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REPORTS OF ORIGINAL INVESTIGATIONS

Minimum protamine dose required to neutralize heparin in cardiac surgery: a single-centre, prospective, observational cohort study

Dose minimale de protamine requise pour neutraliser l'héparine en chirurgie cardiaque : une étude de cohorte observationnelle prospective monocentrique

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Abstract

Purpose *Excess protamine contributes to coagulopathy following cardiopulmonary bypass (CPB) and may increase blood loss and transfusion requirements. The primary aim of this study was to find the least amount of protamine necessary to neutralize residual heparin following CPB using the gold standard assays of anti-IIa and anti-Xa activity. Secondary objectives were to evaluate whether the post-CPB activated clotting time could be used as a surrogate marker for quantifying heparin neutralization.*

Methods *Twenty-eight consecutive patients undergoing elective cardiac surgery were enrolled. Protamine administration was standardized through an infusion pump at 25 mg·min^{-1}. Blood samples were withdrawn prior to and following administration of 150, 200, 250, and 300 mg protamine and analyzed for activated clotting time and anti-IIa and -Xa activity.*

Results *Following a mean (standard deviation) cumulative heparin dose of 67,700 (19,400) units and a CPB duration of 113 (71) min, protamine requirements varied widely. Eight out of 25 (32%) patients showed complete neutralization of anti-IIa and -Xa activity at the first sampling point (150 mg protamine; protamine:heparin ratio, 0.3 [0.1]). A protamine:heparin ratio of 0.5 (0.2) was sufficient for heparin neutralization in > 90% of patients. After CPB, a low to mid-range activated clotting time correlated well with anti-IIa and -Xa activity.*

Conclusions *The protamine:heparin ratio required to neutralize residual unfractionated heparin (UFH) following CPB is variable. A protamine:heparin ratio of 0.3 was sufficient to neutralize UFH in some patients, while a ratio of 0.5 is sufficient to neutralize both residual anti-IIa and -Xa activity in most patients. Larger studies are necessary to confirm these findings and evaluate their clinical implications.*

Study registration *ClinicalTrials.gov (NCT03787641); registered 26 December 2018.*

Résumé

Objectif *L'excès de protamine contribue à la coagulopathie après la circulation extracorporelle (CEC) et peut augmenter les pertes de sang et les besoins transfusionnels. L'objectif principal de cette étude était de déterminer la quantité minimale de protamine nécessaire pour neutraliser l'héparine résiduelle post-CEC en utilisant les tests de référence de l'activité anti-IIa et*

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anti-Xa. Les objectifs secondaires consistaient à évaluer si le temps de coagulation activé post-CEC pouvait être utilisé comme marqueur de substitution pour quantifier la neutralisation de l'héparine.

Méthode *Vingt-huit patients consécutifs bénéficiant d'une chirurgie cardiaque non urgente ont été recrutés. L'administration de protamine par une pompe à perfusion à 25 mg·min^{-1} a été normalisée. Des échantillons de sang ont été prélevés avant et après l'administration de 150, 200, 250 et 300 mg de protamine et analysés pour déterminer le temps de coagulation activé et l'activité anti-IIa et -Xa.*

Résultats *Après une dose cumulative moyenne (écart type) d'héparine de 67 700 (19 400) unités et une durée de CEC moyenne de 113 (71) min, les besoins en protamine variaient considérablement. Huit patients sur 25 (32 %) ont affiché une neutralisation complète de l'activité anti-IIa et -Xa au premier point de prélèvement (150 mg de protamine; rapport protamine : héparine, 0,3 [0,1]). Un rapport protamine/héparine de 0,5 (0,2) était suffisant pour la neutralisation de l'héparine chez > 90 % des patients. Après la CEC, un temps de coagulation activé bas à moyen était bien corrélé avec l'activité anti-IIa et -Xa.*

Conclusion *Le rapport protamine : héparine nécessaire pour neutraliser l'héparine non fractionnée (HNF) résiduelle suivant une CEC est variable. Un rapport protamine : héparine de 0,3 était suffisant pour neutraliser l'HNF chez certains patients, tandis qu'un rapport de 0,5 est suffisant pour neutraliser à la fois l'activité résiduelle des anti-IIa et celle des anti-Xa chez la plupart des patients. Des études plus vastes sont nécessaires pour confirmer ces résultats et évaluer leurs implications cliniques.*

Enregistrement de l'étude *ClinicalTrials.gov (NCT03787641); enregistrée le 26 décembre 2018.*

Keywords activated clotting time · cardiac surgery · cardiopulmonary bypass · heparin · protamine

Transfusions are common in cardiac surgery and are associated with increased morbidity and mortality.[1] Hemorrhage due to lack of surgical hemostasis remains relatively uncommon following cardiopulmonary bypass (CPB) and only a small proportion of patients return to the operating room (OR) for inadequate surgical hemostasis.[2] Microvascular or diffuse capillary oozing, on the other hand, remains by far the most common cause of postoperative hemorrhage.[3] Its etiology remains multifactorial, and management is challenging and resource intensive.[4]

Protamine is used ubiquitously to neutralize unfractionated heparin (UFH) following CPB. It, however, is an anticoagulant in its own right and an excess can contribute to microvascular hemorrhage.[5–9] To mitigate this side effect and to reduce bleeding, the 2017 and 2019 European Association for Cardio-Thoracic Surgery/Anaesthesiology guidelines suggested that protamine should be administered in a protamine to "initial" heparin dose ratio of < 1:1 and 0.8–1:1, respectively (class IIa recommendation; level of evidence B).[8,9] On the other hand, the 2018 the Society of Thoracic Surgeons (STS), the Society of Cardiovascular Anesthesiologists (SCA), and the American Society of Extracorporeal Technology (AmSECT) Clinical Practice Guidelines[7] still recommends a protamine:heparin (PH) ratio of < 2.6:1—i.e., < 2.6 mg protamine to neutralize 1 mg (or 100 U) of "total" UFH administered (class IIa recommendations, level of evidence C). None of the current guidelines specify whether the UFH added to the priming volume of the CPB circuit should be factored in when calculating the protamine dose. Emerging research also shows that protamine can potentially exacerbate post-CPB coagulopathy in much lower doses. For example, in a before-after study using protamine hydrochloride, Goedhart *et al.*[6] showed that a PH ratio of 0.6:1, compared with 0.8:1, was associated with decreased blood loss and transfusions. Furthermore, a PH ratio as low as 0.7 can induce a marked, yet transient, decrease in *in vitro* platelet aggregation.[10] What is less clear is if such low doses of protamine are associated with complete and definitive neutralization of UFH activity as quantified by its gold standard—the anti-IIa activity.[11] Theoretically, protamine is expected to neutralize UFH in a PH ratio of 1–1.3:1 or 1 mg:100 U;[5,7,12] however, the appropriate amount of protamine required to neutralize UFH at the end of CPB for a particular patient remains controversial and ultimately the least amount of protamine required to neutralize UFH following CPB remains unknown.

Lack of specialized point-of-care (POC) tests for monitoring UFH has further impeded the evaluation of different neutralizing strategies. Activated clotting time (ACT), the most widely used POC test globally for monitoring anticoagulation on CPB, has commonly lacked specificity for reflecting ongoing UFH activity.[7] Recently and more controversially, however, post-protamine ACT was shown to correlate well with neutralized anti-Xa activity of UFH.[13] Nevertheless, no data exist for neutralization of anti-IIa activity, which is now the reference standard for UFH.[11] Given the uncertainties, the primary objective of this prospective study was to establish the minimum dose of protamine required to neutralize UFH for routine elective cardiac surgery patients, as determined by its effect on anti-IIa and Xa activity. A secondary objective was to evaluate if post-

CPB ACT could be used as a surrogate marker for UFH neutralization following CPB.

Methods

Following approval of the Institutional Research Ethics Board, trial registration (ClinicalTrials.gov #NCT03787641; 26 December 2018), and written informed consent, 28 consecutive adult patients scheduled for elective cardiac surgery with planned institution of CPB lasting no more than 2–2.5 hr were enrolled in the study from December 2019 to December 2020. Patients were excluded if they were < 18 yr of age, were unable to give informed consent, had a history of heparin resistance, or had prior adverse reactions to protamine. Other exclusion criteria included existing coagulopathy**,** liver dysfunction, previous cardiac surgery, and exposure to heparin (unfractionated or low molecular weight), warfarin, clopidogrel, or other direct thrombin inhibitors in the preceding 14 days.

A baseline ACT (Max ACT, Actalyke MA-ACT; Array Medical, Somerville, NJ, USA) was recorded before surgical incision. Anticoagulation for surgery was initiated with UFH (Sandoz Canada Inc., Boucherville, QC, Canada) (Lot #KC9191) in the dose range of 300–400 $U \cdot kg^{-1}$ to achieve a target ACT of > 480 sec as per the standard of care at the authors' institution. Further heparin (5,000–10,000 U) was administered as necessary prior to and during CPB to maintain the target ACT as per routine clinical practice. Tranexamic acid (bolus: 10 $mg \cdot kg^{-1}$, infusion 1–5 $mg \cdot kg^{-1} \cdot hr^{-1}$) was administered intraoperatively to all patients, as per standard protocol. All patients underwent normothermic CPB using a membrane oxygenator and biocompatible circuits (Xcoating, Terumo, Ann Arbor, MI, USA). The extracorporeal circuit was primed with 800–1,000 mL Plasma-Lyte (Baxter Healthcare Corporation Deerfield, IL), 40–50 g mannitol, and 5,000–10,000 U UFH as per hospital protocol.

Protamine protocol

Protamine sulphate (Sandoz Canada Inc., Boucherville, QC, Canada) (Lot # JY7348) dose was *decided* by the attending anesthesiologist but *administered* according to study protocol. First, a test dose of 50 mg protamine (as per local clinical practice) was administered via an infusion pump at 25 $mg \cdot min^{-1}$ through the central venous catheter over two minutes. Three minutes later, additional 100 mg protamine was administered at the same rate (i.e., 25 $mg \cdot min^{-1}$) and this was followed by a three-minute waiting period at the end of which blood samples were withdrawn. Blood samples followed additional aliquots of 50 mg protamine at the same infusion rate until a total of 300 mg protamine had been administered. This marked the end of the study, with the maximum duration of time from protamine initiation to last sample collection being 27 min. Additional protamine was administered at the discretion of the attending anesthesiologist.

Blood sampling and laboratory tests

Blood samples (up to 49 mL) were drawn from arterial catheters prior to induction of anesthesia (baseline), immediately prior to administration of protamine, and following administration of 150, 200, 250, and 300 mg protamine. Arterial transducer system flushes were free of heparin, and blood samples were drawn after discarding about six dead space volumes of the catheter and tubing. Post-CPB ACT was quantified for all time points immediately and the OR team were kept blinded to the results. For anti-IIa and -Xa assays, blood samples were transferred into sodium citrate vacutainer tubes (3.2%) and were centrifuged twice at 1,500 g for 15 min. Supernatant plasma was removed and centrifuged again for five minutes to recover platelet-poor plasma. It was then aliquoted, stored at −80 °C, and analyzed in batches in the Hemostasis reference laboratory, Henderson Research Centre, Hamilton, ON, Canada.

Plasma anti-IIa (Biophen Heparin Anti-IIa, Aniara Diagnostica, Mason, OH, USA) and anti-Xa (Stachrom anti-Xa assay kit, Stago Canada Ltd, Toronto, ON, Canada) activities were measured, in duplicate, by automated chromogenic assays. These assays determine the functional activity of UFH[14] based on the ability of UFH to accelerate the inhibition of activated coagulation enzymes Xa and IIa (thrombin). Briefly, heparin is analyzed as a heparin-antithrombin (AT) complex formed by interaction of heparin with human AT in the plasma sample. Factor Xa or IIa is then added in excess to the sample and is neutralized by the heparin–AT complex. Residual Xa/IIa activity is quantified with a synthetic chromogenic substrate (with lower limit of detection 0.05 $U \cdot mL^{-1}$). All assays were performed using the included supplemental AT. Average interassay and intraassay coefficients of variation were 5.1% and 3.3% for the anti-IIa assay and 8.7% and 8.1% for the anti-Xa assay, respectively.

Statistical analysis

All analyses were performed in Stata version 16.1 (StataCorp LLC, College Station, TX, USA). A convenience sample of 25 patients was used. Patient demographics were summarized using conventional means.

Residual UFH effect was defined as an anti-IIa or Xa level of ≥ 0.05 U·mL^{-1}. Activated clotting time was considered neutralized post-CPB if the post-protamine values were $\leq 110\%$ of baseline values. For patients ($n = 8$) where UFH activity was neutralized following 150 mg, the PH ratio was calculated as if the protamine dose required for neutralization was 150 mg. Calculation of PH ratios in the analysis included the UFH (5,000–10,000 U, depending on the perfusionist) added to the CPB circuit priming volume.

Boxplots were used to show the values of post-CPB ACT, anti-IIa levels, and anti-Xa levels as a function of protamine dose. The proportion of patients with residual heparin activity was plotted by protamine dose. To correlate the post-CPB low to mid-range ACT (samples drawn after 150 mg protamine), correlation coefficients of anti-IIa levels and anti-Xa levels were computed that accounted for the lack of independence of the repeated measures within patients using the *rmcorr* command in Stata.[15] Inferential statistics, including the use of confidence intervals, was not performed given the small sample size and the lack of comparison groups.

Results

Patient demographics are outlined in the Table. Out of 28 patients, one was excluded from analysis because CPB was reinitiated after administration of the protamine test dose (for management of surgical bleeding). Two additional patients were excluded because of failure to adhere to protocol. Hence, a total of 25 patients were included for analysis.

At the end of CPB, the mean (standard deviation [SD]) ACT was 577 (98) sec and the mean (SD) anti-IIa and anti-Xa were 9.1 (2.1) and 8 (2) U·mL^{-1}, respectively. Out of 25 patients, both anti-IIa and -Xa activity were neutralized at the time of the first sampling point (i.e., after administration of 150 mg protamine) in eight (32%) patients. This subset of patients had received a mean (SD) total UFH dose of 51,125 (26,675) U, making the optimum mean (SD) PH ratio not more than 0.3 (0.1) in this cohort. For the remaining 17 patients, anti-IIa and -Xa levels declined with incremental doses of protamine (Figs. 1 and 2).

Only 8/25 (32%) and 3/25 (12%) patients showed any anti-IIa or -Xa residual UFH activity following a total of 200 (PH ratio 0.4 [0.2]) and 250 (PH ratio 0.45 [0.2]) mg protamine, respectively (Fig. 2). Three hundred milligrams of protamine (PH ratio 0.5 [0.2]) was sufficient to neutralize anti-IIa and Xa activity in $> 90\%$ patients. Overall, 219 (73) mg protamine would have definitively neutralized the circulating UFH, as calculated by neutralization of both anti-Xa and IIa activities, as opposed to the 318 (103) mg that was administered by anesthesiologists in this cohort. Anti-IIa and Xa levels showed good correlation with each other and with the post-CPB ACT (Fig. 3).

Table Demographics

Variable	Value *N* = 25
Age (yr)	65 (7.9)
	66 [61–70]
Male sex	19/25 (76%)
Height (cm)	175 (11)
	174 [168–180]
Weight (kg)	92 (18)
	96 [82–105]
Surgery type	
CABG	15/25 (60%)
Valve	7/25 (27%)
CABG + valve	3/25 (12%)
CPB duration (min)	113 (71)
	90 [73–141]
Aortic cross-clamp duration (min)	88 (64)
	69 [43–107]
Initial UFH dose (10^3 units)	37.8 (11)
	38 [30–40]
Total UFH dose (10^3 units)	67.7 (19.4)
	60 [55–90]
Initial protamine dose (mg)*	310 (104)
	300 [250–350]
Total protamine dose (mg)*	318 (103)
	300 [250–350]
Blood loss (mL)	
4 hr	161 (96)
	150 [120–180]
12 hr	374 (113)
	340 [300–410]
ICU length of stay (days)	1.2 (0.6)
	1 [1–1]
Hospital length of stay (days)	6.1 (1.6)
	6 [5–7]

Numbers are *n*/total *N* (%) or mean (standard deviation) and median [interquartile range]

*The initial and total doses of protamine refer to the doses administered by the anesthesiologist

CABG = coronary artery bypass grafting; CPB = cardiopulmonary bypass; ICU = intensive care unit; UFP = unfractionated heparin

Discussion

This study has several notable findings. In a cohort of 25 consecutive patients undergoing elective cardiac surgery, UFH activity (as measured via its gold standard anti-IIa

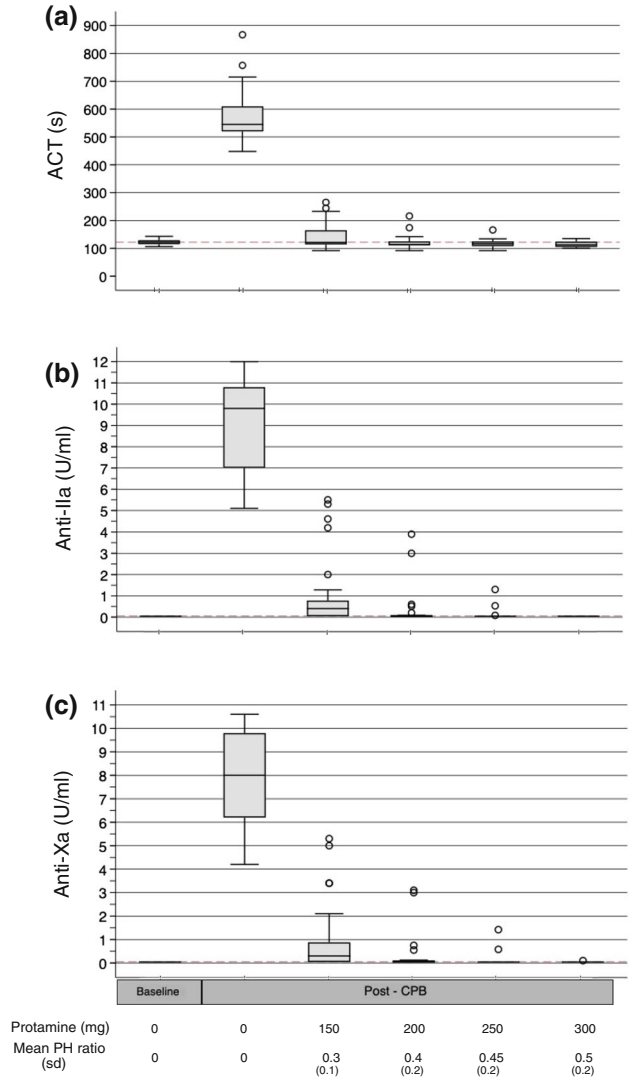

Fig. 1 Boxplots of activated clotting time, anti-IIa activity, and anti-Xa activity are shown as a function of increasing doses of protamine. The boxes depict the median and 25th and 75th centiles. The whiskers depict adjacent values, and the dots depict outside values (as described by Tukey, J. W. 1977. Exploratory Data Analysis. Reading, MA: Addison–Wesley).

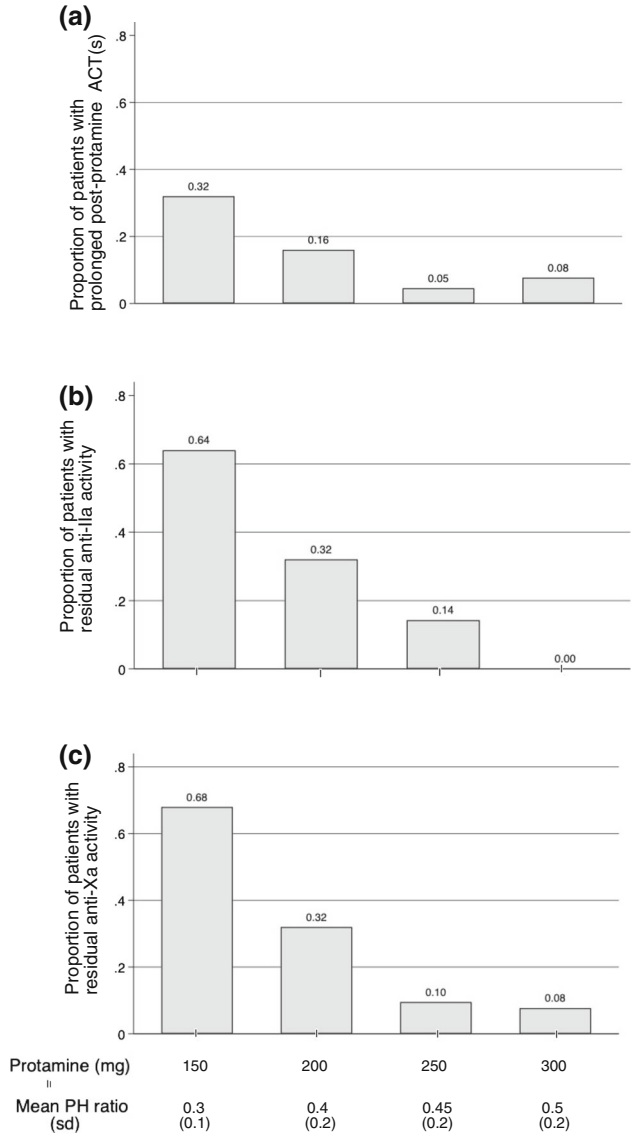

Fig. 2 Proportions of patients with activated clotting time values $> 110\%$ of baseline or any detectable anti-IIa or anti-Xa activity, as a function of increasing doses of protamine.

and -Xa activity) was quantified at 9.1 (2.1) and 8 (2) $U{\cdot}mL^{-1}$, respectively, following termination of CPB. Protamine requirements to neutralize UFH activity varied widely. At the first sampling point (with 150 mg protamine; mean [SD] PH ratio 0.3 [0.1]), both anti-IIa and Xa activity was neutralized in 32% of patients. Incremental protamine was associated with a progressive decline in UFH activity and a PH ratio of 0.5 (0.2) was associated with complete neutralization in 23/25 (92%) patients. Anti-IIa and anti-Xa activity correlated well with each other and with post-CPB low to mid-range ACT.

Anticoagulant properties of protamine have been well characterized in vitro. Protamine can impair platelet function,[10,16] and coagulation factor activation[5,17] which is associated with increased intrinsic clotting times.[18] Administration of a protamine bolus of 0.5 $mg{\cdot}kg^{-1}$ to healthy volunteers can lead to plasma concentrations up to 5 $\mu g{\cdot}mL^{-1}$,[19] and this level is associated with thromboelastography quantified prolongation of coagulation times,[20] delayed clot initiation, and decreased clot strength and duration time, as well as increased clot disintegration.[21] A 250 mg bolus of protamine can lead to levels up to 50 $\mu g{\cdot}mL^{-1}$,[22] and this leads to a consistent dose-dependent decrease in platelet agglutination,[16] and tissue factor mediated thrombin generation[22] by inhibition of factor V.[23] Lower doses of protamine have been associated with reduced postoperative hemorrhage and

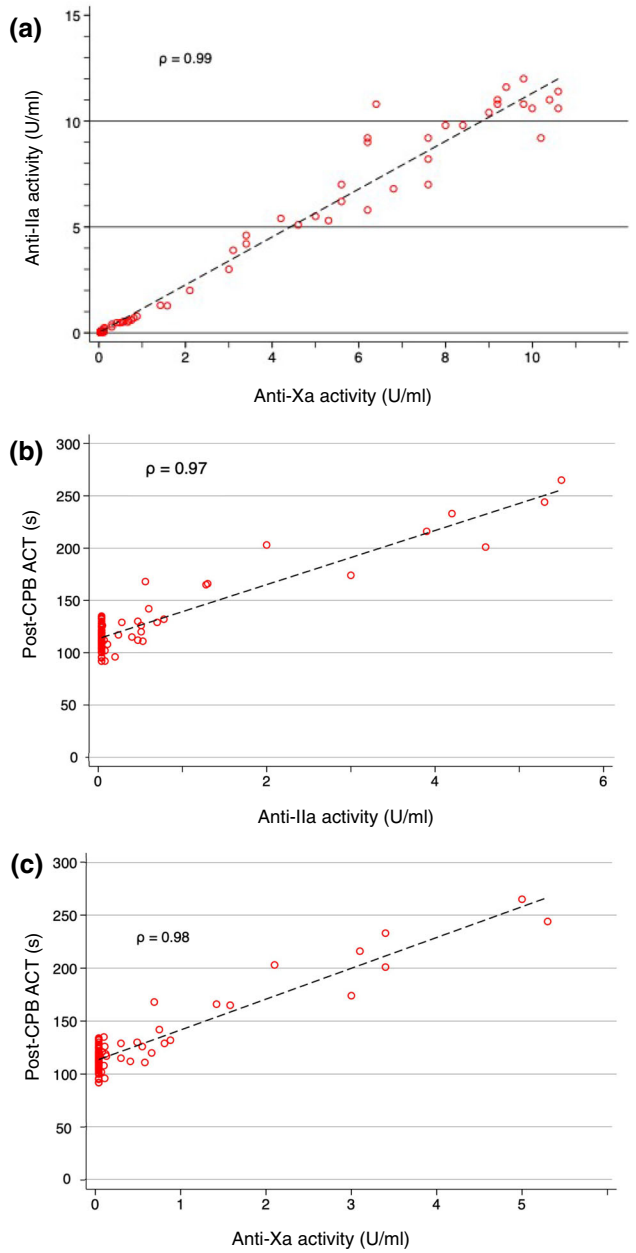

Fig. 3 Correlation coefficients, adjusted for repeated measures using the *rmcorr* command in Stata, for each combination of post-CPB low to mid-range activated clotting time (achieved after 150 mg protamine), anti-IIa activity, and anti-Xa activity.

transfusions by numerous author groups over the years,[6,18,24–28] so limiting the protamine dose in cardiac surgery remains an important consideration.

Unfractionated heparin activity in one third (8 out of 25) of study patients was neutralized with 150 mg protamine. Given that this was the first time point when blood samples were collected for quantifying residual UFH, we cannot be sure of the least amount of protamine required to neutralize UFH in these patients. Nevertheless, this small cohort of patients had received a mean UFH dose of over 50,000 U and the mean (SD) PH ratio required for these patients was at most 0.3 (0.1). The authors do recognize that this was a small subset of patients in the cohort and hence this clearly warrants further investigation, Nevertheless, it should not be surprising that this variability was observed. It is well-known that UFH has a widely varying pharmacokinetic and pharmacodynamic profile, including variable protein binding.[29] Protamine also forms insoluble complexes with plasma proteins[30] and the few pharmacokinetic studies available to date suggest that free protamine concentrations vary widely following its intravenous administration.[19,22] There could be other reasons for varying protamine requirements. The phase of protamine administration is inexorably linked with transfusions of heparinized blood from the CPB circuit reservoir (potentially containing high concentrations of UFH) for treating hypotension or restoring hematocrit; however, it does further support the notion that lower than traditionally calculated doses of protamine may be effective in neutralizing UFH. Other groups have reported similar findings using different POC tests[24,25] and mathematical models,[27,28] and we confirm these findings with measurement of UFH activity through the gold standard anti-IIa and Xa activity. Given the, not unanticipated, wide interindividual variability in the dose-response curve of UFH and protamine, we propose that it may be most logical to titrate protamine in following CPB rather than placing a firm adherence to calculation of protamine dose based on an *a priori* theoretical PH ratio. The 2018 STS/SCA/AmSECT Clinical Practice Guidelines[7] do suggest protamine titration but these recommendations suggest evidence based on monitoring heparin concentrations via Hepcon HMS (Medtronic Inc., Minneapolis, MN, USA) or *in vitro* protamine titrations. Given that the vast majority of cardiac surgery centres still use ACT and not Hepcon HMS[31] as a POC test, incremental doses of protamine by the anesthesiologist and quantification of residual UFH with post-CPB ACT may well be sufficient for confirming its neutralization.

Much attention has been given to the PH ratios for calculating protamine dose. The authors believe that a number of prevailing misconceptions exist regarding the PH ratio, which has been alluded to variably in the literature as 1:1 or 1:100 (1 mg protamine:100 U UFH).[5,7,12,32] Given that the literature traditionally recommends against reference to UFH in "milligrams,"[33,34] it then becomes important to understand the rationale behind the construct "PH ratio = 1:100." The polyanionic UFH combines with protamine, a polycationic basic peptide, almost instantly in a 1:1 stoichiometric reaction.[35] While the precise evidence for using the 1:100 (milligram:units) annotation currently remains elusive to the authors, we suspect the "1:100"

annotation is an extension of common knowledge embedded in the belief that 1 mg UFH contains 100 U UFH. In fact, this was true only in initial studies conducted to characterize UFH in the 1950s, where 1 mg UFH was approximated to 100 Toronto Units[36,37] largely for the sake of convenience. The 2nd International Standard for UFH was concluded in 1959 and at that time 1 mg UFH was calibrated to 130 U.[38] Notwithstanding, we note that, as per the United States Food and Drug Administration and the United States Pharmacopeia, 1 mg UFH currently contains not less than 180 U.[39] As for protamine's efficacy, protamine monographs categorically state that 1 mg protamine neutralizes not less than 100 U UFH,[38] suggesting that protamine is perhaps more potent in neutralizing UFH than suggested by the theoretical PH ratio of 1:1. Not only are there are original reports that emphasize protamine's efficacy,[40] there are also recent data that 1 mg protamine can neutralize 161 U of anti-IIa porcine mucosal UFH activity (0.9 mg UFH).[41] Indeed, if these data can be replicated, it may become necessary to change the way the PH ratio is perceived.

This study has strengths and limitations. Unfractionated heparin activity was quantified using the gold standard anti-IIa and Xa levels as opposed to whole blood heparin concentrations via Hepcon HMS. While the Hepcon HMS offers advantages as a POC test, whole blood heparin concentrations do not correlate with anti-Xa activity[42] and most centres using this modality only target ACTs on CPB, and not whole blood heparin concentrations.[31] We also elected to evaluate residual UFH after fixed protamine doses rather than assessing the effect of increasing individual PH ratios on residual UFH activity. Although this would have been a legitimate alternate study design, it would have required on-the-spot calculations of protamine dose for individual patients in the OR, further complicating an already complex study protocol that involved timed infusions and multiple sampling points. As for the limitations, this was a single-centre study and patient numbers were small because of the complex study protocol. This has precluded a better evaluation of the causes of variability in protamine requirements among patients. We propose that, based on this hypothesis-generating study, there is an urgent need for a systematic evaluation of UFH-protamine interactions that will allow the lowest effective dose of protamine following CPB to be administered to the individual patient. Antithrombin levels were not measured primarily since the anti-IIa and Xa assays included supplemental AT. Postoperative heparin rebound could not be quantified since the total dose of protamine administered to the patient was left to the anesthesiologist's discretion.

Conclusions

The minimum dose of protamine required to neutralize UFH in cardiac surgery patients is variable. A PH ratio as low as 0.3 may be enough in some patients undergoing elective cardiac surgery and a PH ratio of 0.5 may be effective in most patients in neutralizing UFH (as quantified through anti-IIa and Xa activity). Our study suggests that anesthesiologists should adopt a protamine titration approach to administer the lowest effective dose to neutralize UFH following CPB. Larger studies are urgently required to confirm these findings as well as their impact on clinical outcomes.

Author contributions *Ravi Taneja* contributed significantly to this manuscript by making substantial contributions to the conception, design, acquisition of data, analysis of the data as well as drafting and critically revising the manuscript. He approved the submission of the final manuscript. He has no conflicts of interest to disclose. *Daniel Szoke* contributed significantly to this manuscript by making substantial contributions to the acquisition of data, analysis of the data, as well as drafting and critically revising the manuscript. *Zachary Hynes* contributed to this manuscript in conceptual design, data collection, and draft revision of the manuscript. *Philip M. Jones* contributed significantly to this manuscript by making substantial contributions to the conception, design, analysis of the data as well as drafting and critically revising the manuscript.

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