

Contrast-enhanced sonothrombolysis in a porcine model of acute peripheral arterial thrombosis and prevention of anaphylactic shock

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Acute peripheral arterial thrombosis can be threatening to life and limb. Dissolution of the thrombus local catheter-directed intra-arterial infusion of fibrinolytic agents such as urokinase is the standard therapy for thrombosis; however, this method is time-intensive, and amputation of the affected limb is still needed in 10–30% of cases. Furthermore, thrombolytic therapy carries the risk of bleeding complications. The use of small gas-filled bubbles, or ultrasound contrast agents (UCAs), in combination with ultrasound has been investigated as an improved thrombolytic therapy in acute coronary and cerebral arterial thrombosis. The authors describe a porcine model of acute peripheral arterial occlusion to test contrast-enhanced sonothrombolysis approaches that combine ultrasound, UCAs and fibrinolytic agents and recommend a strategy for preventing severe allergic reactions to UCAs in the pigs.

Acute peripheral vascular disease is most often caused by a thrombus blocking an artery. This can lead to acute lower limb ischemia, which is associated with high mortality rates, and with amputation of the affected limb within 30 d after hospital admission in ~10–30% of patients¹. Dissolution of the thrombus, for example, by catheter-directed intra-arterial infusion of fibrinolytic agents such as urokinase proximal to the thrombus site, can restore blood flow to the limb. However, this technique is invasive and time-consuming, requires repeated angiography and carries the risk of major hemorrhagic complications, resulting in high morbidity and mortality rates². Improved therapies are therefore needed.

Sonothrombolysis is a promising technique for the treatment of acute peripheral arterial occlusion that combines thrombolysis with ultrasound^{3,4}. The efficacy of sonothrombolysis in small vessels can be further enhanced with the addition of ultrasound contrast agents (UCAs)^{5–7}. UCAs consist of small gaseous

microbubbles (1–10 µm) with lipid shells that oscillate under the influence of low-intensity ultrasound and even collapse at higher intensities. These oscillations result in mechanical forces on the clot surface, making the thrombus more susceptible to thrombolytics⁸. These particles therefore have been broadly investigated to improve thrombolytic therapy⁹.

A therapeutic approach that combines the use of ultrasound, UCAs and fibrinolytic agents has not been described previously in subjects with acute thrombotic occlusion of large peripheral arteries. To assess the efficacy of contrast-enhanced sonothrombolysis (CEST) for the treatment of acute peripheral arterial occlusion, an appropriate animal model is needed. Because porcine and human coagulation and fibrinolytic systems have similar components¹⁰, we developed a porcine model of CEST by modifying a previously described model of intra-arterial thrombolysis¹¹. Here, we describe a technique for inducing acute peripheral arterial occlusion in pigs for the investigation of

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different CEST protocols and for preventing anaphylactic shock after administration of UCAs.

TECHNIQUE

All procedures were done at the Animal Laboratory of the VU Medical Center under the direct supervision and support of a licensed veterinary staff. All procedures were done in accordance with both the Dutch national guideline for humane animal treatment (Code Of Practice Welzijnsbewaking van proefdieren, 2004) and the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The VU Medical Center Animal Ethics Committee approved all experiments and procedures carried out on the animals.

Twenty-three adolescent female Yorkshire pigs, weighing between 58 kg and 90 kg, were housed at the research facility for acclimatization and quarantine for 1 week before initiation of the experiments.

Anesthesia

We sedated the pigs with an intramuscular injection of 28 mg per kg body weight ketamine (Alfasan, Woerden, The Netherlands), 0.5 mg per kg body weight midazolam (Actavis bv, Baarn, The Netherlands) and 1 mg atropine (Pharmachemie, Haarlem, The Netherlands). We then induced anesthesia with intravenous (i.v.) injection of 20 mg etomidate (B. Braun, Melsungen, Germany), after which the pigs were intubated. When necessary for cannulation of the airway, we repeated the administration of etomidate. During the procedure, we maintained anesthesia with 1.5–2.0% isoflurane (Pharmachemie, Haarlem, The Netherlands) administered endotracheally and with i.v. 50 µg/h fentanyl (Hameln Pharmaceuticals, Hameln, Germany), 50 mg/h midazolam and 20 mg/h pancuronium (Organon, Oss, The Netherlands). We also administered i.v. 5 ml per kg body weight per h 0.9% NaCl. Tidal volumes were set at 10 ml per kg body weight, with a frequency of 15–18/min, and were adjusted depending on capnography to maintain the CO₂ concentration between 35 mmHg and 40 mmHg. We assessed arterial blood gases regularly for pH, partial pressure of CO₂ (pCO₂) and partial pressure of oxygen (pO₂) using the IRMA TruPoint blood analysis system (ITCmed, Edison, NJ). We also measured blood pressure, heart rate, oxygenation and body temperature (Solar 8000 patient monitor, Marquette, GE Medical Systems, Milwaukee, WI) as well as temperature and microcirculation of the affected limb (laser Doppler probe; Periflux 4001 Master, Perimed AB, Järfälla, Sweden) during the entire procedure.

Creation of a stable thrombus

Via a midline laparotomy, we identified the left common and external iliac arteries and ligated the internal iliac artery. We measured blood flow in the iliac artery

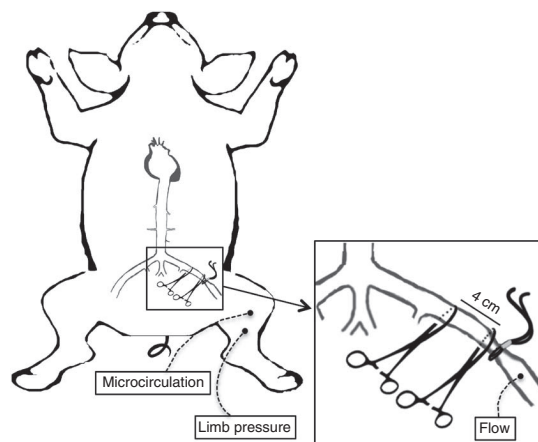


FIGURE 1 | Diagram of the iliac artery. A stenosis was created, the endothelium was damaged and the artery was occluded. Illustrated by H.P. Ebben.

using an ultrasonic flow probe (Transonic-Systems Inc., Ithaca, NY). We created a stenosis in the external iliac artery by reducing the diameter of the vessel with a vascular tourniquet, decreasing the flow in the iliac artery by $50 \pm 10\%$ (Fig. 1). To promote adhesion of a thrombus to the vessel wall, we damaged the endothelium by clamping and declamping the iliac artery with a straight Kocher clamp (Heljestrand, Eskilstuna, Sweden) over a length of 4 cm. We then occluded the artery with the same clamps proximally and distally and injected 100 units of freshly prepared bovine thrombin (Calbiochem, EMD Millipore, Darmstadt, Germany) intraluminally. We removed the proximal clamp after 1 h and the distal clamp after 90 min. When persistent flow in the iliac artery was present, we once again occluded the vessel and administered another 100 units of thrombin. Once a thrombus formed, we left it to stabilize for 30 min.

In the first pig, time to creation of thrombus (TCT) was 295 min, after which the iliac vessel still was not completely occluded. TCT diminished during the study period, however, to 95 min in the last of the four subjects. The main reason for this shortening of TCT may be that we adjusted our technique to increase the amount of endothelial damage given; we increased the force applied to the vessel by fully closing the clamp ratchet when clamping and we increased the amount of time spent causing the endothelial damage. Another adjustment in the procedure was to release the proximal clamp for a short time after injection of the bovine thrombin to allow for extra blood to mix with the thrombin; this prevented the thrombin from replacing some of the blood when it was injected into the vessel, which might originally have made the thrombin less effective.

CEST procedure

To perform sonothrombolysis, we used a Philips Sonos 7500 ultrasound machine with a diagnostic

S3 transducer (Philips Healthcare, Eindhoven, The Netherlands). We placed a balloon filled with saline between the probe and the arterial wall to mimic transcutaneous application of the probe, because, if direct contact with the vessel was made, the probe would be too close to the thrombus to allow for visualization and treatment. The resulting distance between the ultrasound probe and the occluded vessel was 3 cm. We set the mechanical index (MI) to 1.1 with a focus of 3 cm and a frequency of 1.6 MHz.

From this point on, a variety of CEST protocols could be initiated. These protocols varied with respect to the mode of application of the fibrinolytic agent. The UCA that we used was SonoVue (Bracco Imaging SpA, Milan, Italy), which consists of microbubbles with a phospholipidic monolayer shell containing sulfur hexafluoride. UCAs can be administered i.v. or intra-arterially, one vial per several minutes until the UCAs diminished from circulation, which can be controlled by ultrasound. The fibrinolytic agent that we used was urokinase (Lampro bv, Breda, The Netherlands). Pigs received urokinase either i.v. or intra-arterially in a dosage regularly used in clinical setting: a bolus injection of 500,000 U followed by continuous infusion of 50,000 U per h. Microbubbles were administered i.v. and local ultrasound was applied to the occluded vessel. Because of the allergenic effects of the UCAs, therapy time varied from 0 to 180 min and all animals were terminated at the end of the experiment.

In the first pig that was administered UCA, systemic blood pressure dropped less than 2 min later, severe bradycardia occurred and ventilation was no longer possible owing to high airway resistance. We performed external cardiac compressions to no avail, and the pig died within 3 min. Differential diagnosis consisted of pulmonary embolism, anaphylaxis or severe hemorrhage elsewhere in the body. At autopsy there were no signs of pulmonary embolism. There was some intracranial hemorrhage, though not enough to be a likely cause of death. In the second, third and fourth pigs, similar reactions occurred a few seconds after each bolus of UCA, and external cardiac compressions were necessary in all pigs. Symptoms could not be prevented by administering i.v. methylprednisolone (Pfizer, Capelle a/d IJssel, The Netherlands) and tavegy (Novartis, Breda, The Netherlands) 30 min prior to injection of the UCA, but the second, third and fourth pigs did respond to i.v. epinephrine (Pharmachemie, Haarlem, The Netherlands) in various doses (0.1–0.5 mg) and recovered. In these four pigs, severe hemodynamic changes and cardiac arrhythmia were caused by the anaphylactic shock, and the surviving three pigs were excluded from further testing. We did not observe any

cutaneous symptoms of allergic reaction in the pigs, and a literature search identified no previous reports of allergic reactions to UCAs in pigs. Allergic reactions to liposomes have been mentioned previously^{12,13}; Szebeni *et al.*¹⁴ recently stated that “nanoparticulate materials in pigs could lead to acute cardiopulmonary, hemodynamic, hematological, biochemical and dermatological changes within minutes, mimicking the human infusion (or anaphylactoid) reactions” and cited complement activation-related pseudoallergy as a possible cause¹⁴. Therefore, we found it necessary to premedicate the pigs prior to injection of the UCA to prevent possible allergic reactions to the liposome outer layer of the microbubbles. Thirty minutes before injection of UCA in the remaining pigs, we administered 40 mg i.v. methylprednisolone and 500 mg indomethacin (Pharmachemie, Haarlem, The Netherlands) via rectal suppository. Because it takes time to obtain adequate blood levels of indomethacin when administered rectally, we also administered 2.5 g of i.v. acetylsalicylic acid (Sanofi-Aventis, Gouda, The Netherlands) in three doses, each given 5 min prior to administration of a dose of UCA in increments of 15 min. After introduction of the premedication step to the protocol, none of the remaining pigs ($n = 19$) experienced anaphylactic reactions.

CONCLUSIONS

Experiments to evaluate different protocols of CEST are currently being conducted using our porcine model, and results from these protocols are being analyzed. In view of our experience, we recommend that such experiments include premedication to prevent anaphylactic reactions to the lipid shell of the UCA. Our premedication approach was based on recommendations of other researchers in this field, who used an i.v. combination of 40 mg methylprednisone and 5 mg per kg body weight indomethacin. Since the cost of i.v. indomethacin (~\$600 per 1-mg vial) is high, we instead used a suppository form of the drug (~\$2 per 100 mg). Although this is not a new treatment in itself, indomethacin via suppository has not previously been described for this purpose to our knowledge. Using i.v. aspirin (~\$190 per 500 mg) in a dose of 10 mg per kg body weight is another option with a lower cost than i.v. indomethacin and has previously been described as an effective method of preventing adverse reactions to liposomes¹³. Because aspirin has antiplatelet activity, there might be a slight effect on the outcome in a model of thrombolysis; however, patients with peripheral arterial vascular disease will almost always be taking antiplatelet medication. When comparing different methods of thrombolysis, the same systemic medications, including aspirin, should be used in both methods.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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