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Experimental paper

Coagulofibrinolytic effects of recombinant soluble thrombomodulin in prolonged porcine cardiac arrest

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Abstract

Aim: To evaluate coagulofibrinolytic abnormalities and the effects of ART-123 (recombinant human thrombomodulin alpha) in a porcine model of cardiac arrest and prolonged cardiopulmonary resuscitation (CA/CPR).

Methods: Fifteen pigs (n = 5 per group) underwent 8 minutes of no-flow CA followed by 50 minutes of mechanical CPR, while 2 pigs underwent sham arrest. CA/CPR animals were randomized to receive saline or 1 mg/kg ART-123 pre-arrest (5 minutes prior to ventricular fibrillation) or post-arrest (2 minutes after initiation of CPR). Arterial and venous blood samples were drawn at multiple time points for blood gas analysis and measurement of plasma and whole blood markers of coagulation and fibrinolysis.

Results: In saline-treated CA/CPR, but not sham animals, robust and persistent activation of coagulation and fibrinolysis was observed throughout resuscitation. After 50 minutes of CPR, plasma tests and thromboelastography indicated a mix of hypercoagulability and consumptive coagulopathy. ART-123 had a robust anticoagulant effect, reducing both thrombin-antithrombin (TAT) complexes and d-dimer ($p < 0.05$ for each). The duration of anticoagulant effect varied depending on the timing of ART-123 administration. Similarly, ART-123 when given prior to cardiac arrest was found to have pro-fibrinolytic effects, increasing free tissue plasminogen activator (tPA, $p = 0.02$) and decreasing free plasminogen activator inhibitor-1 (PAI-1, $p = 0.04$).

Conclusion: A porcine model of prolonged CA/CPR reproduces many of the coagulofibrinolytic abnormalities observed in human cardiac arrest patients. ART-123 demonstrates a combination of anticoagulant and profibrinolytic effects, depending on the timing of its administration relative to cardiac arrest.

Keywords: Cardiac arrest, Soluble thrombomodulin, Porcine model, Coagulation, Fibrinolysis

Introduction

Approximately one third of the nearly 500,000 patients who suffer sudden Cardiac Arrest (CA) each year in the US[1] survive to hospital admission, but another 60–70% of these survivors die before discharge due to failed recovery of one or more vital organs.[2] Despite decades of research, the factors which contribute to prolonged organ dysfunction in successfully resuscitated patients remain unclear. Direct cellular injury from the initial ischemia and reperfusion is thought to be the most important contributor, but persistent ischemia from inadequate restoration of microvascular blood flow may play an equally important role.[3–5] While the significance of the latter, dubbed the "no-reflow phenomenon", is supported by evidence from both animal models and human CA patients,[6,7] interest in pharmacologic intervention has waned since the failure of the TROICA trial, a multicenter study of tenecteplase in patients with out-of-hospital CA.[8]

There are a number of reasons why the failure of systemic fibrinolytics to improve clinical outcomes should not be taken as strong evidence for or against the role of "no-reflow" in prolonged organ dysfunction after CA. Tissue plasminogen activator (tPA) and its derivatives have proven clinical benefit in stroke, myocardial infarction, and

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other diseases involving thrombosis and occlusion of moderate to large arteries,[9] but no-reflow is believed to be a microvascular phenomenon, with a pathophysiology similar to that of severe sepsis and disseminated intravascular coagulation (DIC).[3,10,11] In these conditions, severe endothelial injury is believed to produce dysregulation of coagulation and fibrinolysis, which in turn result in diffuse microvascular thrombosis and ischemia.[12,13] Systemically administered tPA may clear these microthrombi transiently, but sustained response is likely to require restoration of endothelial function and coagulofibrinolytic balance.[2,11]

An alternate or potentially adjunct therapeutic approach may be modulating the endothelial protein C pathway,[6,14] which couples thrombin generation to the activation of two key zymogens – protein C, an antithrombotic and profibrinolytic protease, and TAFI, an antifibrinolytic carboxypeptidase.[15] Microvascular injury disrupts the delicate balance between these proteolytic cascades – largely due to loss or inactivation of endothelial thrombomodulin (TM) by inflammatory mediators, reactive oxygen species and/or proteolytic enzymes.[16–18] A recombinant, soluble form of human TM, ART-123 (Recomodulin, Asahi Kasei), offers a pharmacologic means of boosting the protein C pathway and has been used clinically in patients with DIC and sepsis-associated coagulopathy.[19] We sought to develop a porcine model of CA and prolonged CPR (CA/CPR) with abnormalities in coagulation and fibrinolysis mirroring those seen in human CA patients, with a central hypothesis that ART-123 would reverse some abnormalities in coagulofibrinolytic biomarkers.

Materials and Methods

All procedures outlined in this study adhered to the eighth edition of the Guide for the Care and Use of Laboratory Animals and were approved by the University of Michigan Institutional Animal Care and Use Committee.

Prolonged CA and CPR (CA/CPR) model and ART-123 treatment

All CA/CPR experiments were performed at the Weil Institute for Critical Care and Innovation at the University of Michigan. A schematic of instrumentation procedures, detailed experimental protocol and data collection are provided in S Fig. 1 Briefly, seventeen male Yorkshire swine (36–44 kg) were randomized to four experimental groups: 1. Sham, 2. CA/CPR + Vehicle control (saline given 5 minutes before VF), 3. CA/CPR + Pre-arrest ART-123 (40 mg given 5 minutes before VF), and 4. CA/CPR + Post-arrest ART-123 (40 mg given 2 minutes after the start of CPR). Allocation of animals to group 2 or 3 (saline vs. pre-arrest ART-123) was concealed from the surgical team, who performed all procedures and collected experimental samples (e.g., plasma) and data (e.g., hemodynamics, blood gas results). Blinding of the surgical team to the remaining animals was not feasible due to obvious differences in experimental protocol. In all cases, investigators conducting biochemical assays of coagulation and fibrinolysis were blinded to experimental group.

Experimental therapeutic

Clinical grade ART-123 (10.9 mg/mL in sterile saline) was provided by the Asahi Kasei Pharmaceutical corporation. Drug and vehicle were infused via central venous catheter.

Blood collection, processing, and analysis

Venous and arterial blood samples were collected from the central venous and arterial catheters, respectively, at baseline, after administration of drug or vehicle, and at 5, 10, 20, 30, 40, and 50 minutes after the initiation of CPR (T5-T50 time points, S Fig. 1B). Details of blood preparation and testing are described in the Supplemental Methods.

Statistical analysis

All quantitative data are presented as mean ± standard error of the mean (SEM). Differences between groups or changes over time were tested with one-way analyses of variance (ANOVA). Statistically significant main effects were followed-up with pairwise contrasts, with Tukey's correction for multiple comparisons.

Results

Pharmacokinetics of ART-123 in healthy pigs

Since the PK of ART-123 had not been previously determined in swine, we tested a series of doses in healthy swine and found a dose of 1 mg/kg was able to generate plasma concentrations above 10 μg/mL (S Fig. 2), a level previously identified as therapeutic based on porcine plasma studies.

Animal characteristics, arterial blood gas, and hemodynamic data

No significant differences were found in the baseline weight, arterial pH, lactate, partial pressure of oxygen (pO_2), mean arterial blood pressure (ABP), central venous pressure (CVP), peak airway pressure (PAP), or capnography ($ETCO_2$) of the animals in the four experimental groups (S Table 1). Arterial lactate and CVP increased, while ABP and pH decreased significantly in animals that underwent CA/CPR, as compared to sham (Fig. 1A-D, $p < 0.05$). Treatment with ART-123 did not affect the changes in any of these variables, but did significantly increase arterial pO_2 and the alveolar-arterial (A-a) gradient of oxygen when given after CA, at least at early time points (T5, T10, T20, and T30, all $p < 0.05$ for post-arrest vs. vehicle) (Fig. 1E-F). PAP and $ETCO_2$ were not significantly different in any of the experimental groups (S Fig. 3).

Measurements of coagulation and thrombosis

We next compared the most common clinically used coagulation tests, PT and aPTT. There were no statistically significant differences in either clotting time at baseline. In vehicle-treated CA/CPR animals, both PT and aPTT were shorter immediately after no-flow and prolonged at later time points, but not statistically different from sham at any time point. Clotting times were significantly elevated in ART-123 treated animals, consistent with prior reports of the effects of the drug on plasma coagulation tests.[20] In the pre-arrest group, only aPTT was significantly different ($p < 0.01$ vs. vehicle at T5-T50), whereas both PT and aPTT were significantly elevated in the post-arrest group ($p < 0.01$ vs. vehicle at T5-T50) (Fig. 2A-B).

We next measured biochemical markers of coagulation and thrombosis, including platelet count, plasma fibrinogen, TAT, and d-dimer. The latter is a measure of both coagulation and fibrinolysis, although commonly used as a marker of clotting.[21] Both platelet count and fibrinogen showed a progressive decline from baseline in all CA/CPR groups, but none of the groups were statistically significantly different from sham (Fig. 3A-B). In contrast, plasma levels of

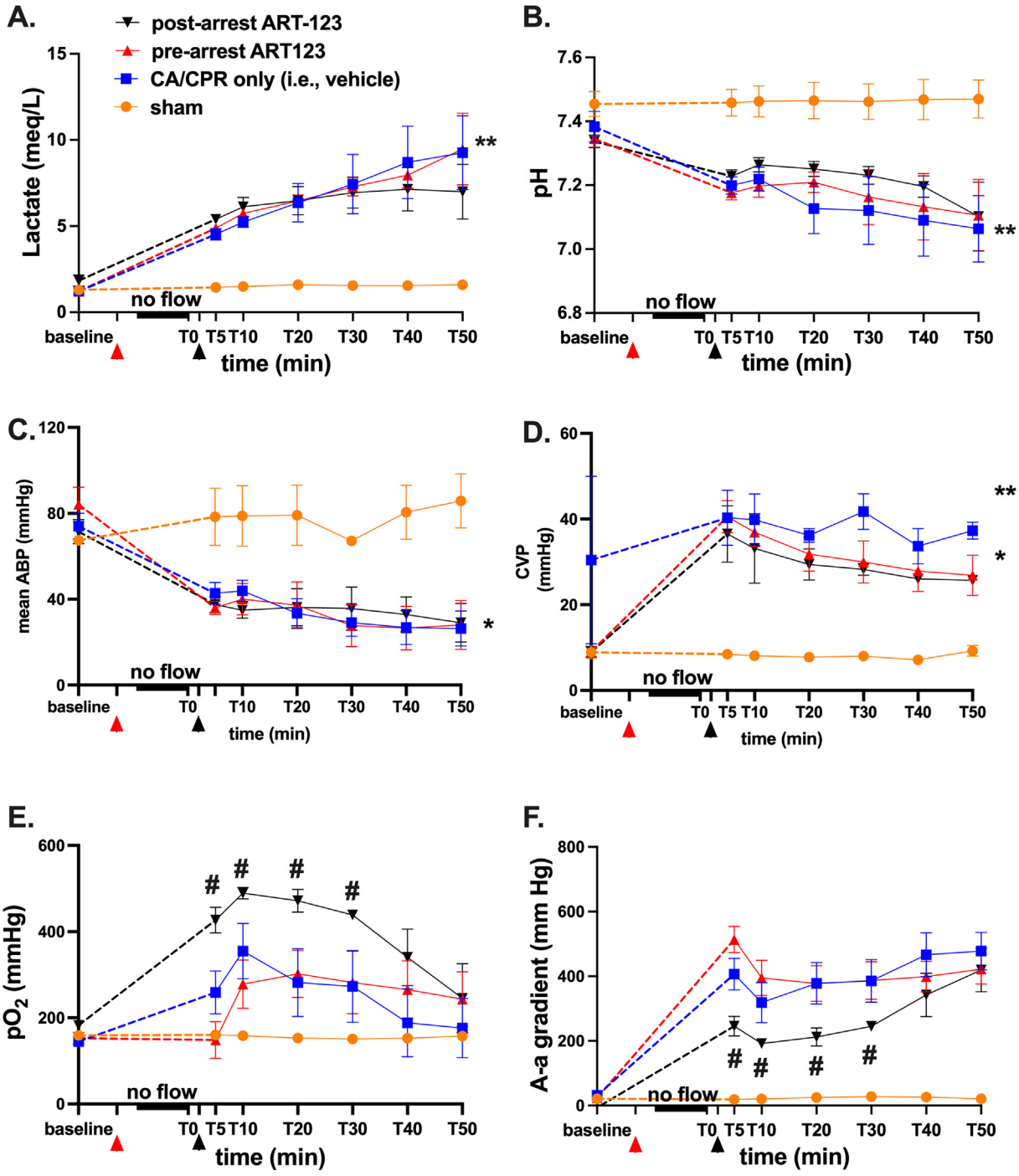

Fig. 1 – Arterial blood gas and hemodynamic values in sham and CA/CPR animals. A and B. arterial lactate and pH, ** – $p < 0.01$ for CA/CPR groups vs. sham (T5-T50), C and D. Arterial blood pressure (ABP) and central venous pressure (CVP), ** – $p < 0.01$ or * – $p < 0.05$ for CA/CPR groups vs. sham (T5-T50), E and F. Partial pressure of oxygen (pO_2) and alveolar-arterial (a-a) gradient, # – $p < 0.05$ for post-arrest ART123 vs. vehicle-treated CA/CPR. Red and black arrowheads indicate timing of pre- and post-arrest ART-123, respectively. $n = 2$ in sham group, $n = 5$ for all other groups. All data presented as mean ± SEM.

TAT were markedly elevated in vehicle-treated CA/CPR animals, from 36.0 ± 8.7 ng/mL at baseline to 363.2 ± 45.4 ng/mL at T5 to a peak of 462.5 ± 46.5 ng/mL at T50 ($p < 0.05$ vs. sham at T5-T50) (Fig. 3C). D-dimer showed a similar pattern, although its increase came slightly later than TAT, from 0.56 ± 0.10 mg/L at baseline to 1.49 ± 0.30 mg/L at T5 ($p = 0.09$ vs. sham) to a peak of 6.97 ± 1.45 mg/L at T40 ($p < 0.05$ vs. sham at T10-T50) (Fig. 3D).

Treatment with ART-123 had a clear anticoagulant effect, significantly decreased both TAT and d-dimer, as compared to vehicle-treated animals. The effect on TAT varied depending on the timing

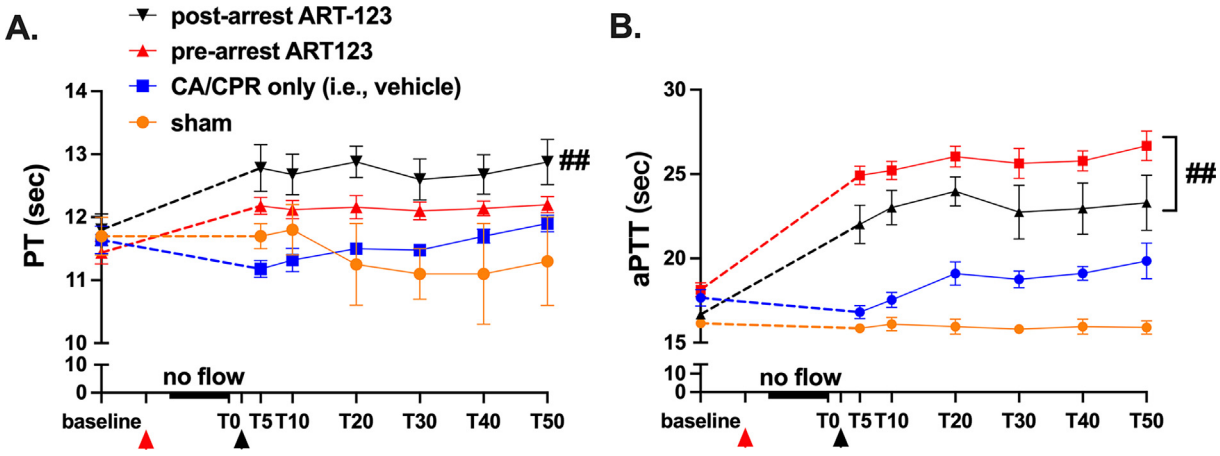

Fig. 2 – Clotting times. A. Prothrombin time (PT), ## – $p < 0.01$ for post-arrest vs. vehicle-treated CA/CPR (T5-FT50), B. Activated partial thromboplastin time (aPTT), ## – $p < 0.01$ for both ART123 groups vs. vehicle (T5-T50). Red and black arrowheads indicate timing of pre- and post-arrest ART-123, respectively. $n = 2$ in sham group, $n = 5$ for all other groups. All data presented as mean ± SEM.

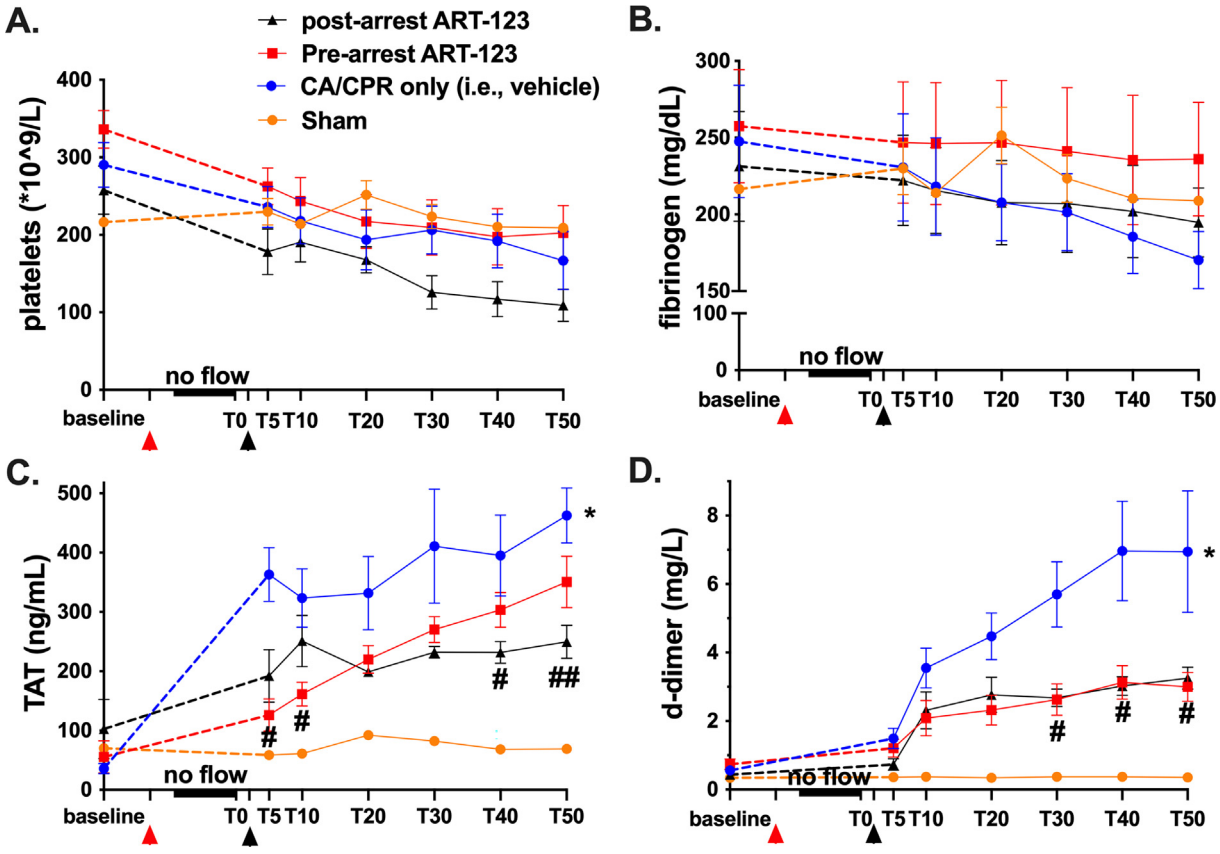

Fig. 3 – Markers of systemic coagulation and/or thrombosis. A and B. platelet count and plasma fibrinogen, C and D. thrombin-antithrombin complexes (TAT) and d-dimer, * – $p < 0.05$ for vehicle-treated CA/CPR vs. sham (T5-T50), # – $p < 0.05$ and ## – $p < 0.01$ for ART123 vs. vehicle. Red and black arrowheads indicate timing of pre- and post-arrest ART-123, respectively. $n = 2$ in sham group, $n = 5$ for all other groups. All data presented as mean ± SEM.

of administration. In the pre-arrest group, ART-123 significantly decreased TAT levels at early time points, T5 (126.1 ± 27, $p < 0.01$ vs. vehicle) and T10 (161.7 ± 20.1, $p = 0.04$ vs. vehicle). In contrast, post-arrest ART-123 seemed to have a more prolonged anticoagulant effect, with significant decreases in TAT at T5 (191.2 ± 44.2, $p = 0.04$ vs. vehicle), T40 (231.8 ± 18.3, $p = 0.04$ vs. vehicle) and T50 (249.8 ± 28.0, $p = 0.01$ vs. vehicle) (Fig. 3C). The effect of ART-123 on d-dimer was largely independent of the timing of administration, with both pre- and post-arrest groups significantly decreased vs. vehicle at T30, T40, and T50 ($p < 0.05$ for each). Of

note, the protein C activity assay – a marker of residual protein C zymogen in the plasma[22] – was not significantly impacted by administration of ART-123, although this was measured only in the pre-arrest group (S Fig. 4).

Measurements of fibrinolysis

We next measured the activation of the fibrinolytic cascade, which begins with release of tPA from endothelial cells in response to a variety of coagulation factors.[23] tPA is quickly complexed by its serpin inhibitor, PAI-1, such that the free enzyme only accounts for a fraction of the total amount in the plasma.[24] During resuscitation, tPA-PAI-1 complexes in the plasma increased markedly in vehicle-treated CA/CPR animals, from 7.82 ± 1.39 ng/mL at baseline to 45.79 ± 8.64 ng/mL at T5 to a peak of 46.59 ± 7.35 ng/mL at T50 ($p < 0.05$ vs. sham at T5-T50) (Fig. 4A). There was a corresponding increase in free tPA, (from 0.41 ± 0.11 ng/mL at baseline to 1.51 ± 0.40 ng/mL at T5) and decrease in free PAI-1 (from 1.93 ± 0.38 ng/mL at baseline to 0.37 ± 0.14 ng/mL at T5), although only the latter was significantly different from sham ($p < 0.01$ at T5). At later time points, free tPA declined and free PAI-1 increased in vehicle-treated CA/CPR animals, such that neither biomarker was significantly different vs. sham (Fig. 4B-C).

The effects of ART-123 on fibrinolysis also varied depending on the timing of administration. While neither pre- nor post-arrest ART-123 significantly impacted the CA/CPR-induced increase in tPA-PAI-1 complexes, free PAI-1 was nearly undetectable in animals that had received pre-arrest ART-123 ($p < 0.05$ vs. vehicle at T20-40). There was a corresponding increase in free tPA at these time points, with a peak of 3.71 ± 0.38 ng/mL at T50 ($p < 0.05$ vs. vehicle at T30-T50). Interestingly, post-arrest ART-123 showed no significant effects on any fibrinolytic biomarkers (Fig. 4A-C).

Effects of CA/CPR and pre- and post-arrest ART-123 on thromboelastography (TEG)

We next evaluated the impact of CA/CPR and ART-123 on blood coagulation and fibrinolysis using thromboelastography (TEG). Fig. 5A shows representative TEG traces from the vehicle-treated CA/CPR group. The "R time", reflecting the time to clot initiation, was shortened at T10 and partially recovered at T50. Conversely the angle (α), which reflects the speed of clot formation, increased at T10 before returning to baseline at T50. The maximum amplitude (MA), which reflects clot strength, was only minimally affected by CA/CPR (Fig. 5D-F). Finally, the clot lysis at 30 minutes after maximum clot strength (LY30), a fibrinolytic parameter, decreased to nearly

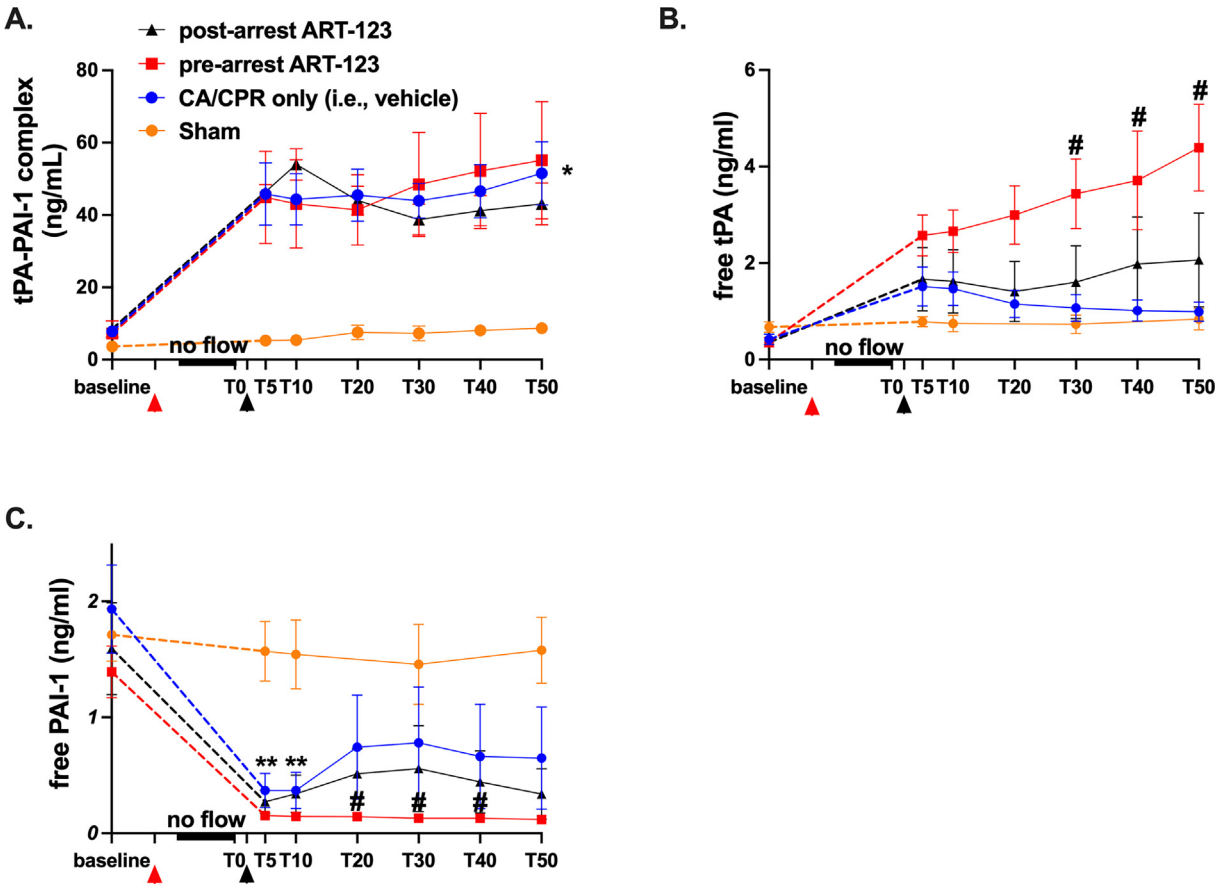

Fig. 4 – Fibrinolytic markers. A. tissue plasminogen activator (tPA)-plasminogen activator inhibitor-1 (PAI-1) complexes, * – $p < 0.05$ for vehicle-treated CA/CPR vs. sham (T5-T50), B. free tPA, # – $p < 0.05$ for pre-arrest vs. vehicle, C. free PAI-1, ** – $p < 0.01$ for vehicle-treated CA/CPR vs. sham, # – $p < 0.05$ for pre-arrest vs. vehicle. Red and black arrowheads indicate timing of pre- and post-arrest ART-123, respectively. $n = 2$ in sham group, $n = 5$ for all other groups. All data presented as mean ± SEM.

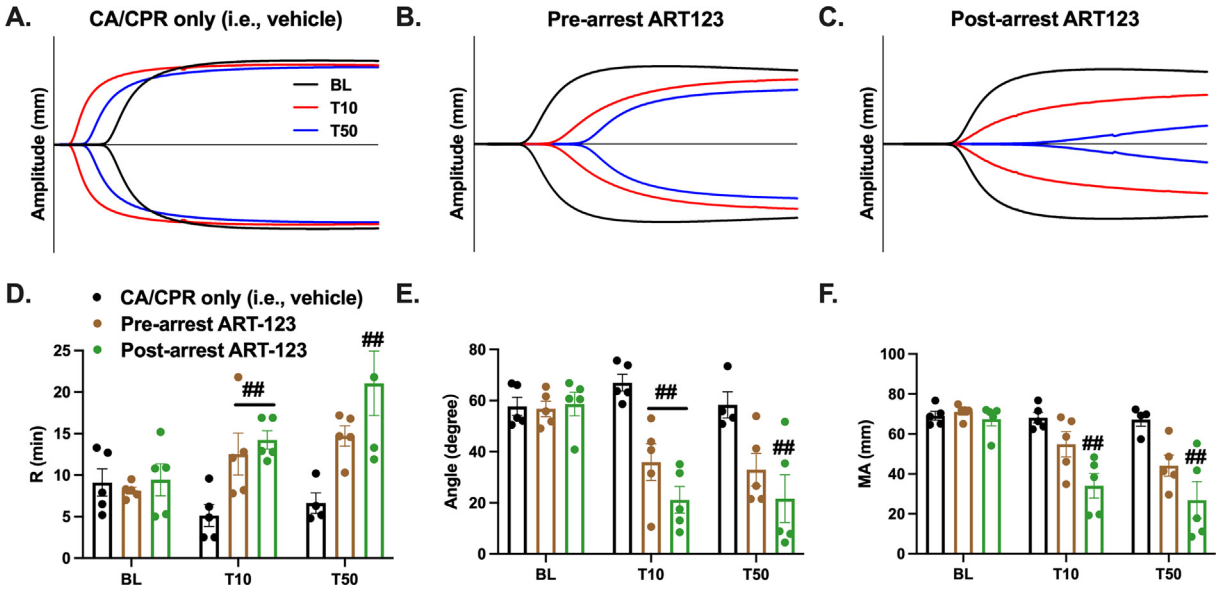

Fig. 5 – Thromboelastography (TEG). Representative traces from A. vehicle-treated CA/CPR animal. B. Pre-arrest ART-123, and C. Post-arrest ART-123. Quantification of parameters D. Reaction (R) time, ## – $p < 0.01$ vs. vehicle. E. Angle, ## – $p < 0.01$ vs. vehicle, F. Maximum Amplitude (MA), ## – $p < 0.01$ vs. vehicle. $n = 5$ for each group, data presented as mean ± SEM.

undetectable levels in vehicle-treated animals after CA/CPR (0.3 ± 0.2 at T10 and 0.2 ± 0.1 at T50) (S Fig. 5).

Both pre- and post-arrest ART-123 had significant effects on TEG parameters, as seen in the representative traces (Fig. 5B-C) and quantified parameters. Post-arrest ART-123 had more prominent effects on coagulation parameters, significantly increased R time and reduced angle and MA at both T10 and T50 ($p < 0.01$ vs. vehicle for all) (Fig. 5D-F). Both pre- and post-arrest ART-123 reduced LY30 to 0% at both T10 and T50 ($p = 0.27$ and 0.13 vs. vehicle, respectively) (S Fig. 5).

Pharmacokinetics of ART123 in CA/CPR animals

We measured plasma levels of ART-123 in pre- and post-arrest groups to allow estimation of drug PK in CA/CPR animals. As shown in Fig. 6, higher initial plasma concentrations were observed in post-arrest ART-123 animals than the pre-arrest group (24.03 ± 1.72 vs. 14.53 ± 1.48 μg/mL, $p = 0.003$) and plasma concentrations in pre-arrest animals reached a slow elimination phase much faster. For both groups, plasma concentrations appeared to level off between 10 and 15 μg/mL.

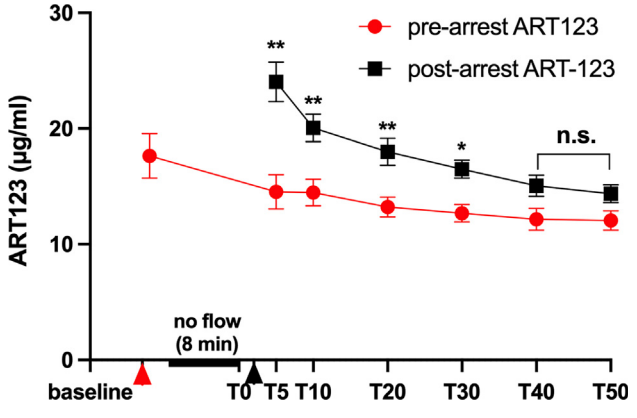

Fig. 6 – Plasma ART123 concentrations in CA/CPR animals treated pre- and post-arrest. Red and black arrowheads indicate timing of pre- and post-arrest ART-123, respectively. ** – $p < 0.01$, * – $p < 0.05$ for pre- vs. post-arrest. $n = 5$ for each group, data presented as mean ± SEM.

Discussion

With improvements in bystander CPR,[25] first responder defibrillation,[26] and extracorporeal cardiopulmonary resuscitation,[27] return of spontaneous circulation is likely to become an increasingly achievable aim in sudden cardiac arrest patients, placing ever greater priority on understanding and treating persistent organ dysfunction after successful resuscitation. While systemic fibrinolysis has been convincingly shown not to improve survival or neurologic outcome, there remains compelling evidence of abnormal endothelial function, coagulation, and fibrinolysis in the post-resuscitation period.[2,8,28] Without effective therapies or clinically relevant pre-clinical animal models in which to study these abnormalities, it will remain challenging to determine the extent to which they contribute to adverse outcomes.

With this in mind, the results of our study have a number of interesting implications for the field. First and foremost, the porcine model of prolonged CA/CPR appears to reproduce many of the coagulofibrinolytic derrangements reported in cardiac arrest patients.[29,30] As in humans, traditional laboratory tests, like platelet count, fibrinogen, and clotting times, were found to be highly variable (despite the controlled laboratory setting) and relatively insensitive in our study. In contrast, specific biochemical assays like TAT and d-dimer rose steadily over the course of CA/CPR, with the latter slightly delayed, consistent with the fact that it reflects a combination of both fibrin deposition and fibrinolysis.[21] Similarly, the biochemical assays of fibrinolysis used in our study, which to our knowledge have not been

previously validated in pigs, offer some insight into the complex series of events which occur during CA/CPR. Based on our results, a substantial release of endogenous tPA occurs during no-flow or just after initiation of resuscitative efforts. This is largely balanced by release of PAI-1, such that plasma levels of tPA-PAI-1 complexes increase without a significant rise in free tPA. Incorporation of ROSC into the CA/CPR model and measurement of additional fibrinolytic biomarkers are likely to provide further insights into the response of the fibrinolytic cascade to cardiac arrest and resuscitation.

Our study also investigated the use of TEG, an assay which offers a more comprehensive measurement of coagulation due to its incorporation of cellular components and clot strength.[31] Generally, TEG measurements were more variable than plasma assays but clearly indicated hypercoagulability during resuscitation. Pseudonormalization of TEG parameters after prolonged CPR, together with prolongation of clotting times suggest a mix of continued hypercoagulability and consumptive coagulopathy. At the same time, these data must be interpreted with caution, given limited information regarding normal porcine TEG parameters and reports of potential differences in TEG and ROTEM in pigs and humans.[32,33]

Apart from helping to define the underlying biology of coagulation and fibrinolysis in CA, our CA/CPR model offers the opportunity to study relevant therapeutic interventions. Our results indicate that pharmacologic interventions aimed at restoring coagulofibrinolytic balance are at least feasible. Systemic coagulation seems to occur throughout resuscitation and not exclusively during no-flow or the initiation of CPR, creating a potential window for therapeutic intervention. While biomarkers like TAT and d-dimer might lag behind the processes they are intended to reflect and their gradual increase could result from slow return of blood from the periphery, these explanations are countered by the effects of ART-123 in our study. Treatment in both pre- and post-arrest phases produced significant decreases in plasma TAT, and the precise effects depended on the timing of administration (pre-arrest animals showing larger differences at early time points and post-arrest animals at later time points). This indicates ongoing coagulation at all phases of CA/CPR and suggests a limited duration of action of ART-123, at least with respect to its anticoagulant effects. The latter finding is surprising, given sustained levels of ART-123 and protein C zymogen in the plasma throughout CA/CPR, and will require further investigation.

Beyond its anticoagulant effects, ART-123 also had significant impact on fibrinolytic biomarkers, and again, the timing of administration significantly impacted these effects. In fact, the therapeutic window may be even more restrictive in this case, with pro-fibrinolytic effects seen exclusively in pre-arrest animals. The underlying mechanism is again somewhat unclear, although APC has been reported to bind and directly inhibit PAI-1, at least in some species.[34] Our data showing complete elimination of free PAI-1 in all animals pre-treated with ART-123 match this mechanism and suggest that the presence of the drug at the time of reperfusion promotes the release of free tPA. Complete understanding of the effects of ART-123 on the fibrinolytic cascade will require additional porcine-compatible assays, as the drug should also increase TAFI activation.[35]

Finally, ART-123 had minimal effects on the hemodynamic and gas exchange parameters measured in our experiments. The sole exception – a significant increase in arterial pO_2 and corresponding decrease in A-a gradient observed in the post-arrest group – is somewhat difficult to explain, given the lack of other differences in these animals. Our sample size was modest, so our study could have been underpowered to detect small differences in other hemodynamic variables. At the same time, it is worth noting that hemodynamic changes during CPR were not necessarily expected, and it is possible that coagulation and fibrinolysis are simply not relevant in this context. Given our overall hypothesis – that ART-123 and other interventions aimed at restoring coagulofibrinolytic balance will improve microvascular blood and organ function after successful resuscitation – it will be more important to determine the effects on these variables in animals which have achieved ROSC.

Of course, it will also be important to test ART-123 and other drugs with more realistic timing of administration – e.g., 12–14 minutes after the start of CPR, which clinical studies have shown is the median time to first IV access[36] – and in the context of other ACLS interventions (e.g., epinephrine). Nonetheless, our results suggest that ART-123 has a different range of activities depending on the timing of its administration relative to cardiac arrest and initiation of CPR. If nothing else, these data should serve as a reminder of the critical importance of testing the therapeutic window of various pharmacologic interventions in high-fidelity pre-clinical models with well-characterized PD biomarkers – especially in complex and time-sensitive critical illnesses, like sudden cardiac arrest.

Conclusion

Our porcine model of prolonged CA/CPR reproduces many of the coagulofibrinolytic derangements reported in human cardiac arrest patients and has utility in testing pharmacologic interventions. ART-123 was found to have both anticoagulant and pro-fibrinolytic effects in this model, although its modes of activity varied depending on the precise timing of administration relative to cardiac arrest.

CRediT authorship contribution statement

Boya Zhang: Investigation, Formal analysis, Writing – original draft. **Brendan M. McCracken:** Investigation. **Carmen Colmenero Mahmood:** Investigation. **Danielle Leander:** Investigation. **Nicholas Greer:** Investigation. **James A. Cranford:** Formal analysis. **Cindy H. Hsu:** Conceptualization. **Mohamad Hakam Tiba:** Supervision. **Robert W. Neumar:** Conceptualization. **Colin F. Greineder:** Conceptualization, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: BZ and CFG received study drug (ART123) and research funding from Asahi Kasei Pharma (AKP) Corporation. AKP was not involved in experimental design, data analysis, or manuscript preparation. BMM, CCM, DL, NG, JAC, CHH, MHT, and RWN have no conflicts of interest to report.

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HL130430). CFG had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resplu.2023.100477.

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