Perfusion of Ischemic Brain in Young and Aged Animals
A Laser Speckle Flowmetry Study

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Background and Purpose—Aging is an important determinant of ischemic stroke outcomes. Both clinical and experimental stroke studies have shown that aging negatively correlates with infarct volumes but is associated with worsened functional recovery after stroke. This may correspond to a differing cellular and molecular response to stroke in the aged versus young brain. It was hypothesized in this study that the smaller injury seen in the aged ischemic brain is because of structural differences in microvasculature with aging or differences in intraischemic tissue perfusion.

Methods—Both young and aged C57BL6 mice were subject to middle cerebral artery occlusion modeling. Laser speckle flowmetry was used to study the functional dynamics of cerebral perfusion, and fluorescein isothiocyanate (FITC)-dextran staining was performed to examine the structural change in microvasculature. In separate cohorts, cresyl violet staining and immunohistochemistry with CD31 and IgG antibodies were applied to further assess the microvascular density and blood–brain barrier breakdown after stroke.

Results—No difference in cerebral blood flow was seen at the baseline, intraischemically, and postreperfusion in young versus aged mice. FITC-dextran and CD31 staining did not show significant differences in the microvascular density between young and aged ischemic brains. More extravasation of IgG through the blood–brain barrier was found in the young versus aged cohort at both 24 and 72 hours after stroke.

Conclusions—Cerebrovascular dynamics and perfusion are not responsible for the different stroke phenotypes seen in the young versus aged animals, which may be more related to different levels of blood–brain barrier breakdown. (Stroke. 2014;45:571-578.)

Key Words: aging ▪ brain ischemia ▪ infarction ▪ microcirculation ▪ middle cerebral artery occlusion ▪ perfusion ▪ regional blood flow

Aging is the most important nonmodifiable risk factor for ischemic stroke1 and an independent predictor of worse stroke outcomes.2 The rate of stroke doubles every decade after the age of 55 years,3 and the burden of this disease on public health is projected to grow with the increasing life expectancy. Many patients are not candidates for tissue-type plasminogen activator (the only Food and Drug Administration–approved treatment for ischemic stroke) therapy because of the short approved time window (3 hours) or contraindications to treatment. Despite the development of numerous promising agents in preclinical models, bench to bed translation of therapies is a challenge for stroke researchers. This may be secondary, in part, by the almost exclusive use of young animals, which may not mimic the hemodynamic and inflammatory milieu of the aged brain.

We have shown that aged male mice have smaller infarct volumes but worse functional recovery when compared with young mice,4,4 which is consistent with clinical data.3 However, the underlying mechanism for this discrepancy in histological and functional outcome is unclear. Aging causes several morphological and pathological changes in the vascular bed, such as atherosclerosis, small vessel disease,8 and altered cerebrovascular reserve.9 Therefore, we hypothesized that structural differences in the microvasculature in the aged or differences in intraischemic perfusion may be responsible for the smaller infarct volumes seen in aged animals. In the present study, we used laser speckle flowmetry (LSF) to measure cerebral blood flow (CBF) before and after middle cerebral artery occlusion (MCAO) in both young and aged mice. Unlike the traditional laser Doppler flowmetry, LSF provides the spatial and temporal pattern of CBF in the brain.10 Therefore, perfusion of the brain through collaterals can also be visualized and quantified. The high-resolution images and flux traces allowed efficient intraoperative monitoring of the CBF and its dynamics in young and aged mice. Fluorescein isothiocyanate (FITC)-dextran, CD31, and IgG staining were also used to evaluate differences in the microvasculature and extravasation of blood–brain barrier (BBB) in the young and aged brains after stroke.

Materials and Methods

Experimental Animals
Young (8–12 weeks) and aged (18–20 months) C57BL/6 male mice were purchased from National Institute of Aging colonies. The mice were housed in sawdust bedding cages and had access to chow and...
Neurological deficit scores were recorded 24 hours after MCAO: 2 minutes of postreperfusion period after 10-minute reperfusion. 50 minutes of preischemic period, 50 minutes of intraischemic period, and 5 minutes of postreperfusion periods. Data were expressed as mean CBF flux of 2 minutes. The imaging was set up at a display rate of 25 Hz, time constant of 1 second, and a camera exposure time of 4 milliseconds. A 2-minute baseline CBF flux was recorded before induction of ischemia. MCAO surgery was performed and live image and flux recordings were made throughout the intraischemic and postreperfusion periods. Data were expressed as mean CBF flux of 2 minutes of preischemic period, 50 minutes of intraischemic period, and 2 minutes of postreperfusion period after 10-minute reperfusion. Neurological deficit scores were recorded 24 hours after MCAO. Briefly, a midline scalp incision was made to an isoflurane-anaesthetized mouse and the skull was exposed; the charge coupled device camera of LSF was installed 30 cm from an isoflurane-anaesthetized mouse and the skull was exposed; animals that did not show any decrease in CBF by LSF after suture insertion were excluded from the study.

Laser Speckle Flowmetry
Laser speckle perfusion imaging of the brain was performed using moorFLPI Full-Field Laser Perfusion Imager (Moor Instruments, Devon, UK) according to the manufacturer’s instructions and as described previously. Briefly, a midline scalp incision was made to an isoflurane-anaesthetized mouse and the skull was exposed; the charge coupled device camera of LSF was installed 30 cm above the skull using an articulating arm. Two identical rectangular regions of interest were selected on each of the 2 hemispheres (Figure 1A). The imaging was set up at a display rate of 25 Hz, time constant of 1 second, and a camera exposure time of 4 milliseconds. A 2-minute baseline CBF flux was recorded before induction of ischemia. MCAO surgery was performed and live image and flux recordings were made throughout the intraischemic and postreperfusion periods. Data were expressed as mean CBF flux of 2 minutes of preischemic period, 50 minutes of intraischemic period, and 2 minutes of postreperfusion period after 10-minute reperfusion. Neurological deficit scores were recorded 24 hours after MCAO: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, no spontaneous locomotor activity or barrel rolling.

Terminal Histopathology and IgG Staining
One cohort of mice was euthanized at 24 hours after MCAO and a second cohort was euthanized at a 72-hour end point. The brains were perfused with cold PBS followed by 4% paraformaldehyde. The brains were then postfixed in paraformaldehyde for 24 hours and placed in cryoprotectant (30% sucrose), frozen and then sliced into 30-μm free-floating sections on a freezing microtome. Every eighth section was stained with cresyl violet stain to evaluate ischemic cell damage, as described previously.

IgG staining shows BBB compromise. Brain sections were stained with IgG, as described previously. Briefly, first endogenous peroxidase activity was blocked using 1% H2O2, and then incubated with biotinylated antimouse antibody (Vector Laboratories) for 30 minutes, rinsed in PBS, and incubated in Vectastain ABC reagent for 30 minutes followed by 1-minute hematoxylin counterstaining. After rinsing in PBS, the reaction product was visualized using 3,3’-diaminobenzidine (DAB) staining and images were captured using the MicroPublisher 5.0 RTV (Q Imaging) brightfield microscope with the Metamorph software (Universal Imaging Corporation). Sections were quantified subsequently by a blinded observer using SigmaScan Pro (version 5.0) image measurement software. The area of DAB signal on the ipsilateral hemisphere was measured and IgG extravasation was calculated as the ratio of DAB signal/total hemispheric area.

FITC-Dextran Staining
A total of 150 kDa FITC-dextran (Sigma) was injected through the femoral vein of mice at 24 hours of MCAO. The mice were then euthanized by cervical dislocation (after 2 minutes of injection) and the brains were harvested and flash frozen in 2-methylbutane on dry ice. Frozen brains were then embedded in optimal cutting temperature embedding solution and cut into 10-μm sections on a cryostat at –19°C and mounted on charged slides.

Immunohistochemistry
Immunohistochemical staining of frozen fixed sections (30 μm) was performed as described previously. Briefly, brain slices were mounted onto gelatin-coated slides, allowed to air dry, and then blocked in 0.1 mol/L phosphate buffer with 0.3% TritonX-100 (Sigma) and 10% donkey serum for an hour. Primary antibody (CD31, Sigma, 1:500) was added overnight. After washes, the sections were incubated with secondary antibody (1:1000) and 4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI, 1:1000, 6,16,17

**Figure 1.** Laser speckle flowmetry (LSF) images in the brain after stroke. **A.** The 2 rectangular boxes indicate regions of interest selected for measurement on the exposed skull of the mice. **B.** LSF flux images (perfusion images) showing cerebral blood flow (CBF) preischemia, intraischemia, and postreperfusion in young vs aged mice. No difference in CBF was noted in young vs aged animals at the baseline (preischemia), intraischemically, and postreperfusion. After middle cerebral artery occlusion the ipsilateral side (right side) shows a reduction in CBF and postreperfusion the CBF is restored. The arrow in young/intraischemia indicates occluded blood vessel. The arrow in young/postreperfusion shows release of obstruction and blood flow through that vessel. Red color, high CBF; blue color, low CBF.
Fluorescent Microscopy

FITC-dextran and CD31 images were acquired with a Zeiss Axiosvert 200 mol/L microscope (Carl Zeiss, Oberkochen, Germany) using a X-Cite 120Q fluorescence illumination system (Lumen Dynamics Group Inc, Mississauga, ON, Canada) and Zeiss image acquisition software (Zeiss LSM 510).

Brain slices were taken at the same distance from bregma (0.5 mm anterior to bregma) and 3 (20×) fields per animal were analyzed by a blinded observer in the penumbral area of the infarct. Microvascular density was evaluated by calculating the integrated pixel density of the images using MacBiophotonics ImageJ software. The average of the integrated pixel density of 3 fields of view (20×) was used for the analysis.

Statistical Analysis

Flux data are presented as mean±SEM. CBF flux percentage was calculated by normalizing the baseline preischemic flux to 100% and expressing the intraischemic and postreperfusion flux as a percentage of the baseline. Two-way ANOVA with factors of aging (young and aged) and stroke (prestroke, intraischemic, and postreperfusion) was used. The ordinal data (neurological score) is expressed as median (interquartile) and was analyzed using Mann–Whitney U test on SPSS software (version 20). Integrated pixel density and IgG immunoreactivity were analyzed by t test with unequal variance. \( P<0.05 \) was considered statistically significant.
Results

CBF Flux in the Brain After Stroke

LSF was performed in both young and aged mice subjected to 60-minute MCAO (Figure 1A and 1B). One mouse that did not show ischemia by LSF was excluded, and the mortality was 5% by the end of this experiment. There was no significant effect of aging on the CBF flux in the ipsilateral hemisphere (Figure 2A; \( P = 0.4; n = 7 \) per young group, \( n = 8 \) per aged group), indicating that there was no difference in CBF in young versus aged mice at the baseline (prestroke measurements), during ischemia and postreperfusion. Not surprisingly, there was a significant main effect of stroke on both the CBF flux (\( F[2, 45] = 50.4; P < 0.001 \)) and CBF flux percentage (\( F[2, 45] = 62.2; P < 0.001 \)) in young and aged mice (Figure 2A and 2B). Intraischemically, there was a significant decrease in CBF flux to 33.8±1.9% (young) and 33.4±6.6% (aged) from the baseline. No collateral vascular flow specific to the aged was seen on the flux images. The CBF returned to 73±6.4% in young and 64.5±9.5% in aged mice 10 minutes after reperfusion.

Similar to the ipsilateral hemisphere, no significant difference in perfusion (CBF flux) was seen in young versus aged mice at baseline, during MCAO, and postreperfusion in the contralateral hemisphere (Figure 3A and 3B; \( P = 0.6 \)). A significant effect of stroke on the CBF flux was observed (\( F[2, 45] = 6.2; P < 0.001 \)), as the perfusion during MCAO in both the young (79.6±4.4%) and the aged (79.9±6.7%) mice was significantly decreased from the baseline.

Neurological Scores Are Worse in the Aged Mice

The neurological deficit was scored 24 and 72 hours after stroke. At 24 hours of stroke, aged mice had significantly worse neurological scores, 4 (0) versus 1 (0.5) in young mice (\( P < 0.05 \)). There was no significant difference in the weight loss between young (5.7±1.5%) and aged (6.8±1.3%) mice at 24 hours after MCAO. The same pattern was also seen in 72-hour cohort.

FITC Dextran and CD31 Show No Difference in Microvascular Density

To confirm the LSF data that there was no difference in CBF flux between young and aged mice after stroke, we further examined the integrity of microvasculature in both cohorts. FITC-dextran staining found dextran localized along the vessels; there was no significant difference in dextran-integrated pix density between the young and aged cohorts (Figure 4B and 4C; \( P < 0.05 \); 9 images from 3 brains were analyzed). We further assessed the microvasculature using CD31 staining. The integrated vessel density was not significantly different in young versus aged mice (Figure 5A and 5B; \( P < 0.05 \); 9 images from 3 brains were analyzed). This suggests that structural differences pertaining to vessel density do not exist in young versus aged mice.
BBB Breakdown Is Higher in Young Mice
Because we found no difference in the perfusion but worse neurological scores in the aged mice, we wanted to explore the integrity of the BBB in these mice using IgG staining. Figure 6A shows representative coronal brain sections of cresyl violet staining. Figure 6B shows IgG staining in mice euthanized at 24 and 72 hours of MCAO, respectively (n=5 in aged and n=6 in young group). Significantly higher IgG immunoreactivity was seen in young versus aged mice post stroke, indicating higher BBB leakage in young brains (P<0.05; Figure 6C and 6D).

Discussion
This study is the first to use LSF to monitor CBF changes in the brains of aged mice peri-ischemia. Several important novel findings were found. First, by the functional analysis of CBF with LSF, we established that the different stroke phenotypes between young and aged animals seen in this study and previous reports are not because of different levels of ischemia in young versus aged brains. Second, the structural analysis of CBF with FITC-dextran and CD31 staining also revealed that the cerebral microvascular density is not different in young versus aged animals after MCAO. Third, our LSF data demonstrated that the unilateral MCAO not only induces infarction in the ipsilateral hemisphere, but also causes CBF reduction in the contralateral hemisphere, indicating a widespread and diffuse change that occurs throughout the brain and vasculature even in focal stroke models. Finally, we found that aged male mice have less IgG extravasation at both the acute and subacute stages of stroke as compared with the young animals, suggesting that there was less BBB breakdown in the aged ischemic brains.

It is increasingly accepted that modeling stroke in aged animals more appropriately mimics patients with stroke. However, stroke studies in aged animals are often not performed because of the high cost of aging animals and technical expertise required for surgeries. The few studies available in the literature have shown varying results in the histological outcomes in young versus aged animals. There have been reports of increased, equal, or decreased infarct volumes in aged versus young animals. The discrepancies may be because of the difference in animal strains, animal sexes, or ischemic models used in the studies. Several clinical studies demonstrated that age negatively correlated with the size of either the ischemic lesion or the penumbral area in patients with stroke, which is consistent with the results from our studies. Despite discrepancies in histological outcomes, there is agreement in most preclinical and clinical studies that functional (behavioral) stroke outcomes are worse in aged cohorts. The underlying mechanism remains elusive and may be because of age-related comorbidities, an increased systemic immune response, autonomic or cardiac instability, and decreased neurogenesis angiogenesis, all of which have been well documented in aged animals.
Aging is associated with structural alterations in the vessel wall (intima and smooth muscle cells), which leads to arterial stiffening, endothelial dysfunction, atherosclerosis, and calcification of vessels.30–32 Therefore, it was physiologically plausible that there may be a structural difference in the cerebral vasculature and also a functional difference in the cerebral perfusion either at the baseline and in the setting of an ischemic injury, leading to smaller infarcts in the aged. However, both functional and structural analysis of CBF perfusion performed in the present study did not support this hypothesis. We did not see differences in the baseline, intraischemic, and postreperfusion CBF between the young and aged cohorts, consistent with clinical studies that showed CBF did not decline with age in the healthy individuals33 and that there were no differences in CBF in young and aged patients with acute stroke.7 Thus, it seems that age-related changes in the vascular bed do not significantly change the perfusion of the brain territories before or after stroke, and neither does the aged brain seem to have enhanced collateral-mediated reperfusion intraischemically, suggesting that other molecular mechanisms downstream to ischemia instead of CBF changes may be involved in mediating the age-dependent stroke injury.3,16 Interestingly, our LSF data revealed that CBF in the contralateral hemisphere also significantly dropped from the baseline after stroke, which exemplifies the fact that stroke is a systemic disease and that the ischemic challenge may be sensed throughout the vasculature. Systemic changes in distant organs (eg, the spleen) induced by stroke have been increasingly reported34,35 and are most likely mediated by activated molecules or metabolites released from the ischemic brain tissue into the circulation.

The amount of plasma protein IgG leakage correlated with infarct volumes. Less IgG extravasation was seen in the aged brain indicating less BBB breakdown, consistent with our previous finding that Evans blue extravasation was significantly less in aging versus young ischemic brains.4 It is interesting that despite similar intraischemic reductions in CBF, the BBB integrity was less compromised in the aged brain. Expression of connexin 43, a major gap junction protein and a key component of BBB, was reduced in young mice but preserved in aging brains after ischemic stroke.18 Although aging has deleterious effect on BBB function under normal conditions,36 young brains may still have more robust BBB breakdown after ischemia because of the enhanced inflammatory responses compared with aged brains.20 However, whether the relatively preserved BBB integrity in the aged brain is a result of less ischemic injury or vice versa remains unknown. Experimental studies37,38 found that the inhibition of matrix metalloproteinase-9, a proteolytic enzyme that degrades the BBB, is neuroprotective by reducing infarct size, suggesting that BBB integrity is important in limiting the ischemic

Figure 6. Cresyl violet (CV) and IgG staining (hematoxylin counterstain) in young vs aged brain sections 24 hours after middle cerebral artery occlusion (MCAO). A, Representative CV staining pictures showing young brains had larger infarct volumes (white) than aged brains. B, Representative IgG staining pictures showing young brains had higher IgG immunoreactivity (brown) than aged brains at 24 and 72 hours end point. C, Microscopic images (×20) of IgG staining in young vs aged brains. Pictures are taken from the area marked with a box in B. Brown shows the IgG staining. Blue is the counterstain with hematoxylin. D, Quantification of IgG immunoreactivity shown in B and C. Young brain shows higher IgG immunoreactivity (brown) than the aged at both 24 and 72 hours after MCAO, *P<0.05 vs young group (n=6 young, n=5 aged).
lesion. The possibility could not be excluded that the less injured BBB in aged ischemic brains may be causative to the smaller infarct compared with young brains in the present study. Future studies are needed to examine age-related BBB changes after ischemia.

There are several limitations to this work that need to be recognized when interpreting our data. We only used LSF for the functional analysis of CBF, as LSF allows us to monitor the CBF change intraschemically in a live animal. 14C-iodoantipyrine autoradiography is a more accurate and quantitative method to examine brain tissue perfusion after ischemia. However, we did not elect to do this because 14C-iodoantipyrine autoradiography is a terminal study and cannot be used to monitor CBF in real-time preischemic, intraschemic, and also postperfusion in the same animal. Moreover, LSF does not inform us about CBF differences in subcortical gray and white matter. Iodoantipyrine autoradiography would be more appropriate for such an assessment. Iodoantipyrine autoradiography, arterial spin labeling, and diffusion tensor imaging are the techniques which can be used in future studies to examine the association between cortical and subcortical white matter CBF. Another caveat is that we only focused on the acute stage of MCAO for structural analysis of CBF; whether microvascular density postischemia changes overtime to cause a difference between young and aged animals needs further investigation. All the animals involved in this study were males, and we did not examine whether there is a difference in CBF changes between young and aged female animals. Sex differences in age-related CBF changes after ischemia could exist as females exhibit different stroke phenotypes than their male counterparts at either young or old age, which is one of the on-going projects by our research group.

In conclusion, young and aged animals exhibit equivalent levels of CBF throughout ischemia, and the age-specific stroke phenotype seems to be unrelated to CBF/perfusion changes in stroke. Microvascular density in the ischemic brain was not different between young and aged animals. Aged brains have less IgG immunoreactivity after MCAO than the young group, which is related to the smaller infarct size seen in aged brains. Other molecular mechanisms that are unrelated to acute CBF changes may underlie age-specific stroke phenotypes.

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Disclosures
None.

References


