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Early disruptions of the blood-brain barrier may contribute to exacerbated neuronal damage and prolonged functional recovery following stroke in aged rats

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Abstract

We examined the effects of age on stroke progression and outcome in order to explore the association between blood-brain barrier (BBB) disruption, neuronal damage, and functional recovery. Using middle cerebral artery occlusion (MCAO), young (3 months) and aged (18 months) rats were assessed for BBB disruption at 20 min post-MCAO, and 24 h post-MCAO with tissue plasminogen activator induced reperfusion at 120 min. Results showed that BBB disruptions in aged rats occurred early and increased nearly two-fold at both the 20 min and 24 h time points when compared to young animals. Neuronal damage in aged rats was increased two-fold as compared to young rats at 24 h, while no neuronal damage was observed at 20 min. Young and aged rats exhibited neurological deficits when compared to sham-controls out to 14 days following MCAO and reperfusion; however, aged rats exhibited more severe onset of deficits and prolonged recovery. Results indicate that aged rats suffer larger infarctions, reduced functional recovery and increased BBB disruption preceding observable neuronal injury.

Keywords

Blood-brain; Ischemia; Aging; Stroke; Permeability; Tissue plasminogen activator

1. Introduction

Stroke is a disease of the elderly. Approximately 72% of people who suffer a stroke are over the age of 65, 25% of the population over the age of 75 will have a stroke, and the incidence of stroke more than doubles with each successive decade for people over the age of 55 (AHA, 2001). Increased infarction volumes and altered physiologic response has been demonstrated in aged rodents following middle cerebral artery occlusion (MCAO) (Sutherland et al., 1996; Rosen et al., 2005; DiNapoli et al., 2006). Young male animals and filamentous methods of occlusion commonly utilized in laboratory investigations may not accurately represent the

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2006.12.007.

stroke patient, as these methods have consistently identified promising neuroprotective candidates that have failed in clinical trials (Fisher, 1999; Gladstone et al., 2002; Fisher and Ratan, 2003).

The disparity between laboratory models and clinical studies may be secondary to changes which occur in the blood-brain barrier (BBB). A number of morphological and functional changes to the BBB occur during the aging process, including remodeling of the extracellular matrix, decreased occludin expression, changes in lipid and protein composition (Burns et al., 1981; Mooradian, 1988; Mooradian and Meredith, 1992; Mooradian and Smith, 1992), alterations in nutrient and protein transport (Banks and Kastin, 1985; Kleine et al., 1993; Shah and Mooradian, 1997), and decreased reactivity to β -adrenergic neurotransmission (Mooradian, 1994). These alterations make the barrier more vulnerable to insult, such as ischemia. Cessation of cerebral blood flow and the subsequent lack of tissue oxygenation can lead to increased BBB permeability by a progressive loss of component antigens from the basal lamina and extracellular matrix, as well as alterations in endothelial cell-cell and cell-matrix interactions. The active hypoxia/ischemia induced following a stroke stimulates the surface expression of adhesion molecules (Haring et al., 1996; Mao et al., 2000) and changes in integrin expression (Wagener et al., 1997) in the first few hours. Activation of the BBB leads to endothelial dysfunction, characterized by increased permeability, extravasation of plasma components, and edema formation (Okada et al., 1996). The effect aging has in this process and how it may affect stroke outcome are not well characterized.

We utilized a clinically relevant model of stroke, based upon selective embolization of the middle cerebral artery (MCA) combined with recombinant tissue plasminogen activator (rt-PA) induced reperfusion, and assessed tissue loss, functional outcomes, histopathology and changes in BBB permeability in young (3 months) and aged (18 months) Sprague-Dawley rats. Our data demonstrated that the BBB in aged animals is disrupted earlier and to a greater degree compared to young animals at 24 h post-MCAO and rt-PA treatment, and 20 min post-MCAO. Aged rats suffer strokes which are larger in size and have more severe functional consequences, providing a potential mechanism by which accelerated breakdown of the BBB during acute stroke leads to worsened outcomes.

2. Materials and methods

2.1. Animals

This study was conducted in accordance with NIH guidelines for the care and use of animals in research. All protocols were approved by the West Virginia University Animal Care and Use Committee. Two groups of female, age-matched, Sprague-Dawley rats were investigated. Young (3-4 months; $n = 68$), and aged (17-18 months; $n = 77$) animals underwent an embolic MCAO.

2.2. Surgical procedure for MCAO

Rats were anesthetized with an intraperitoneal injection of 2.5 mg/kg flunixin, 90 mg/kg ketamine and 5 mg/kg xylazine, or with inhaled isoflurane (4% induction; 1% maintenance) for BBB objectives. A servo-controlled homeothermic heating blanket, utilizing a rectal thermometer, was used to maintain body temperature at 37 °C. A cartridge based arterial blood gas machine (GEM Premier 3000, Instrumentation Laboratory, Lexington MA) was used to monitor arterial P_{aO_2} , P_{aCO_2} , pH and hematocrit. Blood from the femoral artery was analyzed just prior to embolization, during ischemia, and following recanalization. The femoral vein was catheterized for fluid replacement (1.4 ml/h 0.9% saline) and drug delivery.

Rats underwent selective embolization of the right middle cerebral artery (MCA), producing 120 min of ischemia, followed by rt-PA induced reperfusion utilizing a technique recently described (DiNapoli et al., 2006). Briefly, a micro-catheter, outer diameter 0.3 mm, was inserted into the ICA via the ECA stump and advanced until its tip occluded the ipsilateral MCA. This mechanical occlusion was verified by laser Doppler (LD-CBF) monitoring of the MCA perfusion territory. The catheter was retracted until MCA flow was restored around the microcatheter. A 25 mm fibrin-rich, autologous blood clot was then injected directly into the MCA. Ischemia was monitored continuously for 120 min, rt-PA was administered via the femoral vein, and restoration of MCA flow was verified by LD-CBF.

2.3. Functional testing

Rats were handled and habituated to testing procedures in order to establish stable baselines on all measures. Animals were then tested repeatedly in a battery of functional assessments described below, beginning 24 h post-MCAO or sham operation and continuing daily for 1 week. Animals were then allowed to recover for an additional week and final assessments made at 14 days post-insult. Scores were obtained by an observer blind to treatment of subject in order to reduce bias. However, grossly observable differences in the functional performance of young versus aged animals following stroke make true blinding of observer difficult.

2.3.1. Composite functional score—The mNSS is a composite score of motor, sensory, balance and reflex measures and ranges from 1 to 17, with higher scores indicating greater neurological injury (Germano et al., 1994; Chen et al., 2001; Seyfried et al., 2004).

2.3.2. Bracing—Postural adjustments were assessed according to methods previously reported (Schallert et al., 1979). Animals were gently pushed laterally across a stainless steel bench top over a distance of 90 cm, at a rate of approximately 20 cm/s, by the experimenter's hand. Rats will generally adjust their posture, making numerous adjustments with their forelimb on the side to which they are being moved. These adjustments were tallied over two trials and analyzed.

2.3.3. Placing—This is a test of sensorimotor function previously reported to be effected by normal aging in rodents (Marshall, 1982). Animal were held suspended by the investigator, allowing free movement of the unsupported limbs. The animal was then brought to the edge of a bench top, such that its body was parallel to the table's edge and its whiskers brushed against the top surface. The number of times the animal successfully raised its forelimb to the table top was tallied over a series of 10 trials for each forelimb.

2.3.4. Tactile adhesive-removal test—Somatosensory function was tested as previously reported (Schallert et al., 1982, 1983). A small adhesive paper circle ("Color Coding Labels", 1/4 in. in diameter, ACCO brands, Lincolnshire, IL) was placed on the distal-radius area of each forelimb and the animal was placed back in cage. The time taken for the animals to remove to stimulus with its mouth was recorded over a series of three trials, with an inter-trial interval of 1-2 min, and a maximum trial length of 180 s. The means from three trials were analyzed.

2.3.5. Akinesia test—This test assesses the animal's ability to initiate movements with the paretic forelimb (Schallert et al., 1992). The rat was held by the experimenter such that one forelimb was allowed to rest on the table top and move freely. The number of steps taken with the limb over a 30 s trial was tallied for each forelimb.

2.4. TTC staining

Animals were allowed to recover 24 h following MCAO and rt-PA reperfusion, anesthetized with Sleepaway (Fort Dodge Animal Health), the brains were removed immediately and sliced

coronally at 2 mm intervals. Slices were placed in 2%-2,3,5-triphenyltetrazolium chloride (VWR International, West Chester PA) in 0.1 M PBS for 20 min at 37 °C, scanned on a flatbed scanner and analyzed using Adobe Photoshop software (v7.0). Infarction volumes were determined according to the methods described by Yang et al. (1998). Briefly, volumetric calculations were performed by an observer blinded to treatment. Determination of cortical and striatal infarct border, to be included in analysis, was performed according to methods previously reported (DiNapoli et al., 2006) and consistent throughout calculations.

2.5. Tissue collection for frozen sections

Animals were allowed to recover for designated period, anesthetized with Sleepaway (Fort Dodge Animal Health), and transcardially perfused with 100 ml of 0.09% saline followed by perfusion solution (4.0%, w/v paraformaldehyde in 1X SPBS). Brains were removed, placed in fixative for 24 h, cryoprotected in a series of 10, 20 and 30% sucrose w/v in DPBS solution for 24 h per solution and sectioned at 35 µm.

2.5.1. Hematoxylin and eosin staining—Slide-mounted sections from three sham-controls and three randomly selected animals in each age group 24 h post-MCAO and rt-PA reperfusion, were rehydrated in DPBS for 5 min, and incubated in hematoxylin and eosin (Fischer Scientific) stain for 5 min at room temperature. Sections were rinsed for 5 min in running tap water and dehydrated for 5 min each in 75%, 95% and 100% ethanol, cleared in xylene, and coverslipped with Permount (Fischer Scientific). Sections were taken serially at 250 µm intervals, and examined utilizing light microscopy for areas of polymorphonuclear infiltration, hemorrhage and edema.

2.5.2. Fluoro-Jade B staining—Slide-mounted sections from three sham-controls and three randomly selected animals in each age group 24 h post-MCAO and rt-PA reperfusion, and 20 min post MCAO, were stained with Fluoro-Jade B. Slides were immersed in a solution of 1% sodium hydroxide in 80% ethanol for 5 min, followed by immersion in 70% ethanol for 2 min, and distilled water for 2 min. The slides were transferred to 0.06% potassium permanganate for 10 min on a shaker to suppress background staining. Following a rinse in distilled water for 2 min, the slides were transferred to the staining solution for 20 min (stock FJB solution: 10 mg dye in 100 ml distilled water; 0.004% working solution: 4 ml stock solution in 96 ml 0.1% acetic acid). The slides were washed three times for 1 min each in distilled water, and air-dried overnight. The slides were coverslipped directly with DPX (Fisher Scientific).

2.6. Confocal microscopy

At 23 h following MCAO and rt-PA administration, 1 mg each of lysine fixable dextran (70,000 Da; AlexaFluor 488) and fibrinogen (300,000 Da; AlexaFluor 568) dissolved in 0.5 ml of 0.9% saline were injected into the femoral vein. After 1 h, rats were anesthetized and transcardially perfused with 0.9% saline for 5 min then 4% paraformaldehyde/0.1 M sodium cacodylate buffer (pH 7.2). For 20 min assessments, fluorescent markers were injected 40 min prior to clot injection and animals were perfused, as stated above, 20 min post-MCAO. Following perfusion, brains were removed and post fixed in 4% paraformaldehyde overnight. Areas around infarction were cut into 4 mm blocks, sliced into 60 µm sections, and assessed for fluorescent reactivity using a Zeiss LSM 510 confocal microscope and analyzed using Optimas analysis system.

2.7. Evans blue albumin extravasation

Albumin extravasation was quantified 24 h following MCAO and rt-PA reperfusion. Rats were anesthetized with 2.5 mg/kg flunixin, 90 mg/kg ketamine and 5 mg/kg xylazine and the infused with 2% Evans blue (4 ml/kg) via the femoral artery. The Evan's blue was allowed to

circulate for 1 h and the rats were perfused with cold phosphate buffered saline (pH 7.4) for 15 min via the left ventricle. The brains were excised; meninges and ependymal organs removed, hemispheres excised, separated, weighed, and homogenized in 500 μ l of 50% trichloroacetic acid. The tissue was incubated for 24 h at 37 °C, and then centrifuged at 13,000 $\times g$ for 10 min. The supernatants were diluted four-fold with absolute ethanol; fluorescence intensity was measured using a fluorometer at 620 nm excitation, 680 nm emission (Beckman DU 640). Calculations were based on external standard readings and extravasated dye was expressed as ng EB/mg brain tissue.

2.8. Statistical analysis

Data and figures are presented as means $\pm$ S.E. Physiologic parameters and functional data were compared by two-way ANOVA with groups of age (young and aged) by treatment (pre- or post-stroke), followed by Tukey's post hoc evaluation. Infarction volumes and EB data were compared by one-way ANOVA. Post hoc *t*-tests were performed as directed by ANOVA outcomes. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Physiologic variables

Physiologic data for groups subjected to MCAO are presented in Table 1. Significant differences between groups were not observed, and values are within normal physiologic ranges.

3.2. Mortality

Aged animals exhibited a 33% mortality rate at 24 h post-MCAO and rt-PA reperfusion with 12 of 36 animals dying during the first day of recovery, young animals suffered 17% mortality (5 of 29). In the group receiving MCAO only, 4 of 9 aged animals and 2 of 7 young animals died during the 24 h recovery period. There were no deaths amongst sham-operated animals or prior to 20 min sacrifice. The final analyses included 42 sham-operated controls, 65 rats with 120 min MCAO and rt-PA reperfusion, 16 rats with 120 min MCAO and 22 rats with 20 min MCAO.

3.3. Tissue loss is exacerbated in aged animals

Infarction volumes were significantly affected by animal age in both the cortical ($F[2,27] = 8.5$, $P = 0.001$) and striatal ($F[2,27] = 6.9$, $P = 0.004$) regions, 24 h following 120 min MCAO and rt-PA induced reperfusion (Fig. 1C). The volume of infarcted cortex relative to contralateral hemisphere was significantly higher in the aged (Fig. 1B) group ($63\pm 9\%$) when compared to the young (Fig. 1A) ($26\pm 6\%$, $P = 0.002$). Striatal infarction volumes were also significantly greater in the aged group ($53\pm 5\%$) relative to the young ($33\pm 4\%$, $P = 0.006$).

3.4. Increased hemorrhage and infiltration of inflammatory cells into the infarct is observed in aged animals

At 24 h following MCAO and rt-PA reperfusion, brains from three animals in both age groups were harvested for pathological examination. Fig. 2 displays representative photomicrographs (H&E) of slices from a young and aged rat. In the young animal (Fig. 2A), a central area of coagulation necrosis is evident. This area contains sparse aggregates of acute inflammatory cells (PMNs). Penumbra contains swollen neurons, some with vacuolated neuropil. In the aged animal (Fig. 2B), the area of coagulation necrosis is partially obscured by dense inflammatory cell infiltration and petechial hemorrhages. Increased vacuolation of the neuropil is evident.

3.5. Functional deficits are more severe with prolonged recovery in aged animals

All animals were assessed for gross neurologic competence after awakening from anesthesia. Behavioral deficits were grossly apparent in both age groups throughout the 14 days testing period, with the persistence of circling in 4 of 10 young animals and all aged animals. Impairment in normal feeding and grooming behavior was observed by 1 day post-MCAO. A red Harderian gland secretion surrounding the eyes and nostrils was present in most animals during the initial 48-72 h period, indicating significant stress following the insult. Feeding and grooming behavior returned in all young animals by 7 days following MCAO, however, 4 of the 10 aged animals remained severely impaired until time of sacrifice.

Consistent with gross observations, behavioral assays detected significant functional impairment compared to baseline in both the young and aged animals throughout the recovery period (Fig. 3). Animals were tested for neurological competence utilizing the modified neurologic severity scale (mNSS) (Chen et al., 2001), sham-operated animals are represented as day 0. ANOVA demonstrated a significant effect of age on functional severity of stroke ($F[2,27] = 17.07$, $P < 0.001$) (Fig. 3A), with elevated mNSS scores correlating to increased severity of functional impairment.

Results of the bracing test showed a significant effect of treatment on performance of both young and aged rats throughout the 14 days testing period ($F[8,72] = 5.83$, $P < 0.001$) (Fig. 3B). Additionally, age had a significant affect on performance ($F[1,72] = 223.06$, $P < 0.001$). Sham-controls in the aged group (10.4 ± 0.6) demonstrated decreased baseline performance when compared to young sham-controls (13.6 ± 0.24) ($P < 0.01$).

Results of the placing test showed a significant effect of treatment on performance of both young and aged rats throughout the 14 days testing period ($F[8,72] = 24.69$, $P < 0.001$) (Fig. 3C). Additionally, age had a significant affect on performance ($F[1,72] = 933.11$, $P < 0.001$). Young animals fully recovered in this task by day 14, with a peak deficit of 8.6 ± 0.74 responses/10 trials. Aged animals recovered to a mean of 4.4 responses/10 trails by day 14, with a peak deficit of 0 responses on days 1-3.

Results of the tactile removal test showed a significant effect of treatment on performance of both young and aged rats throughout the 14 days testing period ($F[8,72] = 22.64$, $P < 0.001$) (Fig. 3D). Additionally, age had a significant affect on performance ($F[1,72] = 13.2$, $P < 0.001$). Young animals showed significant recovery in this task by day 7 with a mean removal time of 96.3 ± 29.6 s, and a peak deficit of 170.6 ± 9.3 s on day 1. Aged animals did not show meaningful recovery.

Results of the akinesia test showed a significant effect of treatment on performance of both young and aged rats throughout the 14 days testing period ($F[8,72] = 21.5$, $P < 0.001$) (Fig. 3E). Additionally, age had a significant affect on performance ($F[1,72] = 98.8$, $P < 0.001$). Young animals showed moderate recovery in this task by day 14 with a mean of 26 ± 2.7 steps, a baseline of 43.6 ± 0.9 and a peak deficit of 16.4 ± 3.3 steps on day 1. Aged animals did not show meaningful recovery with a mean of 10.6 ± 1.3 steps on day 14, a baseline of 40.4 ± 1.9 and a peak deficit of 7 ± 1.6 steps on day 1.

3.6. BBB permeability 24 h post-MCAO is two-fold higher in the aged brain

BBB permeability in young and aged rats was assessed at 24 h post-MCAO by quantifying extravasation of albumin (Fig. 4A) and visualization of fluorescent vascular markers by confocal microscopy (Fig. 4B frames 3-6). Administration of rt-PA (5 mg/kg) at 2 h after MCAO significantly ($p < 0.01$) reduced albumin extravasation in infarcted hemispheres of both young and aged rats at 24 h post-MCAO (Fig. 4A). Aged animals demonstrated a 212% increase in BBB permeability to albumin in the infarcted hemisphere as compared to young animals at

24 h post-MCAO and rt-PA reperfusion (Fig. 4A; $p < 0.01$). No significant ($p > 0.05$) difference in albumin extravasation at the BBB was noted between contralateral hemispheres of young and aged rats (Fig. 4A) nor in comparing contralateral hemispheres of young and aged rats to age-matched sham control hemispheres (data not shown).

Fig. 4B (frames 3-6) depict a visualization of cerebral microvessels in an area adjacent to the infarct (penumbra) of young (frame 3) and aged (frames 4-6) rats at 24 h post-MCAO and rt-PA reperfusion using two vascular space markers (dextran-70 kDa and fibrinogen-300 kDa). Frame 3 shows fibrinogen outlining the circuitous pattern of microvessels with no apparent detection of dextran in the microvessels or extracellular space. Frame 4 shows fibrinogen outlining microvessels and the appearance of punctate regions of dextran in the extracellular space. Frame 6 is a brightfield image that clearly shows fibrinogen immunofluorescence is confined to the luminal side of the microvasculature and dextran is found only in the extracellular fluid. Frame 5 is an image of the contralateral side of an aged rat 24 h post-MCAO and rt-PA reperfusion.

3.7. Functional disturbance of the BBB by 20 min post-MCAO

BBB permeability in young and aged rats was assessed at 20 min post-MCAO by quantifying extravasation of albumin (Fig. 5A) and visualization of fluorescent vascular markers by confocal microscopy (Fig. 5B frames 3 and 4). Aged animals demonstrated a 175% increase in BBB permeability to albumin in the infarcted hemisphere as compared to young animals at 20 min post-MCAO (Fig. 5A; $p < 0.001$). No significant ($p > 0.05$) difference in albumin extravasation at the BBB was noted between contralateral hemispheres of young and aged rats (Fig. 5A) or in comparing contralateral hemispheres of young and aged rats to age-matched sham control hemispheres (data not shown).

Fig. 5B (frames 3 and 4) depict a visualization of cerebral microvessels in an area adjacent to the infarct of young (Frame 3) and aged (Frames 4) rats at 20 min post-MCAO using two vascular space markers (dextran-70 kDa and fibrinogen-300 kDa). Frame 3 shows fibrinogen outlining the microvessels with no apparent detection of dextran in the microvessels or extracellular space. Frame 4 shows fibrinogen outlining microvessels and the appearance of dextran in the surrounding extracellular space. No difference in dextran extravasation was noted between contralateral hemispheres of young and aged rats or in comparing contralateral hemispheres of young and aged rats to age-matched sham control hemispheres (data not shown).

3.8. Neuronal damage is preceded by BBB disruption

Neuronal damage in young and aged rats was assessed at 24 h post-MCAO and rt-PA reperfusion (Fig. 4B frames 1 and 2) and 20 min post-MCAO (Fig. 5B frames 1 and 2) by Fluoro-Jade B staining. Aged (Fig. 4B frame 1) animals demonstrated increased severity of injury, characterized by increased density and intensity of staining, as well as a greater degree of vacuolation compared to young (Fig. 4B frame 1) rats. The transition from severe neuronal injury within the MCA distribution to survival within the penumbra can be observed.

No difference in intensity of staining was seen between aged (Fig. 5B frame 1) and young (Fig. 4B frame 2) animals at 20 min post-MCAO. Early neuronal damage could not be revealed utilizing the Fluoro-Jade B staining technique in the young or aged animals.

4. Discussion

We demonstrated that aged rats suffered more severe infarcts with increased disruption of the BBB, a greater degree of neuronal damage, and reduced functional recovery following MCAO.

In addition, we found that rt-PA (5 mg/kg) induced reperfusion at 120 min post-MCAO produced a significant decrease in disruption of the BBB to albumin in young and aged animals at 24 h following MCAO. Finally, we report data that suggests BBB disruption occurs prior to observable neuronal damage during MCAO; thus, suggesting a potential mechanism by which alterations in BBB functional integrity during stroke contribute to the degree of subsequent neuronal damage.

A primary concern of this study was the use of rt-PA in rodents. Prior investigations have administered an rt-PA dosage 10-20 times the therapeutic dose for humans. A primary reason for this discrepancy in dose was that rt-PA is produced by human recombinant DNA, and species variations decrease rt-PA efficacy in rodents. In humans, rt-PA is administered at a dosage of 0.9 mg/kg and has a narrow therapeutic window. Since introduction in 1995, rt-PA has remained the only approved method of drug induced thrombolysis for acute ischemic stroke (NINDS, 1995). These breakthroughs have been hindered by unwanted consequences of rt-PA treatment, most prominently the increased risk of hemorrhagic transformation and neurotoxicity (NINDS, 1995; Jiang et al., 2003).

Successful and reproducible thrombolysis was achieved in rodents at half the rt-PA dose which is commonly administered, limiting the deleterious effects. The drug was only administered according to NIH suggested guidelines, and both young and aged rats were routinely screened for intracranial hemorrhage following administration (DiNapoli et al., 2006). Young and aged rats underwent 120 min of stable MCA occlusion, followed by administration of rt-PA for recanalization. Duration of occlusion and successful reperfusion of the MCA territory was verified by laser Doppler; furthermore, the reperfusion profile was similar to that seen in a clinical setting. Previous studies have reported mortality rates (up to 80%) amongst aged animals receiving 60-120 min of filamentous occlusion, which would preclude further use in MCAO models (Futrell et al., 1991; Hachinski et al., 1992; Wang et al., 1995; Wang et al., 2003). This model produced a 33% mortality rate in the aged animals. Administration of rt-PA at 120 min post-occlusion resulted in significantly decreased disruption of the BBB and reduced mortality in both young and aged animals.

Studies have produced varied results concerning the effect of age on stroke outcome. In comparison to young rats, the aged rats in this study showed nearly a two-fold increase in infarction volume. The principal difference in infarction size can be attributed to larger involvement of the cortical brain region in aged animals. The current study produced larger infarctions volumes in all age groups and brain regions, compared to suture occlusion (Rosen et al., 2005; DiNapoli et al., 2006). The average reported volume amongst the controls in five representative papers on focal ischemia was 135.31 mm³ (Wang et al., 1995; Sutherland et al., 1996; Alkayed et al., 1998; McCullough et al., 2001; Yang et al., 2002). This average is nearly identical to the stroke volumes in the 3-month-old animals of this study, while all of the cited studies utilized male animals. Furthermore, neuroprotective benefits of estrogen are localized primarily to the striatal region (McCullough et al., 2001; McCullough et al., 2003) and could not account for the two-fold increase in volume of infarction volume observed in the aged rats of this study. In addition, histopathology of the infarcted region in aged animals distinctly revealed advanced vacuolation of the neuropil, increased infiltration of polymorphonuclear cells and pettichial hemorrhaging not seen in younger animals. These findings demonstrate that cerebral ischemia in aged rodents creates infarcts which are structurally different than those generated by equivalent insults in younger animals.

The intact BBB has negligible transport of albumin into the brain; however, susceptibility of the BBB has been documented in a number of diseases. Evidence has suggested that a damaged endothelial barrier results in BBB disruption contributing to the progression and onset of several neurodegenerative diseases, including Alzheimer's and Parkinson's diseases (Ariga et

al., 1998; Mackic et al., 2002). Under homeostatic conditions, the BBB remains functionally intact during aging (Mooradian, 1988); however, becomes more susceptible to disruption by external factors with increasing age (Daniel et al., 1978; Burns et al., 1981; Shah and Mooradian, 1997).

In this study, we measured Evan's blue albumin extravasation into the brain parenchyma of infarcted and non-infarcted hemispheres in young and aged rats at 20 min and 24 h post-MCAO, and 24 h post-MCAO with rt-PA induced reperfusion. Young and aged rats exhibited a significant increase in BBB permeability to albumin 24 h post-MCAO and rt-PA reperfusion, and this disruption was two-fold greater in the aged compared to the young rats. Confocal microscopy confirmed leakage of albumin sized molecules across the BBB in aged animals. Microscopy demonstrated extravasation of dextran (70 kDa) in an area adjacent to the infarct of aged animals. The failure to observe dextran in the penumbral region of the infarct in young rats suggests that the BBB is still functionally intact. Fluoro-Jade B staining assessed the degree of neuronal injury present, and demonstrated grossly observable damage within the MCA fed territory of young and aged animals. In addition, the aged rats exhibited increased intensity of staining within injured neurons, as well as vacuolation of the neuropil in agreement with histopathology.

A significant increase in albumin extravasation was also noted at 20 min post-MCAO in both age groups. The disruption in aged animals was again nearly two-fold that seen in the young group and was greater than three-fold that observed in the contralateral hemisphere. Confocal microscopy demonstrated the presence of dextran (70 kDa) in the extracellular space surrounding the MCA perfusion territory at 20 min post-MCAO in aged rats, while Fluoro-Jade B staining did not reveal neuronal injury at this time point. These data demonstrate a functional alteration in the barrier's ability to maintain a separation between systemic circulation and the brain parenchyma, ultimately contributing to cytotoxic changes in the extracellular environment. The presence of this disruption preceding observable neuronal injury suggests that exacerbated breakdown of the BBB in aged rats during acute stroke may contribute to the worsened outcomes observed. Furthermore, it may be important to consider the stabilization of the barrier in investigating new therapeutic interventions for stroke.

Evaluation of functional impairment has become a prominent focus in development of preclinical models, as results gained can be correlated to subsequent clinical assessment in stroke victims. Inclusion of aged animals in long-term functional assays has proven problematic due to impractically high mortality rates. Previous reports have addressed this issue by creating an incomplete filamentous occlusion, limiting the amount of tissue loss, lowering long term mortality (Lindner et al., 2003) and improving mortality in the aged animals to approximately 24%. Their experiments produced long-lasting functional deficits amongst the aged rats, and allowed for functional evaluation in the aged group (age 16 months) out to 90 days post-occlusion. This approach certainly provides a compliment to the traditional use of young animals, which show less initial deficit and recover more quickly; however, the previously mentioned shortcomings of filamentous occlusion remain, as well as the inability to study rt-PA reperfusion and infarctions representative of larger strokes.

In utilizing functional assessments previously reported (Lindner et al., 2003), the current study examined functional deficits in young and aged animals following 120 min embolic MCAO and rt-PA induced reperfusion. Functional deficits seen in young animals were very similar to previous reports, showing a moderate initial deficit followed by significant recovery over a 2 weeks period. Aged animals in this study, however, showed severe initial deficits, with a dense and profound paresis of the limbs on the affected side and significantly poorer performance on a battery of functional challenges. Additionally, aged animals never exhibited functional competence equivalent to the young animals at any time point and recovery was prolonged.

Thus aged rats had a functional status very similar to that seen clinically in patients having suffered a stroke.

The need for an accurate preclinical MCAO model that mimics the stroke population and allows for effective, reliable reperfusion is imperative for the successful translation of therapeutic strategies and improved stroke outcomes. Hope for potential neuroprotective agents has spurred intense research interest in basic science investigation and clinical trials, yet no effective agent has been found. This study clearly demonstrated that age has a profound influence on acute brain damage and subsequent functional recovery following an MCAO and rt-PA reperfusion. These data further the consensus that stroke models should be developed in older animals, and that use of aged animals in preclinical models may increase their clinical relevance and predictive validity (Millikan, 1992). Additionally, we begin to explore the role of the aging BBB and how alterations to this structure during acute stroke affect outcome. Early disruption of the BBB in aged animals during stroke provides a mechanism by which breakdown contributes to subsequent neuronal damage, and is a critical element to consider in the development and implementation of effective therapeutic strategies for treatment of elderly stroke victims.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. TTC stained sections representative of young (A) and aged animals (B) infarction distribution 24 h post-MCAO and rt-PA reperfusion. (C) Volumetric analysis of cerebral infarctions. Infarction volumes are expressed as a percentage of the contralateral structure $\pm$ S.E. *Significant difference in 18 month vs. 3 month age group ($P \leq 0.006$). $N = 10$ for all groups.

Fig. 2.

Representative photomicrographs of slices from young (A) and aged (B) animals, 24 h post-MCAO and rt-PA treatment. In the young rats (A), a central area of coagulation necrosis is evident (closed arrowhead). This area contains sparse aggregates of acute inflammatory cells (*) (PMNs). The margin of the infarct contains swollen neurons, some with vacuolated neuropil (arrow). In the aged animals (B), the area of coagulation necrosis (closed arrowhead) is partially obscured by dense inflammatory cell infiltration (*) and petechial hemorrhages (open arrowhead). Vacuolation of the neuropil is evident (arrow) (scale bar = 100 μ m).

Fig. 3.

Performance on functional tests performed from 1 to 14 days post-occlusion to assess neurological deficit. (A) mNSS, A composite functional score, judged on a scale of 0-17 with higher scores correlating to increased severity of functional deficits ($N= 10/\text{group}$). (B) Bracing test reflects number of postural adjustments made by the animals while being pushed laterally toward the paretic side over 90 cm ($N= 5/\text{group}$). (C) Placing test reflects the number of directed paw placements made in response to somatomotor stimulus over 10 trials ($N= 5/\text{group}$). (D) Tactile removal test reflects the time taken for animal to remove somatosensory stimulus placed on distal forearm ($N= 5/\text{group}$). (E) Akinesia test reflects the number of steps made with usable forearm over 30 s trial ($N= 5/\text{group}$). Scores are means $\pm$ S.E.

Fig. 4.

(A) Extravasation of albumin across the BBB assessed by quantification of Evan's blue dye 24 h post-MCAO. Aged group untreated and treated with rt-PA exhibited greater BBB permeability in relation to corresponding young animals and contralateral hemisphere ($*P < 0.01$) ($N=5$). (B) Photomicrographs of Fluoro-Jade B stained sections from a young (frame 1) and aged (frame 2) animal 24 h post-MCAO and rt-PA reperfusion. Damaged neurons within the infarcted region are fluorescently stained (green), while surviving neurons within the penumbra are left unstained (scale bar = 100 μ m). Frames 3-6, Confocal microscopy of vascular permeability markers dextran (70 kDa) and fibrinogen (300 kDa) 24 h post-MCAO and rt-PA reperfusion, taken in the penumbral region of the infarct. Frames 3 and 4, Confocal images of fibrinogen (red) and dextran (green) reactivity in the ipsilateral cortex of young (frame 3) and aged (frame 4) animals. No dextran extravasation is observed in the young animal (frame 3) and is similar to images from contralateral hemisphere of the aged animal (frame 5). Dextran is present in the extracellular space of aged (frame 4) animals at the same time point. Frame 6, Image in bright field that depicts lumen of cerebral microvessel. Fibrinogen can be seen remaining within the lumen of the vessel, while dextran reactivity is present along the outer surface in an aged animal that underwent MCAO and rt-PA reperfusion (scale bar = 5 μ m).

Fig. 5.

(A) Extravasation of albumin across the BBB assessed by quantification of Evan's blue dye 20 min post-MCAO. Aged group exhibited greater BBB permeability in relation to corresponding young animals and the contralateral hemisphere (*) ($P < 0.001$). (B) Photomicrographs of Fluoro-Jade B stained section from a young (frame 1) and aged (frame 2) animal 20 min post-MCAO. Damaged neurons were not observed within the MCA perfusion territory in either young or aged animals (scale bar = 100 μm). Frames 3 and 4, Confocal images of fibrinogen (red) and dextran (green) reactivity in the ipsilateral cortex of young (frame 3) and aged (frame 4) animals, taken in the penumbral region of the infarct. No dextran extravasation is appreciated in the young rats (3) and is similar to images from contralateral hemisphere of the aged rats (not shown). Dextran is present in the extracellular space of aged (4) animals at the same time point (scale bar = 5 μm).

Table 1
Summary of physiological values pre-, during and post-middle cerebral artery occlusion

Groups	pH	PaCO ₂ (mm Hg)	PaO ₂ (mm Hg)	% Hct	MAP
Young					
Pre (10)	7.44 ± 0.07	32 ± 8.1	109 ± 6.8	36 ± 3.2	80 ± 7.8
During (10)					89 ± 11.5
Post (10)	7.39 ± 0.05	37 ± 4.9	97 ± 5.3	35 ± 3.3	102 ± 18.1
Aged					
Pre (10)	7.43 ± 0.07	34 ± 6.8	114 ± 7.3	34 ± 2.5	84 ± 13.7
During (10)					79 ± 13.0
Post (10)	7.41 ± 0.03	32 ± 9.6	116 ± 6.3	32 ± 3.9	98 ± 19.5

Values are means ± S.E.