Peer Review File

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Reviewer A

Comment 1: Introduction: The author states that previous models use inconsistent clot sizes. I don't agree. Clot size is easily adjusted and not decided by route of injection EBUS-TBNI or central venous access. The novel and potential advantage of the method described in the paper is that clots can be delivered more selectively to specific pulmonary artery branches as described in the methods.

Reply 1: Thank you for your comment. 'Inconsistent clot size' was in reference to the fragmentation of clots during delivery, and thus lack of control for clot size to specific areas; we agree this is likely better understood in the context of selectivity. We have corrected the following sentence.

Changes in the text:
Abstract session, Page 3, Line 6
Current massive pulmonary embolism animal models use central venous access to deliver blood clots, which have features of random clot distribution and potentially fatal hemodynamic compromise.

Introduction session, Page 5, Line 5
These models can replicate the disease state well with features of random clot distribution [1, 2] and potentially fatal hemodynamic compromise [3, 4]. However, this variability can make these models challenging.

Comment 2: Clot in figue 4C looks a lot bigger than 3 ml. Please elaborate?

Reply 2: Initially, 3mL of clot was injected into a lower lobe pulmonary artery. If embolus was not clearly seen by EBUS, additional 1mL clots were administered until visualized. The average of injected blood clot volume was 6.6 ± 2.3 ml in this study. In terms of the embolus shown in figure 4C, 7 mL clot was injected into the right lower lobe pulmonary artery. We have added the following sentence to clarify.

Changes in the text:
Results section, Page 11, Line 6
In 10 pigs, all 20 blood clots (average clot size 6.6 ± 2.3 ml, supplemental table1) were successfully injected.

Figure legend, Page 19, Line 6
(C) Postmortem examination confirms embolization of the right caudal lobe pulmonary artery; this specific embolus followed injection of 7 mL clot into the right lower lobe pulmonary artery.

Comment 3: How many pigs were excluded from the study?

Reply 3: No pigs were excluded from the study. We have made this clearer in the manuscript by adding the following modification.

Changes in the text:
Result section, Page 12, Line 6
All animals survived the procedure; no pigs were excluded from the study.

Comment 4: Did you save some blood for BNP, TNI or TNT analysis. Did you evaluate RV strain? This could be of interest to correlate the animal model to clinical presentation (low, intermediate, high-risk/submassive, massive).

Reply 4: Thank you for your comment. BNP, TNI or TNT analysis, and RV strain evaluation were not performed in this study. We agree that these factors would be of interest to correlate the animal model to clinical presentation. As we look to employ this model for more complex analysis of PE treatments, we agree that BNP, TNI or TNT analysis, and RV strain evaluation should be included in any future study.

Changes in the text: No change.

Reviewer B

A discussion of the results and clinical relevance is a serious missing point in this manuscript. Please comment on:

Comment 1: The PE risk group and relevance for further research in the model.

Reply 1: Thank you for your comment. BNP, troponins analysis and RV strain evaluation were not performed in this study. Therefore, stratifying pigs as stable (low-risk) and sub-massive (intermediate-risk) is not possible (high-risk/massive PE did not develop in this study, as reflected by the relative hemodynamic stability of all pigs). We agree that these factors would be of interest to correlate the animal model to clinical presentation. As we look to employ this model for more complex analysis of PE treatments, we agree that BNP, troponins analysis, and RV strain evaluation should be included in any future study. We have added the following sentence to acknowledge this limitation.

Changes in the text:
Conclusion section, Page 14, Line 8
Third, BNP, troponin analysis, and echocardiography were not performed in this study. Therefore, stratifying pigs as stable (low-risk) or submassive (intermediate-risk) was not possible; no pigs developed profound hemodynamic instability that would otherwise be consistent with massive (high-risk) PE. These tests would be needed to more fully correlate the animal model to clinical presentations.

Comment 2: The administration form with 21 G needle does not seem to correlate with

physiologically/naturally PE. Please support your claims with relevant literature.

Reply 2: Thank you for your comment. We agree that clots administered via a 21 G needle do not perfectly mimic 'natural' PE, which is likely better achieved using the central vessel access approaches described in our introduction. Although we believe the more controlled clot formation offered by our model plays an important role for initial development of new technologies/techniques relevant for PE management, we have added the following sentence to acknowledge this limitation:

Changes in the text:
Conclusion section, Page 14, Line 6
Moreover, clot size and shape formed via an EBUS-TBNI approach are likely less 'clinically realistic' than those formed via central vessel access.

Comment 3: How much of the response after 10 min is mechanical and what is vasoconstriction, could the evaluation times/sampling be changed?

Reply 3: It is not possible to distinguish between mechanical resistance from the clot and vasoconstriction with our current protocol; we expect that both likely contributed to the hemodynamic changes, as in the clinical setting. We measured hemodynamic parameters at 10, 30, 60 and 120 mins after clot injection. The hemodynamic response was observed immediately after clot injection. These hemodynamic parameters didn't show remarkable change over time except for heart rate. The rapidity of onset would be in keeping with a predominantly mechanical cause for our results. We have created a supplemental table2 including hemodynamic measurements over 120 mins.

Changes in the text:
Results section, Page 11, line 17
Serial monitoring out to 120min showed stability in all hemodynamic parameters post-clot injection except for heart rate, which gradually normalized over time (Supplemental Table 2).

Conclusion section, Page 12, Line 12
Hemodynamic changers were observed immediately after clot injection; except for the gradual normalization of heart rate, there was little change over (Supplemental Table 2.).

Supplemental Table 2. Summary of hemodynamic measurements

Comment 4: Methodological rigidity is poorly described and potentially missing, especially in terms of clot size and distribution which is contradictive to the introduction.

Reply 4: Thank you for your comment. We have created Supplemental Table 1, which includes clot size, distribution, and pig weight to improve readers' understanding.

Changes in the text:

Results section, Page 11, Line 6
In 10 pigs, all 20 blood clots (average clot size 6.6 ± 2.3 ml, supplemental table 1) were successfully injected.

Comment 5: Age and weight of the pigs is very different. This might have induced variation in the responses and thereby results.

Reply 5: Thank you for your comment. The supplier provides pigs based on weight rather than age, as the pigs will generally gain weight at a consistent pace. We created a supplemental table including pigs' weights to improve clarity. Looking at this data in aggregate, it is clear the 48kg pig is an outlier; use of this pig was delayed due to experimental scheduling that resulted in additional weight gain. Most pigs were within in the low 30kg weight range.

Changes in the text:
Methods section, Page 6, Line 3
Yorkshire pigs (30-48 kg, mean 33kg; Caughell Farms Inc, Fingal, ON, Canada, supplemental table1) were premedicated with intramuscular ketamine (20 mg/kg, Bimeda, Ontario, Canada), midazolam (0.3 mg/kg, Fresenius Kabi, ON, Canada), and atropine (0.04mg/kg, CDMV Inc., QC, Canada) followed by anesthetic induction with isoflurane (Fresenius Kabi, Canada).

Comment 6: The fraction of oxygen at 0.5 could be confounding factor as some studies have shown that oxygen treatment increases vasodilation in the pulmonary arteries, why we could see a different response at physiological oxygen fractions.

Reply 6: Thank you for your comment. We recognized that oxygen treatment could have an effect, hence the use of 50% oxygen rather than the standard 100% oxygen feed at our facility. The focus of this experiment was to keep, as much as possible, the clots themselves as the modifiable variable. We did not want to be changing ventilatory or anesthetic parameters during the experiment as this would confound the analysis, as you have noted. The 50% FiO2 was selected to ensure anesthetic safety but avoid profound hyperoxemia. With the data we now have, we agree that the FiO2 can likely be safely lowered for future experiments, including evaluation of differential effects of FiO2.

Changes in the text: No change.

Comment 7: P 4 L 1; change to "clots".

Reply 7: Thank you for your suggestion. We have corrected the sentence.

Changes in the text:
Abstract section, Page 4, Line 4
Clots.

Comment 8: P 5 L 5-6; This is debatable and it formulated to be general, consider rephrasing.

Reply 8: Thank you for your comment. We have corrected the sentence.

Changes in the text:
Introduction section, Page 5, Line 5
These models can replicate the disease state well with features of random clot distribution [1, 2] and potentially fatal hemodynamic compromise [3, 4].

Comment 9: P 5 L 13; Move line to "Methods" section.

Reply 9: Thank you for your comment. We have moved the sentence line to Methods.

Changes in the text:
Methods section, Page 6, Line 2
We present the following article in accordance with the ARRIVE reporting checklist.

Comment 10: Methods: consider to alter the sections to a chronological order related to the protocol.

Reply 10: Thank you for your comment. We have altered the sections following a chronological order related to the protocol.

Changes in the text:
Methods section, Page 5, Line 17

Methods
Instrumentation and measurements
The study was approved by the University Health Network Animal Care Committee (AUP5928). We present the following article in accordance with the ARRIVE reporting checklist. Yorkshire pigs (30-48 kg; Caughell Farms Inc, Fingal, ON, Canada) were premedicated with intramuscular ketamine (20 mg/kg, Bimeda, Ontario, Canada), midazolam (0.3 mg/kg, Fresenius Kabi, ON, Canada), and atropine (0.04mg/kg, CDMV Inc., QC, Canada) followed by anesthetic induction with isoflurane (Fresenius Kabi, Canada). Pigs were positioned supine, intubated using an endotracheal tube (Mallinckrodt ID 8.5; Covidien, MA, USA) and ventilated with tidal volume 7mL/kg; respiratory rate 14 breath/min and zero end-expiratory pressure. The fraction of inspired oxygen was 0.5. Oxygen saturation (SpO2) by ear pulse oximeter, end-tidal CO2 (ETCO2) and heart rate (HR) by 3-lead electrocardiogram were measured using a multi-parameter monitor (BM3Vet touch; Bionet Co., Ltd., Seoul, Republic of Korea). Tidal volume and respiratory rate were adjusted to achieve baseline ETCO2 35-45 mmHg and SpO2 94-100%. Central vascular access was obtained by dissection of the femoral vessels. A 9-French (F) sheath (I505BF9; Edwards Lifesciences Corp., Irvine, CA, USA) was placed in the right femoral vein for blood collection and introduction of a 7F Swan-Ganz catheter (111F7; Edwards Lifescience, USA). The catheter was advanced into the main pulmonary artery under fluoroscopy. A 20-G peripheral intravenous

catheter (BD 382537; Becton, Dickinson and Company, USA) was placed in the ipsilateral femoral artery for arterial sampling and blood pressure monitoring. Pressure transducers monitored mean systemic pressure (MAP) and mean pulmonary artery pressure (mPAP). Arterial blood gases and lactate were measured using a blood gas analyzer (RAPIDPoint 500; Siemens Healthcare, Erlangen, Germany).

Preparation of pulmonary emboli

Emboli were formed ex vivo from autologous blood collected in 30mL syringes (BD 309650; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) via a central venous catheter (Figure 1A). These were incubated for 2 hours at 37oC (LR19654; Foster Refrigerator, New York, NY, USA) (Figure 1B). No medications were admixed into the blood. After incubation, the clot was dispensed into 1 mL syringes (BD 309628; Becton, Dickinson and Company, USA) for injection (Figure 1C,D).

Experimental protocol

All animals underwent the protocol shown in Figure 3. Approximately 170 mins after anesthetic induction, baseline (B) values were documented. Additional measurements were obtained 10 minutes after clot injection 1 (PE1) and 10 minutes after clot injection 2 (PE2). PE1 and PE2 were administered sequentially. Initially, 3mL of clot was injected into a lower lobe pulmonary artery via EBUS-TBNI (see below). Successful injection was confirmed by EBUS, including Doppler. If embolus was not clearly seen, additional 1mL clots were administered until visualized. This process was repeated for the contralateral lung 20 mins after PE1. All animals were observed for 2 hours after PE2 for documentation of complications including airway evaluation by bronchoscopy (BF-UC180F or BF-160; Olympus). Pigs were then either sacrificed or used for further study, when possible.

Endobronchial ultrasound and ultrasound processor

Convex-probe endobronchial ultrasound (EBUS; BF-UC180F; Olympus, Tokyo, Japan) was performed with a dedicated ultrasound processor (EU-ME1; Olympus). Emboli were injected using a 21-G EBUS-transbronchial needle aspiration (EBUS-TBNA) needle (NA-201SX-4021; Olympus).

Administration of pulmonary emboli via endobronchial ultrasound-guided transbronchial needle injection (EBUS-TBNI)

The pulmonary artery was visualized by EBUS (Figure 3A), including Doppler (Figure 3B). An optimal injection site was selected; features of an ideal injection site included visualization of pulmonary artery branching and a clear path to the artery. Lower lobe arteries were used in this study due to ease of access and clear visualization. A 21-G EBUS-TBNA needle was inserted into the visualized pulmonary artery branch under ultrasound guidance (Figure 3C). Entry into the arterial lumen was confirmed by aspiration of the TBNA needle. Once backflow was confirmed, a pre-formed autologous blood clot was administered into the pulmonary artery using real-time EBUS guidance (Figure 3D).

Statistical analysis

Baseline and post-clot injection data were compared using a repeated measures

ANOVA with Tukey's Honest Significant Difference test. Relation between injected clot volume and mPAP was analyzed by linear regression and correlation. A p-value of <0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software ver.9, San Diego, CA, USA, RRID:SCR_002798). Values are expressed below as mean ± SEM.

Pathologic Evaluation
Lungs and emboli were collected from euthanized animals and fixed in 10% formalin. Representative slides from embolised and normal lung were stained with hematoxylin and eosin.

Comment 11: Results P 10 L 10-16: this is methods, move and scribe further.

Reply 11: Thank you for your comment. We have moved this sentence into methods section.

Changes in the text:
Methods section, Page 9, Line 15
Ultrasonic imaging, radiological imaging, and pathologic evaluation of pulmonary emboli
Post-PE EBUS confirmed the presence of pulmonary emboli at the planned injection sites (B-mode; Figure 3E) with disruption of blood flow (Doppler; Figure 3F). Pulmonary angiography (Figure 4A) and contrast-enhanced cone beam computed tomography (Figure 4B) performed in representative pigs demonstrated intravascular filling defects consistent with emboli. Post-mortem examination revealed significant pulmonary emboli (Figure 4C: The emboli was observed after injection of 7 mL clot into the right lower lobe pulmonary artery). Microscopic examination demonstrated large arterial emboli with adjacent hemorrhagic regions consistent with pulmonary infarction (Figure 4D)

Comment 12: Table 1; please add the analysis used to compare data. Also, consider to add "compared to baseline" in the legend instead of in the figure text.

Reply 12: Thank you for your comment. We have added the following sentence.

Changes in the text:
Table 1
Baseline and post-clot injection (PE1 or PE2) parameters were compared using a repeated measures ANOVA followed by Tukey's Honest Significant Difference post-hoc test.

Reviewer C

Comment 1: A new model is fine and the physiological changes do mimic clinical PE. However, technically it is more difficult to do (presume one need to have advanced bronchoscopic skills and tools). So I can also understand why other research groups would use conventional venous access to deliver blood clots into the pulmonary

circulation. It's cheaper and easier.

Reply 1: Thank you for your comment. We agree that conventional venous access is cheaper and easier than transbronchial access to deliver blood clots into the pulmonary circulation. The potential advantage of this method is that clots can be delivered more selectively to specific pulmonary artery branches. It may allow more controlled head-to-head comparison of new treatments with 'equivalent' clots to guide treatment optimization before larger, more heterogeneous trials.

Changes in the text: No change.

Comment 2: This study is limited by the lack of a suitable control group. Given EBUS is more expensive and more difficult to do, how does this model compare with the traditional model using clots delivered by central venous cannulation?

Reply 2: Thank you for your comment. This new model using EBUS was not compared with the traditional model with clots delivered by central venous cannulation in this study. We will consider comparing the new model with traditional models using the same clot injection volume to evaluate differences in clot distribution, hemodynamic and biochemical parameters including RV function, and complication rate including fatal events, in a future study. However, our view is that the new model and traditional models have different uses for preclinical research. We have added the following sentence.

Changes in the text:
Conclusion section, Page 14, Line 16
Finally, our model was not compared with traditional models, whereby clots are delivered by central venous cannulation. Comparing the two approaches from the perspective of clot distribution, hemodynamic and biochemical parameters, and complications, will be needed in future studies.

Comment 3: I am also uncertain why the authors feel that this model is better – the lack of the control group limits comparison

Comment 3a) : "the traditional model result in random clot distribution inconsistent clot size, and potentially fatal hemodynamic compromise" – why is this a bad thing? There are random clot distribution, various clot sizes and potentially fatal outcomes in clinical PE. I would say that such a model replicates the disease state.

Reply 3a): Thank you for your comment. We agree that traditional models, with random clot distribution, various clot sizes, and potentially fatal outcomes, replicates the disease state. However, when starting early evaluation of a new technique or technology relevant for PE, this heterogeneity can make development extremely difficult. If one experiment is successful and another unsuccessful, was it because different iterations of a prototype device were used? Or was it because the distribution and severity of clot were completely different between two animals? The potential advantage of our presented method is that clots can be delivered more selectively to specific pulmonary

artery branches. Based on our experience, EBUS can reasonably provide access to all lobar pulmonary arteries. Therefore, this model provides a wide range of potential clot injection sites (Figure 6). Researchers can further modify model parameters through control of the injected clot volume at each site or employing multiple injection sites. This in theory allows more **controlled** replication of the full-spectrum of PE, including submassive (right ventricular dysfunction) and massive (systemic hypotension) PE. Once the device/drug protocol/technique is optimized and characterized, the next phase would most certainly be employing the 'traditional models' that, as you correctly noted, better replicate the heterogeneity that is seen in clinical PE presentations.

Changes in the text: No change.

Comment 3b) : "Pigs that had severe pulmonary hypertension, heart failure or hypoxemia with a possibility of death during experiment were exclude from this study (page 8)" – why?
There is a spectrum of outcomes in clinical PE. If we only study patients without pulmonary hypertension, heart failure or at risk of death – then we would not understand the disease. Perhaps this is why the hemodynamic changes were not statistically significant? The sickest animals were excluded?

Reply 3b): No pigs were excluded from the study, and all animals survived the protocol. The sentence was in reference to humane endpoints required for our animal protocol by our Animal Care Committee. The development of such significant findings would warrant sacrifice rather than attempting prolonged resuscitation (and potential animal suffering) just to reach the target 2hr timepoint. Since this sentence is not relevant for our data, we have removed it to avoid any confusion.

Changes in the text:
Result section, Page 12, Line 6
All animals survived the procedure; no pig was excluded from the study.

Comment 3c) : "Our model therefore has greatest utility for early-stage investigation, where more consistent models facilitate device optimization prior to evaluation in more heterogeneous models"
The authors have shown that the porcine pulmonary circulation is different in arrangement and size compared to humans. So I am not sure this statement is true.

Reply 3c): Thank you for your comment. We agree that the porcine pulmonary circulation has some differences in arrangement compared to humans. Based on our experience, EBUS reasonably provided access to all lobar bronchi of pigs with lungs and vessels of similar size to humans, albeit not identical. Researchers can further modify model parameters through control of the injected clot volume at each site or employing multiple injection sites. To our knowledge, all existing animal lung models have significant differences from humans. Therefore, the differences between the lung anatomy in our model and human anatomy is no different from the differences seen in any other animal PE model – despite those differences, animal research will continue to

play a critical role in device/drug protocol/technique development before introduction in human clinical trials. Often, these animal models may be the only way to provide clinical trial participants with meaningful insight into the potential safety and efficacy profile of the intervention before enrolling.

Changes in the text:

Conclusion section, Page 13, Line 6

Based on our experience, EBUS reasonably provided access to all lobar bronchi of pigs with lungs and vessels of similar size to humans, albeit not identical (Figure 6). Researchers can further modify model parameters through control of the injected clot volume at each site or by employing multiple injection sites. This would allow replication of the full-spectrum of PE, including submassive and massive PE. To our knowledge, all existing animal lung models have significant differences from humans. Therefore, the differences between the lung anatomy in our model and human anatomy is no different from the differences seen in any other animal PE model – despite those differences, animal research will continue to play a critical role in device/drug protocol/technique development before introduction in human clinical trials. Often, these animal models may be the only way to provide clinical trial participants with meaningful insight into the potential safety and efficacy profile of the intervention before enrolling.

Comment 3d) : all lobar arteries are reported to be accessible to EBUS but "Lower lobe arteries were used in this study due to ease of access and clear visualization"
The statement needs clarification. Again, the availability of a control group would allow you to compare between different models.

Reply 3d): Thank you for your comment. EBUS provided access to all porcine lobar bronchi, as shown in figure 6, which demonstrates that clear ultrasound windows can be obtained for all lobar pulmonary artery branches. However, access is still technically easier to the lower lobes given the straighter orientation and wider bronchial lumen diameter. Conventional EBUS bronchoscopes are somewhat limited with respect to their size, angulation range, and camera quality.

Changes in the text:

Conclusion section, Page 14, Line 13

Fourth, only the lower lobes were used; although the EBUS bronchoscope could access all lobar bronchi, limitations with bronchoscope size, angulation range, and white light visualization does make accessing other lobes somewhat more challenging.

Comment 3e) : Although large clots are formed, the EBUS method requires the clot to be injected via 21G needles which results in small strands. In the central access cannulation models, larger clots can be delivered by large bore catheters which is the limitation of the EBUS model. As we can see from the Inari data, clinical PE resembles thrombus casts of femoral and popliteal veins rather than large clots formed from strands of thrombus. The physiological impact of this is uncertain.
Reply 3e): Thank you for your comment. We agree that clots administered via a 21 G

needle does not perfectly mimic ‘natural’ PE, which is likely better achieved using the central vessel access approaches described in our introduction. Although we believe the more controlled clot formation offered by our model plays an important role for initial development of new technologies/techniques relevant for PE management, we have added the following sentence to acknowledge this limitation:

Changes in the text:
Conclusion section, Page 14, Line 6
Moreover, clot size and shape formed via an EBUS-TBNI approach are likely less ‘clinically realistic’ than those formed via central vessel access.

Minor comments): Spelling errors:

colts (page 3)

sever (page 8)

exclude (page 8)

Reply): We have corrected all spelling errors. Thank you.