Low Molecular Weight Protamine (LMWP)as Nontoxic Heparin/Low Molecular Weight Heparin Antidote (II): In Vitro Evaluation of Efficacy and Toxicity

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ABSTRACT Patients undergoing anticoagulation with heparin or low molecular weight heparin (LMWH) require a superior antidote that possesses more selective biological actions and a better safety profile than protamine. We had previously developed 2 low molecular weight protamine (LMWP) fractions (TDSP4 and TDSP5) from thermolysin-digested protamine as potential nontoxic, heparin-neutralizing agents. In this, the second article in this series, studies focused on in vitro evaluation of heparin/LMWH-neutralizing efficacy and putative toxicity. These LMWP fractions, particularly TDSP5, were effective and fully capable of neutralizing a broad spectrum of heparin-induced anticoagulant activities (ie, aPTT, anti-Xa, and anti-IIa activities). Additionally, these LMWP fractions could neutralize the activities of commercial LMWH. As assessed by the anti-Xa assay, TDSP5 was as effective as, although less potent than, protamine in reversing the activity of Mono-Embolex (molecular weight 5000-7000) and 2 other different sizes (molecular weight of 3000 and 5000 d) of LMWH preparations. Furthermore, compared with protamine, TDSP5 exhibited a much-reduced toxicity and thus an improved safety profile, as reflected by its reduced ability to activate the complement system and cross-react with the antiprotamine antibodies, which are 2 primary indices of protamine toxicity.

Key Words: Heparin/LMWH neutralization, protamine toxicity, aPTT clotting assay, anti-Xa assay, complement Activation, immunogenicity, cross-reactivity

INTRODUCTION

Protamine from fish sperm consists of a family of highly cationic, heterogeneous proteins with an average molecular weight of 4500 d. It is used clinically as a heparin antidote after cardiovascular surgical procedures to reverse the anticoagulant activity of heparin and to alleviate heparin-induced bleeding risks (1). The use of protamine, however, is associated with adverse effects ranging from mild hypotension to severe or ultimately fatal cardiac arrest (2). The incidence of mild reactions to protamine was reported to be as high as 10.6%, whereas that of severe reactions was between 0.9% and 1.6% (3). Indeed, the combined use of heparin and protamine has been suggested as the major cause of morbidity and mortality for patients undergoing cardiopulmonary bypass operations (4).

The mechanisms of protamine-induced toxicity are complex and still not yet completely clarified. Available data indicate that severe adverse reactions could be mediated by the immune response because protamine is a nonhuman protein. Thus, a large population of diabetic patients who previously received protamine-containing insulins, and who developed antiprotamine antibodies, were at high risk for this immunoglobin-mediated protamine response (5). On the other hand, protamine reactions could also be triggered by a nonimmunological mechanism. The heparin-protamine complexes, such as the antigen-antibody complexes, could activate the classical complement pathway, leading to sequestration of neutrophils in the lungs, formation of thromboxane A2, and release of vasoactive histamine (6).

A number of approaches were attempted to develop a nontoxic protamine substitute for heparin neutralization, but with limited success (2). The ideal heparin antidote that most clinicians would prefer, as stated in a recent review (2), is a compound that provides all the advantages of protamine, yet lacks anaphylactic potential and preserves hemodynamic stability when being infused. To meet such requirements, our laboratory has engineered the novel approach of deriving a chain-shortened low molecular weight protamine (LMWP) product directly from protamine as a potentially less toxic heparin-

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neutralizing agent. The underlying principle of this approach was based on 2 hypotheses that relate to protamine-induced immunotoxicity: (1) It would not require the entire protamine molecule to yield complete heparin neutralization, and (2) a chain-shortened peptide fragment derived from its parent protein is usually associated with significantly reduced antigenicity (ie, the ability to be recognized by an antibody) and immunogenicity (ie, the ability to induce antibody production) (7). In addition, a chain-shortened fragment would likely be deprived of the cross-linking ability of protamine, which is the major contributing factor to complement activation of protamine toxicity mediated by the nonimmunological pathway (8).

In our previous article in this series (9), we discussed the preparation and characterization of 2 LMWP fractions (TDSP4 and TDSP5) that possess the full scale of heparin-neutralizing ability based on the anti-Xa assay. In this second article, we further examine these 2 LMWP fractions *in vitro* for their efficacy in neutralizing both heparin and low molecular weight heparin (LMWH) by using a variety of widely used coagulation and chemical assays. In addition, we examine the potential toxicity of these compounds with regard to their ability to induce complement activation and to react with the mouse antiprotamine antibodies. In vivo evaluation of the efficacy and toxicity of the TDSP5 compound using a sensitive canine model will be presented in the final article in this 3-part series.

MATERIALS AND METHODS

Materials

Protamine sulfate (salmine, Grade X), human antithrombin III (A7388), and LMWH (H3400, molecular weight 3000 d) were purchased from Sigma Chemical Co (St Louis, MO). Porcine intestine heparin (167 IU/mg) and S-2238 substrate were purchased from Pharmacia Hepar Inc (Franklin, OH). Fragmin and Mono-Embolex, 2 clinical LMWH preparations, were supplied by Kabi (Uppsala, Sweden) and Sandoz (Basel, Switzerland), respectively. Freshly frozen human plasma in citrate was obtained from the American Red Cross (Detroit, MI). All solutions were prepared using distilled and deionized water.

Methods employed in the preparation of the LMWP fractions were described in the first article in this series (9). The TDSP5 fraction was composed of a single tetradecyl peptide with the sequence of VSRRRRRRGGRRRR, whereas the TDSP4 fraction consisted of a mixture of 2 tridecyl peptides with sequences of ASRRRRRGGRRRR and VSRRRRRGGRRRR. The TDSP3 fraction was heterogeneous and composed of a group of peptides with the typical sequence structure containing only 1 cluster of arginine residues.

aPTT Clotting Assay

We followed a modified procedure of Byun et al (10) to evaluate the reversal of the aPTT activity of heparin by either protamine or LMWP. In brief, 15 µL of the heparin solution (5 U/mL) were mixed with 15 µL of a solution containing an increasing concentration of protamine (0-30 µg/mL) or LMWP fractions (0-200 µg/mL). To the mixture was added 100 µL of actin cephaloplastin and 100 µL of plasma. After 3 minutes of incubation, 100 µL of 20 mM calcium chloride (preheated to 37°C) was added, and the clotting time was measured immediately using a fibrometer (Fibrosystem; Becton Dickinson Co, Cockeysville, MD).

Anti-Xa and Anti-IIa Assays

Neutralization of anti-Xa activity of heparin by LMWP fractions was determined using the ACCUCOLOR heparin kit (Sigma Diagnostics, St Louis, MO) according to the procedures described in the previous article (9). For the anti-IIa assay, we followed the same protocol as the anti-Xa assay, except that factor IIa (thrombin) and S-2238 were used as the enzyme and substrate, respectively. In brief, 20 µL of heparin (1 U/mL) in human plasma was mixed with 75 µL of human antithrombin III (0.1 U/mL) at 37°C. After 2 minutes of incubation, 75 µL of human thrombin (21 U/mL), 75 µL of S-2238 substrate (1 mM), and 5 µL of protamine (or LMWP) were added. After 10 minutes of incubation, the absorbance at 405 nm was measured by using a plate spectrometer ($PowerWave_{x340}$, BIO-TEK Inc, Winooski, VT). The heparin-neutralizing ability of protamine (or LMWP) was proportional to the absorbance increase at 405 nm.

Complement Activation

Complement activation by protamine (or LMWP) or heparin-protamine (or LMWP) complexes was assessed by a modified hemolytic complement assay

(11) using sensitized sheep erythrocytes (SRBC). In brief, 150 μL of diluted (1:60) serum containing an increasing concentration of protamine (or LMWP) or heparin-protamine (or LMWP) complexes, 75 μL of sensitized SRBC, and 150 μL of 0.02 M triethanolamine buffer saline (TBS) buffer containing 0.15 M NaCl were mixed together. The mixture was incubated at 37°C, with occasional agitation, for 60 minutes. After centrifugation of the cells, 200 μL of the supernatant were inserted by a pipette into the wells of the microplate; the amount of released hemoglobin was determined at 541 nm using a microplate reader. The absorbances of TBS buffer and H2O in the aforementioned mixture were the controls of 0% and 100% lysis, respectively. The degree of complement consumption (Y) was then obtained from this equation: (OD = optical density)

$$Y = (1 - \frac{\text{test OD} - \text{spontaneous lysis OD}}{\text{H}_2\text{O lysis OD} - \text{spontaneous lysis OD}}) \times 100$$

Cross-Reactivity of the LMWP Fractions Toward Mouse Antiprotamine Antibodies

The mouse antiprotamine sera were produced according to the procedure of Cooper and Paterson (12). In brief, mice (ICR strain, 6-7 weeks old) were immunized with 50 μg of protamine in complete Freund's adjuvant (CFA). Four weeks later, the first booster was given using 5 μg of protamine in incomplete Freund's adjuvant (IFA). Later, animals were bled at 2-week intervals and blood samples were collected, allowed to clot, and then centrifuged to obtain the serum samples. Detection of the polyclonal antiprotamine antibodies was performed according to a previously described enzyme-linked immunosorbent assay method (10) using protamine as the capturing ligand. The goat-antimouse IgG-alkaline phosphatase was then used as the detection probe, and the absorbance readings were recorded at 405 nm.

Cross-reactivity of the LMWP fractions toward antiprotamine antibodies was examined using a competitive binding assay. Phosphate Buffered Saline with Tween 20, pH 7.4 (PBS/Tween 20) was purchased from Sigma Chemical Co (St. Louis, MO). In these experiments, the microtiter wells were coated with 100 μL of 50 μg/mL protamine in PBS/Tween 20 buffer (pH 7.5) and subsequently blocked with 120 μL of 1.0% human serum albumin solution. Diluted (1:100) mouse antiprotamine samples containing an increasing concentration of protamine or LMWP (1 to 1000 μg/mL) were then added to the wells and incubated for 2 hours at 37°C. After washing, the goat-antimouse IgG-alkaline phosphatase and *p*-nitrophenylphosphate substrate were added to the wells, and the amount of bound antiprotamine antibodies was measured at 405 nm. The percent of inhibition (I %) was calculated using the following equation:

$$I\,\% = 100\% \times (1 - \frac{\text{OD}_{\text{in samples with the added protamine or LMWP}}}{\text{OD}_{\text{in samples without the added protamine or LMWP}}})$$

The degree of cross-reactivity of the LMWP fractions toward antiprotamine antibodies is represented by the concentration of LMWP added to the serum sample that resulted in 50% of inhibition.

RESULTS AND DISCUSSION

Neutralization of Heparin

Figure 1 shows the dose-dependent neutralization of the aPTT activity of heparin by protamine, TDSP3, TDSP4, and TDSP5. As seen, the aPTT activity of heparin (5 U/mL) was completely neutralized by protamine at a protamine/heparin neutralization ratio of 7.5 μg/U. By comparison, this aPTT activity was also completely neutralized by TDSP4 and TDSP5 at a higher protamine/heparin ratio of 34 and 18 μg/U, respectively. Based on these results, the required dose of LMWP for complete neutralization of heparin aPTT activity was about 2.5- to 4.5-fold higher than that of protamine, which was indeed in good agreement with the previous results estimated by using the anti-Xa chromogenic assay (9). On the other hand, the TDSP3 fraction, which contained microheterogeneous peptide fragments possessing only 1 arginine cluster as demonstrated previously (9), exhibited a markedly reduced efficacy in heparin neutralization because incomplete reversal of the aPTT activity was observed even at a dose that was 20-fold higher than that of protamine.

The ability to neutralize the anti-IIa activity of heparin by protamine or LMWPs was also evaluated using the S-2238 chromogenic assay. As shown in Figure 2, similar patterns of neutralization by protamine and the LMWP fractions were observed. At a heparin concentration of 0.5 U/mL, the anti-IIa activity of heparin was completely neutralized by protamine at a peptide/heparin ratio of about 7.5

μg/U, whereas TDSP5 and TDSP4 required a higher dose ratio of 12 μg/U and 17 μg/U, respectively. Overall, the potency in neutralizing the anti-IIa activity of heparin by TDSP4 and TDSP5 was slightly higher compared to that in neutralizing the aPTT activity. The required doses for complete neutralization of such heparin-induced anticoagulant activities for the 2 LMWP fractions were about 1.5- to 2.5-fold higher than that for protamine.

Heparin preparation is normally heterogeneous and contains components with significant differences in chain length and degree of sulphation. Such structural variations cause the heparin molecules to interact differently with the coagulation enzymes, resulting in blood anticoagulation via complex and different pathways (13,14). Among all the coagulation enzymes, factors Xa and IIa (thrombin) are the 2 key proteases involved in the regulation of the coagulation process. Thus, comparison of inhibitory effects of the LMWP fractions on heparin-induced anti-Xa and anti-IIa activities seems appropriate in evaluating the specificity and efficacy of such LMWP fractions as the heparin antagonist. Based on our previous results (9), both TDSP4 and TDSP5 neutralized heparin substantially with regard to its induced anti-Xa function. The present study also indicates that both LMWP fractions neutralized the anti-IIa activity of heparin quite effectively. The dose required to achieve complete neutralization of the anti-IIa activity by these 2 LMWP fractions, however, was significantly lower than that of the anti-Xa activity. For instance, each unit of heparin required 12 μg of TDSP5 to completely neutralize the anti-IIa activity (see Figure 2), whereas it would require 27 μg of TDSP5 to achieve a full reversal of the anti-Xa activity (see Figure 2 in first article of this series). This finding is somewhat anticipated, based on the binding specificity of IIa and Xa to heparin. It is known that thrombin binds heparin directly via an electrostatic interaction, as reflected by the dependency of this binding on the chain length of heparin (15). However, the anti-Xa activity of heparin is mediated by a strong and specific binding of antithrombin III (ATIII) to a pentasaccharide sequence in heparin (16). Thus, it is not surprising that a higher dose of TDSP5 is required to dissociate ATIII from its specific binding to heparin than to displace thrombin from a nonspecific, electrostatic interaction with heparin.

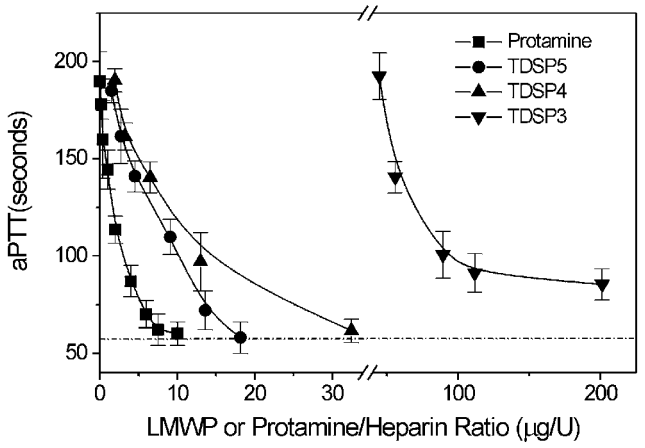

Figure 1 Neutralization of heparin by protamine, TDSP5, TDSP4, or TDSP3, as measured by the aPTT clotting assay. The dotted line represents the baseline clotting time.

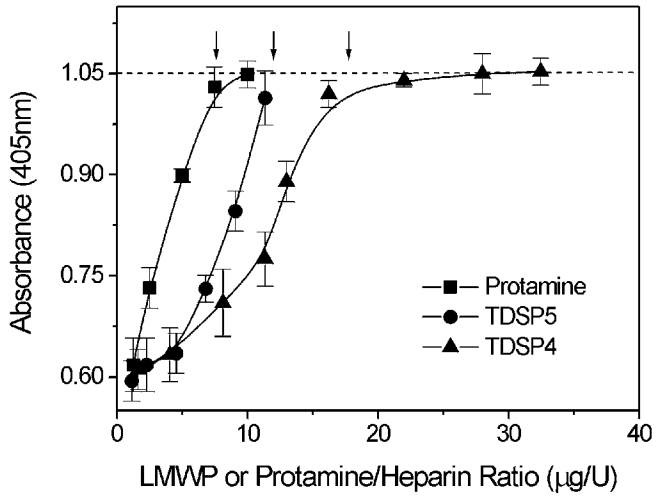

Figure 2 Neutralization of heparin by protamine, TDSP5, or TDSP4, as measured by anti-IIa chromogenic assay. The dotted line represents the control (ie, 100% neutralization).

This in vitro finding is also consistent with the in vivo results reported by many other investigators regarding heparin reversal by protamine (17,18); all cited the difficulty in achieving a complete neutralization of the anti-Xa activity. Despite the requirement of a higher dose, our results nevertheless suggest that the LMWP fractions, particularly TDSP5, are effective and fully capable of neutralizing the overall anticoagulant activities of heparin. The neutralization data shown in Figure 1 provides further support to this conclusion because the aPTT-clotting assay is known to be a general

functional test that measures heparin-induced activation of most of the coagulation factors involved in the intrinsic coagulation cascade.

Neutralization of LMWH

To combat the bleeding risks of heparin and improve its safety, considerable effort has been directed toward developing LMWH fragments as heparin substitutes. Such chain-shortened compounds contain primarily the ATIII binding domain in heparin, thereby reserving the entire anti-Xa function while aborting the anti-IIa activity. These LMWH fragments are used in clinical practice for prophylaxis and treatment of deep vein thrombosis and pulmonary embolism (19). Although LMWH could eventually replace heparin in most conventional uses, it is important to examine if the LMWP under development could also effectively neutralize LMWH. Figure 3 shows the neutralization of the anti-Xa activity of a model LMWH, the commercial Mono-Embolex compound with an average molecular weight of 5000 to 7000 d by protamine, TDSP4, and TDSP5. As seen, neutralization of the anti-Xa activity of Mono-Embolex by these 3 compounds followed a similar pattern; their dose-dependent neutralization curves were all quite alike. Results showed that Mono-Embolex was completely neutralized by either protamine or TDSP5 at relatively similar doses; for each microgram of Mono-Embolex, a plateau was reached by protamine and TDSP5 at a dose of 25 μg and 30 μg, respectively. Neutralization by TDSP4, however, was slightly less effective, as a nearly complete (~90%) reversal was achieved at a slightly higher dose (~35 μg) of TDSP5.

The requirement of a similar dose of protamine and TDSP5 to achieve a complete reversal of the anti-Xa activity of LMWH may offer significant clinical effects and benefits. It has been well documented that, despite displaying improvement over heparin in clinical practice, the LMWH compounds are not exempted from bleeding risks (19). Compounding this problem is the lack of an appropriate agent for reversing the anticoagulant functions of these LMWH compounds (20). As described previously, compared to that of other anticoagulant activities, the anti-Xa activity of heparin is most difficult to neutralize. This difficulty is markedly magnified for the LMWH compounds because the "specific" anti-Xa activity (ie, the activity per unit mass) of these compounds is significantly increased. Although reports in the literature on whether protamine can completely neutralize the anti-Xa activity of LMWH are still contradictory, the consensus is that a considerably higher dose of protamine is required to achieve a full reversal. Consistent with this finding, our results show that it takes more than 20 times the clinical protamine dose (ie, 1.5 mg protamine per mg of heparin [21]) to completely neutralize the anti-Xa activity of the same mass of LMWH (Figure 3). Because protamine-induced toxicity appears to be dose-dependent (22), the need for such a high dose to alleviate the bleeding risk of LMWH may pose a major safety concern as to the use of protamine as

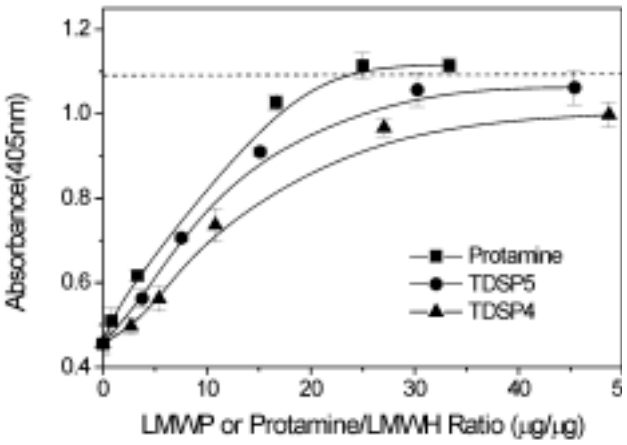

Figure 3 Neutralization of LMWH5000-7000 (Mono-Embolex) by protamine, TDSP5, and TDSP4, as measured by anti-Xa chromogenic assay. The dotted line represents the control (ie, 100% neutralization).

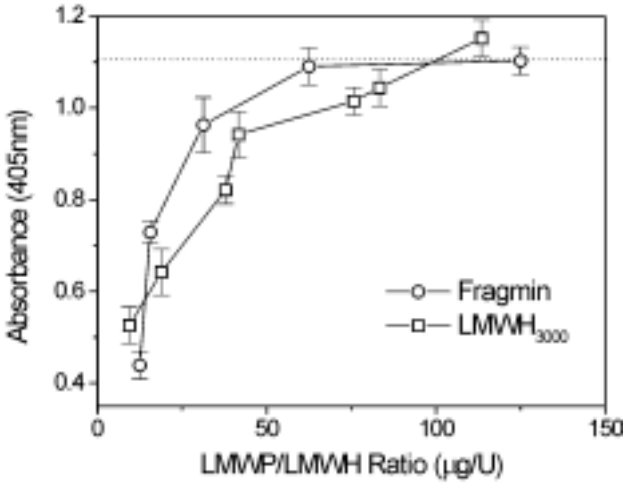

Figure 4 Neutralization of LMWH3000 and Fragmin by TDSP5 as measured by anti-Xa chromogenic assay.

the clinical antidote for LMWH. Thus, the requirement of a similar protamine dose of LMWP, which possesses a markedly reduced toxicity at this dose (as described later in this report), for LMWH reversal would yield significant clinical benefits. Indeed, the combination use of LMWH as the anticoagulant and LMWP as its antagonist may offer a true solution to the establishment of a safe and ideal anticoagulation therapy (ie, with minimal possibility for bleeding risks and adverse effects).

To further validate our findings, neutralization of other commercial LMWH preparations by TDSP5 was also examined. Because it had been shown that certain new heparin antagonists under investigation, such as platelet factor 4, could not neutralize LMWH preparations with smaller molecular sizes (2), Fragmin, a US Food and Drug Administration-approved LMWH preparation with an average molecular weight of 5000 d, and LMWH3000, a laboratory preparation with an average molecular weight of 3000 d, were chosen for our studies. As shown in Figure 4, and consistent with our previous findings, TDSP5 could effectively neutralize both of these LMWH preparations; for each unit of the anti-Xa activity of these 2 LMWH compounds, complete neutralization was achieved at a TDSP5 dose of about 100 µg. These results clearly confirm the general utility of TDSP5 as an effective antidote for the LMWH compounds.

Toxicity Evaluation

Protamine-induced toxicity in general can be mediated by either a nonimmunological or an immunological pathway. For the former, the primary event is the activation of the complement system. Thus, measurements of complement consumption can be used as a key index to assess this type of toxicity of protamine and its derived LMWP analogs. It was reported that protamine by itself at a relatively high concentration could cause the depletion of the complement components *in vitro* (23). In addition, owing to the long chain length and polyionic nature, heparin and protamine were known to form large, cross-linked aggregates with network structures. These large heparin-protamine complexes (HPC), which behaved like the antibody-antigen complexes, were shown to possess even stronger potential in activating the complement cascade both in vitro and in vivo (6,23). Related clinical studies (24), however, suggested that activation of the complement system by HPC required the complex to reach a critical size to bind to C1q—the prelude of the activation event. Previous studies conducted in our laboratory using immobilized protamine appeared to support this hypothesis. The HPC formed with immobilized protamine produced a significantly lower complement consumption than that formed with free protamine (8). This was simply because only a single layer of heparin would be adsorbed on the resin-immobilized protamine, and the HPC thus formed could not reach the critical size required to bind C1q, unlike those large complexes formed between heparin and free protamine. For this reason, the chain-shortened LMWP fragments, which have been largely deprived of their cross-linking ability, are expected to yield markedly reduced potential in inducing complement activation.

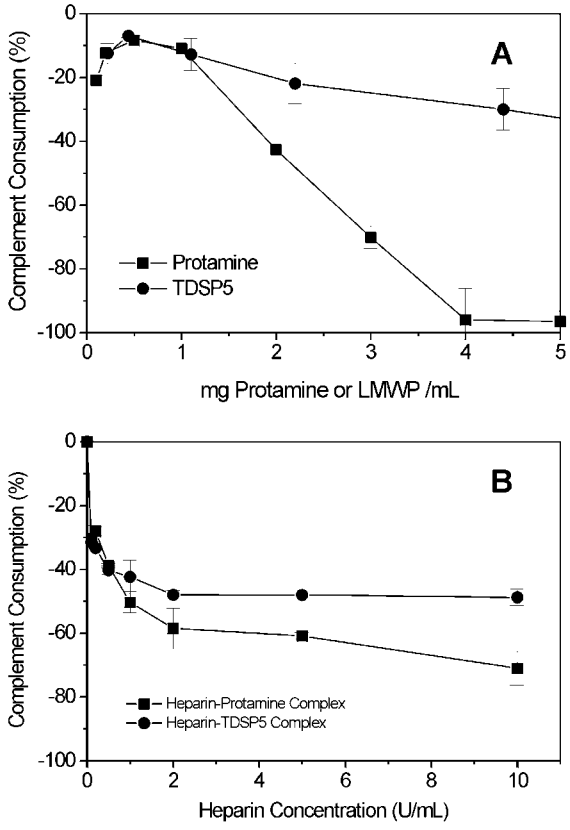

Figure 5 A, Effects of protamine and low molecular weight heparin (LMWP) alone on complement consumption. B, Effects of the heparin-protamine and heparin-LMWP complexes on complement consumption. Complexes were prepared by adding protamine (or LMWP) to serum samples containing an increasing concentration of heparin at the respective neutralization ratio (ie, for each unit of heparin, 10 µg or 22 µg of protamine and TDSP5, respectively, were added for the formation of heparin-[LMW]protamine complexes). For details, see the Material and Methods section.

Figure 5A showed the dose-dependent consumption of the complement components by protamine or TDSP5 alone, as measured by the conventional CH_{50} hemolytic complement assay. Consistent with results reported by other investigators (23), protamine itself did not elicit any detectable complement consumption until the concentration was beyond 1 mg/mL. A nearly complete (100%) depletion of the complement system was observed at a protamine concentration of 4.0 mg/mL. On the other hand, only 30% of the hemolytic activity was seen for TDSP5 at a higher dose of 4.5 mg/mL. These results clearly indicate that LMWP itself was significantly deprived of the toxicity in activating the complement system.

To examine the effects of HPC on the complement system, protamine (or LMWP) was added to serum samples containing an increasing amount of heparin at the dose equivalent to its neutralization ratio (ie, for each unit of heparin, 10 μg and 22 μg of protamine and TDSP5, respectively, were added). Figure 5B showed that at a serum heparin concentration between 2.5 and 10 U/mL, which was the dose normally encountered during cardiovascular surgeries, HPC produced by heparin and protamine yielded 60% to 70% complement consumption (measured by the same CH_{50} hemolytic assay), whereas complexes produced by heparin and TDSP5 yielded only about 50% complement depletion. The differences in complement consumption were all statistically significant ($p < 0.05$). Although the CH_{50} method is a qualitative rather than a quantitative assay for measuring complement activation, these data nevertheless suggest that the TDSP5 LMWP fragment, either alone or in combination with heparin, possesses a much-reduced potential in activating the complement system when compared to the native protamine.

The most severe and life-threatening protamine toxicity comes via the immunological pathway. Protamine is nonhuman protein, so patients who have been previously exposed to protamine are likely to develop antiprotamine antibodies. It was reported that about 40% of the diabetic patients who had received protamine-containing insulin possessed IgG or IgE antiprotamine antibodies (25). A second exposure of such patients to protamine would lead to interaction of protamine with its antibodies on the mast cells and basophils, causing degranulation of these cells and the release of vasoactive mediators

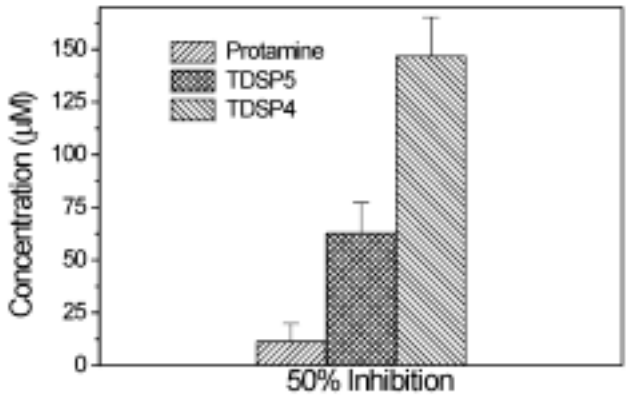

Figure 6 Dose requirement for the inhibition of 50% of the enzyme-linked immunosorbent assay (ELISA) reaction by protamine, TDSP5, and TDSP4. The microplate wells were coated with protamine as the capturing agent. A competitive ELISA assay that involved the addition of protamine or the LMWP fractions (ie, TDSP4 and TDSP5) to mouse serum samples containing antiprotamine antibodies was performed. For a detailed description of this competitive ELISA assay, please see the Materials and Methods section.

such as histamine. As mentioned previously, it is well documented in the literature that a chain-shortened peptide fragment derived directly from its parent protein by enzymatic digestion usually would possess a significantly reduced antigenicity and immunogenicity (7). Although preliminary studies conducted in our laboratory already showed that LMWP yielded a much-reduced immunogenicity (10), the more important issue was to determine if LMWP would actually possess a markedly reduced antigenicity or, in other words, a cross-reactivity toward antiprotamine antibodies. This was because, in reality, the large population of diabetic patients would likely have developed antiprotamine antibodies as a result of the use of protamine-containing insulins. As shown in Figure 6, using the competitive assay described in the Materials and Methods section, it required only 12 μM of protamine to inhibit 50% of the binding between the antiprotamine antibodies in the mouse serum and the protamine coated on the microtiter wells as the antibody-capturing agent, whereas it required 63 and 147 μM of TDSP5 and TDSP4, respectively, to yield the same degree of inhibition. These results clearly demonstrate the presence of a significantly reduced cross-reactivity by the LMWP compounds toward the antiprotamine antibodies. Such a finding could be

of considerable clinical promise because substituting LMWP for protamine in heparin reversal after cardiovascular surgeries may potentially prevent the large population of diabetic patients from the risk of having severe protamine responses. Further animal studies designed to prove this point of interest are currently under way in our laboratory.

The weight (ie, μg) concentration was used to represent the ability of the agents (protamine and LMWP) in heparin neutralization, whereas the molar concentration (ie, μM) was used to represent the immunoactivity of the agents. Current clinical dosing regimens for heparin neutralization are based on the mass unit (ie, 1 mg protamine is used to neutralize 100 U heparin). Therefore, by using the mass unit in heparin neutralization, our results on LMWP can be easily and adequately compared with the clinical databases on protamine. However, it is well documented that the interaction between a compound and its antibodies depends on both the number of antigen sites on this compound and on the affinity of the antigen sites toward the antibodies. For comparison between protamine and LMWP of antigenicity toward the antiprotamine antibodies, it is far more appropriate to use the molar unit instead of the weight unit.

CONCLUSIONS

In this paper, the in vitro efficacy of 2 LMWP fractions in neutralizing both heparin and LMWH were examined. Results showed that, as with protamine, both TDSP4 and TDSP5 could completely neutralize the aPTT and anti-IIa activities of heparin, although it required about 2 to 5 times the dose of protamine (protamine/heparin neutralization ratio of 7.5 μg/U) to reach 100% reversal. In addition, TDSP5 effectively neutralized the anti-Xa activity of the Mono-Embolex LMWH at about the same milligram dose of protamine and was able to completely neutralize 2 other LMWH preparations with significantly lower molecular weights (eg, 3000 d), whereas all currently existing heparin-neutralizing agents failed to do so. Aside from the efficacy in neutralizing the anticoagulant functions, the potential toxicity of these 2 LMWP preparations was also examined. Compared with protamine, TDSP4 and TDSP5 exhibited a significantly reduced ability in activating the complement system and in cross-reacting with the antiprotamine antibodies. Because TDSP5 shows the most promise as a potential nontoxic heparin antidote, it was selected for further animal studies. In the third and final article in this series, investigation of the in vivo efficacy and toxicity of TDSP5 using a sensitive canine model will be reported.

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