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Protamine stimulates platelet aggregation *in vitro* with activation of the fibrinogen receptor and alpha-granule release, but impairs secondary activation via ADP and thrombin receptors

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Abstract

Heparin and protamine are fundamental in the management of anticoagulation during cardiac surgery. Excess protamine has been associated with increased bleeding. Interaction between protamine and platelet function has been demonstrated but the mechanism remains unclear. We examined the effect of protamine on platelet function *in vitro* using impedance aggregometry, flow cytometry, and thrombin generation. Platelets were exposed to protamine at final concentrations of 0, 20, 40, and 80 µg/mL, alone or together with adenosine diphosphate (ADP) or thrombin PAR1 receptor-activating peptide (TRAP). We found that in the absence of other activators, protamine (80 µg/mL) increased the proportion of platelets with active fibrinogen receptor (binding of PAC-1) from 3.6% to 97.0% ($p < .001$) measured with flow cytometry. Impedance aggregometry also increased slightly after exposure to protamine alone. When activated with ADP or TRAP protamine at 80 µg/mL reduced aggregation, from 73.8 ± 29.4 U to 46.9 ± 21.1 U ($p < .001$) with ADP and from 126.4 ± 16.1 U to 94.9 ± 23.7 U ($p < .01$) with TRAP. *P*-selectin exposure (a marker of alpha-granule release) measured by median fluorescence intensity (MFI) increased dose dependently with protamine alone, from 0.76 ± 0.20 (0 µg/mL) to 10.2 ± 3.1 (80 µg/mL), $p < .001$. Protamine 80 µg/mL by itself resulted in higher MFI (10.16 ± 3.09) than activation with ADP (2.2 ± 0.7, $p < .001$) or TRAP (5.7 ± 2.6, $p < .01$) without protamine. When protamine was combined with ADP or TRAP, there was a concentration-dependent increase in the alpha-granule release. In conclusion, protamine interacts with platelets *in vitro* having both a direct activating effect and impairment of secondary activation of aggregation by other agonists.

Keywords

Flow cytometry, impedance aggregometry, platelet function, protamine, thrombin generation

History

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Introduction

Since the 1930s, protamine has been known to reverse the anticoagulant effect of heparin, and its use in connection with cardiopulmonary bypass (CPB) is fundamental to the management of anticoagulation [1,2]. The effect of CPB on hemostasis is multifaceted, and it is not completely understood how and to what degree protamine contributes to the coagulopathy often seen during cardiac surgery [3]. Clinical data have shown a relationship between the protamine heparin ratio and a negative effect on hemostasis, a higher protamine ratio being associated with greater bleeding [4–6]. Protamine has also been described to interact with platelet function [7], but how protamine interferes with platelet function and hemostasis is not fully understood. Many previous studies on hemostatic problems in connection with heparin reversal after CPB have been focused on the dosing of protamine in regard to its negative effects on coagulation, while the effects of protamine on platelet function have been less well studied [8].

There are today several methods available to assess platelet function. Impedance aggregometry is fast and user-friendly and is frequently used during cardiac surgery [9–11]. Flow cytometry allows the study of different aspects of platelet activation [12,13]. Several processes can be analyzed such as activation of the fibrinogen receptor, release of alpha-granules, exposure of phosphatidylserine on the surface of the platelet and disruption of the mitochondrial membrane. Furthermore, thrombin generation can be studied in platelet-rich plasma in order to study platelet interaction with coagulation [14].

We have previously demonstrated a marked decrease in platelet aggregation after the administration of protamine *in vivo* during cardiac surgery [15]. Details of this impairment and possible consequences for postoperative bleeding have not been completely studied. The purpose of this *in vitro* study was to further examine and characterize the mechanisms by which protamine interacts with various aspects of platelet function using aggregometry, flow cytometry, and thrombin generation.

Materials and Methods

The study was performed at the Department of Cardiothoracic Surgery, Linköping University Hospital, Sweden. The study was

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approved by the Regional Ethics Review Board in Linköping, Sweden (Dnr 2012/382-31 and Dnr 2016/20-31) and complied with the principles laid down in the Helsinki Declaration. Written informed consent was obtained from all patients.

Study Population

Thirteen consecutive patients (3 females) aged 56 to 83 (mean 70 ± 7.8 years) undergoing coronary artery bypass grafting (CABG) were studied using impedance aggregometry. Exclusion criteria were; participation in any other conflicting study or previously diagnosed coagulation disorder or platelet dysfunction. Patients on anticoagulation therapy or patients who had taken ticagrelor or clopidogrel during the 5 days prior to surgery were also excluded. All patients were on acetylsalicylic acid 75 mg/day until the day before surgery.

To further investigate how protamine affects platelet function, flow cytometry and thrombin generation analyses were performed on blood from eleven healthy blood donors aged 22–62, not taking any medication affecting coagulation or platelet function.

Blood Sampling and Preparation

Impedance Aggregometry

Prior to induction of anesthesia, blood samples for impedance aggregometry were drawn into hirudin tubes (Roche Diagnostics GmbH, Mannheim, Germany) from an arterial line placed in the radial artery.

To test the effect of protamine on platelet activation, 420 µl hirudinized blood was mixed with saline (0.9%) solution and with protamine (Leo Pharma, Stockholm, Sweden) to final concentrations 20, 40, and 80 µg/mL. A concentration of 40 µg/mL corresponds to what could be expected in a clinical situation and matches what have been used in prior studies [15–17]. The blood samples were analyzed with impedance aggregometry (Multiplate®, Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions and using the commercially available platelet activators in the standard doses: adenosine diphosphate (ADP, final concentration 6.5 µmol), thrombin receptor-activating peptide (TRAP, final concentration 32 µmol), or saline. The results are presented as area under the curve (AUC) in arbitrary units (U) [18]. All stock solutions were pre-diluted to ensure the same dilution factor in all experiments performed.

Flow Cytometry

Blood samples for flow cytometry from the healthy blood donors were drawn into hirudin tubes through venipuncture of an antecubital vein. Experiments were started approximately 1 h after blood collection since platelet activation responses are more consistent at this time [19].

Flow cytometry was performed using a slightly modified version of a recently published protocol [13]. The fluorochrome-labeled platelet activation markers were all from Becton Dickinson (Franklin Lakes, NJ, USA): PAC-1-FITC (a monoclonal antibody that binds to activated fibrinogen receptor GPIIb/IIIa, final concentration 0.56 µg/mL), anti-*P*-selectin-PE (CD62P, clone AK4, final concentration 0.17 µg/mL, used as marker for platelet alpha-granule release), IgG1-PE isotype control, and annexin V-V450 (binds phosphatidylserine, final concentration 2.67 ng/mL). As an indicator of intact mitochondrial membranes, we used the mitochondrial dye 1,1′,3,3,3′,3′-hexamethylindodicarbo-cyanine iodide ($DilC_1(5)$; final concentration 30 nM, Life Technologies, Eugene, OR, USA) and as positive control, we used carbonyl cyanide 3-chlorophenylhydrazone (CCCP; final concentration 100 µM, Sigma, St. Louis, MO, USA), which disrupts mitochondrial membrane integrity. Platelet mitochondrial membrane disruption together with exposure of phosphatidylserine on the surface is a sign of conversion to a procoagulant state [20]. Platelets were identified using anti-GPIIb (CD41)-ECD, which binds to the fibrinogen receptor regardless of the state of activation (final concentration 0.69 µg/mL, Beckman Coulter Inc., Brea, CA, USA).

We used ADP and TRAP to activate the platelets in the same concentrations used for the aggregometry analysis. Each tube with platelet activators had a final volume of 36 µl, of which 3 µl was whole blood derived from the hirudin tube. Blood was incubated for 10 min with markers of platelet activation, without protamine or with protamine in a final concentration of 20, 40, and 80 µg/mL, and platelet activators for 10 min before dilution and analysis with the flow cytometer.

To see if heparin counteracts the effect of protamine on platelet activation, we repeated the experiment described above but with the addition of heparin (Leo Pharma, Stockholm, Sweden) to a final concentration of 11.2 U/mL after 5 min of activation. This concentration of heparin would bind all protamine present at a concentration of 80 µg/mL (according to the manufacturer's specifications that 1 mg protamine binds 140 U heparin). All stock solutions were pre-diluted to ensure the same dilution factor in all experiments performed.

The samples were analyzed with a Gallios™ flow cytometer (Beckman Coulter Inc., Fullerton, CA, USA) equipped with three lasers (405, 488, and 638 nm) and a 10-color configuration. The acquisition was performed using the ultrawide angle of detection (submicron particle setting) for forward scatter and a fluorescence threshold on FL3 (CD41-ECD) to allow detection of all platelet particles. Flow cytometry acquisition was continued until 5000 CD41-positive platelet-sized particles had been collected. Fluorescent control beads (Flow-Check and Flow-Set; Beckman Coulter Inc.) were used to verify that the instrument performance was stable over time. Data analysis was performed using Kaluza v.1.3 (Beckman Coulter). Data are in general presented as percentage positive platelets and as median fluorescence intensity (MFI). In experiments where the highest activation was well below 100%, only percentage is presented and when almost all platelets were activated, we only presented MFI. The platelet gate was placed tightly around the platelet cloud in the sample with resting platelets and adjusted to include single platelets only. Data were processed, analyzed, and exported to Microsoft Excel (Redmond, WA, USA).

Thrombin Generation

Blood samples for thrombin generation were drawn into citrate tubes (0.105 M tri-sodium citrate, BD Vacutainer) from the healthy blood donors through venipuncture of an antecubital vein.

Thrombin generation was monitored using the calibrated automated thrombogram (CAT) method. The experiment was performed using a 96-well plate. Citrated blood was centrifuged at 150 g, 20°C for 15 min to extract platelet-rich plasma (PRP). The rest of the blood was centrifuged at 2500 g, 20°C for 15 min to extract platelet-poor plasma (PPP). The PRP was diluted with PPP to a platelet count of 250×10^9/L and incubated with protamine at concentrations 0, 20, 40, and 80 µg/mL for 10 min before addition of calcium, fluorogenic substrate, and the PRP reagent (Thrombinoscope BV, Maastricht, Netherlands), containing 0.5 pM tissue factor but low amounts of phospholipids. Analysis of thrombin generation was performed on an Ascent FL (Thermo Electron Corporation, Vantaa, Finland) with Thrombinoscope software (Thrombinoscope, Maastricht, Netherlands). All samples were analyzed in triplicate. The Thrombinoscope analysis software calculates all parameters of

the thrombogram and expresses results as nanomoles thrombin with time.

Statistical Analysis

Statistical analysis and graphs were made using GraphPad Prism 8.3.0 for Mac OS X (GraphPad Software, La Jolla, CA, USA). Data are presented as median and interquartile range or mean ± standard deviation as indicated. Student's t-test, analysis of variance (ANOVA), or repeated measures ANOVA were used followed by Dunnett´s correction for multiple comparisons. A p-value <0.05 was considered statistically significant.

Results

Impedance Aggregometry

Impedance aggregometry showed that protamine by itself produced a small increase in platelet aggregation at a concentration of 80 µg/mL compared to control. When blood was exposed to increasing concentrations of protamine and subsequently activated with ADP or TRAP, a concentration-dependent decrease in platelet aggregation was seen (Figure 1).

Flow Cytometry

Activation of the Fibrinogen Receptor Indicated by PAC-1 Binding

To explore any receptor changes associated with the effect of protamine on platelet function, we acquired flow cytometry data and measured the proportion of platelets with active fibrinogen receptors (binding PAC-1). Protamine alone induced a significant concentration-dependent increase in the proportion of platelets with active fibrinogen receptors. The proportion of platelets with active fibrinogen receptors increased from median 3.6% (no protamine) to 97.0% (80 µg/mL), $p < .001$ (Figure 2). The median fluorescence intensity (MFI) also increased with higher concentrations of protamine (Figure 3). Corresponding to the aggregation results from impedance aggregometry, activation of fibrinogen receptors by TRAP or ADP was progressively reduced in the presence of increasing concentrations of protamine up until 40 µg/mL protamine whereafter no further reduction was observed. Platelet MFI levels after ADP and TRAP activation in protamine 80 µg/mL were similar to those seen with protamine 80 µg/mL alone without any other activator (Figure 3). To investigate whether protamine caused platelet agglutination, we also collected the acquisition times and number of platelet-sized particles in the collection gate (which was placed tightly around the platelet cloud in the sample with resting platelets) during that time, to be able to estimate the concentration of platelet-sized particles in the different samples. We found that the numbers of platelet-sized particles per second in 18 analyzed samples from six donors were 351 ± 42 without protamine, 347 ± 42 with 20 µg/mL, 310 ± 50 with 40 µg/mL, and 268 ± 46 with 80 µg/mL protamine, where the numbers at 40 and 80 were significantly lower than without protamine ($p < .001$). However, as the collection gate only included single platelets, all platelet activation results reported in the figures are from the single platelets.

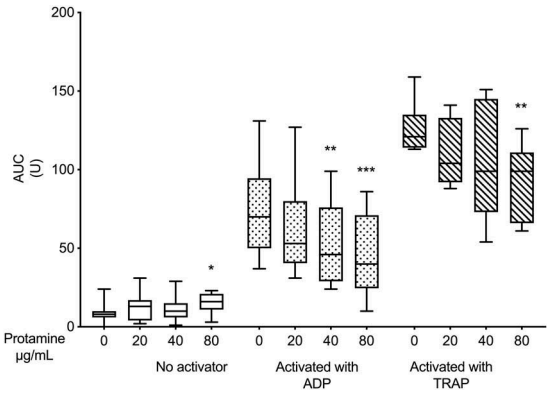

Figure 1. Platelet aggregation in blood exposed to protamine measured by impedance aggregometry. Platelets were exposed to different concentrations of protamine without activator (n = 11) and when activated with ADP (n = 9) or TRAP (n = 7). Results are presented as area under the curve (AUC) in units (U). The box indicates 25–75% quartiles with the line as median and whiskers total range. * denotes $p < .05$, ** denotes $p < .01$, and *** $p < .001$ when compared to 0 µg/mL protamine using repeated measures ANOVA and Dunnett´s correction for multiple comparisons.

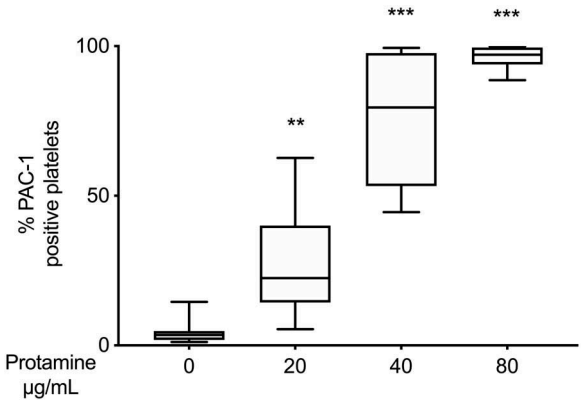

Figure 2. Percentage of platelets with active fibrinogen receptors (binding PAC-1) measured with flow cytometry when exposed to different concentrations of protamine and no other agonist (n = 8). The box indicates 25–75% quartiles with the line as median and whiskers total range. ** denotes $p < .01$, and *** $p < .001$ when compared to 0 µg/mL protamine using repeated measures ANOVA and Dunnett´s correction for multiple comparisons.

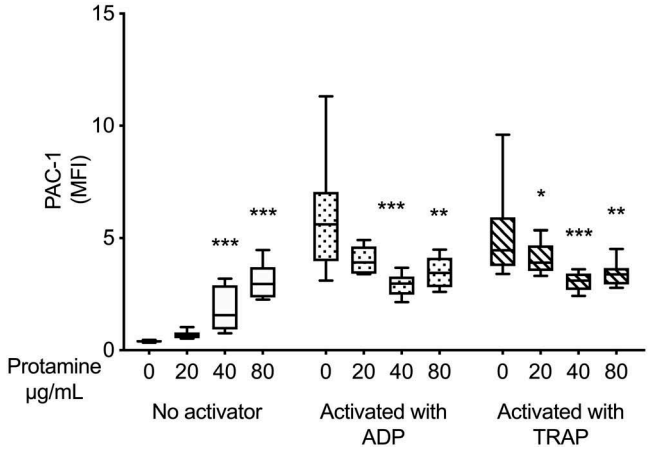

Figure 3. Activated fibrinogen receptors (binding PAC-1) measured with flow cytometry. Platelets were exposed to different concentrations of protamine alone or together with ADP or TRAP (n = 8 for each). Results are presented as median fluorescence intensity (MFI), as all platelets activated with TRAP or ADP were PAC-1-positive. The box indicates 25–75% quartiles with the line as median and whiskers total range. * denotes $p < .05$, ** $p < .01$, and *** $p < .001$ when compared to 0 µg/mL protamine using repeated measures ANOVA and Dunnett´s correction for multiple comparisons.

Release of Alpha-granules Indicated by Surface Expression of P-*selectin*

Protamine exposure led to a concentration-dependent increase ($p < .001$) in the release of alpha-granules, using *P*-selectin as a marker, both when measured as median fluorescence intensity (MFI) (Figure 4) and as percentage *P*-selectin-positive platelets. Protamine alone produced an increase in the mean percentage of platelets expressing surface *P*-selectin from 4 ± 2.7% (0 µg/mL protamine) to 95 ± 3.0% (80 µg/mL protamine), $p < .001$. When protamine was combined with ADP or TRAP, we observed a protamine concentration-dependent increase in the percentage *P*-selectin-positive platelets. For ADP, the percentage *P*-selectin-positive platelets increased from 49.8 ± 17.6% (activated by ADP alone) to 95.7 ± 2.5% (80 µg/mL protamine + ADP), $p < .001$. For TRAP, the percentage increased from 83.8 ± 13.7% (activated by TRAP alone) to 96.1 ± 2.1% (80 µg/mL protamine + TRAP), $p < .001$. MFI values after platelet activation with ADP or TRAP also increased with higher protamine concentrations (Figure 4) and the combination of protamine and ADP or TRAP produced a higher MFI than protamine alone in corresponding concentrations (Figure 4). Furthermore, *P*-selectin MFI values were considerably higher when platelets were exposed to protamine 80 µg/mL alone (10.16 ± 3.09) than when exposed to ADP alone (2.21 ± 0.67 $p < .001$) or TRAP alone (5.73 ± 2.60 $p < .01$) (Figure 4).

Binding of Annexin V and DilC$_1$(5)

The binding of annexin V to the surface of platelets is used as a marker for an activation-induced structural change in the platelet cell membrane leading to the exposure of procoagulant phosphatidylserine. We observed a protamine concentration-dependent increase in the proportion of platelets binding annexin V from 1.8 ± 0.59% (0 µg/mL protamine) to 79.4 ± 20.0% (80 µg/mL protamine) $p < .001$. With ADP and TRAP added there was a non-significant further increase; ADP 1.6 ± 0.4% (0 µg/mL protamine) to 90.2 ± 7.8% (80 µg/mL protamine) $p < .001$ and TRAP 1.1 ± 0.3% (0 µg/mL protamine) to 90.0 ± 8.7% (80 µg/mL protamine) $p < .001$.

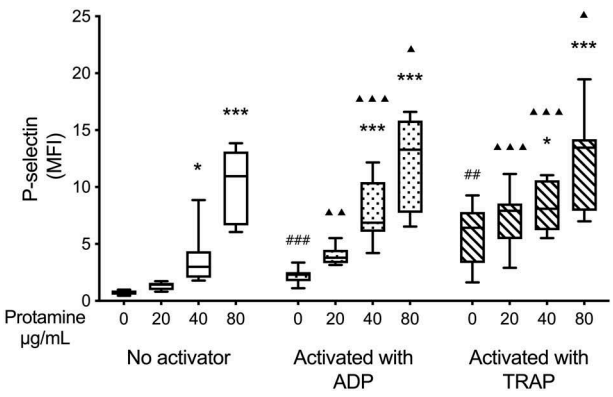

Figure 4. Release of alpha-granules measured with flow cytometry using anti-*P*-selectin-PE as marker. Platelets were exposed to different concentrations of protamine alone or together with ADP or TRAP (n = 8 for each). Results are presented as median fluorescence intensity (MFI). The box indicates 25–75% quartiles with the line as median and whiskers total range. * denotes $p < .05$ and *** $p < .001$ when compared to 0 µg/mL protamine. ▲ denotes $p < .05$, ▲▲ $p < .01$ and ▲▲▲ $p < .001$ when compared to the corresponding protamine concentration with "No activator." ### $p < .001$ when compared to protamine 80 µg/mL without any other activator. Statistics calculated with ANOVA and Dunnett´s correction for multiple comparisons.

In order to check for preserved mitochondrial membranes, we stained platelets with DilC$_1$(5). There was a small but significant dose-dependent reduction in DilC$_1$(5)-positive platelets after protamine exposure (Figure 5). With protamine in the concentration of 80 µg/mL, the mean percentage of DilC$_1$(5)-positive of platelets activated by only protamine had decreased by only 11% indicating a discrepancy between our finding with annexin V as marker, as these two events are tightly linked during the transformation to a procoagulant platelet[12] There was no significant difference when ADP or TRAP was added for further activation. In contrast, the positive control using CCCP caused almost complete disruption of the mitochondrial membrane.

Since the effect of protamine on annexin V binding was more pronounced than the effect on DilC$_1$(5), there was a possibility that annexin V could bind directly to the protamine molecule as well as to phosphatidylserine. To shed light on this, we examined a HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer solution containing neither platelets nor protamine. As expected, there were no annexin V-binding particles. However, when adding protamine in concentrations of 40 or 80 µg/mL, we observed particles smaller than platelets that were annexin V-positive. We also tested other phosphatidylserine markers and observed that protamine also could bind annexin V-APC and lactadherin-FITC. In contrast, protamine did not bind to the antibodies PAC-1-FITC or anti-*P*-Selectin-PE (see Supplemental Figures S1 and S2).

Thrombin Generation

To further evaluate whether protamine affects the role of platelets in plasma coagulation we measured thrombin generation. There was a slight but significant increase in lag time for thrombin generation when platelet-rich plasma was exposed to protamine. The mean lag time increased from 8.50 ± 1.52 min (no protamine) to 10.64 ± 1.07 min (20 µg/mL protamine) $p < .05$, 11.08 ± 1.26 min (40 µg/mL protamine) $p < .01$, and 12.53 ± 2.02 min (80 µg/mL protamine) $p < .01$ (see Supplemental Figure S4). No effect was observed on endogenous thrombin potential (ETP), peak thrombin generation, or time to thrombin peak (data not shown).

The Ability of Heparin to Counteract Platelet Activation by Protamine

We used heparin, as a strong binder of protamine to examine whether the protamine effect on platelets was reversible. Heparin

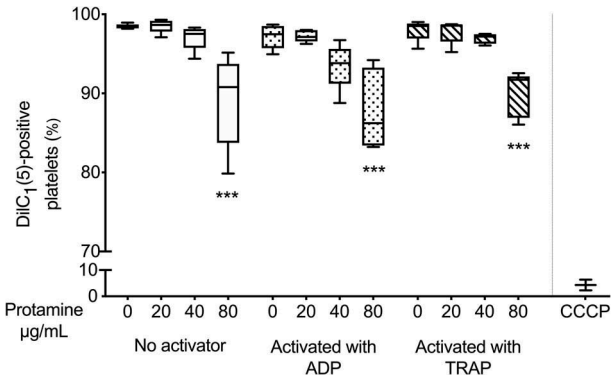

Figure 5. Flow cytometry showing percentage of DilC$_1$(5)-positive platelets (indicating an intact mitochondrial membrane) after activation by protamine alone or together with ADP or TRAP (n = 5 for each except for CCCP, where n = 2). CCCP used as a positive control of mitochondrial membrane disruption. The box indicates 25–75% quartiles with the line as median and whiskers total range. *** denotes $p < .001$ when compared to 0 µg/mL protamine using repeated measures ANOVA and Dunnett´s correction for multiple comparisons. Note break in y-axis.

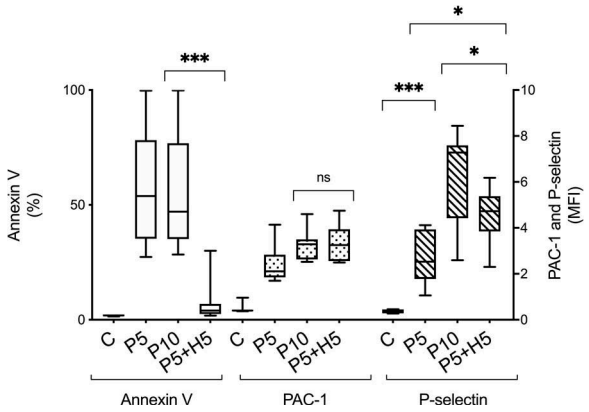

Figure 6. Platelets were exposed to protamine (80 μg/mL) for either 5 min (P5) or 10 min (P10). In the group P5+ H5, platelets were exposed to protamine (80 μg/mL) for 5 min after which heparin was added to bind protamine, whereafter the samples were incubated for another 5 min. C is control without protamine or heparin. Activation of the fibrinogen receptor (binding of PAC-1), release of alpha-granules (*P*-selectin expression), and annexin V binding were measured. Results are presented as MFI for PAC-1 and *P*-selectin and as percentage platelets positive for annexin V (n = 7 for each). The box indicates 25–75% quartiles with the line as median and whiskers total range.

almost completely reversed the annexin V binding indicating that heparin removes protamine from the surface of the platelet (Figure 6 and Supplemental Figure S3). Heparin, however, did not inhibit the activating effect of protamine on the fibrinogen receptor and only partially reduced further release of alpha-granules measured with *P*-selectin (Figure 6). *P*-selectin MFI was less when heparin was added 5 min after protamine activation and the reaction stopped after 10 min, than it was after 10-min activation with protamine without heparin ($p < .05$). However, *P*-selectin expression was still higher than when the reaction was stopped after 5 min ($p < .05$).

Discussion

Platelet Aggregation and Release of Alpha-granule

In this study, we found that protamine *in vitro* interacts with platelets by both a direct activating effect and secondary impairment of function when exposed to other activators. In 1987 Machi et al. [7] showed with high-resolution ultrasound that protamine promotes aggregation of platelets. In our study, we confirm protamine´s aggregatory effect using impedance aggregometry and also show a possible mechanism in activation of the fibrinogen receptor which we show with flow cytometry. We also found that the aggregative response from the known platelet activators ADP and TRAP was progressively reduced with increasing concentration of protamine. This has prior been described with the use of impedance aggregometry [15] and optical aggregometry [16] and we could also show it with diminished activation of the fibrinogen receptor using flow cytometry.

With increasing concentrations of protamine, we also found signs of agglutination of platelets with a reduction of platelet-sized particles up to 25%, consistent with our finding that protamine increases aggregation. However, it is worth emphasizing that the effects on platelets we report using flow cytometry are on single platelets, as the collection gate in the flow cytometer will exclude all larger aggregates.

Protamine exposure also caused the release of alpha-granules with an expression of *P*-selectin on the surface of the platelets in a dose-dependent way, consistent with the increased aggregative response. When ADP or TRAP was added, a further increase of *P*-selectin expression was seen. Thus, in contrast with the aggregative response, protamine did not interfere with alpha-granule release upon platelet activation by other substances. Interestingly, protamine at a concentration of 80 μg/mL was a stronger activator of alpha-granule release than both ADP and TRAP in the concentrations applied. A direct activating effect of protamine with increased expression of *P*-selectin [6] and aggregation [7] has previously been described but the mechanisms of activation including activation of the fibrinogen receptor have not been described. The impairment of aggregation caused by other activators confirms previous *in vivo* and *in vitro* findings of impaired platelet function after protamine exposure [15,16,21,22].

Annexin V, Thrombin Generation, and the Mechanism behind Platelet–Protamin interaction

The discrepancy between the binding of annexin V, as a marker of procoagulant phosphatidylserine exposing state, and DilC1(5), as a marker of mitochondrial membrane disruption, indicated that annexin V may have interacted directly with protamine and we could demonstrate that annexin V binds directly to protamine. As shown in the Supplemental Figures, there was no direct binding between protamine and any of our other markers of platelet activation; thus, the binding to protamine was not via the fluorophore part of the marker in the other analyses. To further rule out a procoagulant effect of protamine we tested how protamine affects thrombin generation. We observed a slight concentration-dependent increase in lag time of thrombin generation in platelet-rich plasma when protamine was added. This supports previous findings of a decrease in thrombin generation by inhibition of coagulation Factor V in the presence of protamine [23]. Since Factor V exercises its role in coagulation at the platelet surface, it is in accordance with a mechanism where protamine binds to the platelet and blocks the access of other factors to the platelet surface. The absence of any other effect on thrombin generation shows that protamine lacks a significant activating effect on procoagulative structure changes in platelets.

We used the strong binding of heparin to protamine to study whether removal of protamine reverses its effect on platelet activation. When adding heparin after 5-min incubation of blood with protamine, we saw an almost complete removal of annexin V indicating removal of protamine from the surface of the platelet and no procoagulant structural change in the platelet. Based on our finding with $DilC_1(5)$, we conclude that protamine alone only affects a small portion of platelets enough to cause them to undergo a structural change with mitochondrial membrane disruption. Heparin, however, did not change the effect of protamine on the fibrinogen receptor and only partially reduced the expression of *P*-selectin compared to the situation without heparin.

Our findings could be explained by a mechanism where protamine, a highly positively charged basic protein, binds to the platelet surface and activates the platelets. Protamine binding could interfere with the binding of other molecules like ADP or thrombin but still allow further activation of alpha-granule release. Removal of protamine leaves the fibrinogen receptor still activated.

Clinical Significance

We observed that protamine *in vitro* diminishes platelet aggregation in response to ADP and TRAP. This could explain the negative effect on platelet function described *in vivo* and contribute to the negative effect on hemostasis seen with excessive use of protamine in cardiac surgery [5,6,15,16,21–25].

Clinical use of protamine is associated with serious side effects [8]. Apart from allergic and anaphylactoid reactions,

protamine can also severely increase pulmonary vasoconstriction with secondary right-sided acute heart failure and circulatory collapse [26,27]. The exact mechanism responsible for this reaction is not known but activation of platelets has been proposed, and a reduction in circulating platelets after protamine administration after CPB is seen [25,26]. Our data showing platelet activation by protamine could be compatible with this hypothesis. Although the concentrations of protamine used in this study are considerably higher than would be expected after total equilibration and binding to circulating heparin at the end of CPB, it is possible to speculate that platelets could be exposed to high concentrations of unbound free protamine during rapid or repeated injections of protamine which could correspond to the empirical knowledge that protamines negative impact on hemodynamics can be avoided by a slow injection.

Limitations

Being an *in vitro* study, any clinical implications should be made with caution. We chose to investigate how protamine affects platelets without first exposing the platelets to heparin in order to study direct protamine–platelet interactions. Clinically protamine is given to reverse heparin that is already present, a major difference from our *in vitro* setting. The *in vitro* setting of this study, however, could mimic the situation where an excess of protamine is administered, or where protamine is given too rapidly, resulting in a high local concentration of free protamine. We also used heparin to examine whether this could reverse the effect of protamine on platelets. Since we did not examine the effect of heparin on platelets without protamine, there could be a possibility that heparin in itself also could affect platelet function.

Another limitation is that we studied healthy volunteers instead of cardiac surgery patients in our flow cytometry and thrombin generation experiments. This was unfortunately a logistic necessity and considering the nature of our experiments we do not think it alters our conclusions.

Conclusions

Protamine *in vitro* interacts with platelets by both a direct activating effect and secondary impairment of function when exposed to other activators. The impairment of aggregometry by protamine is consistent with previous *in vivo* data showing reduced platelet function after protamine exposure, while the direct activating effect is not previously well characterized. Thus, in addition to impairing coagulation, protamine also interferes with platelet function in a complex way and overdosing could in this aspect be harmful.

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Authors' Contributions

Design of study: all authors.
Laboratory and data analysis: all authors.
Preparation and final approval of manuscript: all authors.

Supplementary Material

Supplemental data for this article can be accessed here.

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