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Plasminogen activator inhibitor type 1 derived peptide, EEIIMD, diminishes cortical infarct but fails to improve neurological function in aged rats following middle cerebral artery occlusion

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Abstract

Age is a primary risk factor in stroke that is often overlooked in animal studies. We contend that using aged animals yields insight into aspects of stroke injury and recovery that are masked, or not elicited, in younger animals. In this study, we examined effects of co-administration of a plasminogen activator inhibitor type 1 derived peptide, EEIIMD, with tissue plasminogen activator (tPA) on infarct volume and functional outcome in aged rats following a transient middle cerebral artery occlusion. Results of our study showed aged (18–20 months) rats treated with EEIIMD along with tPA had reduced cortical infarction volume. However, aged rats showed no improvement in total infarction volume, edema formation, or functional outcome as compared to aged rats administered only tPA. Young adult rats (3–4 months) treated with EEIIMD showed significant improvement in cortical and total infarction volumes, edema formation, and functional outcome. Striatal infarction volume was unaffected by EEIIMD treatment in both young adult and aged rats. These findings emphasize that physiological differences exist between young adult and aged rats and suggest that taking aging processes into account when assessing stroke may improve our ability to discern which therapeutics can be translated from bench to bedside.

Keywords

cerebral ischemia; tissue plasminogen activator; cortex; striatum; edema; stroke

1. INTRODUCTION

Treatment of ischemic stroke remains one of the most challenging areas in medicine today. Stroke is the third leading cause of death and the leading cause of long-term disability in the United States. Since FDA approval of tissue plasminogen activator (tPA) in 1995 and despite over 100 failed clinical trials, no other therapeutic has been shown effective in treating acute ischemic stroke. As a thrombolytic agent, tPA improves cerebral reperfusion and increases functional outcome when administered within 3 h of confirmed stroke onset. Unfortunately, tPA has direct and deleterious effects on the neurovascular unit and brain parenchyma that substantially increase the risk of hemorrhage and intrinsic toxicity that limit the use of tPA [Benchenane et al., 1996; Goto *et al.*, 2007; Kidwell *et al.*, 2008]. Thus, only 3–4% of people who suffer an ischemic stroke are eligible for the only approved treatment [Dawson and Dawson, 2006].

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While pursuit of the next clinically viable stroke drug must continue, it would seem prudent to also search for therapeutics that expand the population of patients eligible to be treated with tPA. A recent study reported that hexapeptide, EEIIMD, corresponding to amino acids 350–355 of plasminogen activator inhibitor type 1 (PAI-1), bound non-thrombolytic sites on tPA and reduced edema formation and infarction size and decreased neuronal degeneration following transient middle cerebral artery occlusion (MCAO) in young male rats [Armstead *et al.*, 2006]. While these findings appear promising, reservations and skepticism remain due to the lack of translation of so many other agents that showed promising results in animals but failed in the clinical setting, including calcium antagonists [Funato *et al.*, 1997; Liu *et al.*, 2004], modulation of the glutamatergic and GABAergic systems [Simon and Shirashi, 1990; Turski *et al.*, 1998; McCracken *et al.*, 1993; Shuaib *et al.*, 1993; Snape *et al.*, 1993], free radical scavengers [Schmid-Elsaesser *et al.*, 1998; Imai *et al.*, 2001], anti-inflammatory agents [Bertorelli *et al.*, 1998], membrane stabilizers [Lazzaro *et al.*, 1994], and trophic factors [Sugimori *et al.*, 2001].

We contend that age is the primary risk factor for stroke [D'Agostino *et al.*, 1994] and not accounting for age related processes when evaluating stroke injury in animal models is an important determinant in failure to translate promising findings from animal studies into clinically useful therapeutics. Previously, we reported that aged rats experienced larger and functionally more devastating strokes than young adult rats [Rosen *et al.*, 2005; DiNapoli *et al.*, 2006; DiNapoli *et al.*, 2008]. Ischemic strokes in aged rats are characterized by earlier disruptions of the blood-brain barrier to small molecules, increased infarction volumes, increased neuronal degeneration, higher mortality rates, and decreased functional outcomes as compared to younger rats [Rosen *et al.*, 2005; DiNapoli *et al.*, 2006; DiNapoli *et al.*, 2008]. In this study, we evaluated the effect of PAI-1 mimetic EEIIMD on infarct size and functional outcome in aged rats following MCAO and tPA reperfusion.

2. RESULTS

2.1 EEIIMD reduced cortical but not striatal infarction size in young adult and aged rats following MCAO and tPA reperfusion

Quantification of infarction volume for cortex, striatum and total cerebral hemisphere in TTC-stained coronal sections was measured at 24 h following MCAO and tPA reperfusion (n=6 rats/group). Figure 1 is representative TTC stained sections of brain from young adult (A) and aged (B) rats. For the cortical infarction size, the results showed a significant effect of treatment group [$F_{2,30}=19.97$; $P<0.001$]. In Figure 2, post-hoc analyses demonstrated that the difference in treatment groups was seen in both young adult and aged rats group. In the young adult rats, results showed that the EEIIMD treated group had a significant decrease in cortical infarction size as compared to tPA alone ($P=0.003$) or the EEIIMR treated group ($P=0.007$). No difference between tPA alone and EEIIMR treated young adult rats was noted ($P=0.97$). In the aged rats group, results showed that EEIIMD treated rats had a significant decrease in cortical infarction size as compared tPA treated group ($P=0.019$) or EEIIMR treated group ($P=0.001$). No difference between tPA alone and EEIIMR treated aged rats was noted ($P=0.40$). No interaction between age and treatment was observed [$F_{2,30}=1.14$; $P=0.33$].

For the striatal infarction size, the results showed no significant difference between young adult and aged rats [$F_{1,30}=0.73$; $P=0.40$]. No significant difference was found among treatment groups [$F_{2,30}=0.67$; $P=0.52$]. No interaction between age and treatment was observed [$F_{2,30}=1.04$; $P=0.37$] (Figure 2).

Results showed significant effect of age on total infarction volume [$F_{1,30}=19.54$; $P<0.001$]. Aged rats had a significantly larger total infarction size than young adult rats. This finding was found regardless of treatment. A significant effect was observed among treatment groups

[$F_{2,30}=11.33$; $P<0.001$]. In Figure 2, post-hoc analyses demonstrated that the difference in treatment groups was seen both in the young adult and aged rats group. In the young adult rats group, EEIIMD treated rats showed a significant decrease in total infarction size as compared to the tPA alone treated group ($P=0.006$) and EEIIMR treated group ($P=0.003$). No significant ($P=0.95$) difference between tPA alone and EEIIMR treated young adult rats was noted. In the aged rats group, no significant ($P>0.05$) change in total infarction volume was observed between any treatment group. No interaction between age and treatment was observed [$F_{2,30}=0.28$; $P=0.76$].

2.2 EEIIMD failed to affect edema formation in aged rats following MCAO and tPA reperfusion

Edema formation was measured at 24 h following MCAO and tPA reperfusion ($n=6$ rats/group) (Figure 3). No significant effect of age on edema formation was observed [$F_{1,30}=1.1$; $P=0.30$]. Results showed a significant effect of treatment [$F_{2,30}=3.1$; $P=0.046$]. Post hoc analysis of treatment groups showed no significant ($P>0.05$) difference in edema formation between any treatment group of aged rats. A significant decrease in edema formation was found in young adult rats treated with EEIIMD as compared to age matched sham control ($P=0.003$) and EEIIMR treated ($P=0.004$) rats. No significant ($P=0.99$) difference in edema formation was observed between young adult sham and EEIIMR treated rats. No interaction between age and treatment was observed [$F_{2,30}=1.41$; $P=0.26$].

2.3 EEIIMD did not improve neurological function in aged rats following MCAO and tPA reperfusion

Comparison of neurological function at 24 h following MCAO and tPA reperfusion ($n=6$ rats/group) showed a significant effect for age [$F_{1,30}=41.68$; $P<0.001$] and treatment [$F_{2,30}=4.05$; $P=0.028$]. Aged control rats had significantly increased neurological scores as compared to young adult control rats ($P=0.04$). In Figure 4, Tukey's post-hoc analyses between treatment groups demonstrated that significant difference was only observed in young adult rats, which showed that EEIIMD treated young adult rats had a significant ($P=0.003$) decrease in functional score as compared to the tPA alone treated young adult rats and a near significant ($P=0.010$) decrease as compared to EEIIMR treated young adult rats. No significant ($P=0.84$) difference between tPA alone and EEIIMR treated young adult rats was noted. No significant difference in neurological score was observed in aged rats between treatment groups [$F_{2,15}=0.27$; $P=0.77$]. No interaction between age and treatment was observed [$F_{2,30}=1.27$; $P=0.52$].

3. DISCUSSION

This study, for the first time, showed that administration of EEIIMD significantly improved cortical but not striatal infarct volumes in rats following embolic MCAO and tPA reperfusion. However, our findings also demonstrated that co-administration of EEIIMD with tPA did not improve total infarct volume, edema formation, or neurological functional outcome in aged female rats following MCAO and tPA reperfusion. These findings are contrary to results from a previous study showing EEIIMD protected the brain and improved functional outcome following an embolic MCAO and tPA reperfusion [Armstead *et al.*, 2006].

Consistent with previous studies [DiNapoli *et al.*, 2006; DiNapoli *et al.*, 2008], a mortality rate of $>30\%$ at 24 h following MCAO and tPA reperfusion was observed in aged rats (10 deaths out of 28 aged rats). No improvement in mortality was noted in EEIIMD (4 deaths) or EEIIMR (3 deaths) treated aged rats as compared to control aged (3 deaths) rats following MCAO and tPA reperfusion. Use of ketamine as an anesthetic agent during MCAO is controversial, with some studies showing no neuroprotection [Ridenour *et al.*, 1991; Zhao *et al.*, 2008] and others showing neuroprotection [Zhang *et al.*, 2005], especially when administering tPA [Tsirka *et*

et al., 1995; Yanming *et al.*, 1998]. We used ketamine in this study in order to accurately compare our results with prior studies using EEIIMD [Armstead *et al.*, 2005; Armstead *et al.*, 2006].

While mechanisms for the differential effects observed in brain regions and the relevance of how these findings relate to changes in neurological outcome are still unknown, results of this study reinforce the suggestion that beneficial effects seen in the cortex were due to a direct interaction between EEIIMD and non-catalytic binding sites on exogenous and endogenous tPA [Armstead *et al.*, 2005; Armstead *et al.*, 2006]. Administering a dose of tPA that is 50% lower than used in other studies and only administering tPA at 2 h after MCAO, we observed no signs of hemorrhage at 24 h in either young adult or aged rats, regardless of peptide treatment. No differences in infarct volume, edema formation, or neurological outcome were observed using the PAI-1 decoy peptide, EEIIMR, as compared to control; thus, supporting the suggestion of a direct interaction between tPA and EEIIMD.

The difference in age of the rats between our study and a previous study may explain the discordant results. The previous study used male Sprague-Dawley rats weighing ~250 g, which represented an age between 2–3 months. We argue age is a primary risk factor in stroke and aged rats suffer worsened brain damage and decreased functional recovery as compared to young adult rats. To test our premise, young adult rats (3–4 months of age) were administered EEIIMD along with tPA following MCAO. We showed equivalent results to the previously reported findings with decreased stroke volume and lessened edema formation. Moreover, our study showed that the improvement, which was enough to improve neurological function, was localized to the cortex in young adult rats at 24 h following MCAO. These results also argue against the disparity in findings between the two studies being related to gender, as the results obtained from young adult female rats in our study were comparable to young adult male rats used in the previous study. Thus, we are in agreement with the previous study that EEIIMD improves stroke outcome in young animals; however, relevance of these findings in relationship to the clinical population most at risk from stroke, individuals over the age of 65, suggests that experimental results in our aged model may be more clinically relevant.

To better understand the differences in brain damage following MCAO between young adult and aged rats, we evaluated regional changes in infarct volume. Results showed that the majority of infarct volume was striatal in young adult (83%) and aged rats (65%). This finding has been consistent in our studies showing aged rats not only suffered larger infarcts but a greater percentage of total infarct volume was cortical [DiNapoli *et al.*, 2006; DiNapoli *et al.*, 2008]. Yet in our present study, we found that EEIIMD administration improved cortical but not striatal infarct volume in both young adult and aged rats. This finding seems paradoxical to the functional outcome of the rats and underscores the importance of taking age-related changes in brain physiology into account when assessing stroke damage and recovery. The aged brain is characterized by many changes, including a 5–10% cortical atrophy, a generalized decrease in neurons, increased glia/neuron ratio [Stoll *et al.*, 1998], decreased glucose metabolic rate [Gibson and Peterson, 1981], deficiency in handling of free radicals [Miquel *et al.*, 1983], impaired vascular reactivity and compromised neuronal plasticity [Petcu *et al.*, 2007]. In particular, aging has a number of adverse effects on striatal function, including decreased glutamate regulation [Nickell *et al.*, 2007] and reduced GABAergic and cholinergic neurons [Paul *et al.*, 1988]. Furthermore, aging also affects the functional interaction of the cellular components of the neurovascular unit. Previous studies have shown that cerebral capillaries in aged rodents become frail and lose vascular compliance. In addition, aging arterial vessels have been shown to have reduced elastin and smooth muscle cells and a thickening of the basement membrane [Popa-Wagner *et al.*, 2006; Popa-Wagner *et al.*, 2007; O'Rourke, 2007]. These age-related changes make the cerebral vessels in the area of an ischemic infarct more prone to neurovascular uncoupling [Zaletel *et al.*, 2005; Schroeter *et al.*, 2007], greater susceptibility to tPA neurotoxicity [Akkawi *et al.*, 2006], and increased fragmentation [Knox

et al., 1980], which may lead to morphological changes in the neurovascular unit including loss of pericytes, increased vascular remodeling, and separation of astrocytic endfoot processes from the capillary endothelial cells.

While EEIIMD did show a marked improvement in protecting the cortex from ischemic damage, future studies will need to focus on the implications of striatal infarct toward neurological recovery. Furthermore, researchers may need to implement strategies to differentiate between cortical and striatal damage when assessing neurological damage and recovery following an ischemic brain injury, as modest improvement in cortical injury without concomitant changes in striatal injury may fail to improve functional status.

4. EXPERIMENTAL PROCEDURE

4.1 Chemicals and animals

All chemicals used in this study were of molecular biology grade and purchased from Sigma Chemical (St. Louis, MO), unless otherwise noted. The PAI-1 derived peptide, Am-EEIIMD-Ac, and decoy peptide, Am-EEIIMR-Ac, were synthesized by Biomatik Corporation (Hayward, CA). Two groups of female, age-matched, Sprague-Dawley rats (Harlan; Indianapolis, IN) were housed under 12-h:12-h light-dark conditions and received food and water ad libitum. Young adult (3–4 months) and aged (18–20 months) rats were randomly assigned at 2 h after MCAO to one of three treatment groups [tPA (5 mg/kg) alone; tPA + EEIIMD (1 mg/kg); tPA + EEIIMR (1 mg/kg)] and assessed at 24 h post-MCAO according to procedures detailed below. All protocols involving rats were approved by the West Virginia University Animal Care and Use Committee and abided by National Institutes of Health guidelines.

4.2 Surgical procedure for MCAO

Rats underwent a reversible embolic MCAO as previously described [DiNapoli *et al.*, 2006; DiNapoli *et al.*, 2008]. Briefly, rats were anesthetized with an intraperitoneal injection of ketamine (90 mg/kg; Webster Veterinary; Sterling, MA) and xylazine (5mg/kg; Webster Veterinary) and then administered at 1/5 the initial dose as needed to maintain sedation and analgesia through the duration of the surgical procedure. A servo-controlled homeothermic heating blanket, utilizing a rectal thermometer, was used to maintain body temperature at 37°C. Arterial P_{aO_2} , P_{aCO_2} , pH and hematocrit were monitored prior to embolization, during ischemia, and following recanalization using a GEM Premier 3000 blood gas analyzer (Instrumentation Laboratory; Lexington, MA). A microcatheter, outer diameter 0.3 mm, was inserted into the internal carotid artery via the external carotid artery stump and advanced until its tip occluded the ipsilateral middle cerebral artery (MCA). This mechanical occlusion was verified by laser Doppler 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 injected directly into the MCA. A drop in perfusion greater than 80% of initial baseline was determined to be ischemic. Cerebral perfusion to the MCA area was continuously monitored by laser Doppler for the duration of the procedure. After 2 h of ischemia, reperfusion was elicited by intravenous infusion (10% bolus and 90% infused over 30 min) of tPA (Genentech; South San Francisco, CA). The treatment groups consisted of tPA alone (control), and tPA together with the PAI-1 derived peptide EEIIMD or its decoy peptide EEIIMR. Reperfusion was monitored by laser Doppler and full reperfusion was determined by a return of perfusion to greater than 80% of ischemic baseline.

4.3 Composite functional score

At 24 h following MCAO and tPA reperfusion, functional assessment was examined in rats (n=6) from each treatment group using the modified neurological severity score (mNSS), which

is a composite score of motor, sensory, balance and reflex measures. The mNSS scores ranged from 1 to 17, with higher scores indicating greater neurological injury [Seyfried *et al.*, 2004].

4.4 2,3,5-Triphenyltetrazolium chloride (TTC) staining

Rats (n=6) in each treatment group were allowed to recover for 24 h following MCAO and tPA reperfusion, sacrificed by decapitation, brains removed, and sliced coronally at 2 mm intervals. Sections were incubated in 2% TTC in for 20 min at 37 °C. Following staining, the infarcted area remained unstained, appearing white, making it clearly distinguishable from viable tissue (stained red). All sections were scanned on a flatbed scanner and analyzed using Adobe Photoshop software (v7.0).

4.5 Determination of infarction volume

Following TTC staining, infarction volumes were quantified according to methods previously described [Yang *et al.*, 1998]. On each brain slice, the ischemic area (nonstained) was marked and infarct volume calculated. To avoid overestimation of infarct volume, the corrected infarction volume (CIV) was calculated using the following equation: $CIV = (LA - [RA - RI]) \times d$, where LA was area of the left hemisphere (mm^2), RA was area of the right hemisphere (mm^2), RI was the infarcted area (mm^2), and d was slice thickness (2 mm). Since total brain volume in the treatment groups varied because of different body weight of animals at baseline, we calculated relative infarction volumes expressed as percentage of total brain volume.

4.6 Edema index (%)

Rats (n=6) in each treatment group were allowed to recover for 24 h following MCAO and tPA reperfusion, sacrificed by decapitation, and brains removed. Brain swelling was calculated as edema index (%) according to the following formula by a previously described method [Maier *et al.*, 1998]. Briefly, edema index = $(RV - LV)/LV \times 100\%$, where RV was volume of the right hemisphere (mm^3) and LV was volume of the left hemisphere (mm^3).

4.7 Statistical analysis

Data are presented as mean \pm S.E.M. Physiologic parameters and functional data were compared using two-way analysis of variance (ANOVA) and comparisons between groups determined using Tukey's post hoc analysis. Level of significance was set at $p < 0.05$.

Acknowledgments

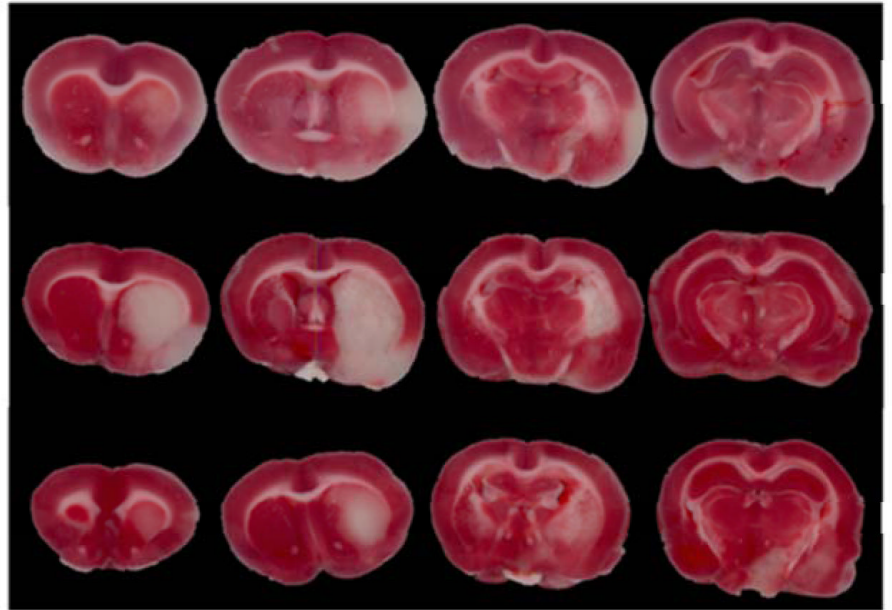
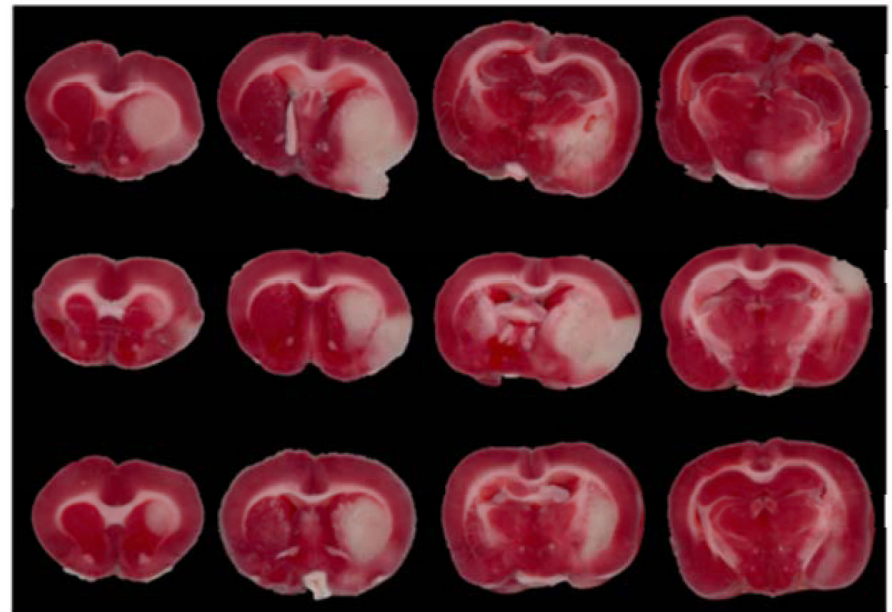
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(A)**tPA****tPA+EEIIMR****tPA+EEIIMD****(B)****tPA****tPA+EEIIMR****tPA+EEIIMD****Figure 1.**

Representative TTC stained coronal sections through the infarcted region of brains in (A) young adult and (B) aged rats following MCAO and tPA reperfusion with and without EEIIMR/EEIIMD co-administration. Using TTC, infarction was identified as unstained (white) and viable tissue was stained (dark).

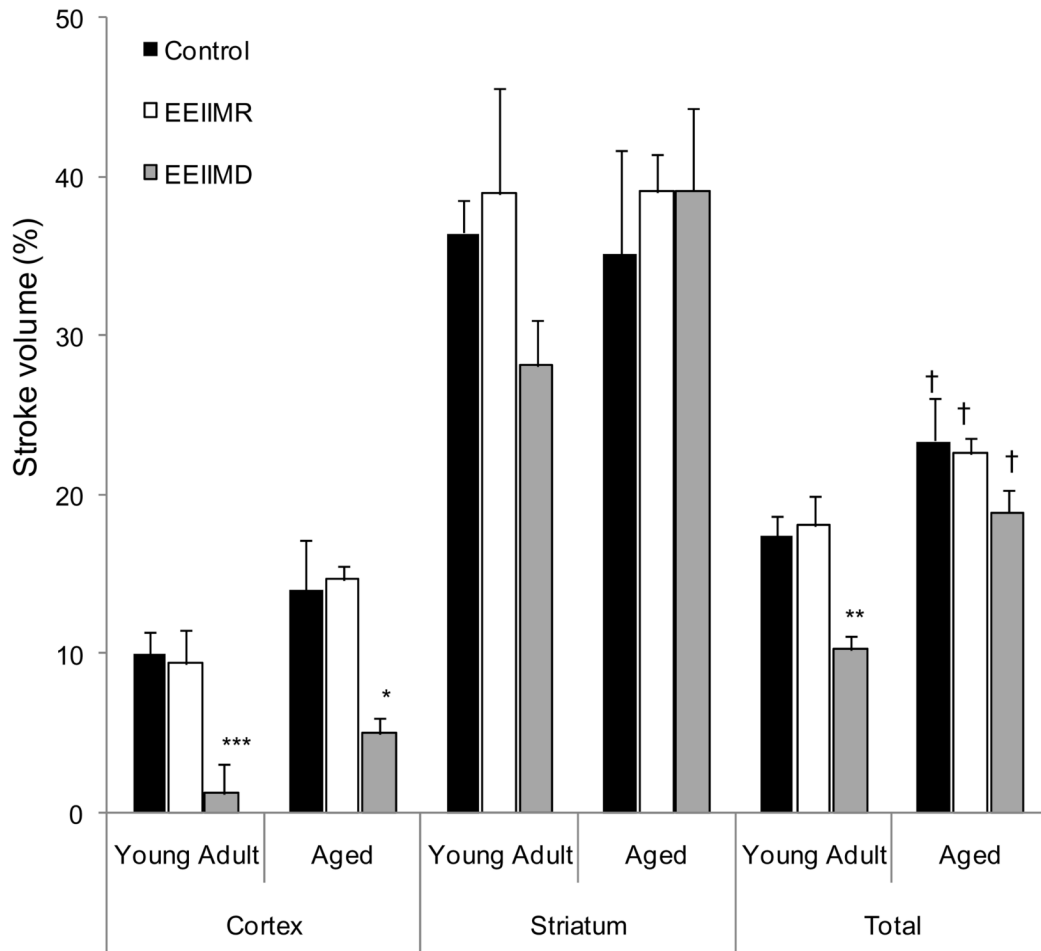


Figure 2.

Measurement of stroke volume of the infarcted cortex, striatum, and total cerebral hemisphere following MCAO and tPA reperfusion. Results showed a significant decrease in cortical stroke volume in both young adult ($1.23 \pm 1.83\%$) and aged ($4.99 \pm 0.95\%$) EEIIMD treated rats as compared to age-matched controls ($9.99 \pm 1.34\%$ and $14.05 \pm 3.11\%$, respectively). No difference in striatal stroke volume was observed in young adult ($28.12 \pm 2.91\%$) and aged ($39.10 \pm 5.18\%$) EEIIMD treated rats as compared to age-matched controls ($36.46 \pm 2.08\%$ and $35.14 \pm 6.52\%$, respectively). Results from total stroke volume showed a significant ($p < 0.01$) decrease in EEIIMD treated young adult rats ($10.26 \pm 0.89\%$) as compared to young adult control rats ($17.46 \pm 1.24\%$). No significant ($p > 0.05$) difference in total stroke volume was demonstrated between EEIIMD treated aged rats ($18.89 \pm 1.42\%$) and aged control rats ($23.39 \pm 2.69\%$). No significant ($p > 0.05$) difference in stroke volume was observed between EEIIMR treated and control rats regardless of age or brain section. Comparison of total infarct volume between young adult and aged rats showed a significant ($p < 0.05$) increase in infarction volume in aged rats as compared to young adult rats regardless of treatment. Bars indicate mean \pm S.E.M. and stroke volume is denoted as a corrected % of total hemisphere. ($n = 6$ rats/groups). * represents $p < 0.05$ as compared to age-matched control, ** represents $p < 0.01$ as compared to age-matched control, *** represents $p < 0.001$ as compared to age-matched control, and † represents $p < 0.05$ as compared to young adult rats within treatment group.

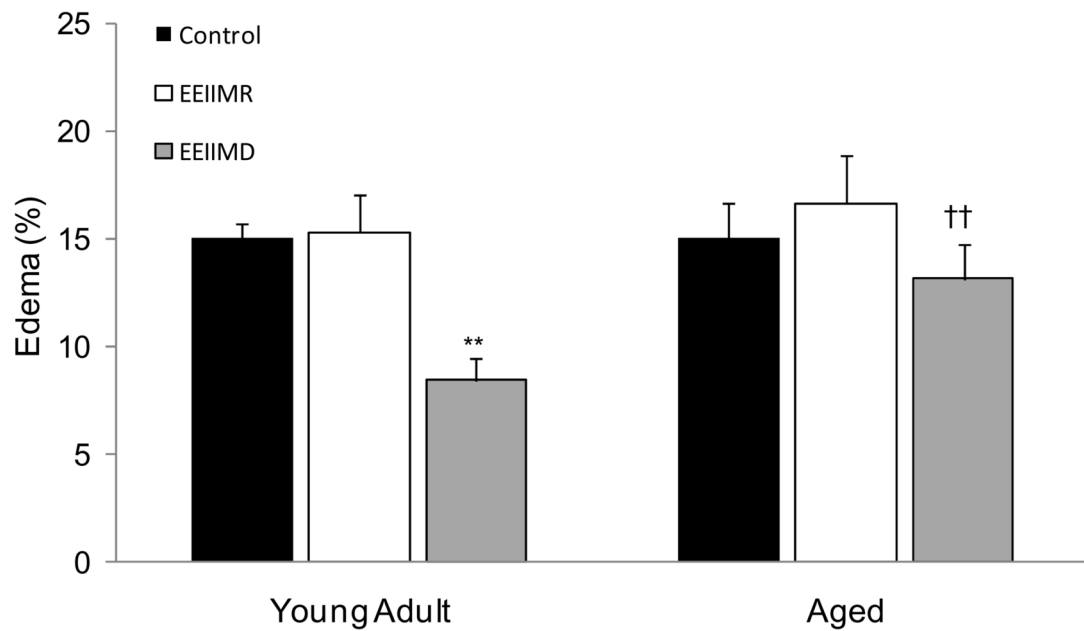


Figure 3.

Measurement of edema formation in the infarcted hemisphere following MCAO and tPA reperfusion. Results showed a significant ($p < 0.01$) decrease in edema formation in the EEIIMD treated young adult rats ($8.42 \pm 1.02\%$) as compared to young adult control rats ($15.03 \pm 0.68\%$). No significant ($p > 0.05$) difference was observed between EEIIMD treated aged rats ($13.13 \pm 1.58\%$) and aged control rats ($15.02 \pm 1.59\%$). No significant ($p > 0.05$) difference in edema formation was demonstrated between EEIIMR treated and control rats, regardless of age. No significant ($p > 0.05$) difference in edema formation was shown between young adult and aged control rats. A significant ($p < 0.01$) difference was demonstrated in edema formation between EEIIMD treated young adult and aged rats. Bars indicate mean \pm S.E.M. ($n = 6$ rats/group). ** represents $p < 0.01$ as compared to age-matched control and † represents $p < 0.05$ as compared to young adult rats within treatment group.

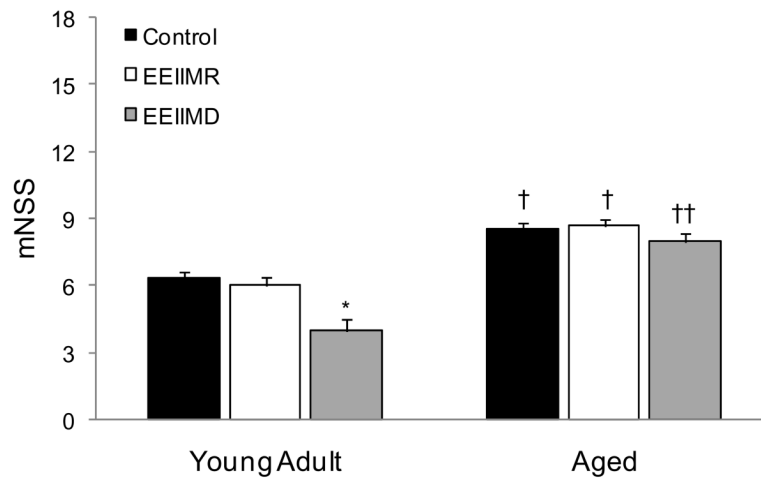


Figure 4.

Assessment of neurological function using a 17 point mNSS following MCAO and tPA reperfusion. Results showed that EEIIMD treated young adult rats (4.0 ± 0.5) had improved mNSS scores as compared to young adult control rats (6.3 ± 0.3). No significant ($p > 0.05$) difference in mNSS scores were demonstrated between EEIIMD treated aged rats (8.1 ± 0.4) and aged control rats (8.5 ± 1.1). A significant difference in mNSS scores was observed between young adult control rats and aged control rats and EEIIMD treated young adult rats and EEIIMD treated aged rats. No significant ($p > 0.05$) difference between EEIIMR treated rats and control rats was observed regardless of treatment. Bars represent mean \pm S.E.M. ($n=6$ rats/group). * represents $p < 0.05$ as compared to age-matched control, † represents $p < 0.05$ as compared to young adult rats within treatment group, and †† represents $p < 0.01$ as compared to young adult rats within treatment group.