主論文

Title:

The N-terminal lectin-like domain of thrombomodulin reduces acute lung injury without anticoagulant effects in a rat cardiopulmonary bypass model

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Abstract

Objectives Systemic inflammation evoked by cardiopulmonary bypass (CPB) leads to acute lung injury (ALI) and respiratory failure. Although recombinant human soluble thrombomodulin (rTM) consists of 3 domains (D1-3) is reported to attenuate systemic inflammation through the N-terminal lectin-like domain (D1), anticoagulant domain (D2) may exacerbate coagulopathy after CPB. We investigated the potential of selective D1 against CPB-mediated ALI free from anticoagulant effects using a rat CPB model.

Methods Rats were divided into 3 groups: Control (CPB alone, n=5), D1 (CPB + D1, n=4), and D123 (CPB + D123, n=6). D1 or D123 were administered to the rats of each group before CPB establishment, respectively. Blood samples are collected at before, during and after CPB, respectively. Blood coagulability was assessed by a coagulation analyzer. Lung samples are collected at 1 hour after the termination of CPB for histological analyses.

Results D123 group exhibited significantly prolonged glass beads-activated clotting time with heparinase after CPB compared to that in Control group, whereas no significant prolongation in Control and D1 group (Control vs D1 vs D123: 65.4 ± 9.2 vs. 65.3 ± 10.9 vs. 83.5 ± 4.6 seconds, p= .036 [Control vs. D123], .99 [Control vs. D1]) indicating the absence of anticoagulant activities of D1. Histological studies revealed less congestion, edema, inflammation, and hemorrhage in both D1 and D123 groups compared to those in control group indicating protective effects of both D1 and D123 against ALI mediated by CPB.

Conclusions N-terminal lectin-like domain of rTM may reduce the risk of ALI without anticoagulant effects.

INTRODUCTION

Cardiopulmonary bypass (CPB) is associated with excessive secretion of inflammatory cytokines due to interaction between blood and artificial surface which can induce coagulopathy and systemic inflammatory response syndrome (SIRS) [1]. Respiratory failure is a crucial complication after cardiac and vascular surgery using CPB associated with increased mortality, and acute lung injury (ALI) triggered by SIRS is one of major causes of post-operative respiratory failure [2]. Although various therapies including alleviation of inflammatory cytokine [3], low-frequency ventilation [4] and the administration of anti-inflammatory organic compounds [5] were reported, no standardized strategy has been established to prevent CPB-derived pulmonary dysfunction associated with SIRS.

Thrombomodulin (TM) is a transmembrane protein expressed in endothelial cells and possesses anticoagulant activity. TM is composed of five domains; the extracellular N-terminal lectin-like domain (D1), epidermal growth factor (EGF)-like domain (D2), O-glycosylation-rich domain (D3), transmembrane domain (D4) and intracellular domain (D5). D1 has anti-inflammatory activities through binding site of high mobility group box 1 DNA-binding protein (HMGB1), one of damage-associated molecular patterns which is known as a cytokine mediator of inflammation [6]. D2 has a thrombin binding site which converts coagulant thrombin to the anticoagulant form and promotes activation of anticoagulant and anti-inflammatory protein C [7]. Recombinant human soluble thrombomodulin (rTM) is composed of active extracellular domains of TM (D1, D2 and D3) and is clinically available for the treatment of disseminated intravascular coagulation [8]. We have previously

reported that rTM prevents ALI through attenuating inflammation and apoptosis during and after CPB in a rat CPB model [9].

Post-operative bleeding tendency is one of noteworthy complications after CPB [10]. The situation is attributed by complex reactions of multiple hemostatic defects including blood dilution due to priming solution for CPB circuit, increased fibrinolytic activity and platelet dysfunction, and deficiency of coagulation factors due to interaction of blood and circuit surface [10-12]. Although the usage of rTM during CPB may alleviate inflammatory responses through D1, the anticoagulant activities of rTM mediated by D2 would be disadvantageous which may exacerbate coagulopathy after CPB.

In the present study, we investigated the therapeutic effect of D1 against lung injury and simultaneously examined anticoagulant effect of D1 using a rat CPB model.

MATERIALS AND METHODS

Animals and reagents

Fifteen male Sprague-Dawley rats (aged 10-11 weeks, 372-400 g of body weight; CLEA Japan Inc., Tokyo, Japan) were used in the present study. All rats were housed in air-conditioned room with free access to food and water at all time. The study protocol was approved by the Kyoto University Ethics Committee for Animal Research. All animals received humane care in compliance with the guidelines prescribed in the Principles of Laboratory Animal Care, formulated by the National Society for Medical Research, and the Guide for the Care

and Use of Laboratory Animals, created by the Institute of Laboratory Animal Resources of the National Research Council and published by the National Academy Press (revised 2011).

D1 and D123 were generously provided by Asahi Kasei Pharma Co. (Tokyo, Japan). D123 and D1 were prepared immediately before use and each dose was homogenized in 1.0 ml of saline. All group rats received the same volume of solution.

Study design and surgical procedure

The rats were randomly divided into 3 groups: Control (CPB alone, n=5), D1 (CPB + D1, n=4), and D123 (CPB+D123, n=6). We originally planned to include 18 rats (n=6 for each group) in the present study. However, we could not complete the 18 experiments because the supply of the membrane oxygenator stopped unexpectedly. Each rat was anesthetized with 3.0 % isoflurane-mixed air inhalation with a vaporizer. After the insertion of a 16-gauge cannula into the trachea, mechanical ventilation using a mechanical ventilator was established during the entire experiment with 8 ml/kg tidal volume, a respiratory rate of 70 cycles per minute, and 30 % of inspired oxygen fraction. Anesthesia was maintained with 1.5-2.0 % of isoflurane with an additional administration of 30 mg/kg intraperitoneal pentobarbital at CPB initiation. All subsequent procedures were performed after sufficient sterilization of skin.

CPB was performed using a surgical technique as previously described in our laboratory with some modifications (Fig. 1) [9, 13-15]. The right femoral artery was cannulated with a 24-gauge intravenous catheter (SURFLO ETFE I.V. Catheter; Terumo, Tokyo, Japan) to monitor systemic arterial pressure. D123 and D1 group

rats received a bolus of D123 (3 mg/kg) or D1 (equivalent to 3 mg/kg of D123) via left femoral vein at 30 minutes before CPB establishment. After systemic administration of heparin sodium (500 IU/kg), left femoral artery was cannulated with a 24-gauge intravenous catheter as an arterial infusion line for the CPB circuit. A 17-gauge multiorifice angiocatheter (Happy Cath 19-gauge inner diameter; Medikit Co, Tokyo, Japan) was introduced into the right external jugular vein and advanced into the right atrium and inferior vena cava. The CPB circuit was primed with approximately 11 ml of hydroxyethyl starch solution (Hespander; Fresenius Kabi Japan, Tokyo, Japan) with 0.35 ml of heparin and 0.5 ml of 7 % sodium bicarbonate solution. Blood was pumped from a venous reservoir through a modified membrane oxygenator (Senko Medical Instrument Mfg. Co., Ltd., Tokyo, Japan) using a tubing roller pump (model RP-VT; Furue Science Co., Tokyo, Japan).

Normothermic CPB with a flow of 70 ml/kg/min was performed for 90 minutes without using blood transfusion or any vasoactive agents [9, 13, 15]. After CPB termination, remaining blood in the CPB circuit was gradually re-transfused in 15 minutes and protamine sulfate (2.5 mg/kg) was administered thereafter. The ventilator management was continued for 60 minutes after termination of CPB, then rats were sacrificed to harvest the lungs. Heart rate and systemic arterial pressure were continuously monitored during the experiments. Rectal temperature was maintained at 37 °C using an electric mattress pad and a heat lamp placed over the animal and the CPB equipment. Arterial blood samples were collected just before administering D1 and D123, 30 minutes after CPB initiation, 15 minutes after and one hour after CPB termination, respectively. Hematocrit, hemoglobin concentration, pH, serum lactate, base excess, and partial pressure of arterial oxygen (PaO_2) and partial pressure of arterial carbon dioxide ($PaCO_2$) were measured by Epoc® Blood Analysis System (Siemens

Healthineers, Erlangen, Germany).

Blood coagulation examination

Blood coagulability was assessed by glass beads-activated test kit with heparinase of Sonoclot® (Sienco Inc., Boulder, USA) with 0.35 ml whole arterial blood samples collected just before administering D1 and D123, 30 minutes after CPB initiation, one hour after CPB termination, respectively.

The Sonoclot Analyzer provided information on the entire hemostasis process in a qualitative graph, known as the Sonoclot Signature (Fig. 2a). The typical Sonoclot Signature consists of first flat waveform representing coagulation reaction phase, second waveform called slope-shaped waveform representing clot formation phase and third waveform called chevron-shaped waveform representing clot retraction phase. The glass beads-activated clotting time with heparinase (H-gbACT) was measured in the first waveform as the time from beginning of the test to the onset of fibrin formation and the end of the liquid phase.

Enzyme linked immunosorbent assay

Concentrations of HMGB1 were assayed by the enzyme-linked immunosorbent assay sandwich method. Blood samples for HMGB1 were collected at 15 minutes after CPB termination. Plasma samples for HMGB1 were collected by centrifugation of whole blood samples at 1000×g for 20 minutes at 4 °C. Plasma levels of HMGB1 (Shino-Test Corporation, Tokyo, Japan) were measured using a commercial enzyme-linked immunosorbent assay kit according to the manufacturer's protocols, respectively. The values of absorbance were measured by

a microplate reader (Bio-Rad Laboratories Inc, Hercules, USA) at 450 nm.

Ratio of Partial Pressure of Arterial Oxygen to Fraction of Inspired Oxygen

PF Ratio was calculated using the collected arterial blood samples just before administering D1 and D123 and 1 hour after CPB termination.

Measurement of Wet-to-Dry Weight Ratio of the Lung

The water content of the lung, representing the severity of pulmonary edema, was measured by calculating the wet-to-dry weight ratio of lung tissues [16]. The upper lobe of the left lung was weighed and dried on a heating device at 75 °C for 72 hours to calculate the wet-to-dry weight ratio of the lung.

Histological Examination

The lungs were harvested from animals 1 hours after CPB, fixed in 4 % phosphate-buffered paraformaldehyde, embedded in paraffin, cut into 4-μm sections, then stained with hematoxylin and eosin. Images were photographed using an all-in-one digital microscope (BZ-X810; Keyence, Osaka, Japan) and assessed using the BZ-X Analyzer (Keyence) software. The extent of lung injury was evaluated by a previously reported method [18]. Twenty-five areas of lung tissue were graded in a blinded manner on a scale of 0 to 4 (0 = abnormalities absent and tissue seems normal, 1 = light, 2 = moderate, 3 = strong, and 4 = intense) for the degree of congestion, edema, inflammation, and hemorrhage. Mean score for each parameter was calculated.

Statistical Analysis

Statistical analysis was performed using JMP Pro release 14.0.0 (SAS Institute Inc. Cary, USA). The values are presented as mean ± standard deviation. Steel-Dwass nonparametric multiple comparison procedure were applied for comparison of each group variables. Exact Wilcoxon-Mann-Whitney nonparametric comparison procedure were applied for comparison of change of each group data with time in PF ratio. Steel nonparametric multiple comparison procedure were applied for comparison of change of each group data with time (control = just before administering reagents). The p value <0.05 was considered statistically significant.

Results

Physiological Parameters and Blood Gas examination before, during and after CPB

All rats of each group survived during the CPB procedures and for 1 hour after the operation. The physiological data and atrial blood gas analysis data are summarized in Table 1. Hemodynamics were stable in all 3 groups during the experiment. During and after CPB, hemoglobin decreased possibly because of hemodilution due to priming solution of CPB circuit. There was no significant difference in the value of hemoglobin during and after CPB between three groups.

Blood coagulation examination

Values of H-gbACT during CPB were significantly longer in D123 group compared to those in other groups, whereas no difference between Control and D1 groups (Control vs D1 vs D123: during CPB: 114.8 ± 10.2 vs. 125.5 ± 7.0 vs. 148.3 ± 13.6 seconds, p = 0.24 [Control vs. D1], = 0.021 [Control vs. D123], = 0.037 [D1 vs. D123]). Although values of H-gbACT at 1 hour after CPB were significantly longer in D123 group than those in Control group, there was no significant difference between Control and D1 groups (65.4 ± 9.2 vs. 65.3 ± 10.9 vs. 83.5 ± 4.6 seconds, p = 0.036 [Control vs. D123], = 0.99 [Control vs. D1]). H-gbACT at 1 hour after CPB was significantly longer than that before CPB in D123 group (before CPB vs. after CPB: 69.0 ± 6.4 vs. 83.5 ± 4.6 seconds, p = 0.033), whereas no significant difference in Control and D1 groups, respectively (Fig. 2b).

Histological Examination of the Lung

Histological evaluations of the lung 1 hour after CPB were shown in Fig. 3. Congestion scores and Edema scores were significantly lower or at lower tendency in D1 and D123 groups compared to those in Control group, respectively Control vs. D1 vs. D123: Congestion: 2.9 ± 0.2 vs. 2.0 ± 0.3 vs. 1.5 ± 0.6, p = 0.052 [Control vs. D1], = 0.022 [Control vs. D123] / Edema: 3.0 ± 0.3 vs. 1.9 ± 0.1 vs. 1.5 ± 0.5, p = 0.052 [Control vs. D1], = 0.022 [Control vs. D123]). Hemorrhage scores and Inflammation scores were significantly lower in D1 and D123 groups than those in Control group, respectively (Hemorrhage: 3.0 ± 0.3 vs. 2.0 ± 0.2 vs. 1.4 ± 0.5, p = 0.049 [Control vs. D1], = 0.021 [Control vs. D123] / Inflammation: 2.8 ± 0.2 vs. 1.9 ± 0.1 vs. 1.6 ± 0.6, p = 0.049 [Control vs. D1], = 0.044 [Control vs. D123]). There was no significant difference between D123 and D1 groups in all of histological evaluation scores (p = 0.404 [Congestion], = 0.292 [Edema], = 0.339

[Hemorrhage], = 0.337 [Inflammation]).

PF Ratio and Wet-to-Dry Ratio of the Lung

To evaluate oxygenation function, we calculated PF Ratio and compared them between before and after CPB. PF ratio significantly decreased in Control and D123 groups, and exhibited a decreased tendency in D1 group at 1 hour after CPB compared to those before CPB (Control vs. D1 vs. D123: before CPB: 463.6 ± 51.8 vs. 451.3 ± 48.9 vs. 487.3 ± 42.1; after CPB: 355.5 ± 53.2 vs. 382.2 ± 33.4 vs. 397.5 ± 38.5, p = 0.015 [Control; before CPB vs after CPB], 0.11 [D1; before CPB vs after CPB], 0.004 [D123; before CPB vs after CPB]). There was no significant difference between 3 groups at each timing.

We calculated wet-to-dry ratio of the lung as a parameter of lung edema. There was no significant difference in wet-to-dry ratio between 3 groups (4.93 ± 0.86 vs. 4.53 ± 1.46, vs. 4.08 ± 0.34 [Control vs. D1 vs. D123]).

Systemic Inflammatory Responses Indicated by Plasma Levels of HMGB1

Plasma levels of HMGB1 was not significantly different between 3 groups (Control vs. D1 vs. D123: 14.1 ± 2.1 vs. 10.6 ± 3.3 vs. 10.3 ± 3.5 ng/ml, p = 0.33 [Control vs. D1], = 0.26 [Control vs. D123]).

Discussion

In the present study, we have evaluated the anti-inflammatory and anticoagulant effects of selective D1 of

thrombomodulin in a rat CPB model. The results suggested that administration of D1 before CPB establishment could reduce CPB-mediated ALI without coagulopathy during and after CPB.

Respiratory failure remains as a serious complication after cardiac and vascular surgery using CPB associated with increased mortality. Filsoufi and colleagues [18] retrospectively analyzed the New York State Department of Health database and reported that the incidence of respiratory failure was 9.1% and the mortality rate of patients with respiratory failure was significantly higher than that without respiratory failure (15.5% vs. 2.4%). Other reports indicated that the mortality rate of patients with ALI was higher than 36.0% [19, 20]. Previously, we have reported that the administration of rTM as D123 attenuated systemic inflammation associated with reduced serum HMGB1 level and prevented ALI in a rat CPB model [9]. Although there was no significant difference in plasma level of HMGB1, PF ratio and wet-to-dry ratio among 3 groups in the present study, D1 and D123 groups showed significantly smaller pathological changes of the lung compared to that in untreated control. These results might suggest that not only D123 but also D1 holds a protective effect against CPB-mediated ALI as well. The results may also indicate that there would be other mechanisms of D1 than the involvement of HMGB1 which affected to the attenuation in pathological changes in lungs and were not clarified in the present study.

CPB is associated with coagulopathy because of multiple factors including hemodilution due to priming solution for CPB circuit, physical damage of blood cells due to negative pressure of blood suction and shear stress of roller pump, dysfunction of platelet and coagulation factors due to interaction of blood and circuit surface, and so on. In the present study, we have shown that the administration of D123 excessively prolonged

H-gbACT during and after CPB compared to those before CPB. On the other hand, D1 administration and untreated control did not affect the duration of H-gbACT. These results may indicate that D1does not affect coagulation system when administrated prior to CPB. It is reported that the activation level of protein C mediated by thrombomodulin is relatively weak in rats compared to that in human [21]. Considering that the administration of D123 in our rat CPB model even caused excessive coagulation activity, it might be possible to estimate that the higher level of H-gbACT after CPB compared to that before CPB in rats treated with D123 would reflect a situation of coagulopathy and to anticipate the occurrence of more severe coagulopathy at human CPB using D123. In this regard, selective D1 may hold promise for safer therapy without possibility of severe coagulopathy.

The present study holds several limitations. First, the membrane oxygenators we used in the present study were custom-made for small animal experiments and the structure is not completely comparable to those in clinical use. This point may raise inconsistencies of data according to the quality of the membrane oxygenators. Clinical studies with clinical-grade CPB would be anticipated to address this concern. Second, there is an interspecific difference between rat and human which raises a possibility that the anti-inflammation effect and the change of the coagulability might not be relevant to those in human. This limitation encourages us to conduct further clinical studies for the standardization of the present strategy in which other parameters than ACT such as clot rate and platelet function should be evaluated as well, and biological effects of each domain of TM should be further investigated considering the broad spectrum of the protein. Finally, experimental volume in the present study might not be enough to fully validate our hypothesis because the availability of membrane

oxygenator was rather limited.

Conclusion

Selective D1 of thrombomodulin may have the potential against CPB-mediated ALI free from anticoagulant effects. The results of the present study may contribute to a new therapeutic strategy for safer cardiac and vascular surgery using CPB.

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Conflict of interest statement

The authors declare that no conflict of interest exists.

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Figure legends

Figure 1: Experimental protocol and procedure.

(a) Schematic illustration of the study protocol. Red allows indicate timings of blood sampling. **(b)** Schematic illustration of the surgical procedure of rat cardiopulmonary bypass model. *ABG* Atrial blood gas, *HMGB1* high-mobility group box 1

Figure 2: Sonoclot® signature and variables.

(a) Sonoclot® signature and ACT. **(b)** Glass beads-activated clotting time with heparinase measured by Sonoclot®. *ACT* Activated clotting time (the time to the onset of fibrin clot formation). *: $p<0.05$ versus Control group, †: $p<0.05$ versus. pre-CPB

Figure 3: Histological evaluations of the lungs.

(**a - c)** Representative hematoxylin and eosin staining images (**a)** Control group, (**b)** D1 group and (**c)** D123 group, respectively. Scale bars = 50 μm. (**d)** Scoring results of the extent of congestion, edema, inflammation, and hemorrhage. *; $p < 0.05$ versus Control group.

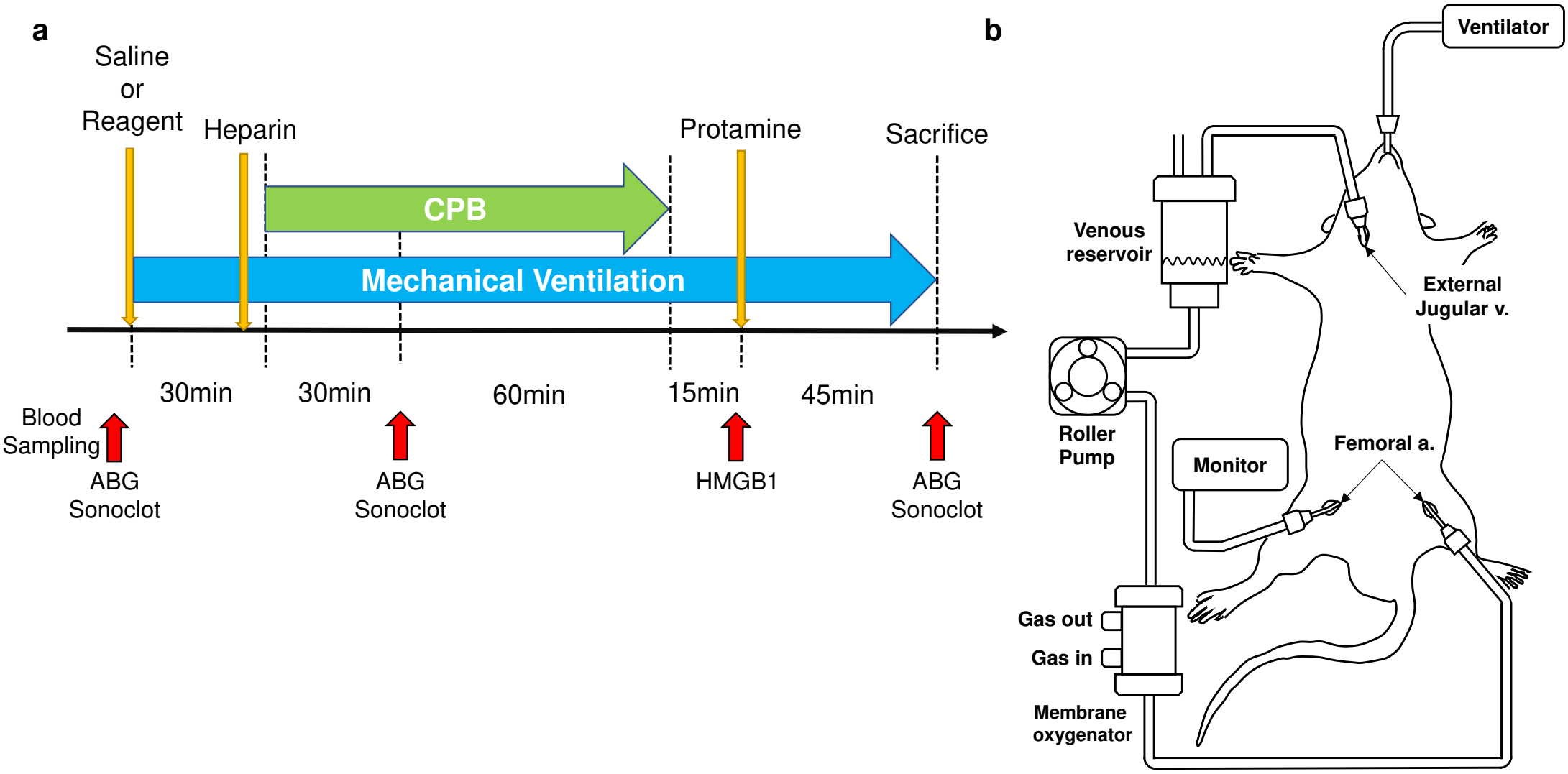
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Fig. 1

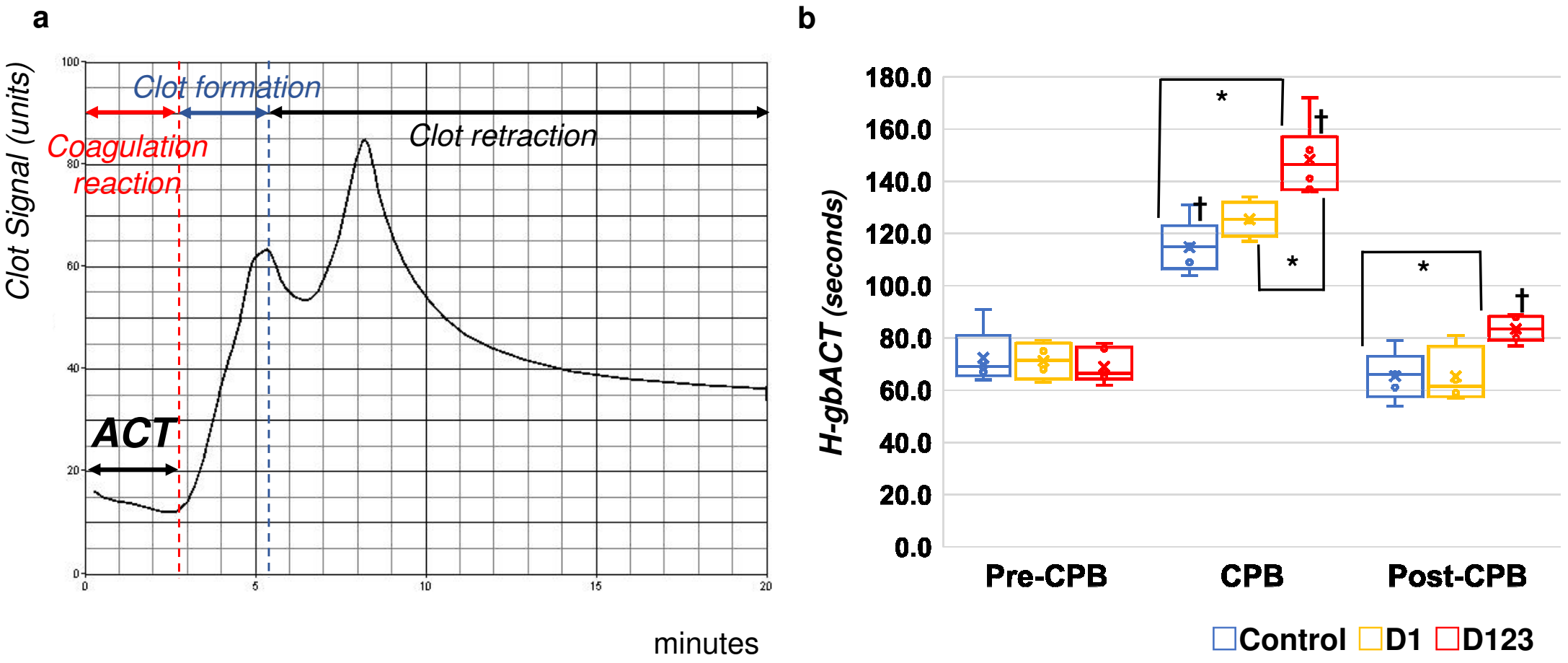
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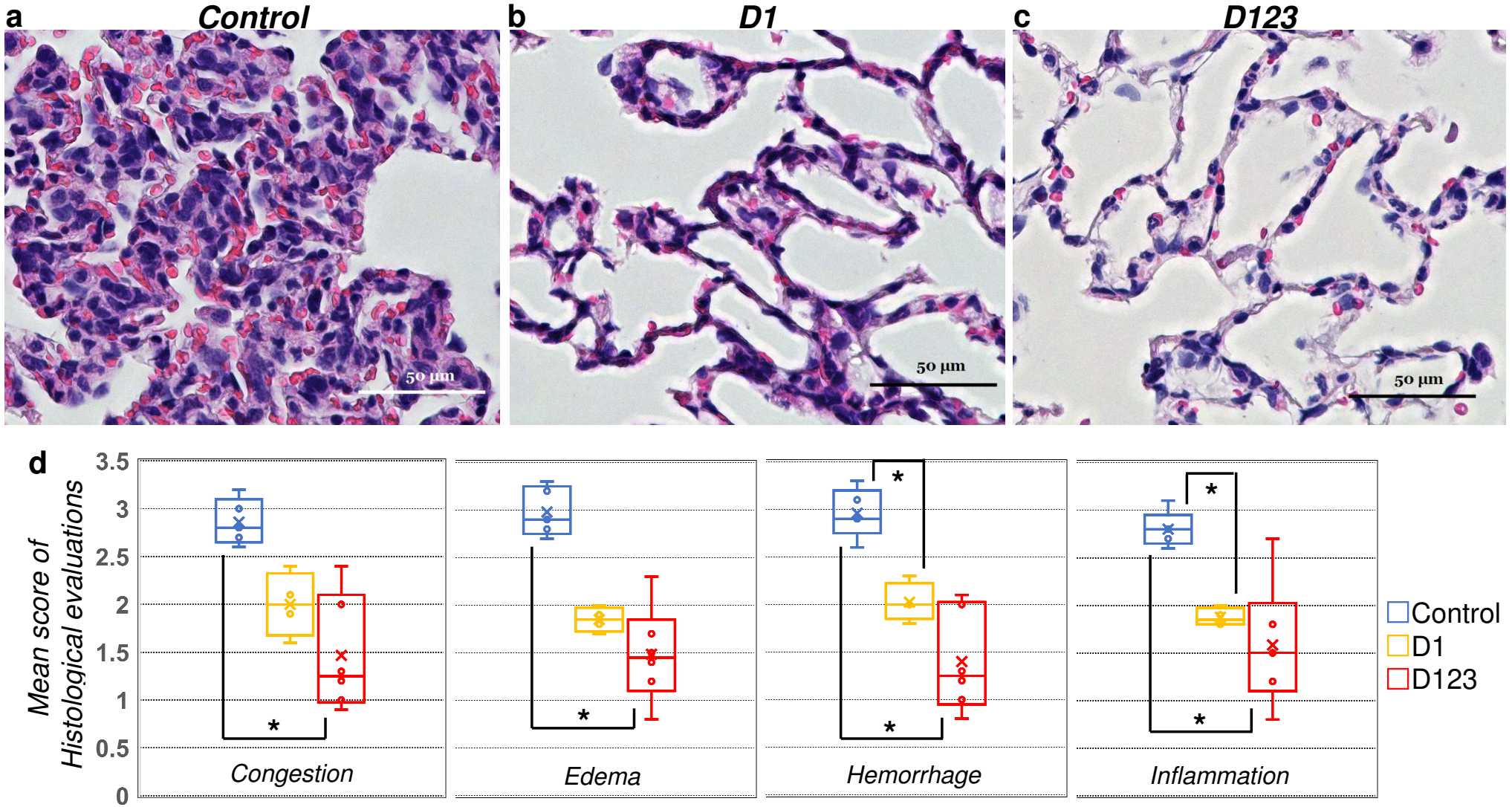
Fig. 2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Control
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Fig. 3