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LOW-DOSE HEPARIN ANTICOAGULATION DURING EXTRACORPOREAL LIFE SUPPORT FOR ACUTE RESPIRATORY DISTRESS SYNDROME IN CONSCIOUS SHEEP

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ABSTRACT—**Background:** Over 32% of burned battlefield causalities develop trauma-induced hypoxic respiratory failure, also known as acute respiratory distress syndrome (ARDS). Recently, 9 out of 10 US combat soldiers' survived life-threatening trauma-induced ARDS supported with extracorporeal membrane oxygenation (ECMO), a portable form of cardiopulmonary bypass. Unfortunately, the size, incidence of coagulation complications, and the need for systematic anticoagulation for traditional ECMO devices have prevented widespread use of this lifesaving technology. Therefore, a compact, mobile, ECMO system using minimal anticoagulation may be the solution to reduce ARDS in critically ill military and civilian patients. **Methods:** We conducted a prospective cohort laboratory investigation to evaluate the coagulation function in an ovine model of oleic acid induced ARDS supported with veno-venous ECMO. The experimental design approximated the time needed to transport from a battlefield setting to an advanced facility and compared bolus versus standard heparin anticoagulation therapy. **Results:** Comprehensive coagulation and hemostasis assays did not show any difference because of ECMO support over 10 h between the two groups but did show changes because of injury. Platelet count and function did decrease with support on ECMO, but there was no significant bleeding or clot formation during the entire experiment. **Conclusions:** A bolus heparin injection is sufficient to maintain ECMO support for up to 10 h in an ovine model of ARDS. With a reduced need for systematic anticoagulation, ECMO use for battlefield trauma could reduce significant morbidity and mortality from ventilator-induced lung injury and ARDS. Future studies will investigate the mechanisms and therapies to support patients for longer periods on ECMO without coagulation complications. **Level of Evidence:** V—therapeutic animal experiment.

KEYWORDS—Acute respiratory distress syndrome, anticoagulation, blood platelet, extracorporeal life support, extracorporeal membrane oxygenation, trauma

BACKGROUND

Acute respiratory distress syndrome (ARDS) can result from pneumonia, sepsis, blast trauma, burns, and inhalation injuries and has an overall mortality of 30–40% (1, 2). Extracorporeal membrane oxygenation (ECMO) is a member of a family of cardiopulmonary support devices increasingly used to support children and adults suffering from severe ARDS (3, 4). ECMO has been used to treat trauma patients successfully since 1972 (5). According to the Berlin definitions of ARDS, over 32% of burned combat causalities develop ARDS with a crude overall mortality of 16.5% (2, 6). Overall, survival for adults on ECMO is over 39% and may be even higher for patients with trauma-induced ARDS (1, 3).

Current ARDS management includes treatment of the precipitating illness, minimal use of supplemental oxygen, and a pressure and volume-limited ventilation strategy (7, 8). However, these ARDS strategies still result in high rates of ventilator-associated lung injury (13%) and mortality (20%) (9, 10). Utilization of ECMO for severe ARDS is increasing because of the positive results during the 2009 influenza A (H1N1) pandemic; publication of the first large controlled clinical trial of modern ECMO technology; and a recent dramatic improvement in technology (4, 11, 12). ECMO

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utilization will continue to gain acceptance, perhaps in conjunction with other promising therapies, such as airway pressure release ventilation and intrapleural steroid injection (13, 14). The current ECMO therapy for ARDS is veno-venous ECMO (VV-ECMO), a method that provides gas exchange using only the venous circulation, a safe and increasingly effective method to improve survival from ARDS (4, 15).

A 2012 study of the 51,000 patients in the Extracorporeal Life Support Organization database revealed that one of the most common complications during ECMO support is clot formation (3). Clot formation is caused by contact activation between blood components and the circuit surface and shear stress generated by the pump, both leading to an inflammatory response and activation of the coagulation cascade (16, 17). Thus, use of systemic anticoagulation is necessary to prevent clot formation and maintain patency of the circuit (18). However, this need for systemic anticoagulation limits the use of ECMO support for trauma patients because anticoagulation may be contraindicated. The solution for the trauma patient may be in the use of state-of-the-art heparin-coated circuits and devices (19, 20). Therefore, there is a compelling need to evaluate the longevity of these new heparin-coated ECLS devices and define the minimal amount of anticoagulation therapy.

Fifteen years ago, Murphy et al. (21) documented the successful use of a low heparin strategy in an ovine model of ARDS using a pump-less arterio-venous carbon dioxide removal circuit (Affinity with TrilliumTM Bio-Passive Surface, Avecor Cardiovascular, Plymouth, MN, USA). Preceding ECMO animal studies also documented success without the need for heparin anticoagulation but they did not induce lung injury (22, 23). Recent clinical studies document the successful management of acute phase trauma patients without anticoagulation therapy using a miniaturized ECMO device; the PLS-Set (MAQUET Cardiopulmonary AG, Hechingen, Germany) (24, 25). However, none of studies fully evaluated the effects of a low heparin strategy on platelet activation, platelet function, and the coagulation system. Therefore, there is an unmet need to understand the hemostatic parameters of low heparin anticoagulation therapy in modern ECMO systems used for severe ARDS. In this study, we evaluated the effects of the CardioHelp ECMO system (MAQUET Cardiopulmonary AG) on advanced coagulation parameters using a standard and a low heparin anticoagulation strategy. We tested these parameters in sheep supported on ECMO before and after the induction of ARDS. Our hypothesis was that a low dose of heparin would not significantly change clinically relevant hemostatic parameters or clot formation in this state-of-the-art veno-venous mobile ECMO device.

MATERIALS AND METHODS

Study design

A prospective cohort laboratory study was performed to compare low versus standard heparin anticoagulation therapy on the coagulation function in sheep with oleic-acid-induced ARDS supported with VV-ECMO. A modern, highly integrated, ECMO device (CardioHelp, Maquet Cardiopulmonary) supported the sheep (n = 9) during 5 h before and 6 h after lung injury, with standard (H+ group, n = 5) or bolus (H− group, n = 4) doses of heparin infusion. We divided the time on ECMO into a pre-injury period, before induction of lung injury, and a postinjury period (Fig. 1). For each animal, there were four time points, which include pre-injury T1 (at the initiation of ECMO), and T2 (5 h of ECMO support) and postinjury T1 (1 h after injury) and T2 (5 h after T1 postinjury). To assess donor variability, baseline cell counts and coagulation function assays were performed before animals were placed on ECMO.

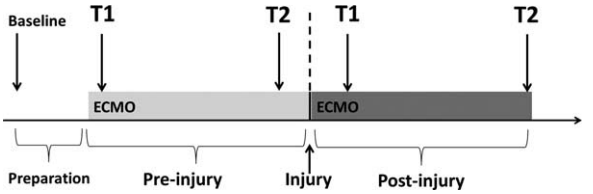

FIG. 1. **Study timeline.**

Animal experiment

The US Army Institute of Surgical Research Institutional Animal Care and Use Committee approved the study, and it was conducted in compliance with the Animal Welfare Act, and the implementing Animal Welfare Act Regulations, and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals. The study design randomly assigned nine crossbred female sheep (45 ± 6 kg) to the standard heparin dose group or the low heparin dose group.

Under general anesthesia (Isofluarane 2–4%), sheep received a tracheostomy and had central catheters placed in the left jugular vein to administer medications. A 23 F bicaval dual-lumen catheter (Avalon Elite, Maquet, USA) was placed through the right internal jugular vein (26) and connected to the VV-ECMO system previously primed with lactated Ringer's. Blood flow through the membrane lung was constant throughout the study at approximately 2 L/min. Sheep were awakened and weaned from mechanical ventilation to continuous positive airway pressure of 8 cmH_2O. Fraction of inspiratory oxygen (FiO_2) was set at 0.5 and 1.0 after injury. To achieve adequate sedation as previously described, we administered buprenorphine (Reckitt Benckiser, UK), Midazolam (Hospira, USA) (27).

To induce ARDS, lungs were injured by intravenous injection of 0.1 mL/kg of oleic acid mixed in 20 mL of whole blood and 300 IU of heparin, repeated up to three times to achieve a PaO_2 to FiO_2 ratio less than 200 (27). In the H+ group, a heparin (APP Pharmaceuticals, USA) infusion maintained the activated clotting time (ACT) between 160 s and 180 s after an initial bolus of 150 IU/kg during the dual-lumen catheter insertion. In the H− group, sheep received only the initial heparin bolus (150 IU/kg) and the oleic acid injection without continuous rate heparin administration (21, 24, 25, 27). The oleic acid is mixed with blood before injection and requires heparin as an anticoagulant. Thus, oleic acid injections also contributed to the heparinization of all study animals. For all time points, central venous blood was drawn directly from the ECMO circuit into appropriate tubes that contained one of the following additives: 3.2% citrate tubes (Vacutainer, BD, USA), EDTA tube (Vacutainer), or a Hirudin tube (Multiplate, Verum Diagnostica GmbH, Germany).

Laboratory analysis

Complete blood count (CBC)—The ADVIA 120 blood counter (Siemens AG, Germany) determined CBCs from samples collected in the EDTA tubes and recorded human settings for platelet count (PLT), white blood count (WBC), and hemoglobin concentration (Hb (mg/dL)). Samples from three animals using specific sheep species settings documented results indicate a very strong correlation with PLT, WBC, Hb parameters run under human settings ($r^2 = 0.97$, $P < 0.0001$).

Thromboelastography—On a TEG 5000 system (Haemonetics, Braintree, MA), blood from the sodium citrate tube was analyzed using heparinase cups. Heparinase (Haemonetics, USA) eliminates the effects of circulating heparin, which was necessary to evaluate the underlying hemostatic potential of blood independent of heparin effects. Following manufacturer directions, each test was completed in duplicate; R (clotting time), alpha (angle), MA (maximal clot strength), and LY60 (lysis rate at 60 min) were recorded.

Aggregometry—To assess platelet function, whole blood aggregation was determined with an impedance aggregometer (Multiplate, Dynabyte Medical GnbH, Germany). According to a previous study (28), only ADP (ADPtest, Verum Diagnostica GnbH, Germany) and collagen (COLTest, Verum Diagnostica GnbH, Germany) were valid for use in sheep, with final concentrations of 4.6 M and 4.5 mg/L, respectively. After 30 min of resting time, we ran in duplicate mixed whole blood in hirudin tubes (Dynabyte Medical GnbH, Germany) to determine the relative area under the aggregation curve (AUC), a measure of aggregation.

Plasma preparation and free hemoglobin—After centrifugation (3,000 g, 10 min, 4°C), platelet-poor plasma (PPP) from citrate tubes was used fresh or flash frozen and stored at −80°C then thawed later to be analyzed. Plasma/LowHB

HemoCue (HemoCue AB, Sweden) determined the free plasma hemoglobin concentration in fresh PPP.

Coagulation testing—Prothrombin time (PT), activated partial thromboplastin time (aPTT), activities of coagulation factors V, VIII, IX, XII, antithrombin III and von Willebrand factor, and concentrations of fibrinogen and d-dimer were measured in previously frozen PPP using the Sta-R Evolution system (Diagnostica Stago, USA). Because of the use of heparin in the animals, we determined aPTT, activities of factors V, VIII, IX, XII, antithrombin III and von Willebrand factor with and without heparinase (6 IU/mL, Haemonetics, USA). ACT was measured on a Hemochron signature elite device (ITC, USA). Plasma concentrations of thrombin/antithrombin complex (TAT) and plasmin/antiplasmin complex (PAP) were measured in previously frozen PPP with the Enzygnost TAT micro ELISA kit (Siemens Healthcare, Germany) and IMUCLONE PAP Elisa kits (Sekisui Diagnostics, USA) according to the manufacturer's instructions.

Flow-cytometry—Flow-cytometry quantified platelet activation using a previously established protocol using a BD FACS Canto (BD Biosciences, USA) (29). Monoclonal antibodies for CD45-FITC (clone 1.11.32), CD62P-RPE (clone Psel.KO.2.7), CD61-Alexafluor 647 (clone PM6/13), and isotypes were purchased from ABD Serotec (UK). A 5-μL volume of fresh whole blood collected in sodium citrate was incubated with antibodies in HBSS at room temperature for 15 min. Specimens then underwent a Duo-lyse cycle on a lyse wash assistant instrument (BD Biosciences, USA) to lyse red blood cells and remove unbound antibody, and then analyzed with a flow cytometer. For phosphatidylserine (PS) analysis, we purchased Bovine lactadherin-FITC from Haematologic Technologies, Inc. (Essex Junction, VT). To perform this analysis, we combined 5 μL of whole blood with 3 μL lactadherin-FITC and 92 μL of HBSS and incubated for 15 min at room temperature. Then, we added 2 mL of HBSS to the sample to analyze immediately by flow cytometry, gating on the platelet population and excluding the red blood cells. Results are given as percentages of P-selectin positive (CD62P marker) and PS positive platelets, and as percentage of double positive for leukocyte population (CD45 marker) and platelets (CD61 marker).

Statistical analysis

Our analysis used a Mann–Whitney test to compare H+ and H− groups for heparin dose, the aPTT, and for all parameters compared between groups at baseline. Two-way ANOVAs on LOG transformed data defined the isolated or combined effects of group (H+/H−) and use of ECMO over time for each factor in the pre- and postinjury periods. A third 2-way ANOVA on LOG transformed data defined the isolated or combined effects of group (H+/H−) and injury for each factor based on the comparison of T2 pre-injury and T1 postinjury time points. For some parameters, repeated measures ANOVA assessed the overall experiment time effect in the two groups. Matched pair t-tests compared the relevant assays with and without heparinase. Statistical significance was set for an alpha risk at 0.05. Results are documented as mean (standard deviation) or median (inter-quartile range). If not expressed, graphs represent mean ± standard error. For all the results, we documented the time effect between T1 and T2 as (h) for healthy animals (pre-injury period) and (s) for sick animals (postinjury period). The injury effect was defined as the differences between pre- and postinjury, represented by the letter (i). For each parameter, we also calculated a time effect combined with a group effect for each pre- and postinjury period (h∗group or s∗group) and over the whole experiment (t∗group).

RESULTS

The median total dose of heparin infused in the H+ group was significantly higher than in the H− group for the duration of the experiment (339 (IQR 293–409) IU/kg vs. 158 (IQR 155–162) IU/kg, $P = 0.02$). The mean total dose of heparin was also higher in the H+ group during the pre-injury period (267 (IQR 245–314) IU/kg vs. 150 (IQR 150–150) IU/kg, $P = 0.0151$) and during the postinjury period (55 (IQR 44–108) IU/kg vs. 8 (IQR 5–12) IU/kg, $P = 0.02$).

Baseline measurements

At the beginning of the experiments, before the animals were on ECMO, H- group had an unexpected lower PLT, PLT/Hb, and WBC ($P = 0.0275$, $P = 0.05$, and $P = 0.0143$, respectively) and higher ADP AUC/Plt and MA/Plt ($P = 0.0275$).

Standard ECMO hemostasis parameters

Standard ECMO hematologic parameters (CBC, PT/INR & aPTT (with and without heparinase), and fPH) measurements are listed in Table 1. ACT was not documented because the heparin titration in the H+ group was stable. To minimize the hemodilution bias, we documented the platelet count as the ratio Plt/Hb and total platelet count. The platelet count was higher in the H+ group during the whole experiment ($P = 0.0292$ and $P = 0.0451$, respectively, for pre- and post-injury periods). Even though there is an injury effect ($P = 0.0006$) and a pre-injury time effect ($P = 0.0449$) causing a drop in platelet count, there was a lack of a combined *time∗group* effect in the pre- and postinjury period signifying that the rate of decline in platelet count is not different between the two groups. Nevertheless, the rate of decrease in overall platelet count for all the groups and phases of the experiment was very significant ($P < 0.001$). Hemoglobin became more concentrated over time with a significant pre- and postinjury *time* effect and *injury* effect ($P = 0.0036$ and $P = 0.0079$, respectively). White blood cells (WBC) also became more concentrated over time and significantly decreased because of injury ($P = 0.0026$), however remained within the normal

TABLE 1. **Routine ECMO hemostatic parameters**

	Pre-injury				Postinjury				
	H+		H−		H+		H−		
Mean (SD)	T1	T2	T1	T2	T1	T2	T1	T2	Effect
WBC (10^3/mm^3)	2.7 (0.7)	5.3 (1.6)	2.1 (0.8)	3.6 (1.0)	3.2 (2.0)	1.6 (1.4)	1.6 (1.1)	3.4 (4.9)	h,i
Hb (g/dL)	8.1 (1.5)	9.9 (1.1)	8.7 (0.3)	9.6 (1.0)	11.3 (1.3)	12.8 (2.1)	11.2 (1.5)	11.9 (0.9)	h,i
RBC (10^6/mm^3)	8.67 (1.75)	10.29 (1.53)	8.72 (0.75)	9.59 (0.65)	11.71 (1.32)	13.16 (2.35)	11.07 (1.16)	11.77 (1.09)	h,i
Plt (10^3/mm^3)	346 (102)	299 (86)	224 (79)	230 (115)	159 (86)	164 (71)	72 (57)	51 (28)	i
Plt/Hb (/ug Hb)	42.4 (6.8)	30.7 (10.5)	25.8 (8.9)	23.2 (8.9)	14.2 (8.5)	13.5 (7.6)	6.3 (4.5)	4.3 (2.4)	h,i
PT (s)	25.2 (2.8)	24.6 (2.9)	25.5 (3.0)	24.4 (2.8)	29.8 (4.9)	35.6 (8.3)	29.9 (4.4)	32.1 (3.2)	h,s,i
aPTT (s)	too long	42.0 (2.8)	too long	32.2 (4.9)	58.4 (12.2)	42.0 (4.3)	33.2 (3.5)	36.3 (4.1)	na
apt Heparinase (s)	28.6 (0.7)	28.2 (1.3)	28.5 (1.8)	26.1 (1.2)	29.7 (1.7)	34.0 (2.8)	29.9 (2.1)	35.3 (3.2)	s,i
fPH (mg/dL)	16 (5.5)	15 (5)	20 (8.2)	14 (4.8)	67 (43)	65 (24)	88 (13)	75 (6)	i

Results for complete blood count (white blood cell count (WBC), hemoglobin (Hb), red blood cell count (RBC), and platelet count (Plt)), standard coagulation tests (prothrombin time (PT), activated partial thromboplastin time (aPTT)), standard coagulation tests with heparinase, free plasma hemoglobin (fPH), and normalized platelet count with hemoglobin (Plt/hb). A significant time effect between T1 and T2 is documented by h or s: h in healthy animals (pre-injury period) and s in sick animals (postinjury period). A significant injury effect between pre and postinjury is documented by the letter i, isolated group effect in both periods is not documented. There was no significant time*group nor injury*group effect by two-way ANOVA. SD, standard deviation.

range throughout the experiment. Overall, no significant combined *time*∗*group* effect on hemoglobin or WBC was observed.

To increase our accuracy in measuring clotting factors consumption because of ECMO time or injury, we documented all changes in coagulation with heparinase. During the pre-injury period in both groups, standard coagulation tests PT and aPTT run with heparinase were relatively stable, showing only a minor decrease in PT (pre-injury period *time* effect: $P = 0.0024$). PT and aPTT increased after the injury (*injury* effect, $P < 0.0001$ and $P = 0.0009$ respectively, postinjury period *time* effect: $P = 0.0036$ and $P = 0.0002$ respectively, Table 1). We also documented that aPTT rose after injury but did not increase in the heparinase treated samples. Plasma-free hemoglobin rose just after the injury (isolated injury effect, $P < 0.0001$, Table 1), and then stayed constant during postinjury ECMO. In both the groups, no animal manifested any clinical or radiologic evidence of venous thromboembolism or bleeding. Moreover, no significant clots formed on any of the oxygenator membranes during the experiment.

Clot formation, platelet function, and coagulation activity—Advanced coagulation and hemostasis assays include thromboelastography, platelet function (aggregation and activation), coagulation factor activity, and complex formation (Table 2). As documented in Figure 2, thromboelastography provided evidence of a significant net hypocoagulopathy after the injury, specifically an isolated *injury* effect of prolonging R ($P = 0.0043$), reducing alpha ($P = 0.0046$), and MA ($P < 0.0001$). Interestingly, MA indexed for platelet count (MA/Plt) increased ($P = 0.0042$). For traditional TEG parameters, no significant change appeared between low or regular heparin groups over time in the pre- and postinjury periods. As expected, the H+ group without heparinase significantly prolonged *R*, data not shown. Moreover, there was no significant clot lysis (LY 60%) during any phase of the experiment. Overall, there was no significant effect of ECMO itself on thromboelastography parameters.

Platelet aggregation stimulated by ADP and collagen are documented in Figure 3. Aggregation increased in healthy animals during ECMO ($P = 0.0004$) for both ADP and collagen agonists. This trend continued in healthy animals even when normalized by platelet count. The increase was significant for ADP and collagen, at $P = 0.0179$ and $P = 0.0056$, respectively. This did not hold true in postinjury animals, as ADP stimulated aggregation actually decreased ($P = 0.0127$). Overall, the global aggregability for ADP and collagen dropped with injury (*injury* effect: $P = 0.0010$ and $P = 0.0009$, respectively). Heparin concentration (H+ and H−) had no effect on platelet aggregation changes during the experiment. The significant rise in platelet activation (percentage of P-selectin-positive platelets) over the course of the experiment (*time* effect, $P = 0.0158$) is presented in Figure 4. There was no statistical difference in platelet activation between heparin groups (H+ and H−). Furthermore, there was no significant difference in P-selectin-positive platelets because of *injury* when analyzed with repeated measures ANOVA.

TABLE 2. **Advanced coagulation and hemostasis assays**

		Pre-injury				Postinjury				
		H+		H−		H+		H−		
	Mean (SD)	T1	T2	T1	T2	T1	T2	T1	T2	Effect
TEG	*R* (min)	8.5 (0.9)	6.9 (1.3)	6.9 (1.2)	6.7 (0.7)	10.8 (3.0)	10.6 (2.6)	10.3 (2.0)	8.9 (0.8)	i
	Alpha (Deg)	62.1 (3.7)	65.7 (3.6)	61.5 (2.4)	60.1 (7.0)	55.5 (6.8)	54.0 (6.1)	51.8 (5.4)	51.6 (5.8)	i
	MA	70.8 (0.8)	69.0 (2.4)	65.3 (3.9)	66.2 (4.9)	64.1 (1.3)	63.4 (2.3)	60.4 (4.6)	60.3 (4.1)	i
	MA/Plt	21.8 (6.0)	25.0 (8.7)	32.0 (10.5)	33.0 (12.0)	49.5 (22.4)	47.3 (26.8)	137.3 (100)	142.6 (60.4)	i
	LY60 (%)	1.0 (1.3)	0.3 (0.2)	0.0 (0.0)	0.0 (0.0)	0.4 (0.9)	0.0 (0.1)	0.0 (0.1)	0.0 (0.0)	.
Multiplate	AUC ADP	139 (56)	244 (104)	148 (45)	223 (67)	173 (94)	92 (86)	117 (55)	89 (87)	h,s,i
	AUC ADP/Plt	43 (23)	97 (76)	74 (42)	106 (36)	121 (75)	54 (33)	215 (103)	166 (85)	h
	AUC Collagen	51 (63)	99 (69)	22 (16)	91 (82)	45 (50)	29 (36)	25 (41)	31 (49)	h,i
	AUC Collagen/Plt	15 (17)	38 (28)	10 (9)	37 (25)	37 (57)	17 (15)	20 (27)	43 (50)	h,i
Circulating activity	Fibrinogen (mg/dL)	169 (16)	174 (16)	167 (33)	159 (30)	163 (7)	167 (8)	145 (21)	160 (13)	i
	D-dimers (ug/mL)	0.29 (0.20)	0.24 (0.14)	0.22 (0.05)	0.13 (0.07)	0.27 (0.12)	0.28 (0.05)	0.82 (0.76)	0.17 (0.04)	.
	PAP (ng/dL)	750 (480)	780 (480)	1850 (1150)	1980 (1350)	720 (470)	800 (570)	1720 (920)	1870 (1190)	.
	TAT (ng/dL)	56 (67)	140 (54)	50 (40)	77 (46)	329 (182)	95 (63)	274 (26)	57 (25)	h,s,i
	vWF (% act.)	96 (10)	108 (17)	86 (9)	100 (25)	81 (16)	73 (11)	60 (12)	66 (14)	h,i
	ATIII (% act.)	59 (4)	63 (9)	53 (15)	56 (15)	67 (10)	62 (9)	61 (13)	62 (15)	h,i
	Factor V (% act.)	296 (23)	248 (34)	272 (107)	248 (95)	181 (21)	192 (26)	190 (52)	231 (36)	h,s,i
	Factor VIII (% act.)	1651 (280)	1788 (339)	1418 (389)	1509 (356)	1244 (427)	1145 (433)	820 (118)	804 (295)	i
	Factor IX (% act.)	370 (83)	489 (79)	301 (131)	359 (147)	262 (54)	235 (51)	214 (80)	184 (70)	h,s,i
	Factor XII (% act.)	86 (18)	97 (58)	86 (46)	66 (34)	75 (21)	72 (22)	53 (24)	44 (18)	.

These assays include measures of clot formation, platelet function, and coagulation factors. Thromboelastography (TEG) performed with heparinase measures clot formation with parameters that include reaction time (R), angle (Alpha), maximal amplitude (MA), MA normalized to platelet count (MA/PLT), lysis at 60 minutes (LY60). Multiplate measures platelet function using impedance aggregometery using stimulants of ADP and collagen documented as area under curve (AUC) and AUC normalized to platelet count (AUC/PLT). Multiple coagulation factor activity performed with heparinase was measured including plasmin/antiplasmin complex (PAP), thrombin/antithrombin complex (TAT), von Willebrand factor (vWF), and antithrombin III (ATIII) percentage activity in comparison to normal human serum (% act). A significant time effect between T1 and T2 is documented by h or s: h in healthy animals (pre-injury period) and s in sick animals (postinjury period). A significant injury effect between pre and postinjury is documented by the letter i, isolated group effect in both periods is not documented. There was no significant time∗group nor injury∗group by two-way ANOVA. SD, standard deviation.

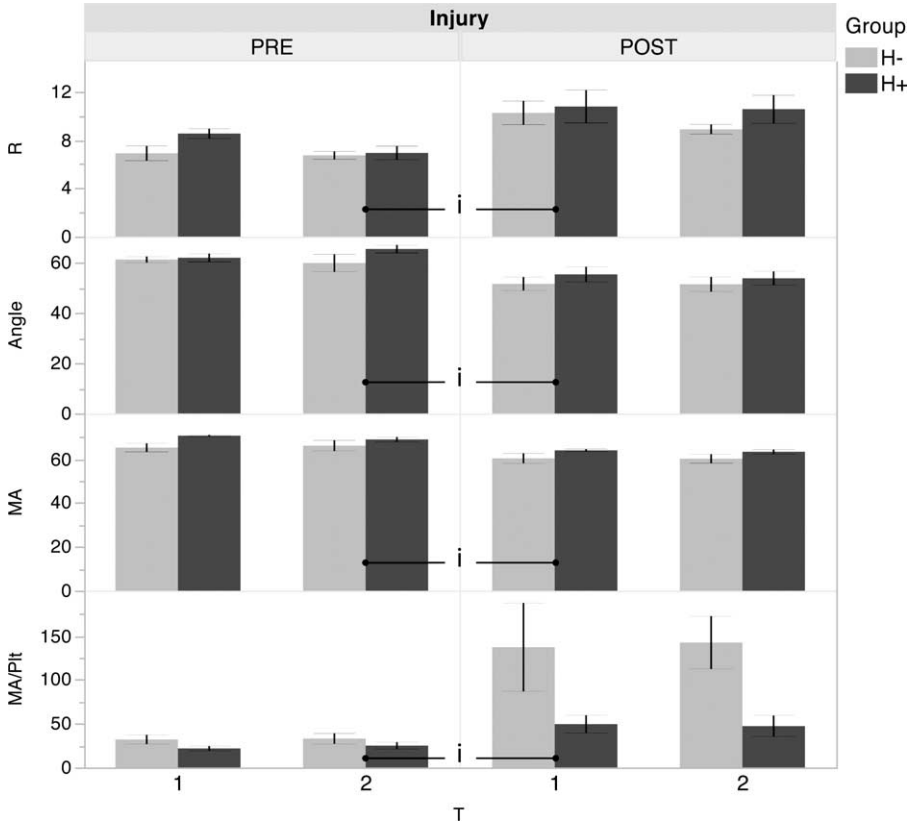

Fig. 2. **TEG parameters for each group of animals.** i: *injury* effect on TEG parameters are R ($P = 0.0043$), alpha ($P = 0.0046$), MA ($P < 0.0001$), and MA/Plt ($P = 0.0042$).

Documented changes in coagulation factor activity and complex formation are given in Figures 5 and 6. There was no clinically significant change in antithrombin III concentration over time, because of injury, or heparin concentration. Activity of coagulation factors V, VIII, and IX dropped after injury with $P = 0.0002$, $P < 0.0001$, and $P < 0.0001$, respectively. Overall, coagulation factor V decreased over time in healthy animals ($P = 0.0193$) and increased over time in injured animals ($P = 0.0046$). Coagulation factor IX activity increased in healthy animals ($P = 0.0003$) and then decreased in injured animals ($P = 0.0053$). vWF activity rose in the pre-injury period ($P = 0.0170$) and then dropped after the injury ($P = 0.003$) with no influence of heparin. The presence or absence of heparinase did not affect the measured activities of ATIII, V, VIII, IX, XII, and vWF, likely because of dilution in the assays (data not shown). The expected effect of heparinase was observed in the correction of aPTT, which does not involve sample dilution as in the factor assays ($P = 0.001$). Overall, no clinically significant reductions in factor activity occurred because of ECMO alone. Thrombin anti-thrombin (TAT) level rose during the pre-injury period ($P = 0.0304$), increased further during injury ($P = 0.0009$), and then decreased in the postinjury phase ($P < 0.0001$). There was no effect of injury or time on the generation of plasmin/antiplasmin complex (PAP). Heparin concentration (H+ and H−) had no effect on PT, heparinase aPTT, TAT, or PAP levels during the experiment ($P > 0.05$).

DISCUSSION

Over 26% of burned critically injured combat causalities develop moderate to severe acute respiratory distress syndrome (ARDS) with an overall mortality ranging from 36 to 43% (2, 6). Mortality from traumatic brain injury (TBI) in the United States is dropping; however, ARDS-related mortality after TBI is still common with over 28% in a recent large retrospective trial (9). ECMO is an effective therapy for severe ARDS failing mechanical ventilation therapy, although adoption of its use for trauma-induced ARDS has been prevented by the need for high-dose heparin anticoagulation and the associated bleeding risk (11, 15). This work evaluated the feasibility of using a low-dose heparin protocol with a state-of-the-art, mobile, compact ECMO device in an animal model of severe ARDS. This builds upon our previous work (27), which documented that spontaneous ventilation of both healthy sheep and sheep with acute respiratory distress syndrome can be controlled via extracorporeal gas exchange. For this study, we tested the effect of lower heparin dose on hemostasis compared with higher dose heparin anticoagulation. The effects of both ECMO circuit and heparin on standard ECMO monitoring tests (CBC, PT, aPTT, ACT, fPH) and coagulation factors were consistent with previous oleic-acid-induced ARDS ECMO and liquid ventilation animal studies (30, 31). Heparin doses in this model were relatively high in both groups because of the initial bolus and the heparin needed for the oleic acid load. Nevertheless, we demonstrated that roughly half of the standard heparin dose, given as an initial bolus, was

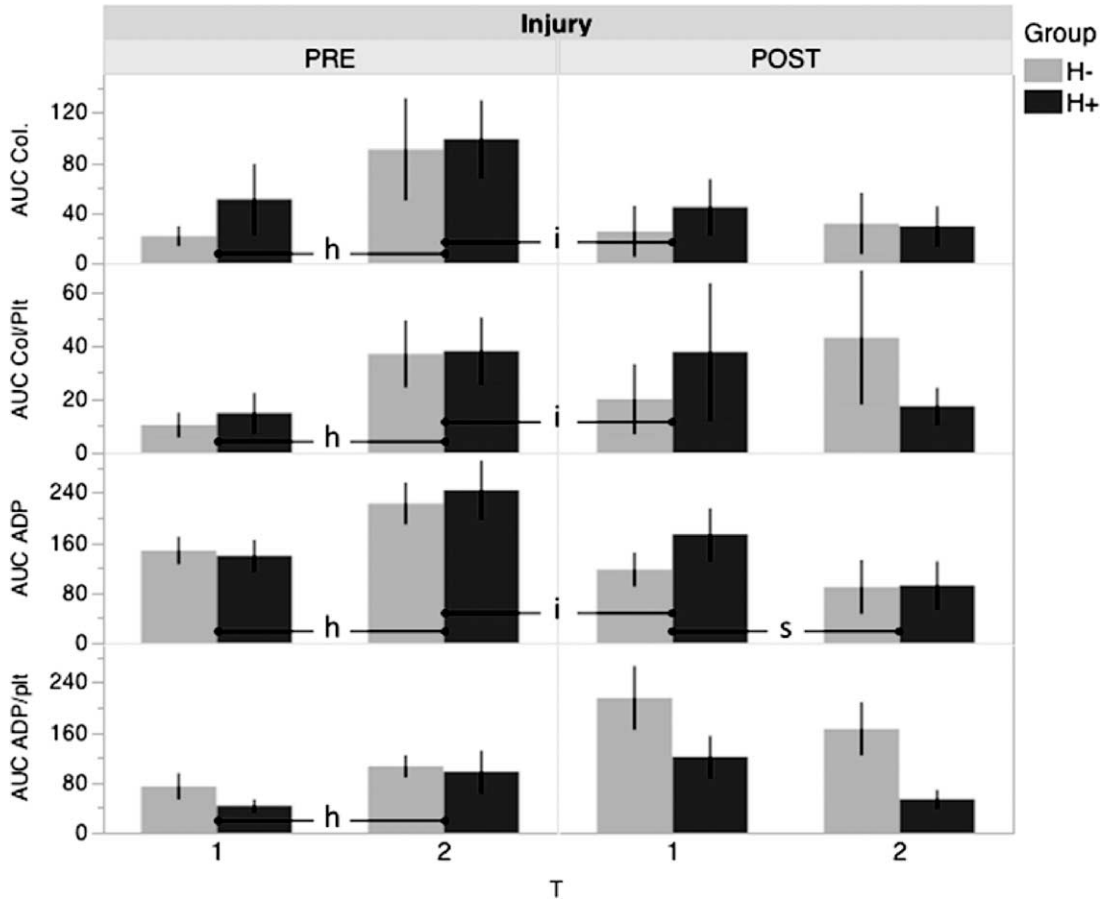

FIG. 3. **Platelet aggregometry function for each group of animals.** Platelet aggregometry stimulated with ADP and collagen with results given in units for area under curve (AUC) or AUC indexed on platelet count (AUC/Plt). h: the *time* effect on pre-injury group for AUC ADP and Col was $P = 0.0004$ for both and for the index values it was AUC ADP/Plt ($P = 0.0179$) and AUC Col/Plt ($P = 0.0056$). s: the *time* effect on postinjury group for AUC ADP was $P = 0.0127$. i: the *injury* effect was $P = 0.0010$ for AUC ADP, $P = 0.0009$ for AUC Col and $P = 0.0347$ for AUC Col/Plt.

adequate to maintain circuit patency in this VV-ECMO system. Moreover, there was little to no change in transmembrane pressures and no clinically relevant thromboembolic events noticed during the experiment. At post-mortem, we identified minor 1–3 mm soft clots on the venous side of the membrane, which were non-confluent.

The largest influence on changes in hemostasis was observed to be because of the lung injury caused by the oleic acid. The injection and the emulsification of the oleic acid can cause a significant amount of hemolysis, documented in our animals by the dramatic increase in the plasma-free hemoglobin concentration immediately after the lung injury. This can be damaging because free plasma hemoglobin release in the plasma causes free heme to react with endogenous hydrogen peroxide, forming toxic-free radicals that are involved in the induction of pro-oxidant damage (32). Furthermore, the nitric oxide scavenging effects of free hemoglobin may contribute to platelet activation and consumption as nitric oxide is an important physiologic regulator of platelet function (33). Indeed, the injury changed coagulation function by inducing a drop in

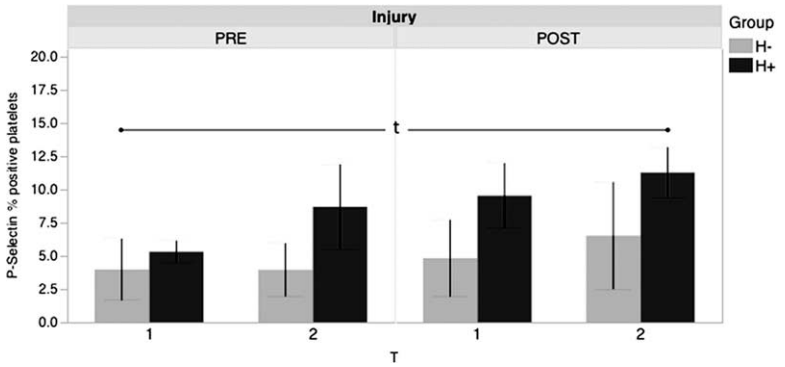

FIG. 4. **Platelet activation for each group of animals.** Results document the percentage of p-selectin expressing platelets. t: the *time* effect was calculated using repeated measure ANOVA, which gave a significant result throughout the whole experiment ($P = 0.0158$).

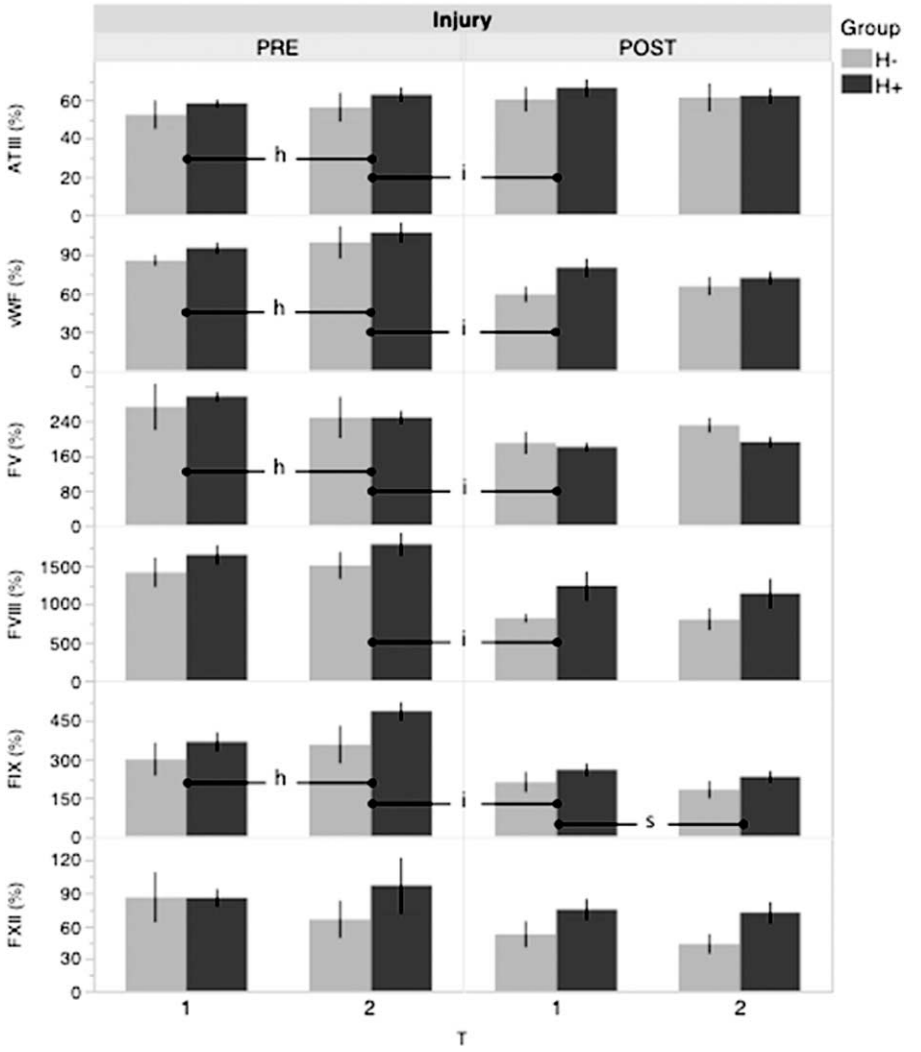

Fig. 5. **Coagulation factors activity for each group of animals.** Coagulation factors activity is represented as a percentage of normal activity for humans. i: the *injury* effect on vWF, ATIII and coagulation factors V, VIII and IX was $P = 0.0003$, $P = 0.0037$, $P = 0.002$, $P < 0.0001$ and $P < 0.0001$, respectively. h: the *time* effect on pre-injury for vWF, ATIII, Factor V and Factor IX was $P = 0.0170$, $P = 0.0332$, $P = 0.0193$ and $P = 0.0003$, respectively. s: the *time* effect on postinjury for Factor IX was $P = 0.0053$.

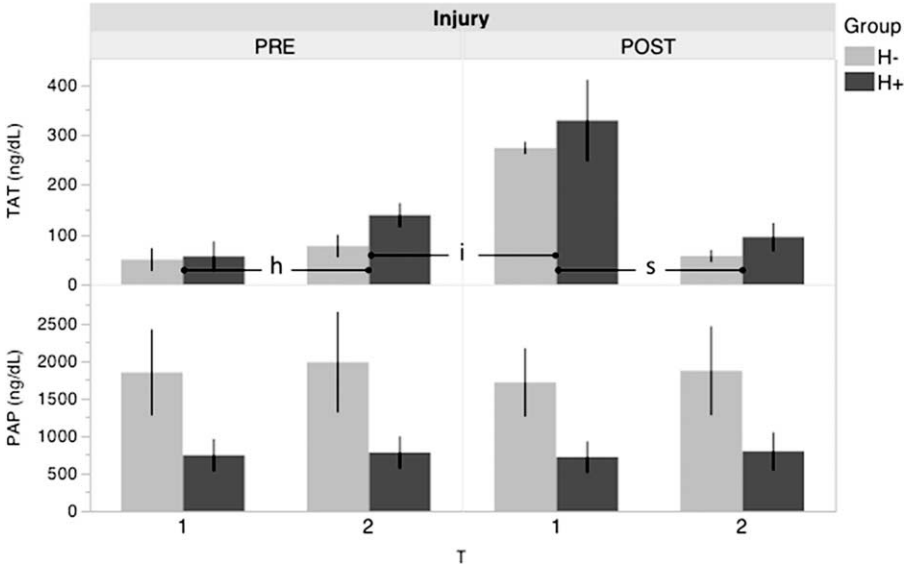

Fig. 6. **Plasma concentration of thrombin/antithrombin complex (TAT) and plasmin/antiplasmin complex (PAP) for each group of animals.** h,s: pre- and postinjury *time* effect was $P = 0.0304$ and $P < 0.0001$, respectively. i: the *injury* effect was $P = 0.0009$. There was no significant effect on PAP concentration.

platelet count, a rise in TAT levels, consumption of coagulation factors, a decrease in thromboelastography parameters, and a decrease in platelet aggregation. The use of heparinase neutralizes any heparin effect on most of the parameters measured, except platelet count and aggregometry. Interestingly, the platelet count was initially overall greater in the H+ group, but decreased at the same rate as the H− group. As expected, the effect of the heparinase was significant for TEG and aPTT, but not for coagulation factor activity. Moreover, coagulation factor activity assays are mixing studies based on changes in activity of PT and aPTT. Therefore, the addition of heparinase may not make a measurable difference because the heparin concentration upon mixing dilutes to subtherapeutic levels. Therefore, we are confident that the results documented here accurately present changes in coagulation as affected by the ECMO system, the injury, or both, and not merely the heparin effect.

The different heparin concentrations did not significantly affect platelet count, activation, or function. The drop in platelet count (Plt/hb) was consistent throughout all the experimental phases, with a significantly larger decrease after injury. The slope of platelet decline remained consistent before and after injury, regardless of heparin concentration or injury ($P = 0.1683$), demonstrating a known effect of all the ECMO systems. An isolated *group* effect on platelet count is observed because of an unexplained higher platelet count in the H+ group at baseline. When the combined *group∗time* interaction was examined, the heparin dose did not affect the rate of platelet clearance. The effect on platelet count by the ECMO system is associated with platelet activation as documented by changes in the p-selectin marker ($P = 0.0158$). Platelet activation and either consumption or clearance by the reticulo-endothelial system can be because of several mechanisms including heparin activation, contact with fibrinogen-coated synthetic surfaces, contact with blood–air interface, damage caused by blood suctioning or shear stress, and exposure to traces of thrombin, plasmin, ADP, or activated complement (34, 35).

The platelet activation and clearance because of ECMO is a well-known phenomenon (35, 36). The main proposed mechanism for platelet activation is contact with anionic foreign surfaces and deposited fibrinogen leading to adherence, as well as activation of the contact pathway and thrombin generation (37). In our study, fibrinogen levels were normal and did not change to a clinically meaningful degree (Table 2) raising the question as to whether platelet adhesion to fibrinogen-coated surfaces plays a significant role in platelet consumption in this system. Moreover, normal fibrinogen levels are not associated with heparin resistance commonly seen in trauma patients (38). Similarly, we saw little evidence of ECMO-driven activation of the contact pathway, as pre-injury changes in Factors VIII, IX, and XII were inconsistent and relatively minor. Another mechanism that causes platelet activation is the shear stress from the blood pump generating increased von Willebrand factor binding to GP1b and outside-in signaling (39–41). Our results support this platelet-led mechanism; the shear stress of the extracorporeal pump (39, 41) activates platelets, unfolding vWF, subsequent aggregation, and clearance (40). Platelet aggregation did increase during the pre- and postinjury phase. However, the oleic acid injection hemolysis significantly affected the response to ADP. Hemolysis can release a large amount of ADP inducing a refractory state to ADP stimulation for the remaining circulating platelets (33). Overall, the results document significant platelet activation and aggregation dependent on time in this state-of-the-art ECMO system, with minimal changes in coagulation factors, ATIII, TEG, and TAT levels. Additionally, adjustments in heparin concentration may not preserve overall hemostatic capacity but could increase bleeding risk while on ECMO support, at least for the short time periods studied in this protocol. Therefore, future studies are needed to target preventing or mitigating decreases in platelet count, function, and clearance. This is clinically relevant because maintenance of platelet count has traditionally been the most effective intervention to prevent hemorrhage on ECMO (38).

This study has several limitations. First, there was an assumption that the significance of combined *group∗time* affect would be the most sensitive predictor of the influence of heparin concentration. However, no statistical *group∗time* effect was found. This may be because of the short perfusion time, and with a longer time, the effect on platelet activation and coagulation factors might have become significant. Second, the use of animals makes the extrapolation of these findings to humans uncertain because of species differences in response to injury. Sheep blood has been documented to be very prone to shear stress resulting in hemolysis compared with human blood (42). Third, the oleic-acid-induced ALI model may not be an ideal model to represent trauma-related ARDS, although a study by Pffeifer et al. documented that oleic-acid-induced ALI model is consistent with other models, reproducible, and will develop early histological characteristics of ALI (43). Last, we compared the H+ group to a low heparin group and not a heparin-free group. Nevertheless, the present results suggest that much lower doses of heparin could be safely used to maintain trauma patients on ECMO during periods of high bleeding risk, thus potentially opening the door to wider use of ECMO for civilian or military transport. It will be important to further quantify the effects of such treatment protocols on platelet function, bleeding, and thrombosis to optimize both anticoagulation and transfusion support.

In conclusion, this study evaluated a two-stage animal model (pre- and postinjury phases) with obvious effects on the coagulation parameters. The largest effect was attributable to the induced acute respiratory distress syndrome, not to the ECMO circuit. During the whole experiment, this new ECMO circuit showed only few and slight effects on coagulation factors and no significant changes with a low dose of heparin. Moreover, additional analysis (data not shown) has demonstrated that heparin concentration does not affect extracorporeal gas exchange, respiratory parameters, blood gases, or chest CT scan imaging (27). However, there were significant increases in platelet activation, a decrease in platelet count, and a minor net decrease in platelet hemostatic capacity, but this did not cause clinically relevant bleeding or clot formation. These results should encourage military and civilian health care providers that ECMO without anticoagulation may be a safe alternative in the early phase of trauma. These findings also highlight the central role of the platelet and its activation in the clotting/coagulation complications that result from ECMO.

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