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In vivo antithrombotic properties of a heparin from the oocyte test cells of the sea squirt *Styela plicata* (Chordata-Tunicata)

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Abstract

In the ascidian *Styela plicata*, the oocytes are surrounded by two types of accessory cells named follicle cells and test cells. A heparin-like substance with an anticoagulant activity equivalent to 10% of mammalian heparin and about 5% as potent as the mammalian counterpart for the inhibition of thrombin by antithrombin was isolated from the oocyte test cells. In the present study, we compared the antithrombotic and hemorrhagic effects of sea squirt oocyte test cell heparin with those of porcine heparin in rat models of venous thrombosis and blood loss. Intravenous administration of the oocyte test cell heparin to Wistar rats (both sexes, weighing ~300 g, N = 4 in each group) at a dose of 5.0 mg/kg body weight, which produced a 1.8-fold increase in plasma activated partial thromboplastin time, inhibited thrombosis by 45 ± 13.5% (mean ± SD) without any bleeding effect. The same dose of porcine heparin inhibited thrombosis by 100 ± 1.4%, but produced a blood loss three times greater than that of the saline-treated control. However, 10-fold reduction of the dose of porcine heparin to 0.5 mg/kg body weight, which produced a 5-fold increase in plasma-activated partial thromboplastin time, inhibited thrombosis by 70 ± 13% without any bleeding effect. The antithrombotic properties of a new heparin isolated from test cells of the sea squirt *S. plicata*, reported here for the first time, indicate that, although sea squirt oocyte test cell heparin was a poor anticoagulant compared to porcine heparin, it had a significant antithrombotic effect without causing bleeding.

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Key words
- Ascidian
- Heparin
- Experimental thrombosis
- Antithrombotics
- Hemorrhage

Introduction

Heparin is a highly sulfated glycosaminoglycan composed of disaccharide repeats of hexuronic acid (α-L-iduronic acid or ß-D-glucuronic acid) linked 1,4 to α-D-glucosamine. Heparin is a heterogeneous mixture of polymers with a similar backbone. The heterogeneity results from variations of D-glucosamine sulfation (N-acetylated, N-sulfated, O-sulfated at C6 and/or C3) and of the uronic acid residue (O-sulfated or not at C2) (1).

Because of its binding to antithrombin by means of a specific pentasaccharide sequence

[GlcNAc($6SO_4$)-GlcA-GlcNS($3SO_4$)-IdoA ($2SO_4$)-GlcNS($6SO_4$)] (2), heparin has a potent anticoagulant activity (3). In the presence of heparin, the rates of inhibition of thrombin, factor IXa, and factor Xa by antithrombin are increased ~1000-fold (4), so that inhibition is essentially instantaneous. Heparin has been used clinically for almost 70 years for the prevention of thromboembolic events frequently observed after surgery, especially pelvic and orthopedic surgery (5,6).

In vertebrates, heparin is present in secretory granules of mast cells and basophils and is released only when mast cells degranulate in response to extracellular signals (1,2,7). Among invertebrates, heparins with different structures, molecular weights and anticoagulant activity have been reported in mollusks, crustacean and annelid (8-11). More recently, a heparin with structure similar to the mammalian counterpart, but with differences in the degree of sulfation has been identified in the intracellular granules of oocyte test cells of the ascidian (commonly known as sea squirt) *Styela plicata* (12). Oocyte test cells are accessory cells located in the perivitelline space of ascidian oocytes, where they remain during egg development until hatching. Sea squirt oocyte test cell heparin is composed mainly of the disaccharide [α-L-IdoA($2SO_4$)-1→4ß-D-GlcN(SO_4)($6SO_4$)-1]n, similar to mammalian heparin. About 25% of the disaccharide [α-L-IdoA-1→4ß-D-GlcN(SO_4)($6SO_4$)-1]n is also present (12). When compared to mammalian heparin, sea squirt oocyte test cell heparin has 10% of its anticoagulant activity measured by activated partial thromboplastin time (aPTT). In addition, it is about 5% as potent as the mammalian counterpart for the inhibition of thrombin by antithrombin. However, it activates heparin co-factor II (HCII) to the same extent as mammalian heparin, as indicated by the IC_{50} for thrombin inhibition in the presence of the inhibitor HCII (12). The prevalence of trisulfated disaccharides containing iduronic acid and N-sulfated glucosamine, the lack of glucuronic acid and N-acetylgalactosamine-containing disaccharides, the antithrombin-mediated thrombin inhibitory activity, and the intracellular localization of the ascidian glycosaminoglycan clearly demonstrate that sea squirt oocyte test cell heparin belongs to the heparin family.

In the present study, we compared the antithrombotic and hemorrhagic effects of sea squirt oocyte test cell and porcine heparins *in vivo* using rat models. We showed that the oocyte test cell heparin has a lower but significant antithrombotic activity and a lower bleeding effect when compared to porcine heparin.

Material and Methods

Extraction and purification of the test cell heparin

Adult specimens of *S. plicata* were collected from Guanabara Bay, Rio de Janeiro, RJ, Brazil, and maintained in an aerated aquarium. The gonads of several ascidians were carefully separated from other tissues under magnifying lenses, and the eggs isolated as described (12). The glycosaminoglycans were extracted from the eggs by papain digestion and ethanol precipitation and the heparin was purified by anion-exchange chromatography on a Mono Q column (Amersham Biosciences, São Paulo, SP, Brazil) as described (12).

Inhibition of factor Xa by antithrombin in the presence of sea squirt oocyte test cell heparin

The inhibition of factor Xa by the sea squirt oocyte test cell heparin in the presence of antithrombin was measured by an amylolytic activity assay using a chromogenic substrate (12). Incubations were performed in disposable UV semi-microcuvettes. The

final concentrations of reactants included 50 nM human antithrombin (Haematologic Technologies Inc., Essex Junction, VT, USA), 15 nM human factor Xa (Haematologic Technologies) and 0-1000 µg/mL heparin in 100 µL 20 mM Tris/HCl, 0.15 M NaCl, and 1.0 mg/mL polyethylene glycol, pH 7.4 (Tris/PEG buffer). Factor Xa was added to initiate the reaction. After 60 s at room temperature, 500 µL 100 µM N-methoxycarbonyl-D-norleucyl-glycyl-L-arginine-4-nitranillide-acetate (Roche, Mannheim, Germany) in Tris/PEG buffer was added and absorbance at 405 nm was measured for 100 s. The rate of change of absorbance was proportional to the amount of factor Xa activity remaining in the incubation mixture. No inhibition occurred in control experiments in which factor Xa was incubated with antithrombin in the absence of heparin, nor did inhibition occur when factor Xa was incubated with heparin alone in the concentration range tested. Porcine intestinal mucosa heparin (porcine-Hep, 180 units/mg; Sigma-Aldrich, St. Louis, MO, USA) and Nadroparin (average anti-Xa activity 112 units/mg; Sanofi, Vitry-sur Seine, France) were used as standards.

Animal procedures

The animal studies were carried out in accordance with the Brazilian Animal Protection Law and the Institutional Guidelines for Animal Care and Experimentation.

***Ex-vivo* anticoagulant action measured by activated partial thromboplastin time**

The effect of sea squirt oocyte test cell heparin on coagulation was determined in Wistar rats (both sexes, ~300 g body weight) anesthetized with an intramuscular injection of 100 mg/kg ketamine (Cristália, São Paulo, SP, Brazil) and 16 mg/kg xylazine (Bayer S/A, São Paulo, SP, Brazil), and supplemented as needed. The right carotid artery was isolated and cannulated with a 22-gauge catheter (Jelco, Johnson & Johnson Medical Ltda., São José dos Campos, SP, Brazil) for blood collection and administration of the heparin. Blood (~500 µL) was collected into 2.8% sodium citrate (9:1, v/v) for aPTT determination before and 10, 20, 30, 40, 50, and 60 min after intravenous administration in a bolus of 0.5 mg/kg of porcine heparin or 5 mg/kg of sea squirt oocyte test cell heparin. At least 4 animals were used in each group.

***In vivo* antithrombotic effect**

Antithrombotic activity was measured in rats using rabbit brain thromboplastin (bioMérieux Brazil, Rio de Janeiro, RJ, Brazil) as the stimulus (13). Briefly, Wistar rats (both sexes, ~300 g body weight) were anesthetized with an intramuscular injection of 100 mg/kg body weight of ketamine (Cristália) and 16 mg/kg body weight of xylazine (Bayer S/A). The abdomen of each animal was opened and the vena cava was carefully dissected. A segment of 0.7 cm was prepared beginning just below the branch of the right renal vein up to and beyond the left renal vein, which was ligated. Porcine heparin (Sigma-Aldrich, 180 units/mg) or sea squirt oocyte test cell heparin at the doses of 0.5 and 5 mg/kg was administered intravenously 2.0 cm below the distal loose suture and allowed to circulate for 5 min. Then, brain thromboplastin (Biolab-Mérieux AS, Rio de Janeiro, RJ, Brazil; 5 mg/kg body weight) was injected slowly intravenously 1.0 cm below the distal loose suture and the venous segment was then clamped, first at the level of the proximal suture and then at the level of the distal suture. After 20 min of stasis, the thrombus formed in the occluded segment was washed with 5% sodium citrate, dried for 1 h at 60ºC and weighed. At least 4 animals per group were used. Mean thrombus weight was obtained for each group and is reported as percent of the weight of

the thrombus in the absence of heparin.

Bleeding

The bleeding effect was determined in Wistar rats (both sexes, ~300 g body weight) anesthetized with a combination of xylazine and ketamine, as described above. A cannula was inserted into the right carotid artery for the administration of heparin (0.5 or 5.0 mg/kg). After 5 min the rat tail was cut 3 mm from the tip and carefully immersed in 40 mL distilled water at room temperature. Blood was collected for 60 min and the blood loss measured on the basis of hemoglobin content of the water solution by spectrophotometry (14). The volume of blood was deduced from a standard curve based on blood dilution by absorbance at 540 nm. At least 4 animals were used per group.

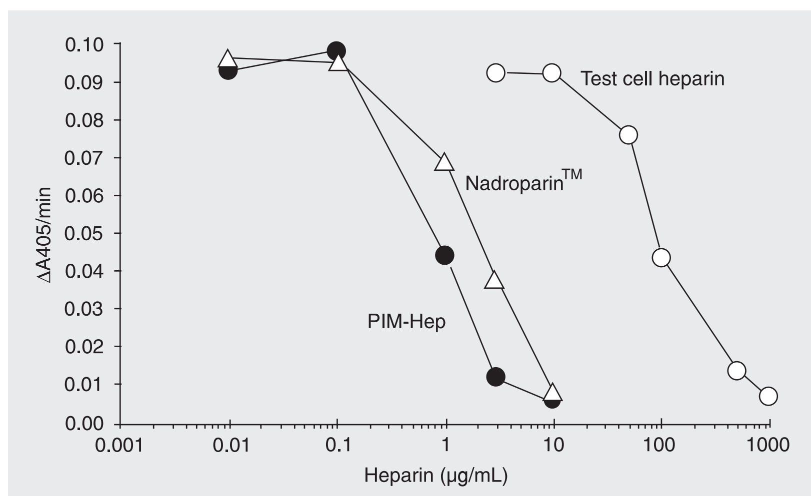

Figure 1. Anti-factor Xa properties of porcine and oocyte test cell heparin. Human antithrombin (50 nM) was incubated with human factor Xa (15 nM) in the presence of 0 to 1000 µg/mL of the different heparins. After 60 s, the remaining factor Xa activity was determined with the chromogenic substrate N-methoxycarbonyl-D-norleucyl-glycyl-L-arginine-4-nitranillide-acetate (100 µM) and absorbance at 405 nm was recorded for 100 s (ΔA405/min). PIM-Hep = porcine heparin (filled circles), Nadroparin (triangles), oocyte test cell heparin (open circles). Results represent the average of two separate experiments.

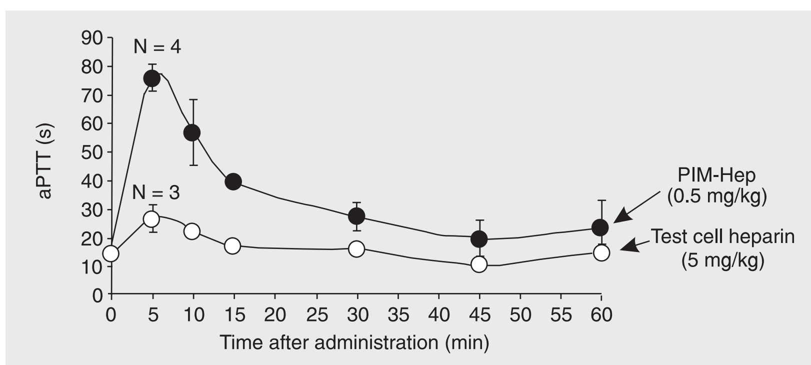

Figure 2. Anticoagulant effect of porcine heparin (PIM-Hep) and oocyte test cell heparin. Blood (~500 µL) was collected into 2.8% sodium citrate (9:1, v/v) before and after intravascular administration in a bolus of 0.5 mg/kg body weight porcine heparin (filled circles) or 5.0 mg/kg body weight of test cell heparin (open circles). Activated partial thromboplastin time (aPTT; mean ± SD) was determined for *ex vivo* rat plasma as described in Material and Methods. Error bars were omitted when they were less than the size of the symbol.

Statistical analysis

Data are reported as means ± SD. Comparisons between two groups were made by the *t*-test and the differences were considered significant when $P < 0.05$.

Results

Anti-factor Xa activity of oocyte test cell heparin

The data in Figure 1 show that the anti-factor Xa activity of the ascidian heparin is equivalent to 1.1% of porcine heparin and 40% of Nadroparin, based on total weight. The IC_{50} for factor Xa inhibition by antithrombin, estimated by the curves in Figure 1, was about 1.0, 2.5, and 90 µg/mL for porcine heparin, nadroparin and test cell-heparin, respectively.

In vivo anticoagulant effect

Figure 2 shows that aPTT increased up to ~5-fold during the first 5 min after injection of porcine heparin. After 5 min, aPTT started to decrease and remained 2-fold higher than the control value for the next 60 min. Oocyte test cell heparin induced a less intense change in aPTT values, with a maximum increase of 1.8-fold at 5 min, returning to normal after 15 min. No significant change in aPTT was observed after the administration of test cell heparin doses lower than 5 mg/kg (data not shown).

Effect on thrombosis

The effect of ascidian and mammalian heparins on thrombosis was investigated using an experimental venous thrombosis model in rats. A single injection of 0.5 mg/kg of porcine heparin in a bolus, given 5 min before the thrombogenic stimulus with rabbit brain thromboplastin induced about 70% inhibition of thrombosis (Figure 3). Administration of the same dose of oocyte test cell heparin did not produce any inhibition of thrombosis (Figure 3). Porcine heparin administered at 5 mg/kg produced 100% inhibition of thrombosis, whereas the same dose of oocyte test cell heparin reduced thrombosis by only 45%. We also evaluated the effect of the mammalian and ascidian heparins on thrombosis at the dose of 2.0 mg/kg. At this dose, porcine heparin inhibited thrombosis by 100%, whereas oocyte test cell heparin had no effect (data not shown).

Hemorrhagic effect

The hemorrhagic effect of porcine and oocyte test cell heparins was assessed after intravascular administration based on blood loss in a rat cut-tail bleeding assay. At the antithrombotic dose of 5 mg/kg body weight, oocyte test cell heparin did not modify the blood loss compared with rats receiving saline (Table 1). The blood loss increased ~2.6-fold in rats receiving the same dose of porcine heparin (Table 1). The dose of porcine heparin needed to achieve 70% inhibition of thrombosis (0.5 mg/kg) did not increase the blood loss.

Discussion

In a previous study, heparin with a different sulfation pattern and anticoagulant activity was isolated from the oocyte test cells of the ascidian *S. plicata* (12). Here we assessed the antithrombotic and bleeding effects of this heparin after intravascular administration to rats, using experimental models of venous thrombosis and bleeding.

The anti-factor Xa activity of the test cell heparin was about 40 times lower than that of a low-molecular weight heparin (Nadroparin), which has an average anti-factor Xa activity of 112 units/mg. Therefore, the anti-factor Xa of the oocyte test cell heparin can be estimated to be about 2.8 units/mg. The anticoagulant activity obtained by the *in vitro* experiments reported here (Figure 1) and in the previous study (12) agrees with the results obtained in *in vivo* experiments (Figure 2), showing that the oocyte test cell heparin has a very low anticoagulant activity. A dose of 5 mg/kg body weight of the oocyte test cell heparin is required to produce a 1.8-fold increase in aPTT in rat plasma.

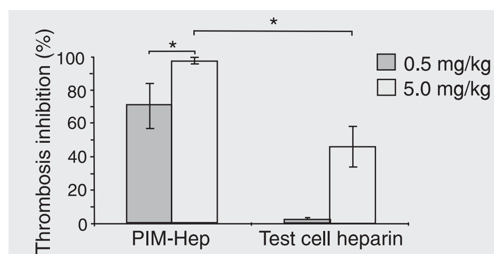

Figure 3. Antithrombotic activity of porcine heparin (PIM-Hep) or oocyte test cell heparin. Antithrombotic activity was determined using a stasis thrombosis model in rats after intravenous administration of the different heparins in a bolus (see Material and Methods). Mean thrombus weight was obtained for each group and then reported as percent weight in the absence of polysaccharide. Percent inhibition of thrombosis (mean ± SD, N = 4) is reported as a function of heparin concentration. *P < 0.05 for the comparisons indicated by the horizontal lines (Student *t*-test).

Table 1. Hemorrhagic effect of mammalian and oocyte test cell heparins.

Treatment	Blood loss (µL)
Saline	32 ± 5.5
Porcine heparin (0.5 mg/kg)	21 ± 5
Porcine heparin (5.0 mg/kg)	87 ± 19
Test cell heparin (5.0 mg/kg)	36 ± 6

Porcine heparin (0.5 or 5.0 mg/kg body weight) or oocyte test cell heparin (5.0 mg/kg body weight) was administered intravenously in a bolus and allowed to circulate for 5 min. The rat tail was cut 3 mm from the tip and immersed in 40 mL distilled water at room temperature. Blood was collected for 60 min and blood loss was determined by measuring the hemoglobin content of the water by spectrophotometry at 540 nm (14). Data are reported as means ± SD for groups of 4 rats.

Porcine heparin at a dose 10 times lower produced a 5-fold increase in aPTT.

The antithrombotic activity of heparin is associated with its ability to induce inhibition of thrombin and factor Xa by antithrombin. It has been shown more recently in animal experiments that anti-factor Xa activity is a prerequisite, although not sufficient by itself, for a thrombosis-preventing effect (15-17). In fact, oocyte test cell heparin inhibited thrombosis to a much lesser extent than porcine heparin at the same dose. At the highest dose tested (5 mg/kg body weight), test cell heparin inhibited thrombosis by only 45% compared to 100% inhibition obtained with the same dose of porcine heparin. This result is probably associated with the very low anti-IIa and anti-factor Xa activities of test cell heparin.

Usually, low-molecular weight heparins, that contain high anti-factor Xa and low anti-IIa activities, have a lower bleeding effect than unfractionated heparin (18-21). However, the mechanism by which heparins and other sulfated glycosaminoglycans contribute to bleeding is unknown. In a previous study (22), we demonstrated that there is a dissociation of the anticoagulant action, antithrombotic activity and bleeding effect in the case of other glycosaminoglycans. We showed that for dermatan sulfates with different sulfation patterns an increase in HCII activity does not result in a parallel increase of the antithrombotic potency or of the bleeding effect (22). Similarly, the effect of another class of sulfated polysaccharides, namely sulfated galactans, on coagulation, bleeding and thrombosis is also not coupled (23). On the other hand, our results regarding the effect of heparins on bleeding indicated that a reduction of the anticoagulant activity of heparin abolished its bleeding effect. However, significant antithrombotic activities (70 and 45% thrombin inhibition for mammalian and oocyte test cell heparin, respectively; Table 1) with no bleeding effect were observed even when oocyte test cell heparin was administered at high doses.

We have reported here the antithrombotic properties of a new heparin molecule from the oocyte test cells of *S. plicata*. Our results indicate that for this oocyte test cell heparin there is a parallelism between the anticoagulant and antithrombotic activities that are dissociated from the hemorrhagic effect.

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