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Extravasation of Microspheres in a Rat Model of Silent Brain Infarcts

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Background and Purpose—We developed a rat model of silent brain infarcts based on microsphere infusion and investigated their impact on perfusion and tissue damage. Second, we studied the extent and mechanisms of perfusion recovery.

Methods—At day 0, 15 µm fluorescent microspheres were injected into the right common carotid artery of F344 rats. At days 1, 7, or 28, the brain was removed, cut in 100-µm cryosections, and processed for immunofluorescent staining and analysis.

Results—Injection of microspheres caused mild and transient damage to the treated hemisphere, with a decrease in perfused capillary volume at day 1, as compared with the untreated hemisphere. At day 1 but not at days 7 and 28, we observed IgG staining outside of the vessels, indicating vessel leakage. All microspheres were located inside the lumen of the vessels at day 1, whereas the vast majority (≈80%) of the microspheres were extravascular at day 7, and 100% at day 28. This was accompanied by restoration of perfused capillary volume.

Conclusions—Microspheres cause mild and transient damage, and effective extravasation mechanisms exist in the brain to clear microsized emboli from the vessels.

Visual Overview—An online visual overview is available for this article. **(*Stroke*. 2019;50:1590-1594. DOI: 10.1161/STROKEAHA.119.024975.)**

Key Words: angiophagy ■ arterioles ■ brain infarction ■ cerebrovascular disorders ■ microcirculation ■ rats

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Silent brain infarcts (SBIs) are defined as ischemic events detected by brain imaging, without any overt clinical symptoms. In recent years, it has become clear that the term silent may be trivializing because SBIs do not cause acute clinical symptoms but are associated with cognitive decline, dementia,[1] and increased risk of stroke and overall mortality.[2] The small lesions may be caused by local ischemia resulting from micro-occlusions and small embolic events.[3]

In addition to the fibrinolytic pathway and hemodynamic forces, Lam et al reported on a newly appreciated mechanism by which the brain vasculature disposes of emboli, coined angiophagy. Using in vivo imaging in mice, they showed that microemboli of different sources were translocated to the perivascular parenchyma through engulfment of the emboli by endothelial cells.[4] This embolus extravasation into the parenchyma leads to vessel recanalization and blood flow reestablishment.[5] However, the exact mechanism of angiophagy is still obscure and deserves further investigation.

Here, we injected microspheres into the carotid artery in rats, which lodge in the small arterioles, and describe the impact on perfusion and tissue damage. In the present work, we confirm the occurrence of angiophagy in this rat model of SBI and took steps to further characterize this process.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

SBI Model

The surgical protocols in this study were performed with the approval of the local committee on the Ethics of Animal Experiments of the University of Amsterdam, Academic Medical Center (permit number: DMF103082). All surgical procedures were conducted under isoflurane inhalation anesthesia mixed with oxygen while the body temperature was maintained at 37°C with a heating pad. Male F344/lcoCrl rats (9–12 weeks old; n=18) were anesthetized with isoflurane (2.5%/L O_2), and SBIs were mimicked by the injection of 15-µm microspheres into the right common carotid artery to disrupt

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The online-only Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/STROKEAHA.119.024975.

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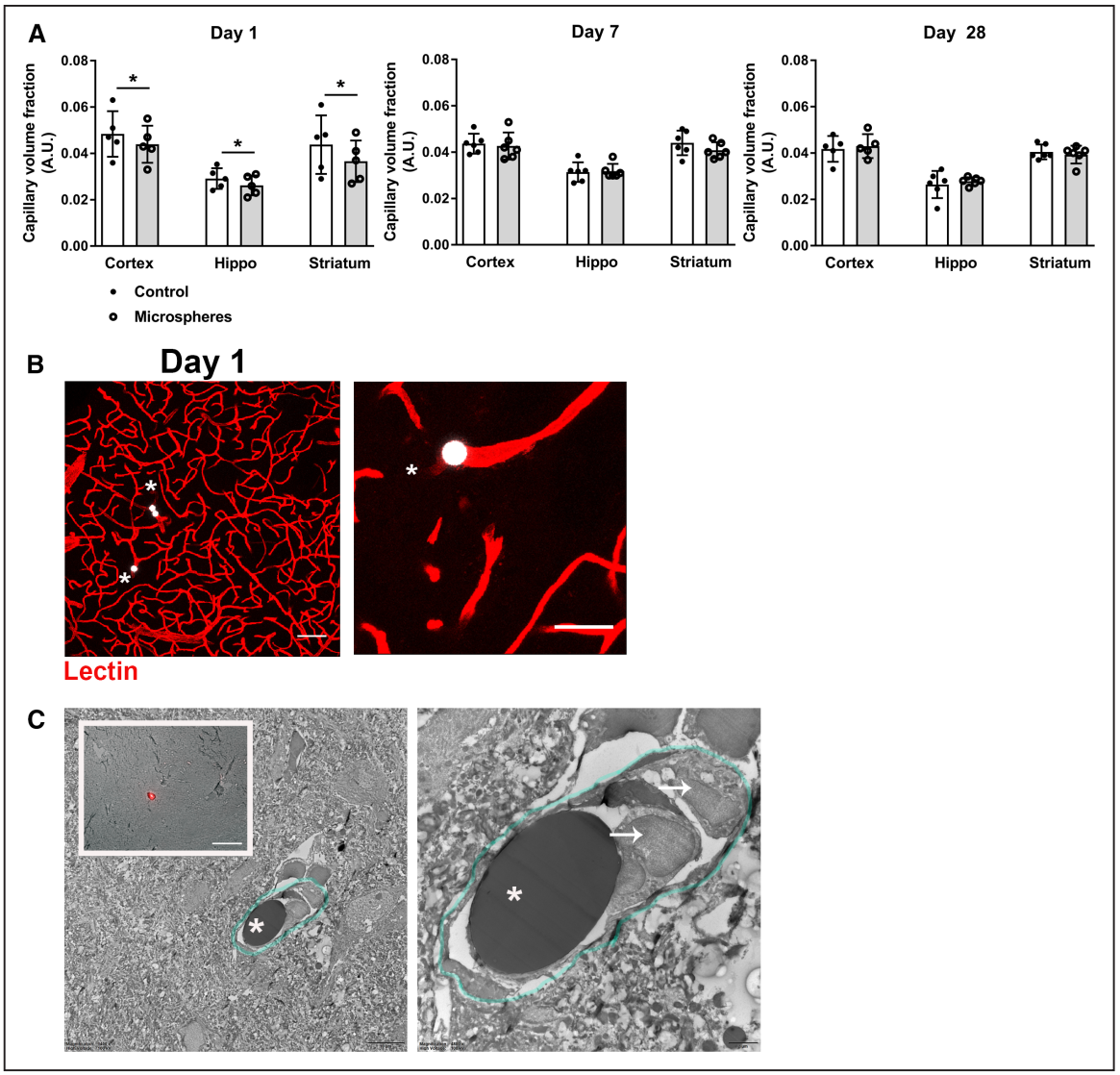

Figure 1. Microspheres cause mild and transient perfusion deficits in the brain. **A**, Injection of microspheres in the right hemisphere caused a small decrease in capillary volume fraction in all brain regions at day (D) 1. n=5 to 6 per time point. **B**, At D1, capillary nonperfusion was occasionally observed near the sites where microspheres had lodged in arterioles, as shown by lectin staining (red). Areas of capillary nonperfusion are indicated by an asterisk (*). Scale bar overview image=75 μm; scale bar enlarged image=35 μm. **C**, Electron microscopy image of an occluded vessel at D1 showed an intact vessel and no tissue damage surrounding the occluded vessel. Cells (most likely leukocytes, arrows) were found inside the occluded vessel. Vessel is outlined in blue. Asterisk=microsphere. Scale bar (**left**; overview)=10 μm, scale bar (**right**; zoom)=2 μm. Boxed inset in the left panel shows the fluorescent microsphere (red) in a semithin section with correlative light and electron microscopy (CLEM). Scale bar (inset)=25 μm.

blood flow in the cerebral arterioles. The left common carotid artery was not injected, and, therefore, the left hemisphere served as the untreated control. Animals were killed on days 1 (n=6), 7 (n=6), or 28 (n=6) after surgery. For the final analysis, 2 animals from day 1 and 1 animal from day 28 was excluded because of technical issues. Detailed Methods are described in the online-only Data Supplement.

Results

Microspheres Cause Mild and Transient Perfusion Deficits in the Brain

To assess to which extent the injection of microspheres impaired capillary perfusion, we stained the vasculature and analyzed perfused vascular density, branching, length, and diameter. Whereas there were no statistical differences in the number and length of branches and vessel diameter between the treated and control hemisphere (data not shown), we found a decrease in capillary volume fraction in all 3 brain regions (cortex, hippocampus, and striatum; Figure I in the online-only Data Supplement) at 1 day after surgery but not at later time points (Figure 1A). The decreased capillary volume fraction indicates locally interrupted capillary perfusion, which was confirmed by the observation of areas with unperfused capillaries near lodged microspheres (Figure 1B). Using electron microscopy (Figure 1C), we observed an intact vessel in which a microsphere had lodged at day 1, with no tissue damage surrounding the occluded vessel. The microsphere was surrounded by cellular material, and several cells were detected near the microsphere inside the vessel, most likely leukocytes.

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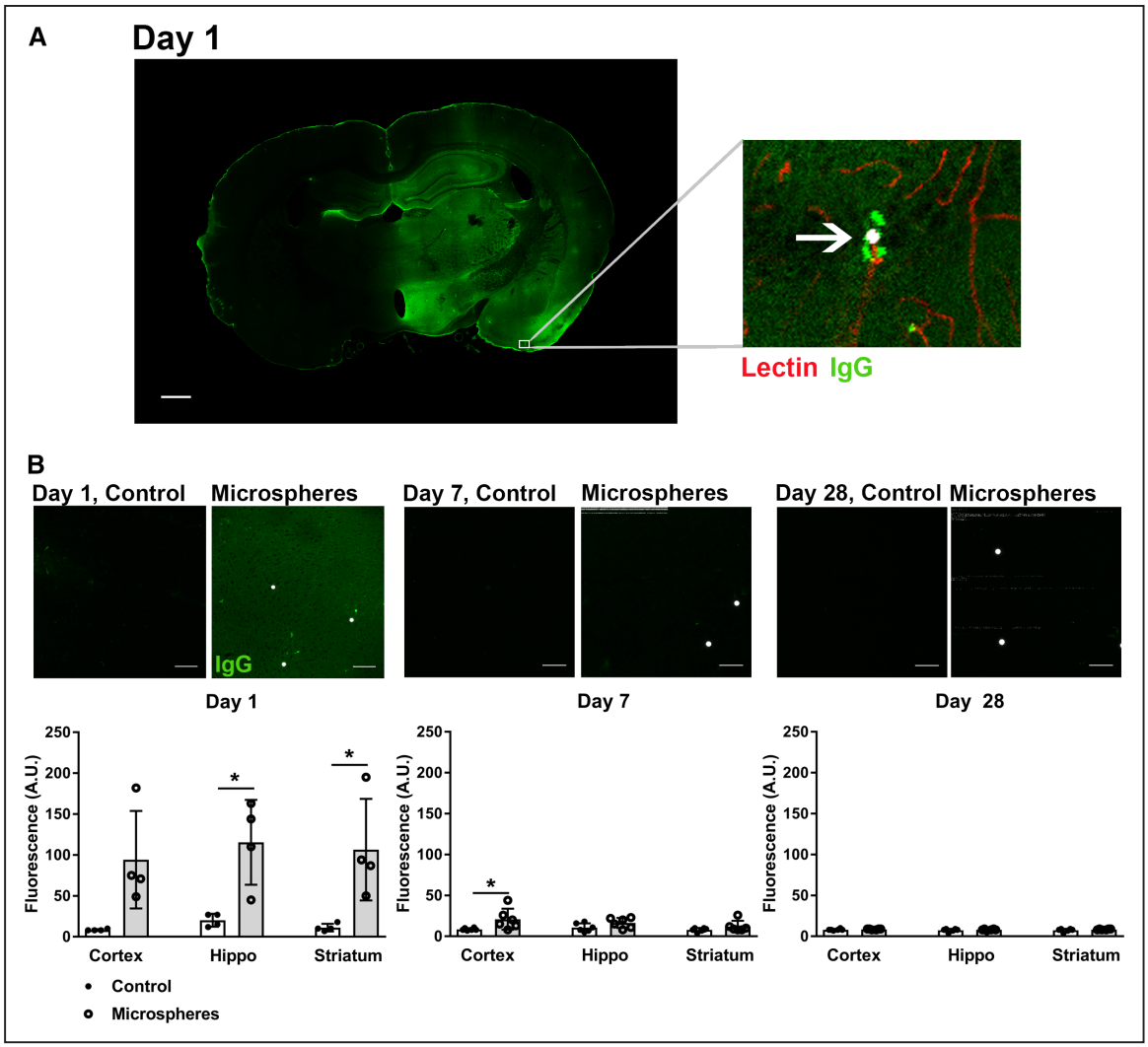

Figure 2. Microspheres cause diffuse leakage of endogenous IgG into the brain. **A**, At day (D) 1, there was diffuse leakage of endogenous IgG (green) out of the vessel lumen (lectin; red) throughout the injected hemisphere and focal leakage spots surrounding microspheres were often seen (white arrow). In contrast, the control hemisphere was clear of IgG extravasation into the parenchyma. Scale bar=1 mm. **B**, Examples of IgG immunoreactivity in the cortex region at D1, D7, and D28. Scale bar=75 µm. Quantification of IgG showed increased leakage in all brain regions at D1, whereas this effect was minimized at D7 and D28. n=4 to 6 per time point. Data are depicted as mean±SD. *P<0.05.

Microspheres Cause Vascular Leakage and Mild Reactive Gliosis

When the blood-brain barrier is disrupted, the brain vasculature becomes permeable, and proteins and other plasma solutes may leak into the parenchyma. In our rat model, there was a profound and diffuse leakage pattern of endogenous IgG in the treated hemisphere, indicating loss of blood-brain barrier integrity. In many cases, we also observed focal spots of IgG leakage close to the microspheres. In contrast, the untreated hemisphere (except for the choroid plexi, which have fenestrated endothelium) was devoid of IgG staining in the parenchyma (Figure 2A). Crucially, this effect was only seen at day 1 (Figure 2B), suggesting a transient opening of the blood-brain barrier after microsphere injection. To assess whether there was reactive gliosis after infusion of microspheres and blood-brain barrier disruption, we stained the brain sections for GFAP (glial fibrillary acidic protein)—a marker of reactive astrocytes and the Iba1 marker to stain microglia. GFAP immunoreactivity was increased in the treated hemisphere at day 1 (Figure IIA in the online-only Data Supplement), although not exclusively around occluded vessels but rather in the whole right hemisphere (Figure IIB in the online-only Data Supplement). Although we observed only a small increase in Iba1 immunoreactivity in the cortex at days 7 and 28 of the treated hemispheres (Figure IIC in the online-only Data Supplement), the morphology of (a subset of) these Iba1-positive cells changed from ramified (with a small cell body and long, thin processes) in the untreated hemisphere to an ameboid morphology in the treated hemisphere (Figure IID in the online-only Data Supplement), indicating activation of microglia.

Microspheres Extravasate From the Vessel Lumen at Later Time Points

Given that we found only little damage caused by the presence of microspheres in the vessels (Figure 1) and that this effect was observed only at day 1, we hypothesized the presence of a repair mechanism that protects the brain from micro-occlusions that occur frequently throughout life. At day 1, almost

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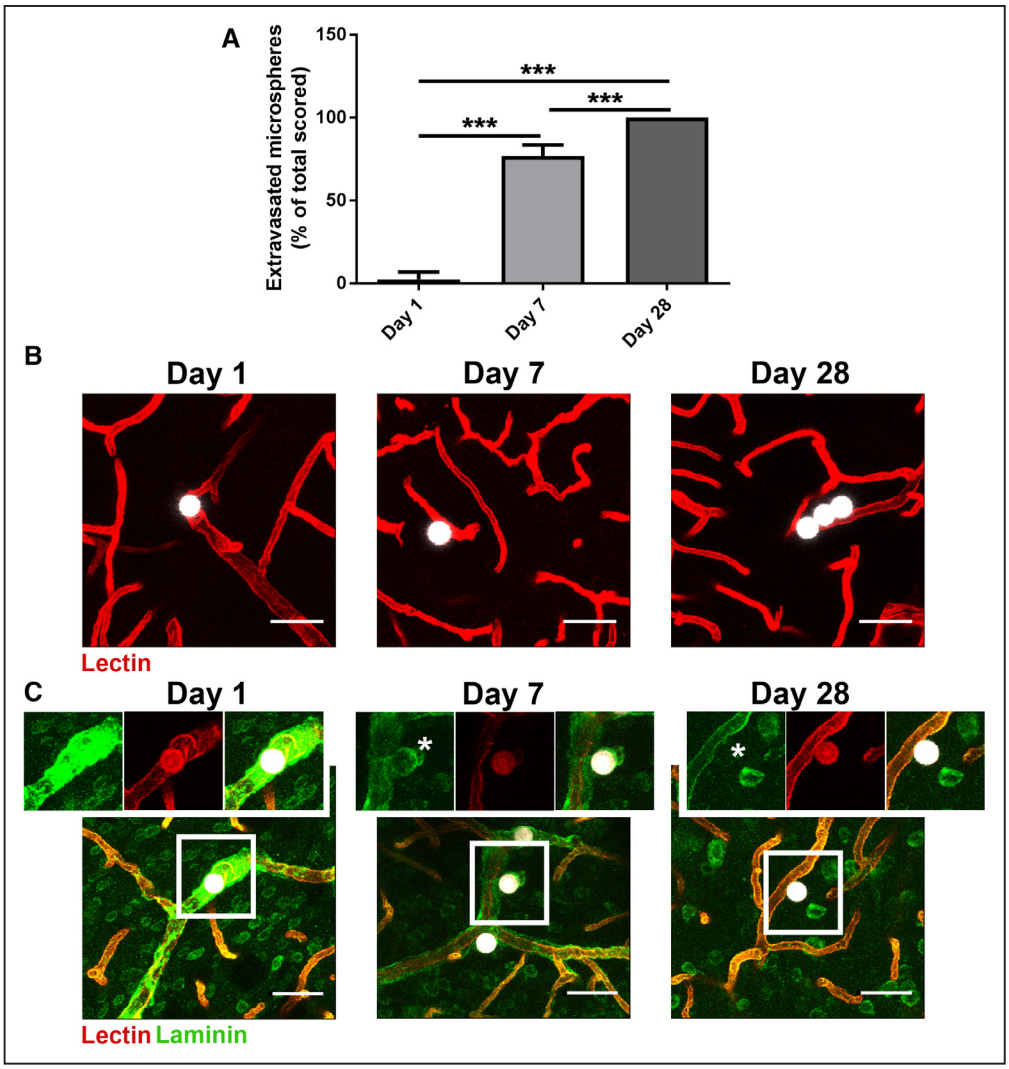

Figure 3. Microspheres extravasate from the vessel lumen. **A**, Quantification of microsphere extravasation days (D) 1, D7, and D28. n=4 animals per time point, 10 to 11 microspheres per animal. Data are depicted as mean±SD. ***P<0.001. **B**, Examples of microspheres confined to the vessel lumen (D1) and extravasated microspheres (D7 and D28), as shown by lectin staining (red). Scale bar=35 µm. **C**, Laminin staining (green) showed that at D7, microspheres that have extravasated, or are in the process of extravasating the vessel lumen, are sometimes still confined within the extracellular matrix (asterisk in inset), whereas at D28, all of the scored microspheres have left the vessel completely (asterisk in inset). Scale bar=35 µm.

all microspheres (n=39 of 40 microspheres in 4 rats) were still lodged inside the arteriolar lumen. In contrast, at day 7, the vast majority of microspheres had extravasated (n=32 of 42 microspheres in 4 rats), and at day 28, all scored microspheres (n=42 of 42 microspheres in 4 rats) had extravasated from the vessels (Figure 3A and 3B). Using immunofluorescent staining for the extracellular matrix protein laminin, we observed that at day 7, some microspheres were still confined within the extracellular matrix of the vessel. This is the time point when the majority of microspheres had extravasated and a small part was still in the process of extravasation. In contrast, microspheres had left the vessel completely at day 28 (Figure 3C). Together, these data indicate that the brain is capable of removing obstructions from the vasculature with great efficiency.

To determine whether extravasated microspheres enter the brain interstititium or remain trapped between endothelial cells and astrocytes, we stained the brain sections for the water channel Aqp4 (aquaporin-4)—a marker of astrocytic endfeet. At points where microspheres extravasated from the vessel lumen, Aqp4 staining was not different from other places, specifically, the microsphere was located in the interstitium and not in between the vessel and Aqp4-expressing astrocytic endfeet (Figure III in the online-only Data Supplement).

Discussion

The principal clearing mechanisms of occlusions in brain vasculature include fibrinolysis and subsequent washout by hemodynamic forces.[6] Yet several occluding materials, for example, cholesterol crystals dislodging from atherosclerotic plaques, are not susceptible to fibrinolytic enzymes. Lam et al[4] were the first to describe the process of angiophagy as a way for the brain to remove microemboli of different sources. We now confirm the occurrence of such a process in cerebral arterioles in rat brain.

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Microspheres caused mild damage to the brain in the form of transient perfusion deficits. Only at day 1, we observed effects on vascular density, likely resulting from capillary nonperfusion. The effect was annulled after 7 days, suggesting that blood flow was reestablished. Indeed, at this point, the majority of microspheres had extravasated. Crucially, opening of the blood-brain barrier and the consequent extravasation of IgG into the parenchyma took place only at day 1, that is, at a time when all the microspheres were still inside the vessel. The diffuse leakage at day 1 suggests that the injection of microspheres caused transient but widespread endothelial dysfunction. The intense IgG signal close to the microspheres could indicate that the endothelium around the microspheres is particularly leaky. Other studies have shown that both microbleeds and serum extravasation cause little-to-no neuronal and functional damage,[7,8] which may explain why the animals had such limited tissue damage in our study, despite the widespread (but temporary) blood-brain barrier dysfunction.

The exact mechanisms through which angiophagy and consequent recanalization take place is not clear. It could be through a process similar to translocation of leukocytes across an endothelial barrier.[9] However, in contrast to leukocytes, microspheres have a fixed size and shape and cannot actively initiate the extravasation process like leukocytes do. Alternatively, the angiophagy process may depend on mechanical forces induced by the complete occlusion of an arteriole, which would generate a pressure gradient between the proximal and distal location of the occlusion.

In conclusion, we characterized a rat model of SBI and show that the effects of microsized emboli on cerebral arterioles are mild and, importantly, transient in nature. This transient effect is likely a result of the occurrence of angiophagy—a process that enables reestablishment of blood flow. These data indicate that the brain is capable of removing obstructions from the vasculature, thereby preventing further damage caused by capillary nonperfusion and consequent hypoxia.

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Disclosures

None.

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