New Method of Embolus Preparation for Standardized Embolic Stroke in Rabbits

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Background and Purpose—Although thromboembolic stroke is caused by red, white, or mixed clots, the emboli previously used in animal studies on thrombolysis were more often red than white. Because this might be one of the reasons why thrombolysis is less effective in patients than in experimental stroke, we developed a new method of preparing highly standardized red and fibrin-rich white emboli.

Methods—The middle cerebral artery of 20 rabbits was embolized with either red or fibrin-rich white autologous emboli. Cerebral perfusion was monitored by MRI.

Results—Red emboli consisted of closely packed erythrocytes within a sparse fibrin net and white emboli of a dense mass of fibrin with only few other blood cells. Infarct volumes were 26±4% (mean±SD) of the ischemic hemisphere with red and 27±6% with white emboli. The relative regional cerebral blood volume dropped below 50% 90 minutes after vascular occlusion with either type of embolus. Late spontaneous lysis and hemorrhagic complications occurred in 37.5% of red but not in white embolus cases.

Conclusions—Emboli prepared by our technique result in standardized cerebral infarctions. Size and composition of the emboli continuously can be adjusted according to the experimental requirements. (Stroke. 2002;33:2329-2333.)

Key Words: animal models ■ thromboembolism ■ rabbits

Thromboembolic stroke models have been used extensively to study the effect of fibrinolytic agents on autologous arterial emboli, predominantly in rabbits. In the current models early treatment with tissue plasminogen activator, for instance, reduced the infarct size by approximately 60% to 77% compared with control values.1–3 Even if started as late as 3 hours after embolization, thrombolysis reduced the infarct size by nearly 30%.4 In contrast, plasminogen activators have proven to be considerably less effective in clinical trials.5–7 It is generally accepted that animal model systems do not directly relate to clinical trial outcomes because of a vast variety of pathophysiological and methodological reasons.8 However, the differences in the outcomes of experimental and clinical studies stress the necessity of searching for differences between the available models and the known human pathophysiology. In this context we want to draw attention to the fibrin content of the emboli used in animal models of thromboembolic stroke. In the current models, thrombosis was induced in polyethylene tubing or the auricular artery, resulting in red clots with a large proportion of closely packed erythrocytes. The parts of these clots that were supposed to serve as emboli were chosen by their gross appearance.2,3,9–12 Thus, the composition of the emboli, in particular their fibrin content, was not known. In the human condition, thromboembolic stroke is caused not only by red clots but also by white clots. White clots were found “frequently” if stroke was caused by carotid artery thrombosis and in 36% of stroke patients with carotid artery occlusion of embolic origin.13–15 Red clots predominantly consist of erythrocytes, few thrombocytes, and some fibrin; white clots consist of fibrin, cellular debris, and platelets but only few red cells.16,17 According to in vitro studies, fibrin-rich white thrombi retract more than red ones, which results in reduced permeability to bulk flow of thrombolytic agents through the clot, increased fibrin content per unit of clot volume, and decreased plasminogen content.18–20 Thus, the proportion of fibrin and erythrocytes within a thrombus may affect the efficacy of thrombolysis. We therefore developed a new technique for preparing autologous spherical emboli of standardized size and composition that can be adjusted without any intermittent steps to yield red emboli consisting of closely packed erythrocytes within a sparse fibrin net or to yield fibrin-rich white emboli consisting of a dense mass of fibrin but only few other admixed blood cells. We discuss the differences between red and fibrin-rich white emboli and their implications in embolic stroke as well as techniques to avoid the pitfalls of existing embolic stroke models.

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Materials and Methods
Twenty-six male New Zealand White rabbits weighing 2 to 2.5 kg were used for randomized occlusion of the middle cerebral artery (MCA) with 2 either red or white spherical autologous emboli that were prepared approximately 20 hours previously. The red emboli had a diameter of 1.02 to 1.07 mm and the white emboli a diameter of 0.70 to 0.75 mm. The experiments were approved by the local animal research committee.

Preparation of Emboli
The day before embolization, 8.5 mL of blood was taken from the rabbit’s auricular artery with a CPDA, Monovette tube, which contained tintrinitromethan as anticoagulant (Sarstedt). After centrifugation at 1300g for 30 minutes, platelet-enchriched plasma (PRP) was pipetted into a separate tube. The concentration of thrombocytes was determined with a CELL-DYN 1700 device (Abbott Laboratories), and fibrinogen was determined according to Clauss with an STA Compact (Roche). The blood was centrifuged for another 10 minutes at 1400g to obtain cell-free plasma. PRP, cell-free plasma, and whole blood were mixed to form a standardized plasma with a platelet concentration of 400 to 500×10^3/μL and a hematocrit of 0.5%. To obtain fibrin-rich white emboli, the standardized plasma and a thrombin solution (3 mg/1 mL 0.9% NaCl; 50 NIH U/mg; Merck KGaA) were each filled into a 1-mL syringe that was connected to a 23-gauge needle. With 2 infusion pumps (Harvard Apparatus syringe infusion pump 22), 5.3 μL standardized plasma and 1.3 μL thrombin solution were ejected and combined into 1 drop that was transferred to another needle, where it retracted to form a fibrin-rich white embolus within the next 15 minutes. Meanwhile, additional drops of standardized plasma and thrombin solution were made and transferred to needles. The retracted emboli were stored in a thrombin solution (1.5 mg/1 mL 0.9% NaCl) for approximately 20 hours at room temperature. For red emboli, 2 μL whole blood and 0.5 μL thrombin solution (3 mg/1 mL 0.9% NaCl) were used after the hematocrit level in whole blood had been regulated to 30% by addition of PRP. Red emboli were transferred into a thrombin solution (1.5 mg/1 mL 0.9% NaCl) after approximately 10 minutes and stored for approximately 20 hours at room temperature.

One hour before embolization of the MCA, emboli were washed in 0.9% saline, and their form and size were checked under a microscope.

Histopathology of Emboli
Approximately 24 and 48 hours after their preparation, emboli were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 4-μm sections. Routine staining was performed with the use of hematoxylin and eosin and the Masson Goldner stain.

Animal Model
The rabbits were premedicated with an intramuscular injection of 0.5 mg atropine and anesthetized with 3.5% halothane in 70% N2O and 30% O2 with the use of a face mask. Anesthesia was maintained with 2% halothane in 70% N2O and 30% O2 and with a single intramuscular injection of ketamine (60 mg/kg body wt; Ketamin; Wirtschaftsgenossenschaft deutscher Tierärzte eG) during transport from the angiography unit to the MRI scanner.

After arteriotomy, a 4F sheath was placed into the left femoral artery. Under angiography (Integris; Philips Medical Systems), a FasTracker-18 MX catheter (Boston Scientific Cork Ltd) was advanced into the proximal part of the right internal carotid artery (ICA). Two red or white emboli were injected slowly and simultaneously into the ICA with 1 mL 0.9% saline over 3 to 5 minutes. Complete proximal occlusion of the MCA was verified by digital subtraction angiography. Blood gases, blood glucose, and blood pressure were monitored before and after embolization.

Measurements of Cerebral Perfusion and Infarct Volume
Perfusion-weighted cranial MRI (Biospec 2.3 T; Bruker Elektronik GmbH) was performed every 30 minutes for a period of 4 hours after embolization. With a field of view of 80 mm and a matrix size of 64×128, the in-plane spatial resolution was 1.25 mm per pixel. The temporal resolution was 0.8 seconds per image. The cerebral perfusion was determined within regions of interest that were placed in the ischemic cortex and the corresponding area of the nonischemic hemisphere. For perfusion imaging, 0.75 mmol gadodiamide (Omniscan, Nycomed Arzneimittel GmbH) was used to avoid transient drops in blood pressure that are known from other contrast agents.

The animals were killed with carbon dioxide 14 hours after embolization. The brains were removed immediately and cut into coronal sections with a thickness of 2 mm. The slices were stained with 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich), fixed in 4% formalin, and photographed with a digital camera. The areas of infarction and of both hemispheres were measured with an image analyzer (NIH Image 1.61). The infarcted areas were corrected for brain edema by multiplying them by the ratio of the total area of the nonischemic hemisphere to that of the ischemic hemisphere at each section level. The infarct volume was calculated as the sum of the corrected areas multiplied by the slice thickness. Outcome measurements were performed by personnel blinded to the experimental groups.

For statistical analysis we used the nonparametric Wilcoxon and Mann-Whitney U tests.

Results
Four of the 26 animals used in this study had a duplication of the MCA, 1 developed vasospasms during angiography, and 1 died before embolization. These 6 animals were excluded from the study. Nine rabbits were embolized with red emboli. This caused an intracerebral and a subarachnoid hemorrhage in 1 rabbit, which was then excluded from further analysis. Eleven rabbits were embolized with white emboli. One of these rabbits was excluded because the emboli occluded the distal part of the ICA. In 2 other rabbits a TTC stain could not be made because 1 rabbit died within 4 hours after embolization and 1 was found dead in its cage. Thus, 8 rabbits remained in each group for statistical analysis.

The content of fibrinogen and the cell counts of standardized plasma and whole blood did not differ between the 2 experimental groups (Table). The physiological parameters after embolization were normal except for a mild respiratory acidosis (Table).

During preparation, both types of emboli retracted to their final size within approximately 3 hours, and the volume of the red emboli retracted 7.8 times less than that of the white emboli. This retraction factor was derived by dividing the ratio of the volumes of the white emboli before and after retraction by the corresponding ratio of red emboli. Approximately 25% of the prepared emboli had the size and shape that were required for the experiments. Red emboli were soft and had a total volume of 0.70 to 0.75 mm^3. Microscopic examination of red emboli (Figure 1A) revealed closely packed erythrocytes within a sparse fibrin net, some of them having a pale appearance due to release of hemoglobin from lysed red blood cells. The white emboli (Figure 1B) consisted of a dense fibrin network with only few blood cells. Despite the use of the PRP, no intact platelets could be distinguished 24 hours after embolus preparation. There were no histological differences between emboli that were 24 or 48 hours old.

Angiography verified complete and proximal occlusion of the MCA in all animals of both groups (Figure 2). The incidence of thrombolysis was higher for red than for white emboli. At
autopsy, red emboli were found in only 5 of 8 rabbits, although angiography had verified complete and proximal occlusion of the MCA right after embolization in all these animals. With white emboli, occlusion of the MCA was permanent in all rabbits. Red emboli were found in the proximal part of the MCA in 3 and in the distal part in 2 cases. With white emboli, autopsy revealed a proximal occlusion in 3 and a distal occlusion in 5 rabbits. Fragments in a downstream location were found with neither red nor white emboli. Although the length of the vascular occlusion was not measured, it appeared to be longer with red than with white emboli. However, perfusion-weighted MRI did not show any differences in the perfusion deficits between the 2 experimental groups during the first 4 hours of vascular occlusion, nor did it show signs of reperfusion of the ischemic area.

The normalized first moment of the signal-time curve, which is closely related to the mean transit time of the contrast agent through the brain parenchyma, was significantly reduced 30 minutes after vascular occlusion ($P=0.004$ for red and $P=0.008$ for white emboli [$P<0.01$ is highly significant]). The relative regional cerebral blood volume decreased below 50% 90 minutes after embolization ($P=0.008$ for red and $P=0.004$ for white emboli [$P<0.01$ is highly significant]). In both groups, the normalized first moment increased and the relative regional cerebral blood volume decreased until 240 minutes after embolization. All animals of both groups developed a cortical infarction in the territory of the MCA. Infarction size was 26±4% of the ischemic hemisphere with red emboli and 27±6% with white emboli. The risk of hemorrhagic complications was higher for infarctions due to red emboli than for those due to white ones: in 2 of the 3 rabbits in which lysis of red emboli occurred, the cerebral infarction was hemorrhagic, and an additional intracerebral hemorrhage had developed in 1 of these 2. A third hemorrhagic infarction was found in a rabbit with permanent occlusion of the MCA with red emboli. None of the infarctions due to white emboli was hemorrhagic or showed an intracerebral hemorrhage.

**Discussion**

In the vast majority of patients, arterial thrombosis occurs in atherosclerotic arteries and is related to impaired endothelium or ruptured atherosclerotic plaque. The composition of the thrombosis depends on local flow disturbances, direction of the rupture of an atheromatous lesion, systemic thrombotic propensity, and many other factors. Some thrombi are homogeneous, whereas others consist of different distinct portions or are laminated depending on whether their growth is constant or episodic. White thrombi consist of varying amounts of cellular debris, platelets, and fibrin but only few red cells. Fibrin clots, which contain some white and red blood cells but no platelets and thus are similar to the white fibrin-rich emboli used in this study, can be found in the small cerebral arteries of approximately one third of patients with recent embolic infarcts. Red thrombi contain fewer massed platelets and fibrin but more red cells. The proportion of erythrocytes within a clot determines the size of the pores between its fibrin fibers and thus may influence thrombolysis, which is dependent on the flow of the fibrinolytic agent through the thrombus. This hypothesis is further supported by the observation that erythrocytes are released from thrombi during fibrinolysis. The retraction of fibrin-rich white clots is purported to hinder fibrinolysis because of reduced permeability to bulk flow, increased fibrin content per unit of clot volume, and extrusion of plasminogen during clot retraction. Because the proportion of erythrocytes and fibrin within thromboembolic material seems to be one of the factors that determine the outcome of thrombolysis, we designed a model with which the composition and size of the emboli can be adjusted continuously within a wide range by varying the volumes of whole blood and platelet-enriched plasma used.

The uniformity of the emboli was ensured by standardizing the plasma, the whole blood, and the thrombin solution, by measuring the volume of each component with infusion pumps, and by checking the form and diameter of the emboli with a microscope. The histological uniformity of the emboli was demonstrated with photomicrographs. Red emboli were very similar to the stagnation thrombi found in patients. Our fibrin-rich white emboli differed from stratified thrombus in that erythrocytes were not arranged in layers but were distributed...
homogeneously. However, we believe that this is a minor drawback compared with the reproducibility of size and high fibrin content of these emboli. The response to fibrinolysis should be determined by the high proportion of fibrin rather than by the physical distribution of the few admixed red blood cells. That we could not differentiate platelets in our emboli despite the use of PRP is in accordance with the observation that platelets disintegrate and fuse when they occur as small aggregates within a retracting fibrin net.25 We accepted the drawback that the red emboli mimic clots of venous rather than arterial origin, whereas the white emboli consist mainly of fibrin, to compare the properties of emboli with distinct differences in the proportion of these 2 components.

It became evident that the ratio of plasma and erythrocytes determines the softness and size of a thrombus, which increase with the number of erythrocytes and decrease with an increasing proportion of fibrin. Soft red emboli lodged much deeper into the vascular tree than rigid white emboli of the same size. Reproducible MCA occlusion, therefore, required different sizes of red and fibrin-rich white emboli. The ratio of the volumes of both types of emboli could not be reduced further than 3:1 because 2 red emboli of a lesser size did not reliably occlude the MCA, and 1 animal had to be excluded from the study because white emboli had lodged in the distal ICA. According to these findings, it is unlikely that an ICA occlusion in patients is due to red emboli unless a high-grade stenosis already exists. This is consistent with the finding that complete thrombotic ICA or coronary artery occlusion that is based on an atherosclerotic stenosis often involves an extensive stagnation thrombus that develops in the low-flow state.13,26

Because reperfusion was excluded by MRI during the first 4 hours after embolization, the lysis of red emboli must have occurred afterward. It was probably a spontaneous systemic thrombolysis because, in vitro, the red emboli were stable for at least 24 hours. The high proportion and density of fibrin and a low plasminogen content18 may have been the reasons why the white emboli did not lyse.
With our model, the length and the location of the occlusion can be varied. The occlusion can easily be lengthened by increasing the number of emboli that are reduced in size, but since the model was designed for studies on thrombolysis, this was not desirable. The approach to embolize the bifurcation of the ICA has 2 drawbacks: because the diameter of the anterior cerebral artery is smaller than that of the MCA and the ICA, complete occlusion of the bifurcation is difficult to achieve, and reperfusion via the anterior cerebral artery commonly occurs. Second, it is not advisable to embolize the distal part of the ICA because such occlusions result in large infarctions that include the basal ganglia so that rabbits often die within the first 6 hours after the onset of infarction. Therefore, we decided to embolize the proximal part of the MCA so that possible proximal branches of the vessel are occluded as well and fluctuation of the infarct size is minimized. Because this approach requires emboli with a highly standardized diameter that exactly match the size of the proximal MCA (this applies in particular to rigid white emboli), we had to abandon the traditional way of producing emboli in polyethylene tubings or the rabbit’s auricular artery.

It is a common problem of animal models for embolic stroke that the emboli occlude vessels contralateral to the side of the injection. Our angiograms revealed that the circle of Willis in rabbits is predominantly supplied by the basilar artery and that the ICAs are relatively small. When contrast medium was injected into the ICA, the anterior parts of the circle were enhanced first, but retrograde flow of most of the bolus through the posterior communicating artery and the basilar artery occurred immediately afterward. Therefore, we did not use >2 emboli and injected them very slowly and simultaneously to prevent retrograde loss through the posterior communicating artery.

Because the angiography and MRI units were in different places, anesthesia had to be maintained with ketamine during animal transport. Although ketamine is a known N-methyl-D-aspartate receptor antagonist that delays ischemic neuronal damage, its effects on the results of this study can be disregarded: all animals received the same dose, and the neuroprotective effect of the drug targeted predominantly selected regions of the hippocampus, which are relatively small compared with the size of the infarctions.

In summary, we have developed a new method of embolus preparation for standardized embolic stroke in rabbits. Size and composition of these autologous emboli are highly reproducible and can be adjusted without any intermittent steps according to the experimental requirements. This model is the basis for experiments to determine differences in the efficacy of thrombolysis of various types of vasocclusive material, depending on the content of fibrin and erythrocytes.

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