G – $GlcNSO_3$-GlcA; ANAc-G – GlcNAc-GlcA; ANS-I2S – $GlcNSO_3$-$IdoA2SO_3$; I2S – $IdoA2SO_3$; I2S-ANH2–$IdoA2SO_3$- $GlcNH_2$; I-A6S – IdoA-$GlcN6SO_3$; I-A – IdoA-GlcN; G-A* – GlcA-$GlcNSO_3$3,$6SO_3$; G-ANS – GlcA-$GlcNSO_3$; G-ANAc – GlcA-GlcNAc; G2S – $GlcA2SO_3$; G-Gal – GlcA-Gal; Gal – galactose; Xyl – xylose.

LMWHs, which are good alternatives for the treatment of procoagulant disorders, are manufactured from porcine heparin using depolymerization methods, which can change the chemical structure of the products, affecting not only their molecular weights.

In this study, bovine heparin (B-UFH) was fractionated by ultrafiltration, a procedure that maintains the native structure of the molecule. The anticoagulant activity of B-HMWH increased relatively to B-UFH, showing that ultrafiltration can be used to obtain a more active fraction. On the other hand, the anticoagulant activity of B-IMWH decreased relatively to B-UFH. It has been known for a long time that the anticoagulant activity measured by aPTT decreases with decreasing molecular weight (Barrowcliffe, Mulloy, Johnson, & Thomas, 1989; Lane, MacGregor, VanRoss, Cella, & Kakkar, 1979; Rosenfeld et al., 1991). The same relation has also been found when measuring the inhibition of thrombin, factor IXa and factor XIa in the presence of AT, whereas the inhibition of factor Xa, factor XIIa and kallikrein is less dependent on the size of heparin (Holmer, Kurachi, & Söderström, 1981). This behavior was also observed for B-IMWH and B-HMWH when inhibiting thrombin and factor Xa, and could explain the relation between their molecular weight and anticoagulant activity.

Despite its lower activity, B-IMWH shows anticoagulant effect when injected subcutaneously. The decrease of the anticoagulant activity from 2 to 3 h after subcutaneous injection of B-IMWH suggests its biodegradation and/or excretion. The anticoagulant effects of both B-HMWH and B-IMWH were inhibited by protamine sulfate. In the case of anticoagulant agents, which are used to control blood clotting in patients with hypercoagulable disorders, it is appropriate that there is a way to neutralize the effect of an overdose.

Therefore, ultrafiltration of B-UFH provides fractions that can have good applications – a more potent heparin (B-HMWH) and a heparin that can be used subcutaneously (B-IMWH).

Heparins are administered to patients in amounts defined in international units (IU). Although B-IMWH has an anticoagulant activity lower than B-HMWH, when equal amounts of IU are used *in vivo* the antithrombotic effects of B-IMWH and B-HMWH are similar. Studying porcine heparins, Doutremepuich, Bousquet and Toulemond (1986) observed that fractions with minor molecular weights (M_w < 5000 Da) have more antithrombotic activity than UFH. However, Ockelford, Carter, Michell and Hirsh (1982) did not observed significant differences between the antithombotic activity of an UFH (M_w = 16,000 Da), an intermediate fraction (M_w = 7600 Da), and a low molecular weight fraction (M_w = 4600 Da).

The compositional comparison of the heparins showed that ultrafiltration of B-UFH does not give only fractions with different molecular weights, but also with different proportion of components. The major abundance of reducing end-units of α-GlcNSO$_3$ (ANS$_{\alpha\text{-red}}$) and α-GlcNAc (ANAc$_{\alpha\text{-red}}$) in B-IMWH is in accordance to its low molecular weight as well as the decrease of this units in B-HMWH is justified by its high molecular weight. The native structural differences between B-UFH, B-IMWH, and B-HMWH probably contribute for their different anticoagulant activities.

Fractionating bovine and porcine heparins by AT affinity chromatography, Naggi et al. (2016) observed a direct relation between G-A* and AT affinity. Studies show that the disaccharide GlcA-GlcNSO$_3$3,6SO$_3$ (G-A*) is an indicative of the presence of the pentasaccharide (GlcNSO$_3$6SO$_3$-GlcA-GlcNSO$_3$3,6SO$_3$-IdoA2SO$_3$-GlcNSO$_3$6SO$_3$), which is considered the binding site to AT. Therefore, G-A* is directly related to the affinity of heparin to AT (Bisio et al., 2009; Kusche, Torri, Casu, & Lindahl, 1990), and thus, to its anticoagulant activity. However, this relation was not observed here to bovine heparins, since the proportion of G-A* in B-IMWH was 1.6 time higher than that of B-UFH, and the anticoagulant activity of B-IMWH was 27% lower than that of B-UFH. Moreover, the proportion of G-A* in B-HMWH was 0.6 time lower than that of B-UFH, and its anticoagulant activity was 8.7% higher than that of B-UFH. Despite having more G-A*, the lower molecular weight of B-IMWH could justify its lower anticoagulant activity.

5. Conclusion

This study showed that: 1) ultrafiltration of bovine heparin can produce a fraction with greater anticoagulant activity (B-HMWH) and another with lower activity (B-IMWH), but that presents effect when subcutaneously injected; 2) B-IMWH and B-HMWH have similar antithrombotic activity *in vivo* and the anticoagulant effect of both can be neutralized by protamine sulfate; and 3) the bovine heparin fractions with different molecular weights, obtained by ultrafiltration, have native structural differences.

Therefore, ultrafiltration could be used to fractionate bovine heparin to give fractions with good clinical applications. This method could probably be used for porcine heparin too. Interestingly, heparin fractions with different molecular weights have native structural differences, which can influence their anticoagulant and antithrombotic properties. More studies are necessary to describe the relationship between the native structures of these heparin fractions and their activities.

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