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Anti-inflammatory properties of a heparin-like glycosaminoglycan with reduced anti-coagulant activity isolated from a marine shrimp

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ABSTRACT

The anti-inflammatory properties of a heparin-like compound from the shrimp *Litopenaeus vannamei* are related. Besides reducing significantly ($p < 0.001$) the influx of inflammatory cells to injury site in a model of acute inflammation, shrimp heparin-like compound was able to reduce the matrix metalloproteinase (MMPs) activity in the peritoneal lavage of inflamed animals. Moreover, this compound also reduced almost 90% the activity of MMP-9 secreted by human activated leukocytes. Negligible anti-coagulant activities in aPPT assay and a poor bleeding potential make this compound a better alternative than mammalian heparin as a possible anti-inflammatory drug.

1. Introduction

The exaggerated and uncontrolled inflammatory response can contribute for the development of chronic and acute inflammatory diseases. In these cases it is necessary an intervention in the immune response with the use of anti-inflammatory drugs. However the prolonged use of some compounds is followed by complications as gastric perforations, stomachic ulcers, and bleeding. Therefore, much work has been focused on finding compounds with reduced collateral effects.

Among various compounds, heparin, a sulfated polysaccharide belonging to the glycosaminoglycans (GAGs) family of molecules,[1] has shown strong anti-inflammatory effects,[2] including inhibition of the complement activation,[3,4] inactivation of chemokines,[5] inhibition of neutrophil-derived elastase[6] and protection of vascular endothelial cells against damaging substances.[7,8] Some observations also suggest that heparin can inhibit the activation and recruitment of inflammatory cells into tissues[9–11] and modulates the synthesis of matrix metalloproteinases (MMPs),[12] a family of zinc- and calcium-dependent endopeptidases which has been reported to play crucial roles in the migration of inflammatory cells through basal membrane components in vitro.[13]

Although the great potential of heparin as anti-inflammatory agent its clinical use is impaired by its strong anti-coagulant activity and hemorrhagic complications. To overcome this problem, many researchers have tried to find heparin analogues or natural compounds with low anti-coagulant activity, which preserve their anti-inflammatory properties. Some chemical modifications from mammalian heparin have been developed,[14,15] but the occurrence of spongiform encephalopathy together with religious restrictions also has limited the use of mammalian heparin derivates. Consequently, it is increasingly motivating the search for non-mammalian sources of heparin, natural polysaccharides with similarities to mammalian heparin. Modifications of natural polysaccharide from *Escherichia coli* K5 strain, besides the new natural sulfated polysaccharides from marine invertebrates have been described,[16–23] however most of researches have focused on the discovery of an ideal anti-thrombotic agent while their possible anti-inflammatory properties are still unexplored. In this paper we have showed for the first time the anti-inflammatory properties

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of a heparin-like glycosaminoglycan isolated from a marine invertebrate, the shrimp *Litopenaeus vannamei*. Using a model of acute inflammation, we studied the ability of this compound, isolated from the shrimp cephalotorax to reduce the inflammatory cells recruitment into the injury site and interfere on activity of MMPs secreted during this process and those secreted by human activated leukocytes. Analysis of peritoneal fluids and culture medium of human leukocytes demonstrate that the compound isolated from crustacean may have a great anti-inflammatory potential.

Litopenaeus vannamei is the species chosen by the northeast coast shrimp farming industry and represents the main exportation product of Rio Grande do Norte state. Since the heparin-like glycosaminoglycan is obtained from the cephalotorax, a material that does not have any commercial value, the *L. vannamei* shrimp can become a source of bioactive compounds with pharmaceutical potential.

2. Results

2.1. Purification of shrimp heparin-like glycosaminoglycan

The compounds isolated after proteolysis and fractionation by ion-exchange resin were fractionated by acetone precipitation at different proportions to obtain three fractions named F-0.5, F-0.7, and F-1.0. The identification of the heparin-like compound was performed by electrophoretic behavior at different buffer systems. The results showed that the fraction F-0.5 had a similar electrophoretic migration to mammalian heparin in all studied systems. In addition, it was fractionated into slow moving and fast moving bands at the discontinuous buffer system barium acetate/diaminopropane (data not shown). The shrimp heparin-like glycosaminoglycan was further purified by anion-exchange chromatography on DEAE–Sephacel using a step-wise salt gradient (Fig. 1). The fraction eluted with 0.8 M NaCl yielded 91.5% of total uronic acid content. It was further desalted by gel chromatography on Sephadex G-25.

2.2. Enzymatic depolymerization

The purified shrimp heparin-like glycosaminoglycan was evaluated by specific enzymatic cleavage with lyases from *Flavobacterium heparinum*. The products formed from shrimp heparin-like compound and mucosal pig heparin by the action of heparinase and heparitinase II are shown in Figure 2. Although in different proportions, the same types of disaccharides are formed from these compounds during the enzymatic depolymerization. Unsaturated pentasulfated tetrasaccharide (ΔU,2S-GlcNS,6S-U-GlcNS,6S) and trisulfated disaccharide (ΔU,2S-GlcNS,6S) are the main products formed from the shrimp heparin-like compound and heparin by the action of heparinase, whereas unsaturated disulfated disaccharide (ΔU-GlcNS,6S/ΔU,2S-GlcNS) is the main product formed from these compounds by the action of heparitinase II. Porcine mucosal heparin contains higher amounts of trisulfated disaccharide than shrimp heparin-like compound. In contrast, this compound liberated higher amounts of products with heparitinase II. In addition, no products resulting by the action of heparitinase I upon shrimp compound were identified and a combined action of heparitinases I and II produced a small amount of monosulfated disaccharide and disulfated disaccharide (results not shown).

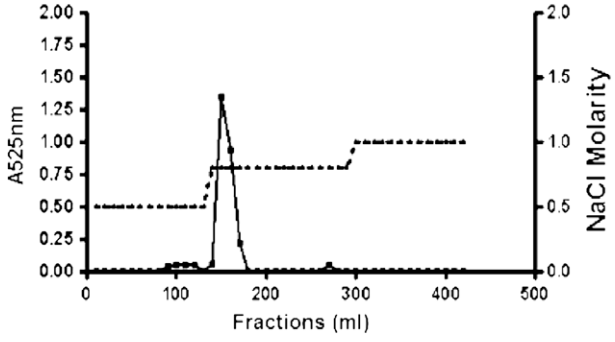

Figure 1. Purification of shrimp heparin-like glycosaminoglycan by DEAE–Sephacel ion-exchange chromatography. The column was eluted with a salt gradient (dotted line).

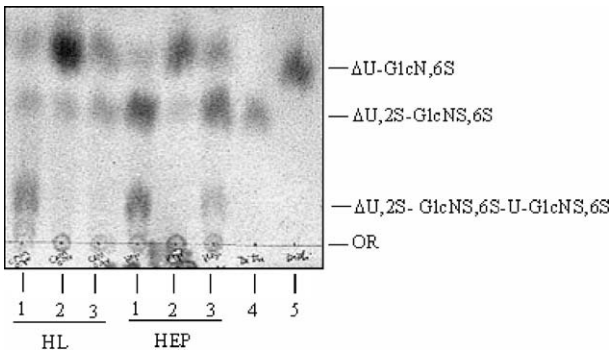

Figure 2. Paper chromatogram of the degradation products formed from heparins by the action of heparinase and heparitinases. One hundred micrograms of shrimp heparin-like compound or mammalian heparin were incubated with heparinase (1) or heparitinase II (2) or a mixture of both enzymes (3). Standard trisulfated disaccharide (4) and disulfated disaccharide (5). Tetra—ΔU,2S- GlcNS,6S-U-GlcNS,6S; Ditri—ΔU,2S-GlcNS,6S; Didi—ΔU-GlcN,6S; HL—shrimp heparin-like compound; HEP—pig heparin; OR—origin.

2.3. Anticoagulant activity

To examine the shrimp heparin-like glycosaminoglycan anticoagulant activity, we carried out aPTT assays using the porcine heparin (190 IU/mg) as reference. As shown in Table 1, the compound isolated from shrimp had dramatically reduced anti-coagulant activity compared to heparin; its aPTT value was similar to that of the control at concentrations ranging from 0.1 to 3 μg/mL. The aPTT value for heparin-like compound, but not heparin, could also be measured at higher concentrations (10–20 μg/mL). These results demonstrate that the compound isolated from crustacean exhibits a reduced anti-coagulant activity when compared to mammalian heparin.

Table 1
Anticoagulant activity[a]

Concentration (μg/mL)	Control	Heparin	Heparin-like
0.1	33.05	33.40	30.65
0.3	33.05	35.55	30.50
0.5	33.05	38.15	32.60
0.8	33.05	39.15	31.00
1.0	33.05	43.25	32.25
3.0	33.05	76.35	35.10
5.0	33.05	171.50	57.77
7.0	33.05	>240[b]	77.45
10.0	33.05		91.15
12.0	33.05		90.95
15.0	33.05		145.00
17.0	33.05		178.00
20.0	33.05		222.50

[a] aPTT (activated partial thromboplastin time) of human plasma containing heparin or shrimp heparin-like at various concentrations.
[b] Values exceeding 240 s could not be determined.

2.4. Effect of shrimp heparin-like compound on synthesis of heparan sulfate by the endothelial cells

Since an increased synthesis of heparan sulfate chains is observed when the endothelial cells are exposed to heparin or other anti-thrombotic agents,[24] we investigated if the shrimp heparin-like compound is capable to stimulate the synthesis of this peculiar heparan sulfate by the endothelial cells. Rabbit endothelial cells were divided in two different groups. One of them was exposed to different concentrations of the heparin-like compound or heparin (100 μg/mL) and [^{35}S]sulfate (150 μCi/mL). The other group (control) was exposed only to [^{35}S]sulfate. After 20 h incubation, we could observe that heparin indeed stimulated the heparan sulfate synthesis by endothelial cells; however the shrimp heparin-like glycosaminoglycan was not capable to stimulate the synthesis in none of the tested concentrations (Fig. 3).

2.5. Hemorrhagic activities

The clinical use of heparin is limited by some undesirable effects as hemorrhagic complications due to its capacity to interfere in the hemostatic balance. Thus, it is of great importance to investigate the effect of shrimp heparin-like compound on haemostasia. Figure 4 shows that mammalian heparin (100 μg/mL) possess a potent hemorrhagic effect while shrimp heparin-like compound at the same concentration showed a negligible potential of bleeding. It is important to note that at the highest concentration (400 μg/mL) the shrimp heparin-like compound still showed an effect about three times lower than mammalian heparin.

2.6. Inhibition of thyoglycollate-induced peritoneal inflammation

Thioglycollate injection into rat peritoneal cavity induces acute inflammation and neutrophil infiltration.[25,26] To compare the inhibitory effects of heparin and heparin-like compound on cell infiltration, these compounds were injected subcutaneously at different dosages 30 min before the inflammation induction. At a low dosage (5 μg/kg), heparin and heparin-like compound inhibited 84.2% and 44.5% of infiltration, respectively (Fig. 5; $p < 0.001$). When a higher dose of heparin-like compound was used (15 μg/kg), there was a reduction of ~60%. Shrimp heparin-like compound was also capable to inhibit 52.5% of the cell influx after 8 h of inflammation, when the neutrophils infiltration is maximal (data not shown).

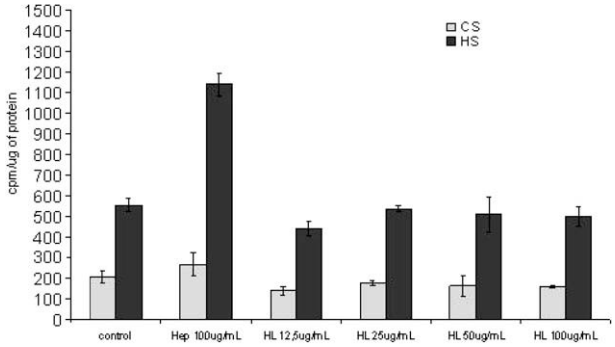

Figure 3. Stimulation of the synthesis of heparan sulfate by endothelial cells exposed to heparin or shrimp heparin-like glycosaminoglycan. Aorta endothelial cells were exposed to 100 μg/mL of heparin (Hep) or different concentration of heparin-like compound (HL) (12.5, 25, 50, and 100 μg/mL) and 150 μCi/mL of [^{35}S]sulfate in F-12 medium. After 20 h of incubation, the heparan sulfate and chondroitin sulfate synthesized by the cells and secreted to the medium were quantified as referred to under Section 5. HS, heparan sulfate; CS, chondroitin sulfate.

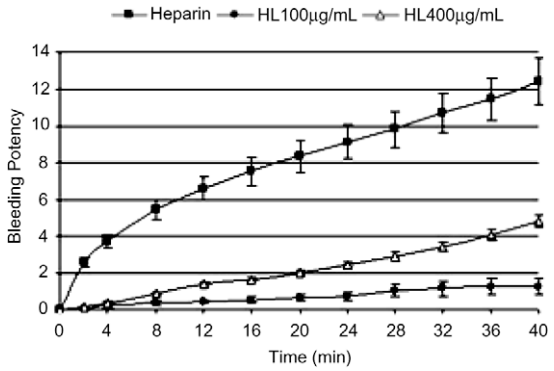

Figure 4. Hemorrhagic activity of the shrimp heparin-like glycosaminoglycan. Heparin (100 μg/mL) or shrimp heparin-like compound (100 or 400 μg/mL) was applied topically, and the bleeding potency was measured after 2 min following saline solution washing. HL, shrimp heparin-like compound.

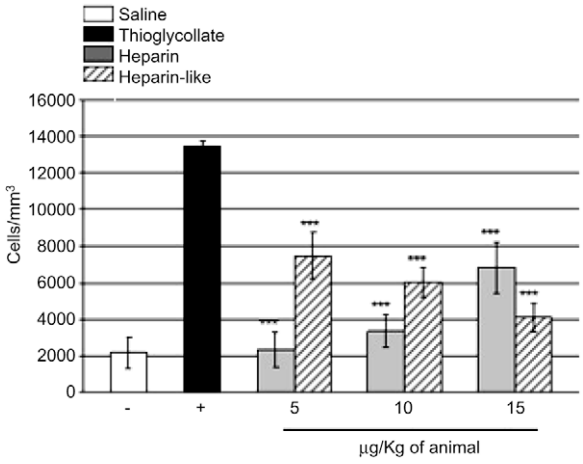

Figure 5. Effect of shrimp heparin-like compound on the total cell infiltration into peritoneal cavity. Groups of five rats received a subcutaneous injection of heparin or heparin-like compound in saline at dosages 5, 10, and 15 μg/kg of animal. After 30 min the animals were injected ip with saline solution alone (−) or 3% sodium thioglycollate (+). $^{***}p < 0.001$ indicates statistically significant difference between the thioglycollate group without treatment and the groups treated with the compounds.

2.7. Shrimp heparin-like compound is an inhibitor of proMMP-2 and MMP-9 activity in peritoneal exudates

In order to investigate the effect of shrimp heparin-like compound on MMPs activity in the acute inflammation, aliquots of peritoneal fluid from the animals treated with heparin or heparin-like compound (5, 10, and 15 μg/kg) were submitted to gelatin zymography. As shown in Figure 6A, in the peritoneal fluid of the animals treated only with thioglycollate there is an increased gelatinolytic activity of MMP-9 and proMMP-2 when compared to the animals which received only saline solution. The pretreatment with shrimp heparin-like compound reduced more than 50% of MMP-9 activity in all tested dosages. At a low dosage (5 μg/kg), heparin promoted just a slight reduction (~20%) of this activity, being more effective at the highest dosages (10 μg and 15 μg/kg) (Fig. 6B). On the other hand, heparin showed a higher effect on proMMP-2, reducing ~70% of its activity at the dosage 15 μg/kg. At low dosage (5 μg/kg) the compound from *L. vannamei* reduced ~20% of the lytic activity of this enzyme, while the treatment with heparin-like

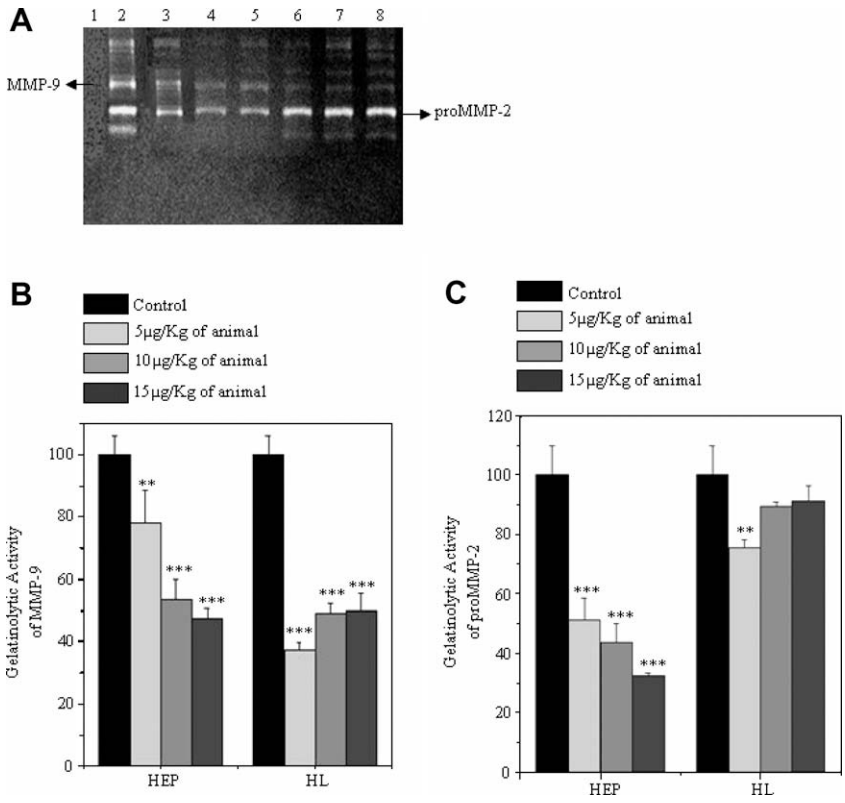

Figure 6. Gelatin zymography for determination of proMMP-2 and MMP-9 activities in the peritoneal liquid of the animals with peritonitis. (A) Gelatinolytic activity of pro MMP-2 and MMP-9 was detected by electrophoresis on polyacrylamide gel containing gelatin. Areas and relative intensities of gelatin digested bands by MMP-9 (B) and proMMP-2 (C) were quantified by densitometry and expressed as percentage of the positive control. Lanes 1 and 2: gelatinolytic activity of MMPs of peritoneal fluid of animals treated with saline alone or thioglycollate, respectively. Lanes 3, 4, and 5: gelatinolytic activity of MMPs of peritoneal fluid of animals treated with heparin (HEP) in dosages of 5, 10 and 15 μg/kg of animal, respectively. Lanes 6, 7, and 8: gelatinolytic activity of MMPs of peritoneal fluid of animals treated with haparin-like (HL) in dosages of 5, 10, and 15 μg/kg of animal, respectively. $^{**}p < 0.01$, $^{***}p < 0.001$.

compound at the highest dosage had no significant effect on proMMP-2 activity (Fig. 6C).

2.8. Effect of shrimp heparin-like compound on leukocytes MMP-9

Since many chronic inflammatory diseases are related to high levels of MMP-9, we investigated if the compound isolated from *L. vannamei* is able to interfere on MMP-9 activity from activated human leukocytes. As shown in Figure 7A and B, heparin and shrimp heparin-like compound were able to reduce this enzyme activity, either in a lower or higher concentration (10 and 100 μg/mL), but the shrimp compound had a pronounced effect on this enzyme activity, reducing almost 90% of its activity.

3. Discussion

Although the anti-inflammatory potential of heparin, its clinical use in the treatment of inflammatory diseases is very limited by its potent anti-coagulant activity. In this paper, we have showed the anti-inflammatory properties of a heparin-like glycosaminoglycan with reduced anti-coagulant activity isolated from the shrimp *L. vannamei*. Structural analysis of this compound, performed by its enzymatic depolymerization, revealed that the difference between mammalian heparin and shrimp heparin-like compound lies mainly in the abundance of different disaccharide units present in these molecules. This is observed by the amount of products formed by the heparinase action (which recognizes 2-O-sulfated iduronic acid residues) and heparitinase II (which acts upon N- and N,O-sulfated glucosaminido-iduronic or glucuronic acid linkages).[27] Shrimp heparin-like compound is more susceptible to heparitinase II when compared to the mammalian one. Based on this enzyme specificity it is possible that the compound isolated from shrimp contains mainly blocks of disulfated disaccharide units containing glucosamine N-sulfated/6-O-sulfated linked to glucuronic acid. These differences have been observed regarding to mollusk and crustacean heparin-like compounds, which also contain large amounts of glucuronic acid residues in their structures.[28,29] Sulfated regions consisting of glucosamine N-sulfated linked to iduronic acid 2-O-sulfated are also present, explaining the identification of disulfated disaccharides (ΔU,2S-GlcNS) produced by the action of heparinase. Moreover, the finding that no product is detected by the heparitinase I action indicates the predominant occurrence of sulfate at C-6 position of the hexosamine moiety, which is impeditive for this enzyme action.

These structural peculiarities, including the higher content of glucuronic acid and disulfated disaccharide units, can be related to the reduced anti-coagulant activity observed in the shrimp heparin-like compound at the aPTT assay. It seems to contrast with heparin-like compound from mollusk, which is rich in glucuronic acid but shows a high anti-coagulant activity.[29,30] Those differences show that the heparin-like glycosaminoglycans pharmalogical properties vary according to its origin. This must be related to some structural peculiarities of these compounds, as the glucosamine residues sulfatation pattern and the distribution of the glucuronic residues. These peculiarities could influence, for example, their capacity to bind to anti-thrombin (AT).

Although its ability to inhibit several proteases of the coagulation cascade,[31] AT is not the only target of anti-thrombotic agents.[32] It is believed that the anti-thrombotic activity of heparin

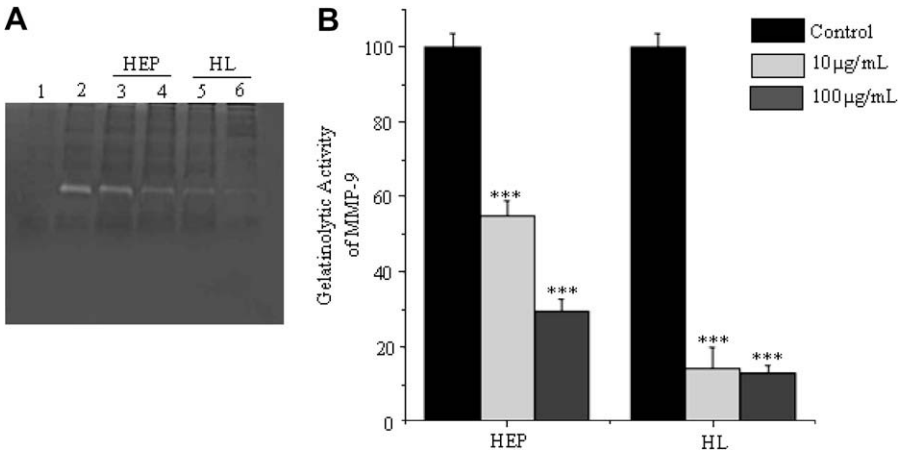

Figure 7. Gelatin zymography for determination of MMP-9 activity secreted by human leukocytes activated with phytohemagglutinin (PHA). (A) Gelatinolytic activity was detected by electrophoresis on polyacrylamide gel containing gelatin. (B) The area and the intensity of the bands digested by MMP-9 were quantified by densitometry and expressed as percentage of the positive control. Lane 1, non-activated leukocytes; lane 2, leukocytes activated with PHA alone; lanes 3 and 4, activated leukocytes treated with heparin (HEP) 10 or 100 μg/mL, respectively. Lanes 5 and 6, activated leukocytes treated with shrimp heparin-like (HL) glycosaminoglycan 10 or 100 μg/mL, respectively. $^{***}p < 0.001$.

and other anti-thrombotic agents are related, at least in part, to their ability to stimulate the synthesis of heparan sulfate chains by endothelial cells,[33,34] therefore, the reduced anti-coagulant activity in vitro of the shrimp heparin-like compound does not exclude the possibility of this compound to possess an anti-thrombotic effect. Heparan sulfate from bovine pancreas, for example, is a powerful anti-thrombotic agent, however, it is dismissed of anti-coagulating activity at the aPTT assay.[35] Despite this, we found that the heparin-like compound from *L. vannamei* was not capable to stimulate the endothelial cells in none of tested concentrations (Fig. 3). The effect of sulfated polysaccharides from marine invertebrates on endothelial cells still was not described in the literature. However, previous studies had shown that other sulfated compounds that show anti-thrombotic activity 'in vivo' like a galactofucan from the brown alga *Spatoglossum schroederi*,[36] dextran sulfate, oversulfated chondroitin sulfates and anti-thrombotic agents[34,37] increase the synthesis of this peculiar heparan sulfate by these cells. Using fragments of heparin obtained by depolymerization with *F. heparinum* lyases, it was shown that the minimum structural requirement necessary to display this activity is a pentasulfated tetrassaccharide.[34] Since its formation is observed when the shrimp heparin-like compound is submitted to the action of heparinase (Fig. 2), the absence of effect on endothelial cells suggests that structural peculiarities of this compound also may exert influence on this activity. Another interesting aspect of shrimp heparin-like compound is the finding that it had a potential of bleeding about three times lower than heparin, which makes it a good model to study its anti-inflammatory properties. Recently, it was showed that a sulfated polysaccharide isolated from the body wall of the sea cucumber *Ludwigothurea grisea* is able to reduce the neutrophils migration during a thioglycollate-induced peritoneal inflammation.[38] Using a similar model with modifications, we demonstrate that the heparin-like compound from the shrimp *L. vannamei* was able to reduce significantly the peritoneal influx of neutrophils in all tested dosages. Although a higher dose (15 μg/kg) of the shrimp compound was more efficient to reduce inflammation, it is interesting to note that it has a low bleeding potential and it was capable to reduce more than half of the cell infiltration. The cell viability of 96%, assessed by Trypan blue exclusion, eliminates a potential toxic effect of shrimp heparin-like compound.

Previous studies showed that the anti-migratory properties of heparin and related molecules depend on interactions with P- and L-selectins and that the sulfation is critical for this interaction.[39] Thus, the differences in dose response for heparin and shrimp heparin-like compound could reflect differences on sulfation pattern of these compounds. The higher content of disulfated disaccharide units can justify the necessity of a higher dose of heparin-like glycosaminoglycan to reduce more than half of cell infiltration when compared to heparin. Despite these differences, the low hemorrhagic effect of this compound represents a great advantage on heparin utilization.

Since the inflammatory response is a very complex process, involving the participation of a great variety of proteins, heparin and shrimp heparin-like compound can have inhibitory effects on multiple components of the inflammatory cascade including selectins, integrins, cytokines, chemokines, and matrix metalloproteinases (MMPs). In this work, we found that the shrimp heparin-like compound was able to reduce the activity of MMP-9 and proMMP-2 in the peritoneal lavage fluids of inflamed animals. Although these enzymes seem not to be directly related to neutrophil transmigration,[40] they can be important effectors in other events of the inflammatory response, modulating the activity of cytokines and chemokines. In fact, MMP-2 and MMP-9 can cleave and activate the IL-1β precursor, a powerful pro-inflammatory cytokine.[41] These gelatinases can also process and activate the tumor necrosis factor (TNF) in vitro.[42,43] Besides, MMP-9 is also biologically important for the basal lamina penetration by leukocytes[44] and the capacity of shrimp heparin-like compound to reduce almost 90% of this enzyme activity secreted by human activated leukocytes is extremely important since these cells accumulation in tissues is an early event in many chronic inflammatory diseases immunophatogenesis.

The mechanism involved in the heparin inhibitory effects on MMPs activity is not completely understood. It has been demonstrated that heparin is able to suppress the gene expression of some of these enzymes, including MMP-9 and this mechanism seems to involve the protein kinase C pathway and the transcription factor AP-1 (activator protein-1), an important activator of MMPs transcriptor.[11] In addition, Gogly and co-authors have reported that heparin caused a downregulation of MMPs human gingival fibroblasts.[45] If the heparin acts inside or outside the cell it is still not fully understood, but since several transcription factors contain heparin-binding sequences, it is reasonable that heparin regulates gene activity at nuclear sites binding to transcription factors.[46] Whether the reduction of MMP-9 activity observed in cells

and animals treated with the shrimp heparin-like compound is due to transcriptional inhibition, decrease in the amount of protein synthesized, or inhibition of the already synthesized protein is still unclear.

Many inflammatory disorders are related to gelatinolytic activity excess and some inhibitors of matrix metalloproteinases are used as anti-inflammatory drugs.[47] Therefore, the capacity to interfere on MMPs activity represents an important mechanism of intervention in the immune response mediated by heparin-like compound from the shrimp *L. vannamei*.

Further studies involving mono- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy are being performed to define the shrimp heparin-like compound detailed structure. The complete structural characterization will contribute to lead the development of more specific therapeutic agents for the treatment of several inflammatory diseases.

4. Conclusion

The structural features of shrimp heparin-like compound, as the high content of glucuronic acid and disulfated disaccharide units, may contribute to the reduced anti-coagulant and hemorrhagic activities observed and the lack of effects on endothelial cells. The leukocytes migration inhibition and the effects on MMP-9 and proMMP-2 activities demonstrate that the shrimp heparin-like compound may interfere in different inflammatory response events. Taken together, the results presented in this study have provided strong evidences indicating the anti-inflammatory potential of the compound isolated from the shrimp *L. vannamei*. The findings that this compound had no hemorrhagic activity make it a better alternative than mammalian heparin for a possible anti-inflammatory drug. Besides, products from mammalian origin have risk of contamination for pathogens, like a prion. Therefore, a compound with less undesirable side effects and a natural occurrence could lead to a wide variety of therapeutic applications for a range of inflammatory diseases.

5. Experimental

5.1. Chemicals

Heparan sulfate from bovine pancreas was gift from the late Dr. P. Bianchini (Opocrin Research laboratories, Modena, Italy). Heparitinases I and II, and heparinase (heparinase I, EC 4.2.2.7) were prepared as previously described.[48] Thioglycollate broth was purchased from Sigma. Carrier-free [^{35}S]inorganic sulfate was purchased from Instituto de Pesquisas Nucleares (São Paulo, SP, Brazil).

5.2. Animals

Wistar rats (150–180 g) were housed in temperature-controlled rooms (22–23 °C) until use. Food and water were supplied ad libitum. Each experimental group included five animals. The animal experiments were conducted in accordance with international guidelines.

5.3. Extraction and purification of heparin-like glycosaminoglycan from the shrimp

Ten kilograms of shrimp heads (which contain the viscera) were homogenized with acetone (10:1 v/v) and left during 24 h at room temperature to eliminate lipids. The precipitate formed was dried (955.86 g), triturated and suspended in 1.0 M NaCl (3:1 v/v). The pH of the mixture was adjusted to 8.0 with NaOH. The material was submitted to proteolytic digestion with 38.2 mg of superase (Biocon Laboratories, Sao Paulo, Brazil). After 24 h of incubation at 60 °C with agitation and periodic adjustments of pH, the mixture was filtered through a cloth. The filtrate received ion-exchange resin, Amberlite IRA 900 (Rohm and Hass, Sao Paulo, Brazil) and the resulting mixture was agitated during 24 h at room temperature under a layer of toluene to prevent bacterial growth. The suspension was filtered again through the same cloth and the resin was recovered. The resin was then washed with water at 50 °C (2:1 v/v) and subsequently washed with 1.0 M NaCl (10:1 v/v) at room temperature. The washed resin was suspended in 1 L of 3.0 M NaCl and agitated during 6 h and filtered. The filtrate was maintained during 48 h at 4 °C after addition of cold methanol (2:1 v/v). The precipitate collected by centrifugation (708 mg) was denominated fraction R3. This fraction was fractionated by precipitation with acetone as follows: ice-cold acetone (0.5:1 v/v) was added to the solution under gentle agitation and maintained at 4 °C during 24 h. The precipitate formed (F-0.5 fraction) was collected by centrifugation (5000*g*, 30 min) and yielded 88.5 mg. This fraction was dissolved in 4 mL of distilled water and applied to a column (2.4 × 3.1 cm) of DEAE–Sephacel (Pharmacia). The column was eluted with 0.5 and 1.0 M NaCl. Effluents were analyzed by carbazole assay to detect the GAGs presence. The fraction eluted with 1.0 M NaCl was fractionated again on DEAE–Sephacel and the fraction eluted with 0.8 M NaCl contains the shrimp heparin-like compound used in this work. This compound was desalted by gel filtration through a Sephadex G-25 column (2.5 × 150 cm) by eluting with 10% ethanol. After lyophilization, the purified shrimp heparin-like compound (22.5 mg) was analyzed.

5.4. Electrophoresis

Agarose gel electrophoresis of the isolated shrimp heparin-like glycosaminoglycan was performed at two different buffer systems: 0.05 M 1,3-diaminopropane acetate (PDA), pH 9.0 and the discontinuous buffer barium acetate/PDA as previously described.[49] Briefly, aliquots (5 μL) were applied to a 0.5% agarose gel and run for 1 h at 100 V in 0.05 M 1,3-diaminopropane/acetate (PDA), pH 9.0. The GAGs in the gel were fixed with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide solution. After 2 h, the gel was dried and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v).

5.5. Enzymatic digestion

Enzymatic digestion was performed as previously described.[27] About 100 μg of glycosaminoglycan were digested with 0.1 U of heparinase and/or heparitinases I and II, in 0.05 M ethylenediamine acetate buffer, pH 7.0 in a final volume of 50 μL. The mixture was incubated during 18 h at 30 °C. The reaction was terminated by boiling during 30 s. The incubation mixtures were spotted on Whatman No. 1 paper and subjected to chromatography in isobutyric acid: 1.25 M NH_3, 5/3.6, v/v during 24 h. The unsaturated products formed were detected by short wave UV lamp and silver nitrate staining.[50]

5.6. Pharmacological activities

The activated partial tromboplastin time (aPTT) assay was performed according to the method of the kit APTTest (Rosario, Argentina). Heparin and shrimp heparin-like compound studied were dissolved in physiological saline to the appropriate concentrations (generating a volume of 10 μL) and incubated with 90 μL of plasma at 37 °C during 3 min. Then, 100 μL of bovine cephalin was added and incubated at 37 °C. After 3 min of incubation, 100 μL of pre-warmed 0.25 M $CaCl_2$ solution were added to the mixture and the clotting time was measured in triplicate using Quick Timer Coagulometer (Drake Eletrônica Comércio Ltda, Sao Paulo, Brazil).

5.7. Effect of shrimp heparin-like compound on the synthesis of heparan sulfate by the endothelial cells

Rabbit aorta endothelial cells were exposed to 100 μg/mL of heparin or different concentrations of shrimp heparin-like compound (12.5, 25, 50, and 100 μg/mL) and 150 μCi/mL of [^{35}S]sulfate in F-12 medium. After 24 h at 37 °C and 2.5% of CO_2 the culture medium was removed and analyzed. Radioactive heparan and condroitin sulfate glycosaminoglycans were prepared from culture medium by proteolysis with Maxatase (4 mg/mL) during 24 h at 60 °C. Radioactive glycosaminoglycans synthesized by the cells and secreted to the medium were identified by agarose gel electrophoresis. The radioactive [^{35}S]-sulfated glycosaminoglycans were located and quantified by exposure of the gels (after fixation, drying and staining) to a scanner of radioactivity. All the experiments were performed in triplicate.

5.8. Residual hemorrhagic effect

The residual hemorrhagic effect of shrimp heparin-like compound was analyzed by a modified model of topical scarification in rat tail.[51] Following anesthesia with ketamine and xylazine in a proportion of 1:1 (v/v) a scarification was made with a surgical blade in the tail distal portion. Soon after, the scarificated tail was dipped vertically in isotonic NaCl, scraped with gauze and dipped again in fresh saline to observe bleeding. After that, the tail was dipped in a solution containing shrimp heparin-like compound or heparin at different concentrations during 2 min and washed extensively with saline solution. The treated tail was immersed in new isotonic saline solutions during 40 min and the amount of blood was determinated by Bradford protein determination method.[52] The results were expressed as the sum of the protein values of each tube minus the amount of protein present before the exposure to the test substance.

5.9. Thyoglycollate-induced peritoneal inflammation

Rats received subcutaneous injections of 0.5 mL sterile saline solution with or without heparin or shrimp heparin-like compound (5, 10, or 15 μg/kg of rat weight). Thirty minutes later, the animals were injected intraperitoneally with 2 mL of 3% thioglycolate broth (Sigma–Aldrich) or sterile saline solution. Rats were sacrificed after 3 h. In another experiment, rats treated with heparin or shrimp heparin-like compound were sacrificed after 8 h. The peritoneal cavity was washed with 10 mL of 0.9% saline solution. After a gentle massage, the peritoneal fluid was aspirated and centrifuged at 300*g* during 5 min at 4 °C in order to separate cells and supernatant. The cell pellets were resuspended in 0.5 mL of 0.9% saline solution. Aliquots (20 μL) were diluted with Turk solution (20:1) and counted in hemocytometer. Supernatants were kept frozen at −20 °C until assayed.

6. Cell viability

Following centrifugation of the peritoneal lavage fluid, cell pellets were resuspended in 2 mL of PBS balanced salt solution. Aliquots (25 μL) were diluted with a 0.16% Trypan blue solution and cell number and viability determined by microscopic examination.

6.1. Gelatin zymography

Gelatin zymography was used to determine activities of proMMP-2 and MMP-9 in peritoneal fluid. Aliquots were collected and their protein contents were determined by Lowry protein determination method.[53] After normalizing the protein content, equal amounts of proteins were electrophoresed under non-reducing conditions on 10% polyacrylamide gels containing 1.5 mg/mL gelatin. Following electrophoresis, polyacrylamide gels were washed with 50 mM Tris–HCl (pH 7.5) containing 2.5% Triton X-100 to remove sodium dodecyl sulfate. Gels were then incubated overnight at 37 °C in a developing buffer containing 10 mM $CaCl_2$, 50 mM Tris–HCl, and 150 mM NaCl to digest gelatin by MMPs. Areas of gelatin hydrolyzed by MMPs were visualized as clear zones against blue background stained by Coomassie blue and the intensities of the bands were estimated by densitometry using the software Scion Image Beta 4.03 (Scion Corporation, USA).

6.2. Leukocytes isolation and culture

Peripheral blood was collected from healthy human donors and diluted with RPMI medium not supplemented on proportion 1:1 (v/v). This mixture was overlaid onto 3 mL of Ficoll-Paque™ Plus in centrifuge tubes and centrifuged at 1000*g* at room temperature for 30 min. Leukocytes were collected from the interface layer with Pasteur pipette, washed twice in RPMI 1640 medium, counted, distributed into 6-well plates (1×10^6 cells/mL) and cultured in the same medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% streptomycin, 0.02% fungizone, and β-mercaptoethanol.

6.3. Gelatinolitic activity of leukocytes metalloproteinases

Leukocytes cultured as described above were cultured alone or incubated with heparin or shrimp heparin-like compound (10 or 100 μg/mL) at 37 °C in a 5% CO_2 humidified atmosphere during 2 h. Subsequently, cells were stimulated with phytohemagglutinin (PHA) (5 μg/mL) and incubated during 48 h. After that, the cell suspension was centrifuged and the supernatants collected by aspiration were analyzed through gelatin zymography in order to determine the MMP-9 activity, as described in Section 6.1.

6.4. Statistical analysis

Results were analyzed by one-way ANOVA and Tukey's post-test. Values of $p < 0.001$ were considered indicative of statistical significance.

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