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OPTIMIZATION OF CLOT FORMATION METHODOLOGY FOR ASSESSMENT OF NEUROTHROMBECTOMY DEVICES IN LARGE ANIMAL THROMBECTOMY MODELS

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ABSTRACT

Neurothrombectomy devices are commonly evaluated for potential clinical success in porcine models of neurothromboembolism. The majority of preclinical evaluations for these devices are performed in the vasculature of swine or dog utilizing clots created *ex vivo*. This investigation was conducted to develop a faster, more reliable method for creating clots ex vivo for model development. Neurothrombectomy devices are designed to perform recanalization of arterial occlusions that cause acute ischemic stroke [1]. Recanalization can be achieved via clot disruption, aspiration, or retrieval using one or more mechanical devices. In order to evaluate these devices *in vivo*, a fast and reliable method for creating and delivering clots to a desired artery, thereby simulating a target site for neurothrombectomy, is essential.

Two types of clot analogs (soft or firm) were created using two different methods in order to compare both their mechanical properties and their ability to reliably occlude selected arteries. Utilizing both methods, pre-formed clots were qualitatively compared *in vitro* to evaluate elasticity, stiffness, and functionality of delivery through a catheter. These evaluations were performed prior to *in vivo* assessment of the effectiveness of the analogs occlusion of selected arterial vasculature.

Keywords: neurothrombectomy, *ex vivo* clot formation, thrombus, medical, device

NOMENCLATURE

NS	0.9% NaCl in water
PBS	Phosphate Buffered Saline pH 7.4
MT	Mechanical Thrombectomy

1. INTRODUCTION

Mechanical thrombectomy (MT) is an emerging method for the treatment of acute ischemic stroke. The current gold standard for treatment is intravenous thrombolysis using tissue plasminogen activator (rt-PA) [2]. Five clinical trials demonstrating the efficacy of MT in treating acute strokes were published in 2015. This caused medical device manufacturers to realize the potential benefits of MT over the previously established pharmacological methods. [3,4]. More recent studies have focused on the efficacy of using retrieval devices alone, or in combination with an aspiration catheter, as well as aspiration catheter use alone [5]. The publication of these trials illustrates the need for a fast and reliable *in vivo* model of neurovascular occlusion.

A porcine model was selected due to notable similarity of available target arterial sites in the swine that appropriately simulate neurovascular targets commonly observed in the clinic. Targeted sites included the maxillary, lingual, APA, and renal arteries. These arteries were chosen based on similarities between porcine and human anatomy, physiology, blood coagulation, and healing characteristics following vascular injury. These similarities allowed for simulation of clinical settings and are far superior to use in available small animal models. Furthermore, clots created and used in the porcine model can closely resemble the cellular composition and physical properties seen in humans [6].

Two types of clots were chosen based on clinical relevance: soft (red), and firm (aged) clots. Soft clots are richer in RBCs and are more elastic; firm clots are richer in fibrin, less elastic, and more prone to fragmentation when compared to the soft clots [7]. *Ex vivo* soft and firm clots were prepared based on a

modification of the clot creation method previously described by Gounis *et. al.* [1]. This method, which will be referred to as method 1 (M1), requires an hour of incubation time for creating the soft clots. Additionally, in order to make the firm clots, a 15-minute centrifugation step is required. These time-consuming steps are not optimal for a typical *in vivo* research laboratory setting where the animal is anesthetized in preparation for implant.

In an attempt to improve the efficiency of model creation for such studies, American Preclinical Services (APS) has developed its own soft and firm clot creation method. Our method was selected after conducting a thorough review of internal study data and combining the beneficial aspects of historical methods used for clot creation. This review, in addition to the results of several internal trials to improve clot morphology, resulted in a method that consumes less time and provides more reliable and radiopaque analogs than those made using M1.

APS has performed numerous evaluations with devices for emboli aspiration and MT using M1 and other methods to produce analogs for porcine model occlusion creation *in vivo*. The goal of this research was to demonstrate that the APS analogs are comparable to the M1 analogs and justify the future use of the APS clot creation method. We compared the clots in terms of their elasticity, stiffness, ability to occlude a selected artery, radiopacity, and their ability to resist fragmentation during delivery. We also performed a histological assessment comparing the analogs created using M1 and the APS method for clot creation. We were unable to collect samples of our final APS method soft clot; therefore, we relied on qualitative comparisons to determine the similarity between the M1 and APS method soft clots.

2. MATERIALS AND METHODS

2.1 Method 1

Sixty mL porcine blood was collected and anticoagulated with acid-citrate dextrose (9:1) maintaining sterility. This anticoagulated blood was used for preparing both soft and firm M1 clots. All solutions were filter sterilized using a 0.22 µm syringe filter prior to use. The final clot formation mixture was injected into silicone tubing (Tygon® S3™ E-3603, US Plastics). For all methods, the inner diameter (ID) of the tubing ranged from 2-6 mm. Tubing sizes are selected based on the estimated ID of the target artery. The tubing containing the final clot analog was cut to approximately 1 cm length for evaluation and delivery.

2.1.1 M1 Soft Clot Creation

For soft clot creation, a 30 U/mL thrombin (Sigma) solution was prepared in PBS + $CaCl_2$ (0.133 mg/mL). Anticoagulated porcine blood was combined with 30 U/mL thrombin solution at a 5:1 ratio. The blood/thrombin mixture was immediately pushed into silicone tubing and allowed to incubate at room temperature for 1 hour prior to use.

2.1.2 M1 Firm Clot Creation

For firm clot creation, a 42.4 mg/mL fibrinogen (Sigma-Aldrich) in 0.9% saline (NS) solution was made in addition to a 100 U/mL thrombin in PBS + $CaCl_2$ (0.133 mg/mL) solution. 35.0 mL anticoagulated blood was centrifuged at 1,500 x g for 15 minutes at room temperature. 10.0 mL concentrated red blood cells (RBC) were mixed with 1.0 g barium sulfate. Mixing was performed using two syringes connected with a female-female luer connector. One component was aliquoted into each syringe followed by vigorous mixing back and forth.

Next, three syringes were prepared, each containing the correct amount of the desired clotting component. A 3-way stopcock was used for the mixing of clotting components. Two of the ports were used to connect syringes for mixing and the third port was connected to a luer-barb fitting inserted into silicone tubing of desired ID.

Syringe 1 was filled with 0.700 mL 42.4 mg/mL fibrinogen. Syringe 2 was filled with 1.8 mL pre-mixed RBC/barium sulfate mixture. Syringe 3 was filled with 0.5 mL 100 U/mL thrombin solution. Using the stopcock to connect the syringes, Syringe 1 was vigorously mixed with Syringe 2 for approximately 5 seconds. Using the same technique, the resulting mixture was vigorously mixed with Syringe 3 for up to 2 seconds and was immediately pushed through the stopcock into the tubing.

2.2 APS Method

Twenty mL porcine blood was collected without the addition of any anticoagulants. Blood was drawn immediately prior to clot creation due to the absence of anticoagulants. 10 mg/mL and 50 mg/mL fibrinogen (Sigma-Aldrich) in NS solutions were prepared for the soft and firm clots respectively. A 500 U/mL thrombin (Sigma) in NS solution was prepared for use in both soft and firm clots. All solutions were filter sterilized using a 0.22 µm syringe filter prior to use. The tubing was allowed to incubate at room temperature for approximately 60 seconds before tubing was cut. Clots stored in tubing are viable for at least one hour after thrombin addition.

2.2.1 APS Soft Clot Creation

Four syringes were prepared with the following contents: 5mL fresh, non-anticoagulated blood; 1.670 mL fibrinogen (10mg/mL); 0.2 g barium sulfate; and 0.075 mL thrombin (500U/mL). The fibrinogen was vigorously mixed with barium sulfate until uniformly suspended. Next, the blood and the fibrinogen/barium sulfate mixture were homogenized. Finally, the thrombin was mixed in vigorously for no more than two full oscillations of the syringe and the silicone tubing was filled with the mixture.

2.2.2 APS Firm Clot Creation

Four syringes were prepared with the following contents: 5mL fresh, non-anticoagulated blood; 3.25 mL fibrinogen (50 mg/mL); 0.2 g barium sulfate; and 0.150 mL thrombin (500 U/mL). The process outlined above for the formation of the APS soft clot was also used for the APS firm clots.

2.3 Evaluation

Using the clot creation methods outlined above, soft and firm clots were prepared using M1 and the APS method. The physical properties of the clots were qualitatively compared with their counterparts. The structural stability of the clots were evaluated visually prior to, and post-delivery. Clots were drawn into ~6" of 1/4" OD silicone tubing using NS as a delivery vehicle. The tubing was connected to an 8Fr catheter and delivered onto a gauze pad using 15-30 mL NS. Clots were determined to be structurally sound if they resisted breaking into multiple pieces during delivery.

After confirming that soft and firm clots formed using both methods were comparable in terms of their physical properties, clots of various diameters were delivered *in vivo*. M1 clots were compared to APS clots in order to determine whether the APS clots could be delivered to target arteries, and the extent to which the clots occluded the arteries.

3. RESULTS AND DISCUSSION

Our first attempt at clot creation using both methods was successful. From the initial comparisons, we found that the APS firm clot was comparable to the M1 firm clot in relative elasticity, stiffness, and post-delivery survivability and no adjustments were needed. Contrary to the success of our APS firm clot, we found that the initial APS soft clot was not comparable to that of M1. The APS soft clot was too stiff and not as able to conform to the small vasculature during delivery when compared to the M1 soft clot. The M1 soft clot does not include the addition of fibrinogen, which makes the clot very soft and upon delivery, fragmentation of the M1 soft clot was commonly observed.

In an attempt to create a viable APS soft clot that would better conform to the artery without fragmenting, the effects of varying the fibrinogen concentration on the final physical properties of the clot were evaluated. The volume of fibrinogen solution added remained the same, whereas the concentration of fibrinogen was decreased. Concentrations ranging between the original 50 mg/mL and 10 mg/mL were prepared. We found that as the concentration of fibrinogen decreased, the firmness of the clot decreased as well. The 10 mg/mL fibrinogen solution resulted in a soft clot that was physically most similar to the M1 soft. After determining the concentration of fibrinogen that yielded the most similar soft clot characteristics, we proceeded with the assessment of post-delivery survival.

On the benchtop, analogs were loaded into a length of tubing attached to a syringe filled with NS. The clots were then delivered through the catheter onto a piece of gauze. Assessments were performed comparing the pre- and post-delivery shape and degree of fragmentation. We found that the soft APS clot made using 10 mg/mL fibrinogen was most similar to the M1 soft analog, in both physical characteristics and post-delivery survivability. The APS soft clot was better able to retain its shape with less fragmentation than the M1 soft analog, suggesting similar or superior performance using the APS method.

One key benefit the APS clots have over the M1 clots is their ability to be visualized fluoroscopically. The M1 soft analogs did not have a radiopaque component; therefore, they could not be directly observed in the artery post-delivery. The addition of barium sulfate to the APS soft clot allowed for faster confirmation of clot location and degree of occlusion. This saves significant time for the interventionalist performing the procedure and reduces the total time the animal spends under anesthesia. Although the clots are relatively easy to visualize by the performing interventionalist during deployment using high quality fluoroscopy, depiction of deployed clots is best obtained using subtractive digital imaging. Figure 1 depicts a typical clot deployed in a swine internal maxillary artery and demonstrates the radiopacity obtained by the barium addition.

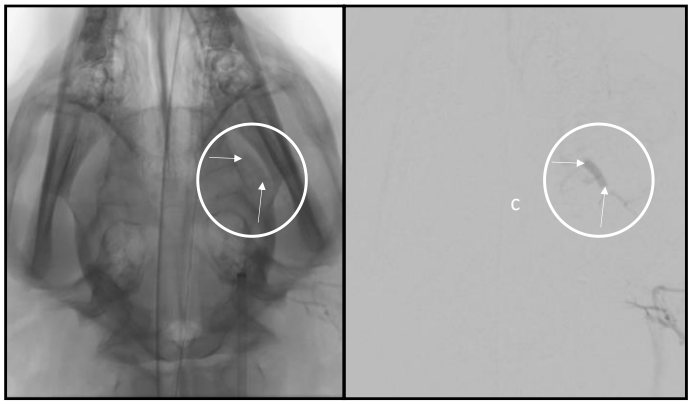

FIGURE 1: *Left*, Fluoroscopic image of the pig head. *Right*, A subtractive digital image showing the location of the barium containing clot in the internal maxillary artery.

Clots were delivered to the arteries typical in size and tortuosity to those treated in neurovascular device evaluations. Our aim was to compare the ease with which clots could be delivered to the target sites and how well the clots were able to occlude selected arteries. We found that the soft and firm clots prepared by both methods were comparable in their deliverability and their ability to occlude the target arteries.

When evaluating histological results, it is important to note that we were unable to evaluate samples of the APS soft analog made with 10mg/mL fibrinogen. However, Figure 1 shows that the main difference between the two firm clots being the RBC and fibrin seen in the APS firm clot (Figure 2, *left)* are more heterogeneously distributed throughout the clot. Both the APS and M1 firm clots were comprised of the same components. The foreign material mentioned is presumed to be insoluble barium sulfate, which was present in both firm clots (Figure 2, *bottom*). Although the RBC and fibrin present in the APS firm clot is more heterogeneously dispersed, it was determined that the two firm clots were physically similar in their elasticity, stiffness, stability, and ability to occlude the selected artery.

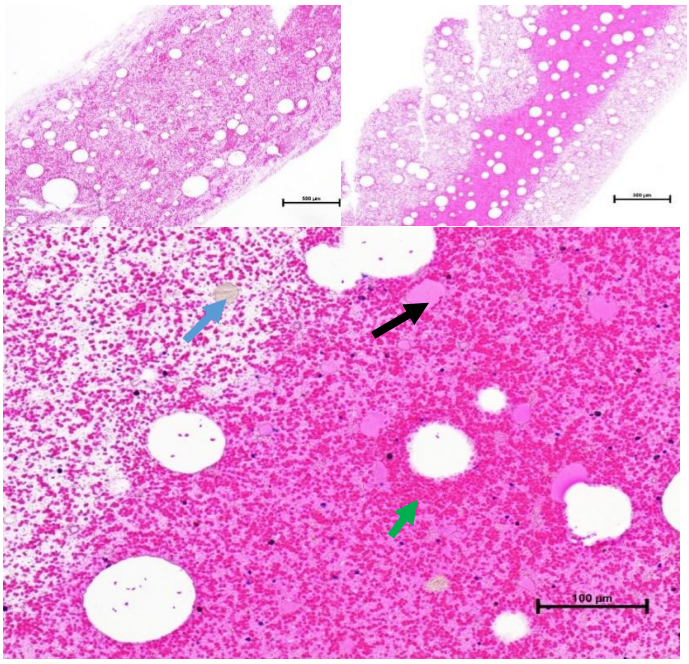

FIGURE 2: *Left*, APS firm clot. *Right*, M1 firm clot. *Bottom*, M1 firm clot: RBC (green arrow), foreign material, likely barium particulates (blue arrow), and fibrin (black arrow).

CONCLUSION

We were able to develop a novel method for *ex vivo* clot creation that was found to be comparable to the previously established M1 method of soft and firm clot production. This new method, which we call the APS method, was developed using methods described in the literature and studies performed at APS. The APS clots were found to be comparable to M1 clots in elasticity, stiffness, and structural stability both pre- and post-delivery. These qualities were found to be true both *in vitro* and *in vivo*. Additionally, the APS clots were able to be delivered to and occlude the targeted arteries as well as the M1 clots. The main benefits of using the APS clot creation method are the reduced time required to create clots in various sizes, the reliability of the method, the consistency of the APS soft clot, and the radiopacity of both the APS firm and soft clots.

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