Factor XII silencing using siRNA prevents thrombus formation in a rat model of extracorporeal life support

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Abstract

Background: Heparin anticoagulation increases the bleeding risk during extracorporeal life support (ECLS) and thus must be limited and monitored carefully. This study determined whether factor XII (FXII) silencing using short interfering RNA (siRNA) can provide ECLS circuit anticoagulation, maintain normal tissue coagulation, and reduce the need for careful anticoagulation monitoring.

Methods: Adult male, Sprague-Dawley rats were randomized to four groups ($n = 3$ each) based on anticoagulant: (1) no anticoagulant, (2) heparin, (3) FXII siRNA, or (4) non-targeting siRNA. In group 1, no anticoagulant was administered. In group 2, heparin was administered intravenously before and during ECLS at an activated clotting time of 180–250 seconds. In groups 3 and 4, siRNA was administered intravenously three days before the initiation of ECLS via lipidoid nanoparticles. The rats were placed on pumped, arteriovenous ECLS with a small 3D-printed mock-oxygenator, and blood flow was maintained at 2 mL/min for eight hours or until the blood flow resistance reached three times its baseline resistance.

Results: A single dose of 3 mg/kg siRNA led to a reduction of plasma FXII to $26\% \pm 3\%$ (mean $\pm$ standard error) of baseline levels three days after treatment. Without anticoagulant, mock-oxygenator resistance tripled within 7 ± 2 minutes. The resistance in the heparin group was significantly ($p < 0.05$) elevated in the first hour of the experiment before returning to a lower, normal value for the rest of the experiment. The resistance in the FXII siRNA group did not change significantly with time ($p = 0.94$). There were no significant differences in resistance or mock-oxygenator thrombus volume between the FXII siRNA and the heparin groups ($p > 0.99$). However, the bleeding time in the FXII siRNA group (3.4 ± 0.6 minutes) was significantly shorter than that in the heparin group (5.5 ± 0.5 minutes, $p < 0.05$).

Conclusions: FXII silencing using siRNA provided simpler anticoagulation of ECLS circuits with reduced bleeding risk as compared to heparin.

Background

Extracorporeal life support (ECLS) provides respiratory and cardiac support to patients with severe cardiac and/or respiratory failure until recovery or donor organ availability for transplantation [1]. The ECLS circuit contains an artificial lung to exchange oxygen and carbon dioxide from the blood, being commonly referred to as an “oxygenator.” Oxygenators have a high rate of occlusive thrombus formation and thus fail within a few weeks [2]. All oxygenators utilize a densely-packed fiber bundle membrane with a $1.2\text{--}1.9\text{ m}^2$ surface area to provide efficient gas exchange [3], but the high degree of blood-artificial surface contact leads to rapid coagulation cascade activation and thrombus formation leading to a fiber bundle occlusion, an increase in blood flow resistance, and a decrease in gas transfer [4]. To slow thrombus formation and preserve ECLS function, anticoagulation is necessary.

Currently, unfractionated heparin is the most widely used anticoagulant [5]. However, heparin is associated with intracerebral and pulmonary bleeding as well as bleeding at the cannulation and surgical sites [6–9]. ECLS practitioners struggle with the balance of hemostasis and thrombosis in critically ill patients, even when monitored carefully. A higher dose of anticoagulant is associated with lower rates of thrombotic complications but a simultaneous increase in risk of bleeding complications [10–12]. Thus, major improvements in anticoagulant are needed to limit both bleeding and thrombosis complications and reduce the need for careful coagulation monitoring, particularly during the COVID-19 pandemic [13].

Thrombus formation during ECLS is initiated primarily by three synergistic processes: (1) activation of factor XII (FXII) and the intrinsic coagulation cascade [14–17], (2) platelet binding and activation to fibrinogen that is adsorbed to the artificial surfaces of the circuit, and (3) shear-induced activation of platelets caused by high resistance circuit components and the pump. Each of these modes of activation accelerates activation of the common coagulation cascade and, ultimately, fibrin formation. Heparin anticoagulation inhibits these processes by inhibiting thrombin and factor X of the common coagulation cascade without any direct platelet inhibition. Direct inhibition of FXII could serve a similar role, with the additional advantage of not inhibiting tissue hemostasis. Factor XII has little to no role in normal hemostasis, and thus, people with an inability to produce FXII live normal lives without excessive bleeding [18–21]. This makes FXII or activated FXII (FXIIa) inhibition an attractive, safe alternative to heparin during ECLS.

To date, several FXII and FXIIa inhibitors have been developed including (1) natural inhibitors, (2) small molecule inhibitors, (3) monoclonal antibodies, (4) antisense oligonucleotides (ASO), and (5) aptamers [22–29]. Among these, the recombinant fully human FXIIa-blocking antibody 3F7 and small-molecule inhibitor FXII900 have shown that FXIIa inhibition can significantly reduce thrombus formation without bleeding complications during ECLS [23, 29, 30]. As an alternative, long-acting anticoagulation would be achievable by eliminating hepatic FXII synthesis for days to weeks with a single administration using oligonucleotide-based drugs, including ASO, aptamer, and short interfering RNA (siRNA). In one study, a FXII-specific ASO attenuated catheter-induced thrombosis up to 35 days in rabbits [31]. However, this ASO needs to be administered twice a week and has a delayed onset of action (four weeks) that limits its utility for ECLS. In contrast, siRNA has a relatively rapid onset and longer duration of action from a single dose. siRNA is a synthetic RNA duplex consisting of two unmodified annealed 21–23-mer oligonucleotides. Once siRNA enters the cytosol, it binds to the RNA-induced silencing complex and degrades target messenger RNA (mRNA), preventing mRNA translation into a specific protein [32].

This study determined whether FXII silencing using siRNA can provide sufficient anticoagulation for ECLS, maintain normal tissue coagulation, and reduce the need for careful anticoagulation monitoring. This hypothesis was examined by performing simulated ECLS in rats receiving either (1) no anticoagulant (negative control), (2) standard heparin anticoagulant (positive control), (3) FXII siRNA, or (4) non-targeting siRNA.

Methods

This study was approved by the Allegheny Health Network Institutional Animal Care and Use Committee (Project No. 1071). Adult male Sprague-Dawley rats (Taconic Biosciences, Germantown, NY) were used and received humane care in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals [33].

Formulation of siRNA-loaded lipid nanoparticles

FXII siRNA was purchased from Sigma-Aldrich (SASI_Rn01_00106775_s, St. Louis, MO). MISSION® siRNA Universal Negative Control #1 (SIC001, Sigma-Aldrich) was used as an untargeted siRNA control. Lipidoid nanoparticles (LNPs) were used as the delivery vehicle for siRNA, as they have been previously identified as an effective and safe siRNA delivery material [34–37]. LNPs were formulated by mixing the ionizable lipidoid 306O₁₀ [34, 37], cholesterol (Millipore Sigma, Burlington, MA), 1, 2- distearoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL), and 1, 2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (C14PEG2000)

(Avanti Polar Lipids) at a molar ratio of 50: 38.5: 10: 1.5, as previously described [34–37]. The lipidoid 306O₁₀ was synthesized by Michael addition reaction using an isodecyl acrylate to 3,3-diamino-N-methyldipropylamine stoichiometric ratio of 4:1, as previously described [34, 37]. To yield a lipid solution composed of 90% (v/v) ethanol and 10% citrate buffer, additional ethanol and 10 mM sodium citrate buffer (pH 4.2) were added [34]. The siRNA solution was prepared by diluting siRNA in 10 mM sodium citrate to yield a final weight lipidoid: siRNA of 5:1 [34]. Equal volumes of lipid solution and siRNA solution were rapidly mixed together using a vortex mixer to form nanoparticles [36]. LNPs were further diluted in phosphate-buffered saline (PBS) and then dialyzed against PBS for 90 minutes in 3500 MWCO cassettes (Pierce/Thermo Scientific, Rockford, IL) to remove ethanol [36].

Extracorporeal life support (ECLS) Circuit

The efficacy of using FXII siRNA as long-term ECLS anticoagulation was evaluated in a rat ECLS model [38]. The ECLS circuit was established using methods similar to that described in reference 38. Briefly, the ECLS circuit, with 2.5 mL priming volume, consisted of 1/16-inch inner diameter (ID) Tygon tubing (E-3603, Fisher Scientific, Hampton, NH), a roller pump (505U Digital Peristaltic Pump, Watson Marlow, Wilmington, MA), and a custom-designed mock-oxygenator (additional file 1, supplementary Fig. 1). The mock-oxygenators were designed using SolidWorks (Dassault Systemes SolidWorks Corp., Waltham, MA) with 7.3-mm width, 3-mm height, and 20-mm length, and then manufactured using a DLP™ 3D printer (Ember Autodesk, San Rafael, CA) (additional file 1, supplementary Fig. 2), which is incapable of transferring gases. However, its low prime volume (0.3 mL), blood flow patterns within the device, and surface area to blood volume ratio (0.005 m²/mL) make it ideal for evaluating thrombus formation [38–40].

Efficacy of anticoagulation during ECLS

Rats were randomized based on the means of anticoagulant and placed on ECLS. Four groups (n = 3 in each group) were tested, based on the means of anticoagulant: (1) no anticoagulant (negative control 1), (2) heparin (positive control), (3) FXII siRNA (treatment), or (4) non-targeting siRNA (negative control 2). In group one, no anticoagulant was administered. In the heparin group, intravenous 50 IU/kg heparin was administered to achieve an activated clotting time (ACT) of 180–250 seconds before initiating ECLS. Afterward, heparin was administered continuously at 50 IU/kg/h for one hour and then at 25 IU/kg/h for seven hours [38]. In the siRNA groups, 3 mg/kg of FXII or non-targeting siRNA was administered three days before ECLS. A small incision of approximately 0.5 x 0.5 cm² in length was made in the middle of the tail to expose the lateral tail vein, and a 24-gauge (G) catheter (Insys™ 0.7 x 19 mm, BD, Franklin Lakes, NJ) was inserted. Prior to siRNA administration, a blood sample was taken to evaluate FXII concentration (0.3 mL), ACT (15 µL), and activated partial thromboplastin time (aPTT) (0.4 mL). Thereafter, the siRNA LNPs were administered slowly, followed by a 0.3 mL saline bolus. The dose of 3 mg/kg was decided based on a preliminary (n = 2) kinetic study evaluating the effect of siRNA dose and time on FXII concentration, ACT, and aPTT (additional file 2, supplementary Fig. 3, 4).

In all cases, the ACT was measured using whole blood (15 µL) via a Hemochron signature Elite (Accriva Diagnostics, San Diego, CA) coagulation analyzer. The aPTT and FXII concentrations were measured using platelet-poor plasma. For aPTT measurements, blood samples (0.4 mL) were collected into tubes containing 44 µL of sodium citrate (Santa Cruz Biotechnology, Dallas, TX) and centrifuged for 10 min at 2000 g. The aPTT was then evaluated using a Diagnostic Stago Start 4 (Siemens, Munich, Germany) coagulation analyzer. For FXII

measurements, blood samples (0.3 mL) were collected into tubes containing 33 μ L of sodium citrate and centrifuged for 15 min at 1000 g. The FXII concentration was measured using an enzyme-linked immunoassay (ELISA; Mybiosource, San Diego, CA). For the presentation and statistical analysis, FXII concentration was normalized to the percentage of the baseline FXII concentration prior to siRNA delivery.

To initiate ECLS, rats were anesthetized with intraperitoneal administration of ketamine (70 mg/kg) and xylazine (7.7 mg/kg). After anesthesia was confirmed, a small rectangular incision (0.5 x 1 cm²) in the middle of the tail was made to expose the lateral tail vein, and a 24-G catheter (Insyte™ 0.7 x 19 mm, BD) was inserted into the vein to maintain anesthesia with continuous intravascular administration of ketamine (1 mg/kg/min) and xylazine (0.03 mg/kg/min). Buprenorphine (0.01 mg/kg) was administered subcutaneously once for pain relief. When sufficient anesthesia was achieved, the animals were positioned supine on a circulating warm water blanket (Homeothermic Blanket Systems with Flexible Probe, Harvard Apparatus, Holliston, MA) to maintain core body temperature at 37.5–38.5°C during ECLS. The surgical sites were prepared by shaving and disinfecting with povidone-iodine. Rats were tracheostomized for mechanical ventilation (Small Animal Ventilator, Model 683, Harvard Apparatus) with a tidal volume of 7 mL/kg and 65 breaths per minute.

Rats were placed on pumped arteriovenous (A-V) ECLS by cannulating the left carotid artery for access using a 24-G catheter (Insyte™ 0.7 x 19 mm, BD) and the right jugular vein for return using a 20-G catheter (Insyte™ 1.1 x 30 mm, BD), as previously described [38]. This is not a typical mode of clinical ECLS attachment, but it provides more effective blood drainage and thus is an easier way to evaluate circuit coagulation in small animals [38, 41]. The cannulation of the left femoral artery using a 24-G catheter (Insyte™ 0.7 x 19 mm, BD) was performed to monitor systemic arterial pressure and collect arterial blood samples. The cannulation of the left femoral vein using a 22-G catheter (Insyte™ 0.9 x 25 mm, BD) was performed to administer methylprednisolone sodium succinate (50 mg/kg) before ECLS and sodium bicarbonate (0.3 mEq/h) during ECLS. The ECLS circuit was flushed with 2 L/min of carbon dioxide for 10 minutes and then primed with saline. ECLS was initiated at a flow of 2 mL/min and maintained for eight hours unless the circuit blood flow resistance tripled.

During ECLS, mean arterial pressure (MAP), heart rate (HR), and peripheral oxygen saturation (SpO₂) were monitored continuously by using the BIOPAC MP150A-CE data acquisition system (BIOPAC Systems, Inc., Santa Barbara, CA) and a Heska VetOx Plus 4800 Vital Signs Monitor (Heska, Loveland, CO). MAP was maintained over 60 mm Hg by administering norepinephrine (0–0.5 mcg/kg/min). The targeted HR was 250 to 350 beats/minute, and targeted SpO₂ was more than 94%. Any rat out of all these ranges was euthanized. To monitor occlusive clot progression in real time, the mock-oxygenator inlet and outlet pressures were monitored continuously using a Transpac IV pressure transducer (Transpac IV, icumedical, San Clemente, CA) connected to the BIOPAC MP150A-CE data acquisition system and recorded at baseline (approximately three minutes after flow initiation), 5, 10, and 30 minutes, 1, 2, 3, 4, 5, 6, 7, and 8 hours after flow initiation. Blood flow resistance was calculated in standard fashion as (inlet pressure - outlet pressure)/(blood flow).

The bleeding time was also measured immediate before (at baseline) and at the end of ECLS. A 23-G needle was inserted by 1 cm into the tail vein starting from 1 cm from the tip of the tail, withdrawn immediately, and blood was blotted every five seconds with a filter paper (Whatman™, Fisher Scientific) until the bleeding stopped. The bleeding time was defined as the time at which no blood is blotted on the filter paper. Blood samples were also taken before initiating ECLS (at baseline) and at the conclusion of ECLS to measure ACT (15 μ L), aPTT (0.4 mL), and platelet count (Plt) (0.3 mL). Methods for ACT and aPTT measurement were described previously. The Plt was measured using a Genesis™ (Oxford Science, Oxford, CT) hematology analyzer. For presentation, the Plt was corrected for

hemodilution during circuit priming using the formula corrected Plt = raw Plt x (hematocrit before ECLS)/ (hematocrit at the end of ECLS).

The experiment was continued for eight hours or until the mock-oxygenator blood flow resistance reached three times its baseline resistance and was considered failed. In either case, a final blood sample was taken to measure aPTT, ACT, and Plt, as described previously, the circuit was disconnected and gently flushed with heparinized saline (2 IU/ml), and the rats were euthanized using pentobarbital (175 mg/kg). The volume in the mock-oxygenator was then weighed, and the difference between the pre- and post-experiment mock-oxygenator volume was recorded as the thrombus volume. The saline in the mock-oxygenator was flushed using air for 10 seconds before and after the experiment, and the amount of flushed volume was measured by weight (g) and converted to volume using the density of saline (1.0 mg/ml). Then the difference between the pre- and post-experimental volume was determined to be the clot volume. From the clot volume, the percent of the mock-oxygenator filled with the clot was calculated.

Statistical analysis

All data are presented as mean $\pm$ standard error. A paired *t*-test was used to examine the effects of the siRNA and LNP delivery vehicle on the FXII level, ACT, and aPTT. A one-way analysis of variance (ANOVA) was used to examine the effect of different anticoagulation groups on ECLS thrombus volume. A linear mixed model was used to assess differences in ACT, aPTT, blood flow resistance, and bleeding time during ECLS. The anticoagulant type (FXII siRNA, heparin, etc.), time, and the interaction of anticoagulant and time were used as fixed effects, and the animal ID was used as a random effect. For post-hoc correction, Bonferroni correction was used for all pairwise comparisons. A one-way ANOVA was used to compare the Plt before ECLS and the loss in platelets during ECLS between groups. The Kaplan-Meier method was performed to compare mock-oxygenator failure rates using log-rank as the statistical test for significant differences in failure rate. All statistical analyses were performed using SPSS software (version 27.0; SPSS Inc., Chicago, IL, USA). All statistical tests were two-sided, and a *p* value less than 0.05 was considered statistically significant.

Results

The mean weight of the rats was 549 ± 5 g in the no anticoagulant group, 516 ± 25 g in the heparin group, 586 ± 24 g in the siRNA group, and 538 ± 6 g in the non-targeting siRNA group. The differences in weight were not statistically significant (*p* = 0.11).

Effect of siRNA on coagulation

LNP-encapsulated FXII siRNA of 3 mg/kg reduced plasma FXII concentrations to $26\% \pm 3\%$ of baseline three days after treatment (*p* < 0.05) (Fig. 1a). Accordingly, a 3 mg/kg dose of FXII siRNA caused a more than 4-fold prolongation in aPTT from 21.4 ± 0.6 seconds before treatment to 93.6 ± 10.0 seconds three days after treatment (*p* < 0.05) (Fig. 1b). A single dose of 3 mg/kg siRNA also caused a significant ACT prolongation from 82 ± 5 seconds before treatment to 139 ± 2 seconds three days after treatment (*p* < 0.05) (Fig. 1c).

In contrast, a 3 mg/kg dose of non-targeting siRNA had no effect on plasma FXII concentration ($102\% \pm 3\%$ after treatment, *p* = 0.48), indicating that the reduction of FXII using FXII siRNA was specifically due to the siRNA and not the LNP delivery vehicle (Fig. 1a). The non-targeting siRNA also had no effect on aPTT (20.5 ± 0.6 seconds before

treatment and 20.7 ± 2.7 seconds after treatment, $p = 0.91$) (Fig. 1b). However, the 3 mg/kg dose of non-targeting siRNA unexpectedly caused a statistically significant ACT prolongation from 75 ± 4 seconds before treatment to 123 ± 2 seconds three days after treatment ($p < 0.05$, Fig. 1c). Therefore, the combination of no effect on FXII concentration or aPTT with an increase in the ACT suggests an effect on the coagulation system outside the coagulation cascade factors.

Anticoagulation during ECLS

The aPTT during ECLS for each anticoagulant group is shown in Fig. 2a. The aPTT before and at the end of ECLS in the FXII siRNA group was 93.6 ± 10.0 seconds and 102.3 ± 8.9 seconds, respectively, which were statistically prolonged when compared to the no anticoagulation (22.2 ± 1.8 seconds before and 26.5 ± 3.5 seconds at the end of ECLS, $p < 0.05$) and the non-targeting siRNA groups (20.7 ± 2.7 seconds before and 23.3 ± 2.9 seconds at the end of ECLS, $p < 0.05$). Similarly, the use of heparin created a statistically significant increase in aPTT (135.2 ± 31.3 seconds before and 46.9 ± 5.2 seconds at the end of ECLS) compared to no anticoagulation ($p < 0.05$) and non-targeting siRNA ($p < 0.05$). There was no significant difference in the aPTT between the FXII siRNA and the heparin groups ($p > 0.99$). The non-targeting siRNA group showed no significant difference in aPTT when compared to the no anticoagulant group ($p > 0.99$).

The ACT for each anticoagulant group is shown in Fig. 2b. The ACT for the heparin group was significantly prolonged (202 ± 8 seconds before and 170 ± 5 seconds at the end of ECLS) when compared to the FXII siRNA (139 ± 2 seconds before and 137 ± 11 seconds at the end of ECLS, $p < 0.05$), the non-targeting siRNA (123 ± 2 seconds before and 126 ± 3 seconds at the end of ECLS, $p < 0.05$), and the no anticoagulant groups (104 ± 9 seconds before and 120 ± 3 seconds at the end of ECLS, $p < 0.05$). The ACT before and at the end of ECLS in the FXII siRNA group was also prolonged compared to that in the non-targeting siRNA and the no anticoagulant groups, but without statistical significance ($p > 0.99$ and $p = 0.14$, respectively).

Thrombus formation

Fig. 3a shows mock-oxygenator blood flow resistance throughout the experiments. The no anticoagulant group exhibited a significantly greater mock-oxygenator blood flow resistance than the FXII siRNA ($p < 0.05$) and the heparin groups ($p < 0.05$). For all rats not receiving an anticoagulant, mock-oxygenator resistance rapidly increased to greater than three times the baseline resistance before 10 minutes had elapsed. However, the blood flow resistance in the FXII siRNA group did not change significantly during the experiment ($p = 0.94$), and no devices reached three times the baseline resistance. The blood flow resistance in the FXII siRNA group was 633 ± 73 mm Hg/(mL/min) at baseline and averaged to 499 ± 25 mm Hg/(mL/min) throughout the experiments. The blood flow resistance in the heparin group was 833 ± 130 mm Hg/(mL/min) at baseline and averaged to 582 ± 62 mm Hg/(mL/min) throughout the experiment. This suggests early, mild coagulation within the first few minutes of blood contact that was lysed thereafter. Accordingly, no heparin group mock-oxygenators reached three times the baseline blood flow resistance, and there was no significant difference between the FXII siRNA and the heparin groups resistance ($p > 0.99$). Unexpectedly, the resistance of all devices in the non-targeting siRNA group did not increase as rapidly as in the no anticoagulant group. The mock-oxygenator failure curve exhibiting significant differences ($p < 0.05$) between the four groups is shown in Fig 3b. The mean time from initiation of ECLS until the

oxygenator failure in the no anticoagulant and the non-targeting siRNA groups was 7 ± 2 minutes and 113 ± 27 minutes, respectively.

Fig. 3c shows thrombus volume in the mock-oxygenator after circuit detachment. The thrombus volume in the FXII siRNA, heparin, no anticoagulant, and non-targeting siRNA groups were $5\% \pm 5\%$, $0\% \pm 2\%$, $49\% \pm 4\%$, and $15\% \pm 2\%$ respectively ($p < 0.05$). The thrombus volume in the FXII siRNA group was significantly lower than that in the no anticoagulant group, demonstrating the antithrombotic effect of FXII siRNA on the mock-oxygenator ($p < 0.05$). There was no significant difference in the thrombus volume between the FXII siRNA and the heparin groups ($p > 0.99$). The thrombus volume in the non-targeting siRNA group was also significantly lower than that in the no anticoagulant group ($p < 0.05$), mirroring the unexpected ACT and blood flow resistance results.

Bleeding complication

The bleeding time for each anticoagulant group is shown in Fig. 4. The bleeding time for the heparin group was significantly prolonged (6.5 ± 0.3 minutes before and 5.5 ± 0.5 minutes at the end of ECLS) compared to the FXII siRNA (2.8 ± 0.4 minutes before and 3.4 ± 0.6 minutes at the end of ECLS), the non-targeting siRNA (1.7 ± 0.4 minutes before and 2.0 ± 0.3 minutes at the end of ECLS), and the no anticoagulant groups (0.7 ± 0.2 minutes before and 0.8 ± 0.2 minutes at the end of ECLS) ($p < 0.05$). The bleeding time before and at the end of ECLS in the FXII siRNA group was significantly prolonged compared to that in the no anticoagulant group ($p < 0.05$). There was no significant difference between the non-targeting siRNA and the no anticoagulant groups ($p = 0.45$) and between the FXII siRNA and non-targeting siRNA groups ($p = 0.18$).

Platelet consumption

Before ECLS, the Plt in the siRNA group was lower than that in the other groups, but this was not statistically significant (no anticoagulant group: $571 \pm 102 \times 10^3/\mu\text{L}$, heparin group: $705 \pm 37 \times 10^3/\mu\text{L}$, FXII siRNA group: $433 \pm 25 \times 10^3/\mu\text{L}$, non-targeting siRNA group: $590 \pm 107 \times 10^3/\mu\text{L}$, $p = 0.18$, Fig. 5). In all groups, the Plt decreased after initiating ECLS (no anticoagulant group: $17\% \pm 1\%$, heparin group: $42\% \pm 4\%$, FXII siRNA group: $14\% \pm 9\%$, non-targeting siRNA: $16\% \pm 10\%$). The decrease in the heparin group was higher compared to the others, but this was not statistically significant ($p = 0.07$).

Discussion

This study determined that silencing FXII using siRNA could provide sufficient anticoagulation for ECLS. A single intravenous dose of siRNA (3 mg/kg) administered three days before the initiation of ECLS reduced plasma FXII concentration to 26% of the normal level, and this prevented thrombus formation within the mock-oxygenator over eight hours, similarly to heparin anticoagulation. At the same time, this had a lesser effect on prolonging tissue bleeding when compared to heparin. Thus, replacing heparin with FXII silencing could reduce bleeding complications that occur in approximately 5% to 56% of ECLS cases [6–12, 42, 43].

Moreover, the ability of FXII siRNA to provide anticoagulation for multiple days would greatly simplify anticoagulant management. During clinical ECLS management, heparin is given continuously and titrated to balance the risk of thrombotic and bleeding complications. This requires regular measurement of clotting times and subsequent adjustment of heparin infusion rates. In the current study, a single intravenous dose of siRNA provided a significant

effect that lasted for three days and did not require management or titration during ECLS. Clinically, siRNA could be repeated on some regular schedule with little or no required measurement of clotting times. The combination of sufficient ECLS anticoagulation and a reduction in bleeding complications will make ECLS safer to employ, and the simplicity in once every 3–4 day dosing would simplify patient care and reduce ECLS costs. This safety and simplification will be of great benefit in helping to move ECLS out of the intensive care unit to a general ward when used as a longer-term bridge to transplantation for patients with chronic lung disease.

Unfractionated heparin is the dominant anticoagulant used during ECLS. Heparin generates anticoagulation in two ways (Fig. 6). First, heparin binds to anti-thrombin (AT), and the heparin-AT complex predominantly inactivates thrombin and activated factor X (FXa), with lesser impacts on other coagulation factors [16, 17, 44–47]. Heparin also increases endothelial cell the tissue factor pathway inhibitor (TFPI) release. TFPI binds to FXa and inactivates it [47]. Furthermore, TFPI inhibits FX activation via the activated factor VII (FVIIa)-tissue factor (TF) complex [47]. Therefore, heparin inhibits coagulation predominantly via the common coagulation cascade factors, FXa and thrombin, and thus, inhibits FXII-activated coagulation (intrinsic pathway) at artificial surfaces and TF-activated coagulation (extrinsic pathway) in tissues. This case was observed in the current study, in which heparin inhibited both oxygenator thrombus formation and tissue coagulation, as demonstrated via blood flow resistance and thrombus volume and bleeding time, respectively. The resistance of the device without anticoagulant increased to greater than three times its baseline within 10 minutes, whereas the device with heparin showed no significant increase in resistance. The thrombus volume in the heparin group was also significantly lower (0%) than that in the no anticoagulant group (49%), while the bleeding time at the end of ECLS in the heparin group (5.5 minutes) was significantly prolonged compared to the no anticoagulant group (0.8 minutes).

Because heparin inhibits coagulation in tissues, its delivery must be limited and constantly monitored, and even the ideal, recommended level of heparin anticoagulation (usually 1.5 times the normal ACT) [48] cannot eliminate bleeding complications. Unlike heparin, inhibition or elimination of FXII does not affect the formation of the FVIIa-TF complex via the extrinsic pathway, and thus, tissue coagulation can still occur normally. Accordingly, the bleeding time in rats treated with FXII siRNA (2.8 ± 0.4 seconds before and 3.4 ± 0.6 seconds at the end of ECLS) was shorter than that in rats treated with heparin (6.5 ± 0.3 seconds before and 5.5 ± 0.5 seconds at the end of ECLS). Thus, FXII silencing is a potential approach to generate ECLS circuit anticoagulation while reducing bleeding.

Previous studies of shorter-acting FXIIa inhibitors have provided similar results. Wilbs et al. demonstrated that FXII900, a bicyclic peptide inhibitor of FXIIa, reduced thrombus formation and blood flow resistance in oxygenators over four hours in rabbit veno-venous ECLS model when compared to no anticoagulation [29]. In that study, the oxygenator thrombus volume was significantly lower ($p < 0.05$) for the FXII900 treated rabbits (10%) than for the untreated rabbits (37%) [29]. The resistance of the oxygenator remained at a baseline of 50 mm Hg/(L/min) in animals with FXII900, but it increased from 50 mm Hg/(L/min) to 100 mm Hg/(L/min) within 30 minutes in rabbits not receiving an anticoagulant [29]. Moreover, FXII900 provided better resistance to thrombus formation than heparin [29]. Naito et al. further demonstrated 94% less oxygenator thrombus formation when FXII900 was delivered intravenously with a polycarboxybetaine surface coating compared to anticoagulation with heparin at ACT of 220–300 seconds [30]. Similarly, Larsson et al. found that the recombinant human FXIIa-blocking antibody (3F7) significantly reduced thrombus formation and increases in oxygenator pressure gradient in a rabbit veno-arterial ECLS model during six-hour testing when compared to no anticoagulant use [23]. Fibrin deposition in the oxygenators was significantly reduced ($p < 0.05$) in 3F7-treated rabbits (4%) than in saline-treated rabbits (100%) [23]. The pressure gradient remained less than 15 mm Hg throughout the experiment in animals with 3F7,

but it increased to more than 500 mm Hg within three minutes in animals not receiving anticoagulants [23]. Of similar importance, the Wilbs, Naito, and Larsson studies demonstrated that the FXIIa inhibitor preserved normal tissue bleeding times, unlike heparin [23, 29, 30].

In contrast, FXII silencing also provides several days of anticoagulation with a single dose. The FXII siRNA used in this study provided at least three days of effective silencing in rats at a dose of 3 mg/kg. FXII900 had to be continuously administered as it was eliminated by the kidney, although it could be bound to long-chain polymers (e.g., polyethylene glycol, polycarboxybetaine) to increase its half-life to as long as a few days. In contrast, siRNA provides a long-lived reduction in FXII concentrations. In our preliminary study, siRNA showed a dose-dependent reduction of plasma FXII and prolongation of aPTT on day 3, but these returned to nearly baseline values on day 7. Further optimization of the siRNA sequence and delivery vehicle could lead to more effective, longer-lasting silencing. Cai et al. observed that 0.01 mg/kg of siRNA provided an 82% reduction in FXII and 1 mg/kg of siRNA provided a 99% reduction in FXII on day 7 [49]. These 82% and 99% FXII reductions led to a 69% and 99% reduction in thrombus formation in an A-V shunt thrombosis model, respectively [49]. Similarly, a single dose GalNAc-siRNA ALN-F12 provided a dose-dependent maximal FXII suppression of 51%–55% at 0.3 mg/kg and 93% at 1 mg/kg on day 10, lasting for 64 days [50, 51]. This holds the promise for single dosing during traditional ECLS or infrequent dosing during extended ECLS. This would be similar in nature to the use of 3F7. The long-term effectiveness of 3F7 has not been documented, but it is likely to be effective for days.

Additionally, the current study demonstrated an increase in the bleeding time in rats treated with the FXII siRNA unlike FXIIa inhibition using the 3F7 and FXII900 and silencing via GalNAc-siRNA targeting FXII (ALN-F12) [23, 29, 30, 51]. Similarly, Cai et al. also reported that rats treated with FXII siRNA delivered via cationic LNPs displayed a statistically significant increase in bleeding time [49]. Thus, there may be direct side-effects on platelet function or off-target gene silencing on other proteins in the hemostatic system [52–54] when using this delivery method. Ultimately, the time course and effectiveness of the FXII silencing will depend on the delivery vehicle, siRNA sequence, and potential chemical modification of the siRNA. Before testing can be attempted in clinical (human) ECLS, this strategy should be proven safe and effective in typical animal models of ECLS. Thus, further optimization of the delivery vehicle and siRNA sequence may be necessary for these animal models.

This study had limitations that affected data interpretation. First, the ability to anticoagulate the ECLS circuit was evaluated in a miniaturized system with components different from a clinical ECLS system and for a period of only eight hours. An initial, short-term, small animal study was deemed necessary, however, to provide proof of concept prior to embarking on significantly more expensive long-term studies in larger animals. Studies in rats also required the use of a miniaturized, small-volume system to avoid dangerous hemodilution. Thus, this model used a microfluidic device that simulates the densely-packed, hydrophobic surfaces of oxygenators but does not include polymethylpentene (PMP) surfaces of hollow fibers in clinical ECLS oxygenators' gas exchange membranes. The simulated fibers in our devices are solid urethane acrylate posts. Urethane acrylate and PMP are both hydrophobic polymers, and thus, the mechanisms causing clotting (e.g., FXII activation, fibrinogen adsorption, platelet adhesion, etc.) are similar. However, the rate of thrombus formation might be slightly different due to the different polymer chemical structures. Additionally, a rat has a Plt three to six times higher than humans, and thus, the rate of thrombus formation and thrombus composition may be accelerated [55], even at the same ACT and aPTT. Therefore, the rate of circuit coagulation for all groups is likely different from that in clinical devices, but the benefits of silencing FXII still hold.

Second, we measured only Plts, ACT, aPTT, and FXII concentrations due to restrictions on blood sampling volumes in rats. The etiology of thrombosis and bleeding is multifactorial. The activation of platelets [56–58], other coagulation cascade factors [59], and fibrinolysis [60] impacted our results and could present a more detailed picture of the impact of siRNA delivery. Future, large-animal studies could further elucidate these effects when large volume blood sampling is possible. Third, we have not yet optimized the siRNA sequence for rats and the optimal delay between delivery and initiation of ECLS.

Lastly, we have not fully evaluated the side effects of LNPs. The non-targeting siRNA showed mild prolonged ACT and a mild reduction in ECLS thrombus formation for approximately two hours. These LNPs demonstrated no toxicity in mice and rats [34, 35]. However, a more detailed examination of their effects on the coagulation cascade might indicate some unknown mild effects, as seen in our study. Potentially, an optimized siRNA sequence can be used to lower the overall dose of LNPs such that these off-target effects are negligible.

Conclusions

In conclusion, FXII silencing can provide simpler, long-acting anticoagulation in ECLS circuits. Due to the reduced bleeding complications and need for coagulation monitoring, FXII silencing using siRNA would enable simpler, safer ECLS. If made simpler and safer, this can also enable long-term ECLS outside an intensive care unit, potentially outside a hospital for destination therapy respiratory support. Permanent destination therapy with artificial lungs cannot be envisioned currently, as the home-based management of heparin delivery will be impossible, and the bleeding and thrombosis complication rates are too high. Therefore, the effectiveness of FXII siRNA must be proven in animal models of ECLS over a typical course of ECLS (5–7 days). This work will require additional optimization of siRNA sequences, doses, and potentially, delivery vehicles for this setting.

Abbreviations

ACT: activated clotting time; ASO: antisense oligonucleotides; ANOVA: analysis of variance; aPTT: activated partial thromboplastin time; AT: anti-thrombin; A-V: arteriovenous; ECLS: Extracorporeal life support; FXII: factor XII; FXIIa: activated FXII; FXa: activated factor X; FVIIa: activated factor VII; G: gauge; HR: heart rate; ID: inner diameter; LNPs: Lipidoid nanoparticles; MAP: mean arterial pressure; PBS: phosphate-buffered saline; Plt: Platelet count; PMP: polymethylpentene; siRNA: short interfering RNA; SpO₂: peripheral oxygen saturation; TF: tissue factor; TFPI: tissue factor pathway inhibitor

Declarations

Ethics approval and consent to participate

The study was approved by the Allegheny Health Network Institutional Animal Care and Use Committee (Project No. 1071).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

NU, SI, SA, KW, and KC conceived and designed the study. NU, SS, JM, and KR performed the experiments and collected data. NU, AL, SI, and KC analyzed the data. DS and NC provided lipidoid nanoparticles. NU, AL, and SI drafted the manuscript. KW and KC revised the manuscript critically for important intellectual content. All authors have read and approved the final manuscript.

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Figures

Figure 1

Effect of siRNA on coagulation. Effect of FXII and non-targeting siRNA on (a) FXII plasma level, (b) aPTT, and (c) ACT three days after administration. FXII siRNA had a significant effect on FXII concentrations, aPTT, and ACT three days after treatment. In contrast, non-targeting siRNA had no effect on plasma FXII concentration and aPTT. Error bars represent the standard error of the mean. ACT, activated clotting time; aPTT, activated partial thromboplastin time; FXII, factor XII; siRNA, short interfering RNA.

Figure 2

Anticoagulation levels during ECLS. (a) aPTT during ECLS for each anticoagulant group. The aPTT in the FXII siRNA and heparin group were statistically prolonged when compared to the no anticoagulation and the non-targeting siRNA groups. (b) ACT during ECLS for each anticoagulant group. ACT for heparin group was significantly prolonged when compared to the FXII siRNA, non-targeting siRNA and no anticoagulant groups. Error bars represent the standard error of the mean. Asterisk indicated $p < 0.05$. P value was calculated by a linear mixed model. ACT, activated clotting time; aPTT, activated partial thromboplastin time; ECLS, extracorporeal life support; FXII, factor XII; siRNA, short interfering RNA.

Figure 3

Thrombus formation. (a) The resistance of the mock-oxygenators over the course of eight hours in each anticoagulant group. The no anticoagulant group exhibited a significantly greater resistance than the FXII siRNA group ($p < 0.05$) and the heparin group ($p < 0.05$). There was no significant difference between the FXII siRNA and heparin groups resistance ($p > 0.99$). (b) Mock-oxygenators failure curve plotted over the course of eight hours in each anticoagulant group. The mock-oxygenator in the no anticoagulant and the non-targeting siRNA groups failed before eight hours. (c) Thrombus volume inside the mock-oxygenators in each anticoagulant group after the experiment. The thrombus volume in the FXII siRNA group was significantly lower than that in the no anticoagulant group ($p < 0.05$). There was no significant difference in mock-oxygenator thrombus volume between the FXII siRNA

and the heparin groups ($p > 0.99$). Error bars represent the standard error of the mean. P value in (c) was calculated by one-way ANOVA. FXII, factor XII; siRNA, short interfering RNA.

Figure 4

Bleeding time during ECLS. This is a figure of bleeding time. The bleeding time in the FXII siRNA group was significantly shorter than that in the heparin group ($p < 0.05$). Error bars represent the standard error of the mean. Asterisk indicated $p < 0.05$. P value was calculated by a linear mixed model. ECLS, extracorporeal life support; FXII, factor XII; siRNA, short interfering RNA.

Figure 5

Change in platelet count from baseline measurement. The platelet count decreased throughout the experiment but showed no significant difference between the four groups. Error bars represent the standard error of the mean. ECLS, extracorporeal life support; FXII, factor XII; siRNA, short interfering RNA.

Figure 6

Overview of medical device-induced thrombosis and mechanism of action of heparin. Unfractionated heparin (UFH) bind to anti-thrombin (AT), and the UFH-AT complex predominantly inactivates thrombin and activated factor X (FXa), with lesser impacts on other coagulation factors. Tissue factor pathway inhibitor (TFPI), released from endothelial cells by UFH, inhibits FXa and activated factor VII (FVIIa)-tissue factor (TF) complex. FXII, factor XII; FXIIa, activated FXII; FXI, factor XI; FXIa, activated FXI; FIX, factor IX; FIXa, activated FIX; FX, factor X.

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