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Title Page

Title

Heparin-binding copolymer as a complete antidote for low-molecular-weight heparins in rats

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RUNNING TITLE PAGE

Running Title: Reversal of low-molecular-weight heparins in rats

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List of non-standard abbreviations: AT, antithrombin; DLS, dynamic light scattering; HBC, heparin-binding copolymer; ITC, isothermal titration calorimetry; LMWHs, low-molecular-weight heparins; PEG, poly(ethylene glycol); PMAPTAC, poly(3-(methacryloylamino)propyl trimethylammonium chloride); RAFT, reversible-addition fragmentation chain transfer polymerization; UFH, unfractionated heparin; UHRA, Universal Heparin Reversal Agent

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ABSTRACT

Bleeding resulting from the application of low-molecular-weight heparins (LMWHs) may be treated with protamine sulfate but lacks efficiency; its action against anti-factor Xa activity is limited to ~60%. Moreover, protamine sulfate can cause life-threatening hypersensitivity reactions. We developed diblock heparin-binding copolymer (HBC) that can neutralize the anticoagulant activity of parenteral anticoagulants. In the present study, we explored the safety profile of HBC and its potential to reverse enoxaparin, nadroparin, dalteparin, and tinzaparin in human plasma and at *in vivo* conditions. HBC-LMWHs complexes were characterized using zeta potential, isothermal titration calorimetry, and dynamic light scattering. The rat cardiomyocytes and human endothelial cells were used for the assessment of *in vitro* toxicity. Male Wistar rats were observed for up to 4 days after HBC administration for clinical evaluation, gross necropsy, and biochemistry and histopathological analysis. Rats were treated with LMWHs alone or followed by short-time intravenous infusion of HBC, and bleeding time and anti-factor Xa activity were measured. HBC completely reversed anti-factor Xa activity prolonged *in vitro* by all LMWHs with an optimal weight ratio of 2.5:1. The complexes of HBC-LMWHs were below 5 μm. We observed no effects on the viability of cardiovascular cells treated with HBC at concentrations up to 0.05 mg/mL. Single doses up to 20 mg/kg of HBC were well-tolerated by rats. HBC completely reversed the effects of LMWHs on bleeding time and anti-factor Xa activity *in vivo* after 20 minutes, and retained ~80% and ~60% of reversal activity after 1 hour and 2 hours, respectively. Well-documented efficacy and safety of HBC both *in vitro* and *in vivo* make this polymer a promising candidate for LMWHs reversal.

SIGNIFICANCE STATEMENT

Over the last decade, there has been significant progress in developing antidotes for the reversal of anticoagulants. Until now, there is no effective and safe treatment for patients with severe bleeding under low-molecular-weight heparin therapy. Based on our *in vitro* and *in vivo* studies, heparin-binding copolymer seems to be a promising candidate for neutralizing all clinically relevant low-molecular-weight heparins.

INTRODUCTION

Low-molecular-weight heparins (LMWHs) represent a group of parenteral anticoagulants for prevention and treatment of several thrombotic disorders, i.e., unstable angina, myocardial infarction, and deep vein or cancer-associated thrombosis. The global parenteral anticoagulant market is dominated by LMWHs and is still growing. LMWHs offer numerous advantages over standard unfractionated heparin (UFH) including more predictable pharmacodynamics and pharmacokinetics, less restrictive monitoring, safer subcutaneous administration, and more favorable overall safety profile with reduced risk of non-hemorrhagic side effects (Kearon et al., 2012).

LMWHs are not a homogenous group of anticoagulants, and may differ in their anticoagulant profiles, pharmacokinetic properties, and recommended dosing regimens (Kearon et al., 2012). LMWHs are obtained from UFH by different methods of depolymerization. The principal mechanism of the anticoagulant action of LMWHs is similar and involves antithrombin (AT). All LMWHs catalyze AT-mediated inactivation of coagulation factors. Because not all molecules of LMWHs contain 18 saccharide units that are necessary for factor IIa (thrombin) inhibition, LMWHs have anti-factor Xa to anti-factor IIa activity ratios between 1.6:1 and 8:1. Differences in anti-factor Xa/anti-factor IIa activity ratio between LMWHs depend on their average molecular weight and molecular size distribution (Gray et al., 2008). It was also shown in a cohort study of more than 12,500 patients that LMWHs differ in the frequency of bleeding episodes (van Rein et al., 2017). In the most clinical trials involving the management of thrombotic disorders with LMWHs, the incidence of major bleeding was between 1.5% and 4.7% and depended mainly on the type and duration of anticoagulation used and on the indication claimed (Simonneau et al., 1997; Hull et al., 2000; Turpie et al., 2002; Petersen et al., 2004; Mismetti et al., 2005; Crowther and Warkentin, 2008).

Severe bleeding related to LMWHs may be treated with intravenous administration of protamine sulfate. Protamine sulfate fully neutralizes LMWHs' anti-factor IIa activity, but its action against anti-factor Xa activity is limited to ~60% (Garcia et al., 2012). In the retrospective study, LMWHs-induced active bleeding was effectively stopped in 66% of patients who received protamine (van Veen et al., 2011). Protamine sulfate can also cause life-threatening hypersensitivity reactions such as hypotension, pulmonary hypertension, noncardiogenic pulmonary edema, cardiovascular collapse, and pulmonary vasoconstriction. Additionally, several risk factors for severe hypersensitivity reactions to protamine have been described, including rapid administration, previous administration of protamine or protamine insulin, fish allergy, left ventricular dysfunction, and previous vasectomy (Sokolowska et al., 2016). Therefore, each administration of protamine should be carefully considered, and better alternatives for LMWHs reversal are required.

Several new reversal agents for LMWHs (mainly enoxaparin and tinzaparin) are being developed. Ciraparantag is a cationic, synthetic small-molecule designed as an antidote for direct oral anticoagulants, UFH, and enoxaparin (Laulicht et al., 2013). Andexanet alfa, a recombinant derivative of factor Xa, has been designed to reverse the anticoagulant effects of direct and indirect factor Xa inhibitors (Lu et al., 2012). However, andexanet alfa has received approval only for uncontrolled and life-threatening bleeding in patients treated with oral anticoagulants: apixaban and rivaroxaban (Heo, 2018). Another antidote currently in the preclinical phase of development is Universal Heparin Reversal Agent (UHRA). UHRA, a synthetic dendrimeric polymer, has been designed to reverse the anticoagulant activity of all available heparins (Shenoi et al., 2014; Kalathottukaren et al., 2017). In a recently published *in vitro* comparative study, UHRA directly bound both UFH and enoxaparin. Andexanet alfa and ciraparantag bound the above heparins relatively weakly. For andexanet alfa and UHRA, binding correlated positively with the neutralization activity. The authors did not observe the

reversal activity of ciraparantag in assays typically used to assess anticoagulant effects (Kalathottukaren et al., 2018). None of the 3 above antidotes has been approved by the Food and Drug Administration for the management of LMWHs-induced bleeding.

Therefore, to address the significant and still unmet clinical need, we developed a heparin-binding polymer (HBC, PEG41-PMAPTAC53), a reversal agent for parenteral anticoagulants. HBC is a diblock synthetic polymer that directly binds anticoagulant molecules and effectively neutralizes their effects in the living organism (Kalaska et al., 2016, 2018, 2019). In this work, we further explored the potential of HBC to safely reverse LMWHs widely used in the clinical practice, i.e., enoxaparin, nadroparin, dalteparin, and tinzaparin at *in vivo* conditions and human plasma.

MATERIALS AND METHODS

In vitro neutralization assay

Enoxaparin, nadroparin, dalteparin, and tinzaparin were purchased from Sanofi-Aventis (France), Aspen Notre Dame de Bondueville (France), Pfizer Europe MA EEIG (UK), and Sigma-Aldrich (Germany), respectively. The neutralization of the above LMWHs was analyzed by measuring anti-factor Xa activity in a 96-well plate reader (Synergy HTX, BioTek, USA) according to the modified kit manufacturer instructions (BioMedica Diagnostics, Canada), as described previously (Kalaska et al., 2016). In brief, sodium citrate-anticoagulated pooled plasma was obtained from healthy rats or human volunteers by centrifugation of whole blood at 3500 x g for 20 min at 4ºC. Two hundred microliters of pooled plasma were mixed with 10 μL of LMWH (20 μg/mL of plasma for enoxaparin and tinzaparin; 2 U/mL for nadroparin and dalteparin). After 150 s of incubation (37°C), 10 μL of a solution containing increasing concentrations of HBC (0.01-0.1 mg/mL of plasma) was added. After another 150 s of incubation (37°C), anti-factor Xa activity was measured at 405 nm. All the procedures involving healthy human volunteers were approved by the Local Ethics Committee of the Medical University of Bialystok (Permit Number R-I-002/193/2019).

Zeta potential measurements

Zeta potential of all HBC-LMWHs complexes in ratios based on the *in vitro* neutralization assay was measured using a Zetasizer Nano-ZS (Malvern Instruments, UK). The samples were prepared in phosphate-buffered saline (PBS) (pH 7.4), filtered through a Chromafil filter (0.45 μm) and measured at 25°C. The data were analyzed using the Malvern software.

Isothermal titration calorimetry (ITC) measurements

All ITC measurements were made using Malvern MICRO-CAL PEAQ-ITC. HBC was dissolved in PBS; its concentration was 12 g/L (0.001078 mol/L). LMWHs' commercial solutions were also diluted in PBS to concentration about 125 units/mL or 1.25 mg/mL. The measurement temperature was 25°C, and the mixing speed was 750 rpm. The measuring chamber contained 270 µL of LMWH solution to which 19 portions of HBC solution, 2 µL each, were added.

The binding constant (K_a) between a molecule of HBC and a free binding site of a respective LMWH was calculated using the formula:

$$K_a = \frac{[bound\ HBC]}{[free\ binding\ sites\ of\ LMWH][free\ HBC]}$$

Dynamic light scattering (DLS) measurements

The size of complexes formed by HBC and LMWHs in ratios based on the *in vitro* neutralization was studied with the DLS technique. LMWHs were dissolved in PBS and bovine serum. To that mixture, the HBC solutions in PBS were added, and the formation of complexes was monitored by DLS using a Zetasizer Nano-ZS (Malvern Instruments, UK).

Cell lines used and cell viability assay

Primary human umbilical vein endothelial cells (HUVECs, ATCC-1730) and non-transformed embryonic rat heart-derived cells (H9c2(2-1), ATCC CRL-1446) were used as healthy control cell lines for assessment of the cardiovascular system toxicity of HBC. HUVECs were cultured in M199 medium containing 20% fetal bovine serum with cell growth supplements at 37°C in the atmosphere containing 5% CO_2 and at 95% humidity. All experiments were performed with mycoplasma-free cells. The ATPlite luminescence test (Perkin Elmer Life Sciences, USA) was used to assess the proliferation and viability of HUVECs *in vitro*. Cells were seeded in 96-well

plates, and after overnight incubation, the cells were treated with increasing concentrations of HBC for 72 h. Cells treated with formulation buffer were used as a control. H9c(2-1) were cultured in DMEM medium containing 10% fetal bovine serum at 37°C in the atmosphere containing 5% CO_2 and at 90% humidity. The experiment was carried out for 24 h after adding HBC to cell media. To assess cell viability, Crystal Violet (CV) assay was used (Popiołek et al., 2019). Briefly, cells were seeded into 24-well plates at the density of about $1.1 \cdot 10^4$ cells/cm^2. After incubation the medium was removed, and the cells were washed and fixed using 1 mL of 4% v/v formaldehyde/PBS and treated for 2 min with a CV solution. Then, unbound CV was removed by rinsing with water. After drying, to each well the destaining solution was added and left for 30 min. Finally, absorbance of the obtained solution at 540 nm was measured which is proportional to the number of living cells.

Animals

Male Wistar rats were obtained from the Centre of Experimental Medicine in the Medical University of Bialystok, grouped cages as appropriate, and allowed to have ad libitum access to sterilized tap water and standard chow in specific pathogen-free conditions. All the procedures involving animals were approved by the Local Ethical Committee on Animal Testing (Permit Numbers for the safety study 38/2017 and 85/2019; Permit Numbers for the efficacy study 15/2013 and 60/2018) and conducted in accordance to the Directive 2010/63/EU of the European Parliament and the Council on the protection of animals, ARRIVE guidelines, and the national laws.

Safety study in rats

Thirty-five male Wistar rats were randomly assigned to 5 groups. HBC was administered into the tail vein of 6 rats at the initial dose of 5 mg/kg. The rats were observed continuously for the

first 30 min after HBC administration for clinical signs of toxicity. Then they were kept under observation for up to 4 days after HBC administration (intermittently for 4 h and then at 6 h, 24 h, 48 h, 72 h and 96 h after HBC administration). Body weights were recorded before HBC administration and before the gross necropsy on day 4. Following a minimum of 1 day without severe clinical findings, HBC was administered to a group of 6 rats at a dose of 10 mg/kg. These rats were observed for clinical signs of toxicity. This procedure was repeated in 6 rats treated with HBC at a dose of 20 mg/kg and 10 rats treated with HBC at a dose of 40 mg/kg. PBS was also administered to 7 animals as a control group. On the 4th day, 4 rats treated with PBS and 4 rats treated with HBC at the dose of 40 mg/kg were anesthetized by an intraperitoneal injection of pentobarbital (45 mg/kg), and blood was collected from the heart, centrifuged, and serum was used for the measurement of alanine aminotransferase and aspartate aminotransferase activities and total bilirubin, urea, and creatinine concentrations. Immediately after the blood collection, the whole liver, lungs, kidney, and thymus were removed for routine histological analysis using hematoxylin and eosin staining.

Efficacy study in rats

In the first procedure, 56 male Wistar rats were randomly assigned to 7 groups, anesthetized by an intraperitoneal injection of pentobarbital (45 mg/kg), and placed in a supine position on a heated operating table. The body weights ranged from 200 to 250 g just before anesthesia induction. Nadroparin (800 U/kg), dalteparin (800 U/kg) or tinzaparin (8 mg/kg) were administered into the right femoral vein 10 min before the measurement of bleeding time alone or followed by short-time, lasting 5 min, intravenous infusion of HBC (20 mg/kg). Ten min after LMWH administration, bleeding time was measured, as described previously (Kalaska et al., 2015). If bleeding was still present at the end of the 480 s observation period, a value of 480 s was ascribed for the sake of statistical analysis. Twenty min after anticoagulant

administration, anti-factor Xa activity was analyzed with ELISA technique using a microplate reader (Synergy HTX, BioTek, USA) according to the kit manufacturer instructions (BioMedica Diagnostics, Canada). If anti-factor Xa activity was more than or equal to 1 U/mg, a value of 1 U/mg was ascribed for the sake of statistical analysis.

In the second procedure, 43 male Wistar rats were randomly divided into 6 groups and anesthetized in the same manner as described in the first procedure. The body weights ranged from 190 to 220 g just before anesthesia induction. Nadroparin and HBC were also administered in the same doses and manner as described in the first procedure. Bleeding time was measured 10 min before blood collection. Anti-factor Xa activity was analyzed 1 h or 2 h after anticoagulant administration. Bleeding time and anti-factor Xa activity were measured as described above.

Statistical analysis

In the *in vivo* study, *n* refers to the number of animals in each experimental group. For each test, the experimental unit was an individual animal. The data are shown as mean ± SD or median with lower and upper limits and analyzed with GraphPad Prism 6 software (GraphPad Prism 6 Software, USA) using the unpaired Student *t*-test or Mann-Whitney test. P values less than 0.05 were considered significant.

RESULTS

The synthesis of HBC (PEG41-PMAPTAC53) by controlled radical polymerization

The synthesis of HBC by reversible-addition fragmentation chain transfer (RAFT) polymerization technique and selection strategy of the leading compound was previously described (Kalaska et al., 2016). RAFT is one of the controlled/living radical polymerization processes, allowing synthesis of very well-defined polymers in terms of molecular weight and molecular weight dispersity. Briefly, a diblock copolymer with the degrees of polymerization of poly(ethylene glycol) (PEG) and poly(3-(methacryloylamino)propyl trimethylammonium chloride) (PMAPTAC) blocks equal to 41 and 53, respectively, was synthesized (Fig. 1). The cationic PMAPTAC198 homopolymer was used as a control to assess the toxicity-reducing activity of the PEG block.

Cell viability reduction

HUVECs and cardiomyocytes were treated with the diblock HBC and a cationic PMAPTAC198 homopolymer. HUVECs viability was not negatively affected by HBC and PMAPTAC198 at concentrations up to 0.05 mg/mL. Both studied polymers significantly impaired HUVECs viability at concentrations of 0.15 mg/mL (Fig. 2A). The conjugation of PMAPTAC with PEG significantly reduced the negative effect of the polycation PMAPTAC block on cardiomyocytes viability (Fig. 2B). At 0.15 mg/mL, the maximum concentration studied, the viability of the cardiomyocytes treated with HBC was still about 100%, while the viability of those treated with PMAPTAC198 decreased to about 50%.

Efficacy in the *in vitro* study

HBC concentration-dependently and completely reversed anti-factor Xa activity *in vitro* of enoxaparin, nadroparin, dalteparin, and tinzaparin in rat and human sodium citrate-

anticoagulated plasma with an optimal ratio of 0.05 mg of the polymer for 0.02 mg of both enoxaparin and tinzaparin and 2 U of both dalteparin and nadroparin (Fig. 3). We chose the concentration and dose of HBC for further *in vitro* and *in vivo* studies based on the estimated ratios: 2.5 mg of HBC per 1 mg of enoxaparin and tinzaparin; 2.5 mg of HBC per 100 U of dalteparin and nadroparin.

Zeta potential of HBC-LMWHs complexes

Based on the effective neutralization ratios found, we next investigated the zeta potential of the HBC-LMWHs complexes. In all cases, the zeta potential of complexes had a value close to zero (Table 1). The polycation completely neutralized the negative charge of LMWH molecules. Such a result was to be expected when the proportion of polymers in the mixture corresponded to the total binding of LMWH by polycation. DLS measurement showed a relatively large diameter of complexes particles due to potential values close to zero and the lack of electrostatic stabilization of the particles. In the case of tinzaparin, the complexes are the smallest.

Calorimetric measurements and thermodynamic parameters of the complexation reaction

Thermodynamic parameters of the interactions between all studied LMWHs and HBC are given in Table 1**,** and the integral enthalpies of interaction are shown in Figure 4. For enoxaparin and nadroparin, the drop inflection points in the respective curves are less clearly defined than for dalteparin and tinzaparin (Fig. 4, A and B vs Fig. 4, C and D) which may be due to higher dispersity of the molecular weight of the former LMWHs.

The size of LMWHs-HBC complexes

The size of complexes formed by LMWHs and HBC in PBS and bovine serum was studied using DLS technique. The particle size of complexes in PBS and serum have diameters increasing in order: tinzaparin (120 nm and 220 nm), dalteparin (0.5 μm and 1 μm), enoxaparin (0.8 μm and 5 μm), nadroparin (in both cases, two populations 0.4/>5 μm and 0.2/>5 μm). The presence of the polycation itself in the serum only slightly affects the size of the objects present in the mixture, and this effect can be considered negligible. The particle size of the complex formed in the serum is, in all cases, smaller than the diameter of the capillary blood vessels. A relatively large dimension equal to a fraction of the size of blood cells will promote the rapid elimination of complexes from the blood system (Fig. 5).

Safety in animals

There were no significant HBC-related effects on the body weight gain in rats treated for up to 4 days at the doses of 5, 10, and 20 mg/kg. A 5% reduction in body weight occurred in the group treated with HBC at the dose of 40 mg/kg. Administration of HBC at the dose of 20 mg/kg resulted in slightly decreased locomotive activity and apathy at approximately 3 h postdose in 50% of rats. We observed transient mild nasal swelling and edema of the limbs in 5 rats. All rats treated with HBC at the dose of 40 mg/kg had a decreased locomotive activity and apathy (both 10-15 min following HBC administration); 40% of rats had an increased thirst (14-20 min following HBC administration); 50% of rats had an increased secretion of porphyrins (2-3 h following HBC administration). All these findings were resolved within approximately 24 h postdose. Furthermore, mild nasal swelling and edema of the limbs were observed in 40% of rats treated with HBC at the dose of 40 mg/kg. These findings appeared immediately postdose and were resolved within approximately 6 h. HBC at the dose of 40 mg/kg resulted in kidney congestion and color heterogeneity in 40% of rats. Gross necropsy

findings in 30% of rats included the presence of urinary white sediment. Severe gross necropsy findings occurred in 1 rat and included lung congestion, kidney congestion, and color heterogeneity, thymus congestion, and liver abnormalities. All rats survived the experiment (Table 2). We found higher serum concentrations of urea and creatinine in 2 out of 4 rats treated with HBC at the dose of 40 mg/kg (Table 3). The same 2 rats presented significant histopathological changes of acute tubular necrosis in kidney tissue, whereas 2 others presented mild tubular toxicity which should not impair renal function. There were small areas of slight congestion and inflammatory cell infiltration in the lungs. HBC did not cause significant histopathological changes in other studied tissues (Fig. 6).

Efficacy in animals

All currently *in vivo* studied LMWHs (tinzaparin, dalteparin, and nadroparin) prolonged bleeding time, and increased anti-factor Xa activity at all studied time-points. HBC completely reversed the effects of LMWHs on bleeding time and anti-factor Xa activity to the approximate control values in a 20 min window (Fig. 7). Our previous study demonstrated that HBC also reversed an increased bleeding time and anti-factor Xa activity by enoxaparin in the same animal model (Kalaska et al., 2016). We observed an almost complete reversal of bleeding time and anti-factor Xa activity increased by nadroparin 1 h from the administration, whereas more than 60% of reversal activity was maintained after 2 h (Fig. 8).

DISCUSSION

We have previously shown the ability of HBC to neutralize UFH, enoxaparin, and fondaparinux. The complete reversal of enoxaparin demonstrated the potential advantage of HBC over protamine sulfate (Kalaska et al., 2016). In this work, we evaluated the safety profile of HBC and its reversal activity against dalteparin, nadroparin, and tinzaparin. We found that HBC bound all tested LMWHs forming the smallest complexes with dalteparin and tinzaparin. The efficacy and safety studies in rats showed that HBC could be a universal antidote for all LMWHs. We also confirmed the neutralization of all LMWHs by HBC in human plasma.

Our *in vitro* studies investigated the cardiomyocyte viability after exposure to HBC and PMAPTAC198 as a control to check the cytotoxicity reduction of the block copolymer compared with homopolymer. Reversal of heparins by cationic protamine sulfate can cause right ventricular failure and bradycardia (Ocal et al., 2005; Sokolowska et al., 2016), and may induce left ventricular dysfunction because of its direct effect on cardiomyocyte contractile processes (Hird et al., 1995). Due to the negative mitochondrial membrane potential in cardiomyocytes, cationic compounds may be taken up by mitochondria and may, therefore, lead to cardiomyocytes damage (Hasinoff et al., 2003, 2016). Indeed, cationic PMAPTAC198 homopolymer decreased cardiomyocytes viability whereas HBC had no significant effect on cell viability, confirming a well-known strategy of copolymerization or functionalization with PEG to reduce polycation toxicity (Pasut and Veronese, 2012). Although HBC and PMAPTAC198 did not affect human endothelial cells viability *in vitro* at concentrations up to 0.05 mg/mL, endothelial cells seem to be more sensitive than cardiomyocytes to the effects of both polycationic compounds applied in the higher concentration. The reduced viability may result from their interaction with the anionic surface of endothelial cells, as it was showed in the case of protamine sulfate (Chang et al., 1989).

HBC sufficiently reversed anti-factor Xa activity increased *in vitro* by studied LMWHs in the concentration of 0.025 mg/mL. In contrast, protamine sulfate was partially effective and showed an anticoagulant effect at concentrations higher than 0.05 mg/mL (Shenoi et al., 2014). The efficacious concentrations of HBC were 3-6 times lower than those maximum studied in the cardiomyocytes viability experiment. UHRAs demonstrated complete tinzaparin neutralization at similar ratios (Shenoi et al., 2014). Andexanet alfa in the concentration of ~3 μM also completely reversed enoxaparin (Lu et al., 2013). However, the authors focused on one LMWH (tinzaparin or enoxaparin), and have not investigated the cardiotoxicity.

We applied DLS and ITC techniques to understand the mechanism of interaction between HBC and LMWHs. The enthalpy of binding is positive indicating an endothermic interaction. It was a bit unexpected for electrostatic interaction. However, endothermic interactions between oppositely charged polyelectrolytes have been reported (Pei et al., 2009). On the other hand, entropy values are positive indicating that the neutralization process is entropy-driven. The values of n vary from 0.38 to 0.69 indicating that one chain of HBC may bind more than one molecule of an LMWH (from 1.4 to 2.6 on the average). This is not surprising taking into account that one HBC chain bears on the average 53 cationic groups while the chains of the LMWHs bear from about 22 to 32 negative sulfate groups (assuming they all have the same charge density equal to 2.7 sulfate groups per disaccharide repeat unit, i.e. a charge of -5/kDa) (Kalathottukaren et al., 2017). It means that the number of HBC cationic groups per chain is about 1.6 – 2.5 times greater than that of LMWHs anionic groups which is close to $1/n$, i.e., the number of LMWH chains complexed by one HBC chain. Moreover, the value of n for HBC-enoxaparin can be compared to the respective value for dendrimeric UHRA, for which the n value vary from 0.17 to 0.28 (Shenoi et al., 2014), and indicates that one UHRA dendrimer may bind from 3.6 to 5.9 chains of enoxaparin. It is about two times more than the number of enoxaparin chains complexed by one HBC molecule. Considering that the MW of

UHRAs is >23 kDa (i.e. almost twice that of HBC) the ability to bind enoxaparin of UHRAs and HBC is comparable in terms of enoxaparin chains bound per kDa of a polycation. The binding constants for HBC-LMWHs complexes are of the order of $10^7 – 10^8$, so the binding is rather strong, also when compared to UHRA-enoxaparin binding for which K_a was of the order of $10^4 – 10^5$ (Shenoi et al., 2014).

The development of a reversal agent for LMWHs is a multi-step process involving an evaluation of its safety. The nonclinical safety evaluation mainly includes a characterization of toxic effects, gross necropsy observations, dose dependence, and relationship to exposure. It should support first-in-human clinical trials (ICH, 2009). Nonclinical toxicity studies of the most promising LMWH-reversal agents, ciraparantag and andexanet alfa, have been conducted in different animal models. Ciraparantag was evaluated to perform the core safety pharmacology studies in rat and dog models. Based on the lack of adverse systemic toxicity and mortality, doses of ciraparantag up to 35 mg/kg in dogs and 40 mg/kg in rats were chosen as maximum tolerated doses. Ciraparantag in the single dose of 40 mg/kg was well-tolerated in dogs. There were mild signs of hypersalivation and loss of balance. In rats, ciraparantag in a single dose of 40 mg/kg caused a decreased activity and red-stained nares (Sullivan et al., 2015). Similar treatment-emergent adverse events were observed in healthy volunteers later (Ansell et al., 2016). The authors have not investigated LMWH-reversal effect of ciraparantag in humans. Andexanet alfa was tested in a single and repeat-dose toxicity study. It was administered to rats or monkeys alone and in combination with apixaban, rivaroxaban or enoxaparin. There were no systemic or tissue pathologies in studied animals after single intravenous injections of up to 60 mg/kg. There were five mortalities of rats treated with andexanet alfa twice daily for 14 days (Faulcon, 2016). In monkeys, they found neither significant safety concerns or unexpected outcomes. All observations were transient or reversible and included transient hypoactivity, labored respiration, vomiting, and anaphylactic

reaction (Lu et al., 2017). Similarly to other antidotes, we tested the toxicity of HBC administered without heparin. Besides mild and occasional clinical and gross necropsy findings, single doses up to 20 mg/kg of HBC were well-tolerated. We observed changes in rats' behavior and signs of organ damage in the group of rats treated with a dose of 40 mg/kg of HBC. In some of the rats, we performed additional biochemistry and histopathological analysis, which revealed a sign of acute tubular necrosis and increased biochemical markers of the kidney damage in 2 cases. Nephrotoxicity may result from the binding of cationic HBC with an anionic surface of renal epithelial cells, similarly to the postulated chemical interactions between crystals and epithelial cell surfaces in kidney stone formation (Lieske et al., 1997; Wesson and Ward, 2006). Similarly to protamine, the efficacious dose of HBC for LMWHs reversal is closely related to the dose of studied LMWH and is based on the estimated optimal mass ratios. In the previous study (Kalaska et al., 2016), we used HBC at the dose of 6 mg/kg that corresponded to 3 mg/kg of enoxaparin. If we assume 3 mg/kg as a therapeutic dose of enoxaparin in rats, the multiple to the NOAEL (no-observed-adverse-effect-level) and adverse level for HBC would be ~4 and ~8, respectively. LMWHs were administered in supratherapeutic doses to induce clinically relevant major bleeding. Indeed, anticoagulation by LMWHs increased bleeding time and anti-factor Xa activity. The strong anticoagulant effect was observed in 100% of the animals. HBC administered in dose corresponding to 2.5:1 mass ratio, but not exceeding safety dose of 20 mg/kg, completely reversed the effects of LMWHs on bleeding time and anti-factor Xa activity to the approximate control values in a 20 min window. A similar study has been conducted to determine the reversal effect of andexanet alfa on enoxaparin. However, administration of andexanet alfa only partially reversed anti-factor Xa activity and decreased bleeding in ~80% of the animals in a rat tail-transection model (Hollenbach et al., 2012). We further evaluated time-related changes of the reversal activity of HBC against nadroparin as an example, since HBC effect was comparable for all studied

LMWHs. The almost complete neutralization of bleeding time and anti-factor Xa activity was observed for 1 h after a 5-minute infusion of HBC, whereas more than 60% of its reversal activity was maintained after 2 h. Based on the literature data (Ibrahim et al., 2017), the inhibition of factor Xa activity by LMWH expires after ~6 h from intravenous administration into rats. Thus, if a reversal of anticoagulation longer than 2 h is needed for surgical intervention or during uncontrolled bleeding, HBC should be probably administered second time or, similarly to andexanet alfa, should be kept infused till the end of surgery or bleeding. Andexanet alfa due to the short half-life (~1 h) had to be infused additionally to nonbleeding healthy volunteers to reverse activity of enoxaparin (Crowther et al., 2014, Siegal et al., 2015). In the ANNEXA-4 trial andexanet alfa was administered as an intravenous bolus followed by a 2 h infusion to 352 patients who had major acute bleeding after administration of rivaroxaban, apixaban or enoxaparin. Of the 15 patients treated with enoxaparin who could be evaluated for anticoagulation efficacy, 13 (87%) were adjudicated as having at least good hemostatic efficacy 12 h after andexanet alfa infusion (Connolly et al., 2019). Thus, a 2-h interval seems to be a convenient therapeutic window for the reversing of LMWHs in patients, especially with a high risk of thrombosis, in which restoring of anticoagulant protection is needed. In the case of LMWHs reversal, more clinical trials are required to assess the effects of andexanet alfa on this drugs-related bleeding (Sartoni and Cosmi, 2018). Ciraparantag, another candidate for LMWHs reversal, was evaluated in rats and healthy volunteers. It stopped tail bleeding in rats treated with enoxaparin, and the effect was stronger than that of protamine sulfate (Laulicht et al., 2013). In healthy volunteers, ciraparantag entirely and rapidly reversed the clotting time induced by enoxaparin but also caused adverse events in more than half of patients (Ansell et al., 2016).

Based on the previous studies on enoxaparin, one could assume that also other LMWHs will be neutralized by HBC. However, it would be impossible to conclude that HBC is an efficient

and safe reversal agent for all main LMWHs without clear direct experimental evidence, especially because other antidotes were less effective against heparins with shorter chains. We also expanded the panel of safety studies in rodents and the toxicity in cardiomyocytes and characterized in more detail the mechanism of binding. We confirmed the efficacy of HBC against LMWHs in human plasma and its safety in HUVECs. It seems that the reversal activity of HBC after a single injection could last longer than the effect of andexanet alfa, which needs to be infused to maintain activity. Nevertheless, the second injection or prolonged infusion of HBC may be required in some clinical scenarios depending on how much time has passed since LMWHs injection and how much time is needed for surgical intervention. Thus, the evaluation of repeated-dose toxicity together with the estimation of the optimal route of HBC administration in non-human primates is required before the administration of HBC to patients. To sum up, we presented here a new pharmacological tool for binding all heparins. The studies with protamine, andexanet alfa and ciraparantag showed that there is still space for a more efficient, safer, and cost-effective reversal agent indicated for LMWHs, and yet no such antidote has been currently registered. The results of safety experiments point a therapeutic potential of HBC administered in doses up to 20 mg/kg. We estimated the neutralization weight ratio of HBC to LMWH for 2.5:1 in rats. Then, we confirmed it in human plasma. Importantly, HBC stopped bleeding from tail of heparinized rats and brought to control level typical coagulation parameters used in the clinical practice. HBC retained ~80% and ~60% of reversal activity after 1 hour and 2 hours, respectively. Based on this study we may expect similar effect of HBC in patients treated with LMWHs.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Kalaska, Szczubiałka, Mogielnicki.

Conducted experiments: Kalaska, Miklosz, Kamiński, Swieton, Jakimczuk, Yusa, Mogielnicki.

Contributed new reagents or analytic tools: Kalaska, Miklosz, Kamiński, Swieton, Yusa, Szczubiałka, Mogielnicki.

Performed data analysis: Kalaska, Swieton, Kamiński, Mogielnicki

Wrote or contributed to the writing of the manuscript: Kalaska, Miklosz, Kamiński, Swieton, Jakimczuk, Yusa, Pawlak, Nowakowska, Szczubiałka, Mogielnicki.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Chemical structure of heparin-binding copolymer (HBC, PEG41-PMAPTAC53) (Kalaska et al., 2016).

Fig. 2. Effect of heparin-binding copolymer (HBC) and PMAPTAC198 on human umbilical vein endothelial cells (HUVECs, A) and cardiomyocyte viability (B). Results are shown as mean ± SD.

Fig. 3. Neutralization of enoxaparin, nadroparin, dalteparin, and tinzaparin by heparin-binding copolymer (HBC) in sodium citrate-anticoagulated plasma with the anti-factor Xa assay. Results are shown as mean ± SD.

Fig. 4. Estimated heat of binding for the titration of heparin-binding copolymer (HBC) into enoxaparin (A), nadroparin (B), dalteparin (C), and tinzaparin (D).

Fig. 5. The size profile of enoxaparin-heparin-binding copolymer (HBC, A), nadroparin-HBC (B), dalteparin-HBC (C), and tinzaparin-HBC (D) complexes in the aqueous solutions (PBS, pH 7.4) and bovine serum, and PBS and HBC (E) in bovine serum obtained by the dynamic light scattering analysis.

Fig. 6. Representative micrographs of tissue sections of liver (A and B), lung (C and D), renal cortex (E and F), renal medulla (G and H), and thymus (I and J) 4 days after intravenous administration of PBS (left column; A, C, E G, and I) and HBC at the dose of 40 mg/kg (right column; B, D, F, H, and J) in rats. Magnification 100x; hematoxylin and eosin staining.

Fig. 7. Neutralization of nadroparin, dalteparin, and tinzaparin by heparin-binding copolymer (HBC) in rats as measured by bleeding time (A) and anti-factor Xa activity (B). ***$P < 0.001$ vs vehicle; $^{###}P < 0.001$ vs appropriate low-molecular-weight heparin, unpaired Student *t*-test. Results are shown as mean ± SD and analyzed with GraphPad Prism software 6.

Fig. 8. Time-course of reversal of nadroparin by heparin-binding copolymer (HBC) in rats as measured by bleeding time (A and B) and anti-factor Xa activity (C and D) 1 h or 2 h after nadroparin administration. ***$P < 0.001$ vs vehicle; $^{#}P < 0.05$, $^{###}P < 0.001$ vs nadroparin, unpaired Student *t*-test. Results are shown as mean ± SD and analyzed with GraphPad Prism software 6.

TABLES

TABLE 1

Zeta potential of LMWHs-HBC complexes and thermodynamic parameters for the interaction of LMWHs with HBC.

	Zeta potential (mV)	**Stoichiometry /number of binding sites, n**	**Estimated binding constant $K_a \times 10^{-7}$ [M^{-1}]**	**Estimated heat of binding, ΔH [kcal/mol]**	**Gibbs free energy, ΔG [kcal/mol]**	**Change in entropy $-T\Delta S$, [kcal/mol]**
Enoxaparin-HBC	1.49±0.63	0.47±0.02	92.4±19.9	8.35±0.54	-6.87	-15.2
Nadroparin-HBC	-3.93±0.29	0.49±0.01	10.8±2.14	8.01±0.22	-8.14	-16.2
Dalteparin-HBC	-0.13±0.08	0.38±0.01	1.42±0.64	9.86±0.22	-9.35	-19.2
Tinzaparin-HBC	-5.29±1.29	0.69±0.01	6.51±1.68	8.68±0.26	-8.45	-17.1

TABLE 2

Selected clinical and gross necropsy observations during safety study in rats.

Dose (mg/kg)	**0**	**5**	**10**	**20**	**40**
Selected clinical findings					
Decreased locomotive activity	0/7	0/6	0/6	3/6	10/10
Apathy	0/7	0/6	0/6	3/6	10/10
Increased thirst	0/7	0/6	0/6	0/6	4/10
Secretion of porphyrins	0/7	1/6	1/6	0/6	5/10
Mild allergic reactions	0/7	1/6	0/6	5/6	4/10
Mortality	0/7	0/6	0/6	0/6	0/10
Selected gross necropsy findings					
Lung congestion	0/7	1/6	0/6	1/6	1/10
Kidney congestion	0/7	0/6	0/6	0/6	4/10
Kidney color heterogeneity	0/7	1/6	0/6	0/6	4/10
Thymus congestion	0/7	0/6	0/6	1/6	1/10
Duodenum congestion	0/7	0/6	0/6	1/6	0/10
Pancreas congestion	0/7	0/6	0/6	1/6	0/10
Liver abnormalities	0/7	0/6	0/6	1/6	1/10
Urine sediments	0/7	0/6	0/6	0/6	3/10

The values indicate a total number of observations/number of animals concerned.

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TABLE 3

Biochemical parameters in rats treated with HBC at the dose of 40 mg/kg.

	Vehicle	**HBC**
Alanine transaminase, U/L	34.5 (25.0-40.0)	26.0 (19.0-34.0)
Aspartate aminotransferase, U/L	86.5 (76.0-94.0)	105.0 (67.0-147.0)
Total bilirubin, mg/dL	<0.15	<0.15
Urea, mg/dL	42.0 (39.0-44.0)	229.0 (38.0-448.0)
Creatinine, mg/dL	0.20 (0.20-0.30)	0.95 (0.20-3.40)

Results are shown as a median with lower and upper limits.

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FIGURES

Fig. 1

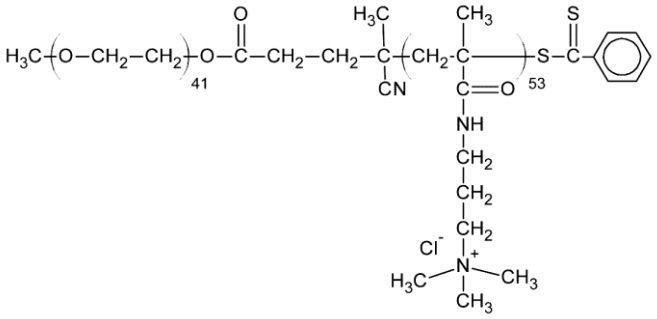

Fig. 2

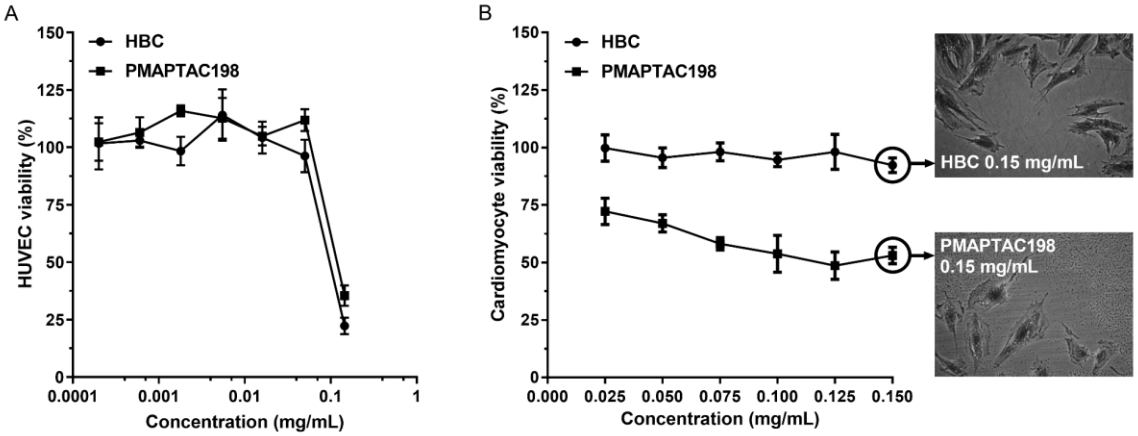

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Fig. 3

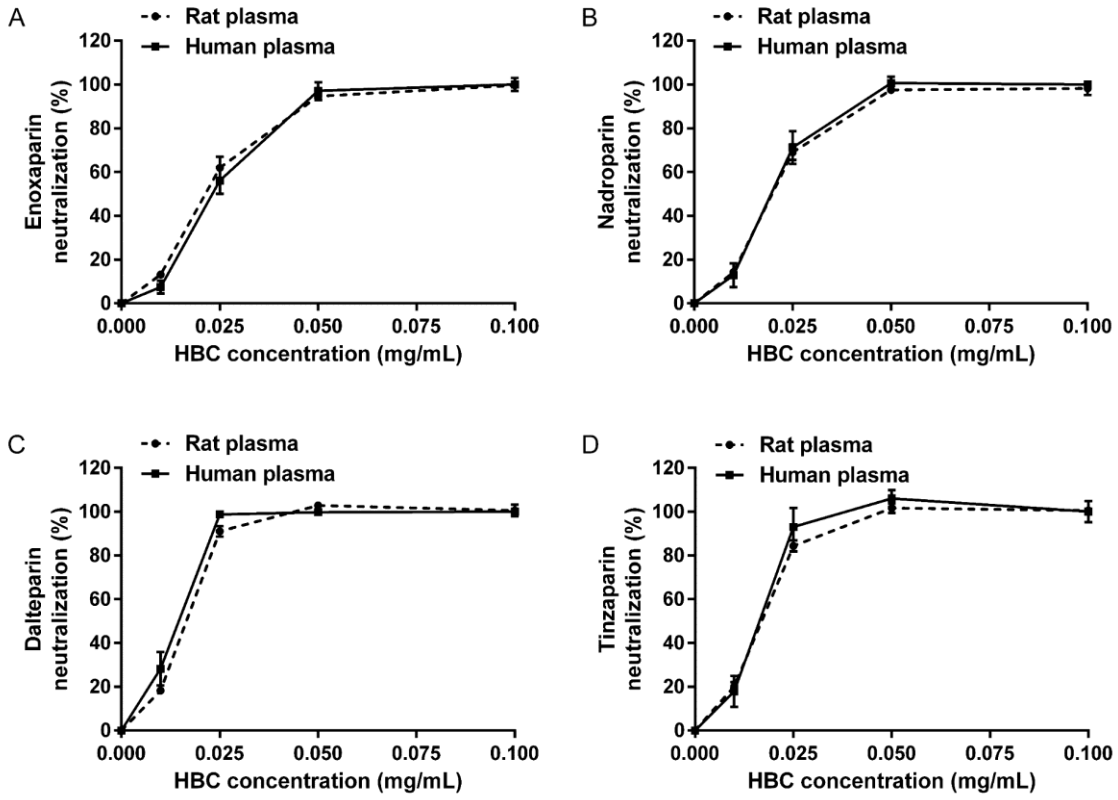

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Fig. 4

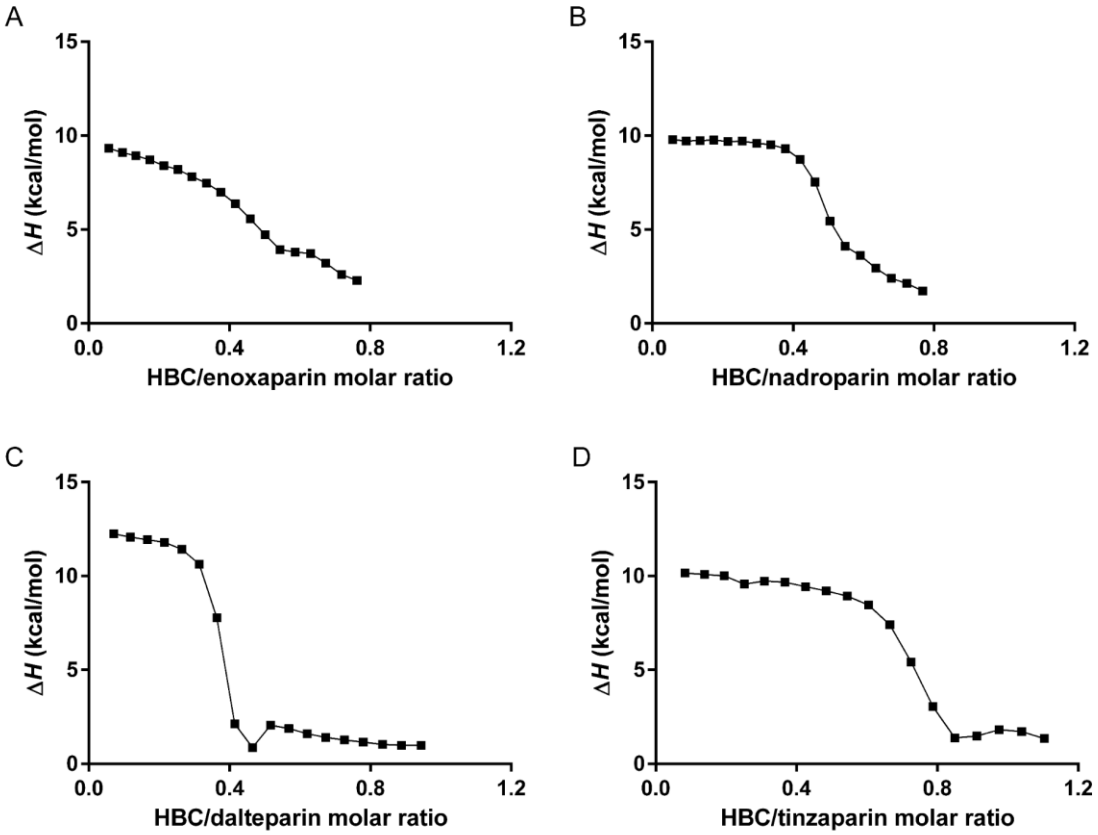

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Fig. 5

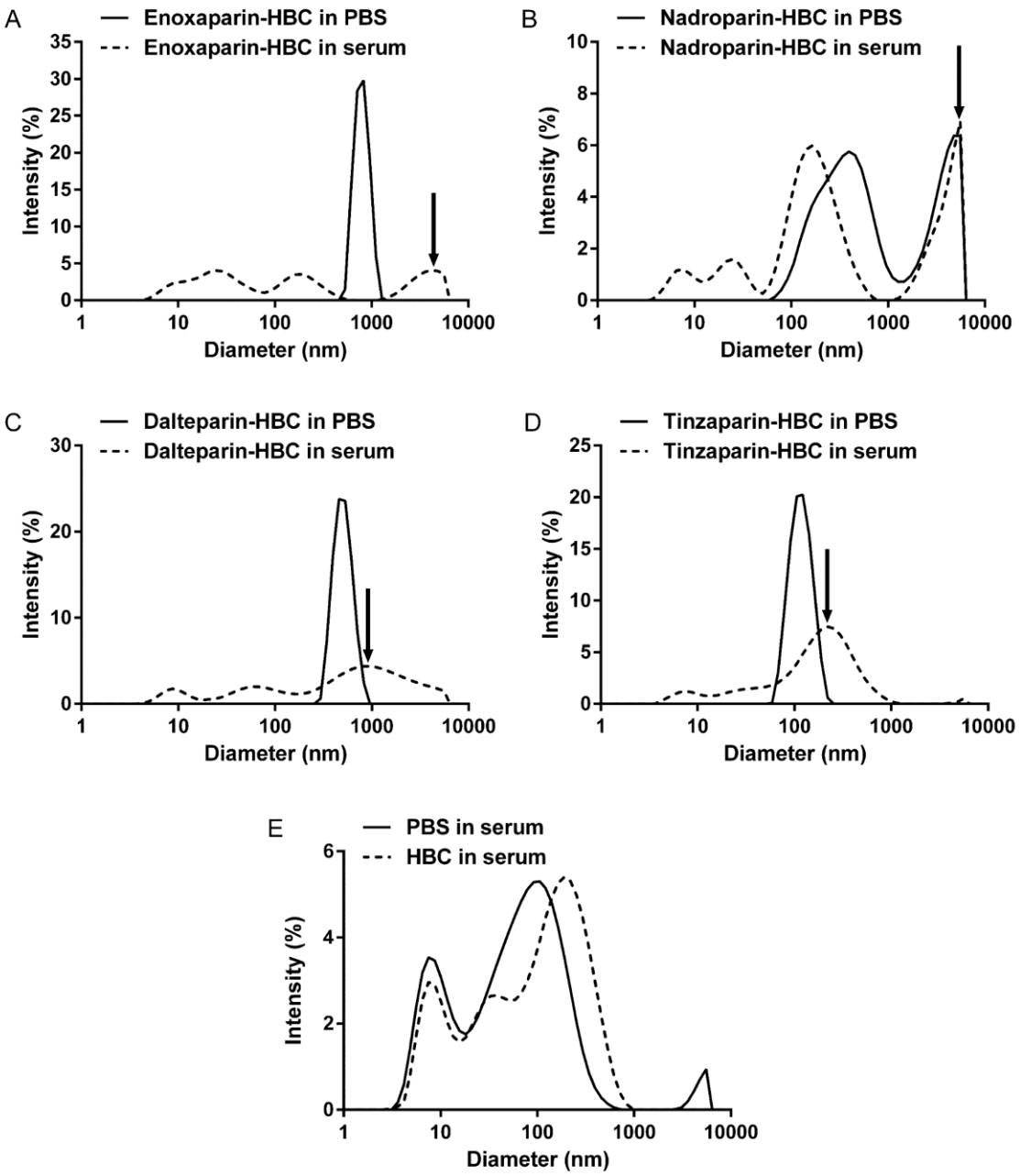

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Fig.6

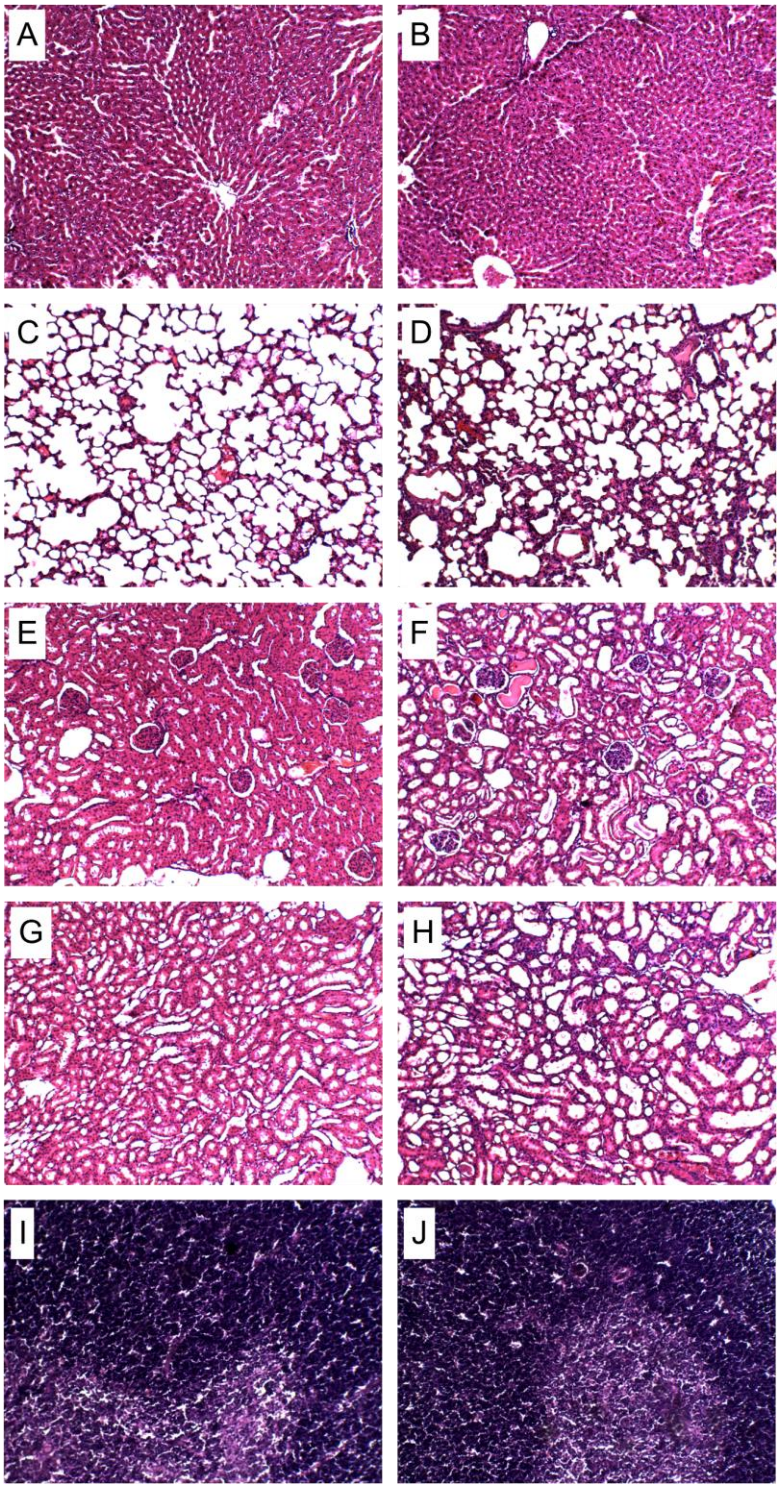

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Fig. 7

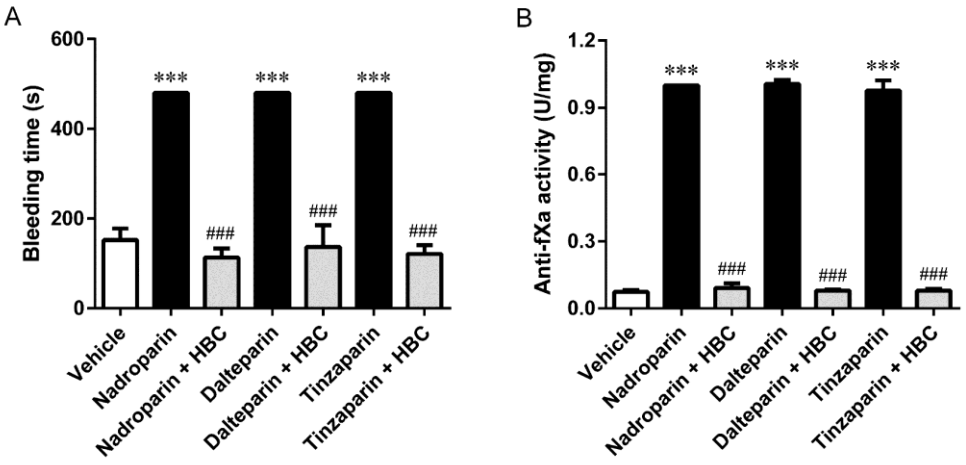

Fig. 8