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Intra-arterial combination therapy for experimental acute ischemic stroke

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Abstract

Acute ischemic stroke continues to devastate millions of individuals worldwide. Current treatments work to restore blood flow but not rescue affected tissue. Our goal was to develop a combination of neuroprotective agents administered intra-arterially following recanalization to target ischemic tissue. Using C57Bl/6J male mice, we performed tandem transient ipsilateral middle cerebral/common carotid artery occlusion, followed by immediate intra-arterial pharmacotherapy administration through a standardized protocol. Two pharmacotherapy agents, verapamil and lubeluzole, were selected based on their potential to modulate different aspects of the ischemic cascade; verapamil, a calcium channel blocker, works in an acute fashion blocking L-type calcium channels, whereas lubeluzole, an N-methyl-D-aspartate modulator, works in a delayed fashion blocking intra-cellular glutamate trafficking. We hypothesized that combination therapy would provide complimentary and potentially synergistic benefit treating brain tissue undergoing various stages of injury. Physiological measurements for heart rate and pulse distention (blood pressure) demonstrated no detrimental effects between groups, suggesting that the combination drug administration is safe. Tissue analysis demonstrated a significant difference between combination and control (saline) groups in infarct volume, neuronal health, and astrogliosis. Although a significant difference in functional outcome was not observed, we did note that the combination treatment group had a greater percent change from baseline in forced motor movement as compared with controls. This study demonstrates the safety and feasibility of intra-arterial combination therapy following successful recanalization and warrants further study.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Treatment of emergent large vessel occlusion (ELVO) traditionally includes administration of intravenous thrombolytic and/or endovascular thrombectomy (ET). Although both works to restore blood flow, neither addresses the cellular death associated with ELVO. Multiple studies have attempted to address the

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issue of rescuing dying neurons in the core and penumbra but most have failed. Furthermore, compounds that demonstrated positive results in animal models failed in clinical trials, leaving only thrombolytic and ET as treatment.

WHAT QUESTION DID THIS STUDY ADDRESS?
The objective of this study was to develop a combination of neuroprotective agents administered intra-arterially (IA) following recanalization to target ischemic tissue. In combination, verapamil, an L-type Ca2+ channel blocker, and lubeluzole, an N-methyl-D-aspartate modulator, demonstrated no negative physiological side effects, decreased infarct volume and inflammation, and increased neuronal survival. Although there was no difference in functional outcome, this is the first study to demonstrate that combination therapy for the treatment of ELVO is safe and feasible.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
This is the first study to combine two pharmacotherapy agents for the treatment of ELVO and demonstrate their safety and efficacy in treating affected tissue.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
Prior ELVO therapy studies looked to one drug. Stroke is not a one-pathway disease but a multifaceted disease needing treatments on multiple fronts. Using the US Food and Drug Administration (FDA) approved drugs with established safety and efficacy profiles removes years of testing. Furthermore, our model of IA drug delivery mimics the clinical condition, allowing compounds to move more easily from the bench to bedside.

INTRODUCTION

Acute ischemic stroke is the second leading cause of death worldwide, affecting 6.5 million individuals.[1,2] A subset of ischemic stroke, emergent large vessel occlusion (ELVO), affects the major cerebrovascular arteries (internal carotid artery, M1 and M2 of the middle cerebral artery [MCA], anterior cerebral artery, posterior cerebral artery, basilar artery, and vertebral artery), and is the most disabling and life-threatening.[3–5] Currently, there is only one US Food and Drug Administration (FDA) approved drug therapy for ELVO, intravenous tissue-plasminogen activator (i.v. tPA), but it has shown limited effect on large vessel occlusions,[6] and has significant exclusion criteria (most notably an administration window of 4.5 h from last known normal).[7–9] Recent clinical trials using mechanical thrombectomy (MT), or the endovascular removal of the clot, have demonstrated increased recanalization rates with fewer exclusion criteria leading to greater patient benefit.[10–12] Although i.v. t-PA and MT have increased the chance of stroke survival through recanalization, they do not confer neuroprotection/neurorepair. There is a critical need for neuroprotective and neuroreparative compounds post-stroke, especially as adjuncts to thrombectomy. A number of compounds have shown positive preclinical results (NXY-059, magnesium, and citicoline), but none have successfully transitioned to the clinic.[13–16] This is attributed to such issues as poor study design, failure to recanalize, and delayed drug administration.[17–19] The completed ESCAPE trial demonstrated significantly improved functional outcomes and decreased mortality when patients received MT compared to i.v. tPA.[20] Recanalization via MT allows neuroprotective/neuroreparative compounds to be administered directly to the site of ischemia.

Using prior studies as a guide, we selected a transient model of ischemia (tandem ipsilateral common carotid/middle cerebral artery occlusion [MCAo]) with confirmed recanalization. This mimics a distal M1 occlusion in humans. Further, we developed an intra-arterial (IA) model of pharmacotherapy administration 5 minutes post-recanalization, similar to the moment immediately following thrombectomy recanalization where IA drug therapy would be clinically feasible.[21] This route of administration also ensured our drug of interest reached the ischemic tissue mitigating negative systemic effects and potential crossover to the contralateral hemisphere.[21,22] Using this methodology, we have previously tested two compounds individually to assess neuroprotection; verapamil, an L-type calcium channel blocker,[22] and nitroglycerin,[23] a known vasodilator.

Although we saw significant histological, pathological, and behavioral outcome benefits with each agent, perhaps unsurprisingly, neither agent succeeded in completely reversing the effects of a stroke.[22,23] Therefore, in view of the fact that multiple pathways are known to induce damage in ischemia, we conclude that one therapeutic compound is unlikely to be sufficient for post-stroke neuroprotection. Rather, a combination of compounds targeting more than one pathway, and, perhaps, acting in different time scales and/or synergistically, would be optimal. To select potential neuroprotective compounds, we established specific criteria: (1) prior successful preclinical stroke studies and (2) mechanisms of action that target different phases of cellular apoptosis. Based on the aforementioned criteria, verapamil and lubeluzole were selected.[22,24–28] Verapamil inhibits the release of intracellular calcium by blocking L-type calcium channels, one of the earliest channels to open during cellular apoptosis. Lubeluzole inhibits extracellular glutamate release through modulation of the N-methyl-D-aspartate (NMDA) receptor, which is downstream of the L-type calcium channels in cellular apoptosis. Our combinational approach will allow us to target both the core and penumbra, two regions of the brain undergoing different stages of cellular apoptosis. The core, or tissue directly fed by the occluded MCA, will begin cellular apoptosis immediately due to lack of blood flow. Whereas the penumbra, or tissue receiving blood flow from a watershed region will have a delayed cellular apoptosis. Using a combinational approach, we are targeting tissue regions undergoing different phases of cellular apoptosis. We hypothesize that combination therapy with verapamil and lubeluzole prevent the various stages of cellular apoptosis in the affected tissue, providing neuroprotection and improved brain health.

MATERIALS AND METHODS

Animals

Experiments adhered to protocols on file with the Institutional Care and Use Committee at the University of Kentucky, and ARRIVE guidelines. Animal studies used 16-week-old C57Bl/6J male mice (Jackson Laboratories). Groups were separated into control ($n = 7$; MCAo surgery with IA saline injection), and combination ($n = 7$; MCAo surgery with IA verapamil/lubeluzole injection). Verapamil IA dose was calculated using current clinical dosing of 10 mg in a 70 kg person; dose corresponds to 0.15 mg/kg.[22,27,29,30] Lubeluzole IA dose was calculated using preclinical

dosing of 0.63 mg/kg.[24] Flow rate (2.5 µl/min) and injection volume (10 µl) was determined in prior studies.[21] Animals were randomized and the experimenter was blinded for analysis of perfusion, physiological measurements, behavioral testing, infarct volume, and immunohistochemistry. Exclusion criteria were rupture of MCA or common carotid artery (CCA) during surgery, if the animal died during recovery, or did not meet health standards and were euthanized prior to study end. Past study morality rates for MCAo surgery and IA injections are less than 5%, the mortality rate for the current study was 0%.

Stroke induction: MCAo

To induce an ischemic event, animals were anesthetized using a ketamine/xylazine cocktail, and focal ischemia was induced for 1 h by MCAo.[22,23,27] Briefly, with the mouse in the supine position, a midline incision was made along the trachea and the left common carotid artery was exposed. Next, an incision was made between the lateral portion of the right eye and medial portion of the right ear, and the temporalis muscle reflected. Then a small burr hole was made over the MCA, but not rupturing the dura. Occlusion consisted of a wire filament placed underneath the MCA and then a vessel clip was attached to the CCA. Ischemia was confirmed using a laser Doppler Periflux System 5000 with 2-millimeter tip (Perimed). Reperfusion included removal of the wire filament underneath the MCA and CCA vessel clamp.

IA injection

The IA pharmacotherapy injection occurred 5 min post-reperfusion. Briefly, 10 min prior to reperfusion the external and internal carotid arteries (ECA/ICA) were exposed allowing access to the bifurcation from the CCA. The distal ECA and ICA were temporarily ligated with 6–0 suture, and a small nick was made in the ECA distal to the CCA bifurcation. Micro-angio tubing attached to a Hamilton syringe was inserted into the nick of the ECA and threaded to the bifurcation. The tubing was temporarily secured using 6–0 suture. The temporary suture at the distal ECA and ICA were removed. Following reperfusion (5 min), IA injection commenced for 4 min. The methods were previously described.[21–23,27] Physiological measurements and body temperature during the MCAo and IA injection procedures were monitored using MouseOx Plus (thigh sensor and rectal probe).

Behavioral testing

Rotor Rod (forced motor movement) was assessed at baseline (BL; 1 day prior to MCAo and IA pharmacotherapy administration), and post-stroke days (PSDs) 1 and 7. Rotor Rod consisted of three trials on a rotating rod (0–40 rpm) accelerating over 5 min, trials were averaged for each testing day, PSDs 1 and 7 were compared to BL to determine percent change. Open field (free roam movement) was assessed at the same time points (BL, and PSDs 1 and 7). Open field consisted of a single mouse placed inside an open box (90 × 90 × 40 cm) for 5 min and allowed to explore the environment. Total distance (cm) travelled was captured using Noldus Ethovision XT software and an infrared camera.

Infarct volume

Mice were euthanized on PSD 7 via cervical dislocation and the isolated brains were flash frozen and sectioned (Bregma −2, 0, 2, and 4) with a Leica CM 1950 cryostat at 20 μm onto glass slides. Brain sections were stained using cresyl violet, scanned, and infarct volume measured with Image J software (National Institutes of Health [NIH]), as previously described.[21,22,26]

Immunohistochemistry

Astrogliosis was evaluated using glial fibrillary activating protein (GFAP; 1:1000; Sigma) immunohistochemistry. Mature neuron survival was evaluated using NeuN (1:500; Abcam) immunohistochemistry. A Nikon Eclipse T*i* microscope with attached to the charge coupled device (CCD) camera and Nikon NIS Element BR software was used to image stained sections at 20× magnification. Imaged brain sections were analyzed for stain intensity via positive pixel density quantification using Photoshop software. Stroke-affected region was morphologically identified and corresponds to the core and peri-infarct, a 500-μm boundary extending from the edge of the infarct core, medial and lateral to the infarct within the cortex.

Statistical analysis

Measured variables presented as mean ± SEM. A Student's *t*-test was performed for comparison between treatment groups (infarct volume and immunohistochemistry). A two-way repeated measures analysis of variance (ANOVA) was performed for time course comparisons (MouseOx, behavior). Significance is defined as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$.

Compliance with STAIR

To maximize the applicability of our results, we designed and conducted the study according to STAIR recommendations for preclinical neuroprotection research.[31] Experimenters were blinded and animals were randomized to groups. As this was an exploratory rather than confirmatory study, we used young single-sex animals, with a plan for future experiments with mixed sexes and older animals with comorbidities mimicking clinical stroke conditions.

RESULTS

Perfusion

Baseline (pre-occlusion) perfusion measurements (laser Doppler) were set to 100% (solid line; Figure 1a). Post-occlusion measurements, 5 min after MCAo induction, demonstrated a decrease of 79.57 ± 2.63% for the control group and 75.63 ± 1.63% for the combination group (Figure 1a). Although groups were not separated into control or combination until the IA drug injection following reperfusion, there was a significant reduction ($p \leq 0.0001$) in MCA blood flow for both groups following occlusion, with no difference between groups.

Physiological measurements

MouseOx Plus physiological measurements for heart rate (bpm; Figure 1b) were combined for all mice for 0–5 min (pre-occlusion; 147.98 ± 0.34), and 60–65 min (post-reperfusion; 145.91 ± 0.37), and demonstrating a nonsignificant decrease of 2 bpm. Groups separated at 70–75 min (post IA drug injection) into control (149.55 ± 0.44) and combination (150.55 ± 0.71) groups. Again, there was no significant difference between groups following IA drug injection.

Physiological measurements for pulse distention (μm; Figure 1c) were also combined for all mice for 0–5 min (28.59 ± 0.15), and 60–65 min (32.02 ± 0.23), demonstrating a significant increase ($p \leq 0.0001$) of 4 μm. Groups separated at 70–75 min into control (27.66 ± 0.30) and combination (30.68 ± 0.25) also show a significant difference ($p \leq 0.0001$) between groups. Although this significant difference in pulse distention

FIGURE 1 (a) Laser Doppler flowmetry measurements of MCA for control ($n = 5$) and combination ($n = 7$), black line indicates baseline (100%). MouseOx plus physiological measurements of combined (black), control (red), and combination (blue) groups. (b) Heart rate in beats per minute (bpm), 0–5 min (baseline), 60–65 min (recanalization), and 70–75 min (post IA drug injection). (c) Pulse distention measuring vessel diameter (μm), 0–5 min (baseline), 60–65 min (recanalization), and 70–75 (post IA drug injection). Functional measurements of control (red) and combination (blue) groups. (d) Rotor rod percent change from baseline. (e) Open field percent change from baseline. *Indicates a $p < 0.001$. IA, intra-arterially; MCA, middle cerebral artery; PSD, post-stroke day

was observed, all mice survived and recovered within normal guidelines.

Behavioral testing

Rotor rod testing for baseline and PSDs 1 and 7 is presented as a percent change from baseline. On PSD 1, both the control (98.71 ± 18.83) and combination (72.77 ± 25.80) groups decreased compared to BL, but there was no difference between groups compared to BL. On PSD 7, performance increased from BL and PSD1 in both the control (131.44 ± 24.30) and combination (175.25 ± 28.36) groups. Although no significant difference was observed between groups, there was a trend toward greater performance in the combination group (Figure 1d).
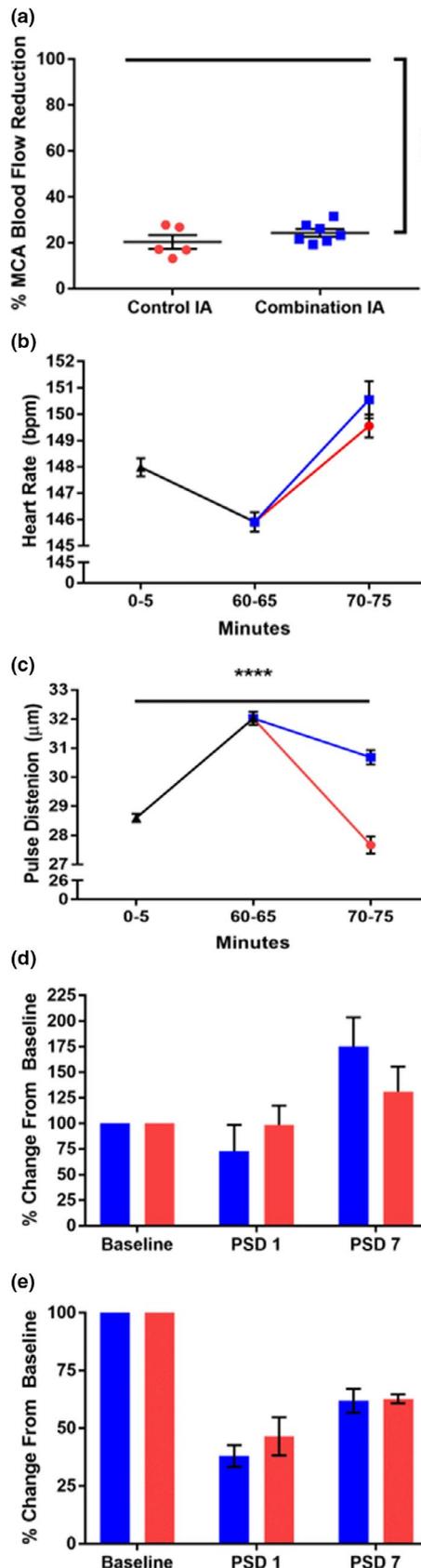
Open field testing is presented as percent change from BL. On PSD 1, both control (46.50 ± 2.33) and combination (38.04 ± 4.64) groups decreased compared to BL with no significant difference between groups. On PSD 7, both control (62.78 ± 1.79) and combination (61.94 ± 5.16) groups increased compared to PSD 1 but did not recover to BL values. No significant difference was observed between groups at any testing time point (Figure 1e).

Infarct volume

Infarct volume analysis using cresyl violet-stained coronal brain sections from PSD 7 demonstrated a significant difference ($p \leq 0.05$) between control IA (20.99 ± 3.51 mm^3) and combination IA (8.27 ± 3.81 mm^3) groups (Figure 2a–c).

Immunohistochemistry

Immunohistochemistry analysis was performed on PSD 7 using 20 μm (3 sections/mouse) coronal brain slices stained with GFAP (astrocyte marker) and NeuN (neuronal marker). Positive Pixel Density analysis for GFAP (Figure 2d–f) demonstrated a significant difference ($p \leq 0.005$) between control IA ($16,492.28 \pm 2029.77$)

and combination IA (6,515.57 $\pm$ 844.34) groups. Positive Pixel Density analysis for NeuN (Figure 2g–i) demonstrated a significant difference ($p \leq 0.01$) between control IA (7071.07 $\pm$ 1505.12) and combination IA (19,347.53 $\pm$ 2763.41) groups.

DISCUSSION

Using a clinically relevant transient mouse model of stroke, we previously developed an IA model of pharmacotherapy administration following recanalization that ensures our drug of interest reaches the ischemic tissue.[21] Although our prior single drug studies showed some success,[22,23] the likelihood that a single drug therapy would have significant clinical impact on the complex pathological cascade of stroke is unlikely.

Therefore, we hypothesized that a combination of compounds targeting acute and delayed phases of apoptosis would provide greater neuroprotection. We selected both verapamil for the acute phase with a terminal half-life of 3–5 h[32,33] and lubeluzole for the delayed phase with a terminal half-life of 20–28 h.[28,34,35] Verapamil inhibits intracellular calcium release through membrane bound L-type Ca^{2+} channels, some of the first to open when blood flow to the brain diminishes, causing a shift in intracellular calcium concentration. This represents the acute phase of stoke-induced cellular apoptosis and would target the ischemic penumbra still receiving watershed blood flow. Lubeluzole inhibits extracellular glutamate release through membrane bound NMDA receptor modulation. These receptors are downstream of the L-type Ca^{2+} channels, and represent the delayed phase of stroke-induced cellular apoptosis targeting the ischemic core, which has no blood flow.
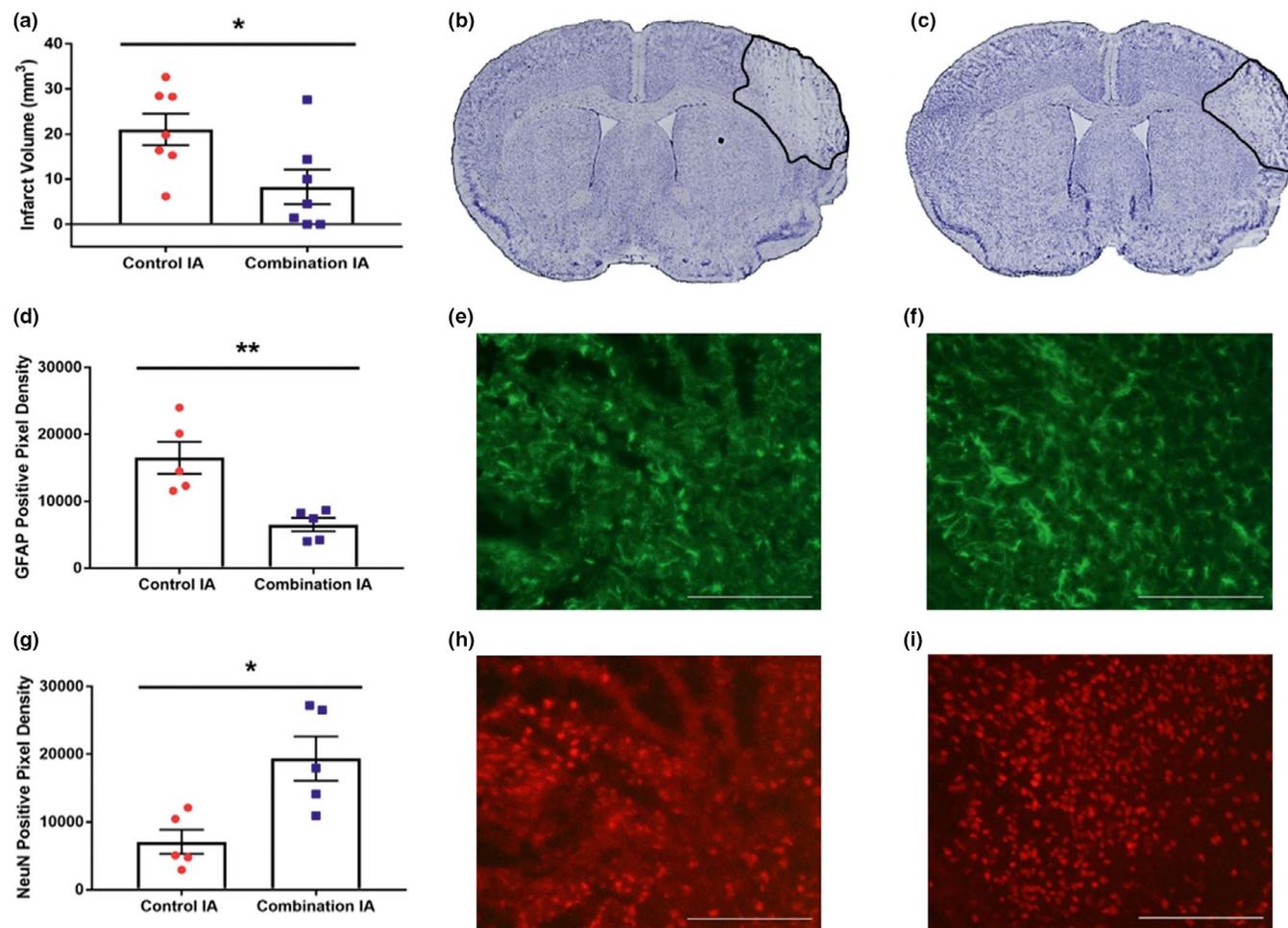

FIGURE 2 (a) Quantification of infarct volume and representative images of cresyl violet sections for (b) control IA ($n = 7$) and (c) combination IA ($n = 7$) groups. (d) Quantification of Positive Pixel Density for GFAP within the infarcted region and representative images for (e) control IA ($n = 5$) and (f) combination IA ($n = 5$) groups. (g) Quantification of Positive Pixel Density for NeuN within infarcted region and representative images for (h) control IA ($n = 5$) and (i) combination IA ($n = 5$) groups. *Indicates a $p < 0.05$, **indicates a $p < 0.01$ Scale bar = 100μm. GFAP, glial fibrillary activating protein; IA, intra-arterially

The goal of potential neuroprotective compounds is to decrease infarct volume through neuron preservation leading to functional abilities equal to pre-stroke measurements. Here, preliminary results using a small "n" demonstrated that combination treatment was safe, feasible, and neuroprotective when compared to saline control, and importantly, was physiologically well-tolerated in terms of both heart rate and pulse distention. Whereas combination animals had a greater pulse distention following IA administration, all animals recovered to BL values. Functional outcomes demonstrated greater recovery for combination animals compared to controls in forced motor movement. This is attributed to the combination having a significant reduction in infarct volume compared to controls. Likewise, the combination had a significant increase in mature neuron survival and a significant reduction in astrogliosis compared to controls.

Present study results were compared to previous study results to determine if combination IA had greater benefit than verapamil IA, and nitroglycerin IA.[20,21] All three studies demonstrated positive results; significant reduction in infarct volume, significant increase in mature neuron survival, significant reduction in astrogliosis, and improved functional outcome. In regard to infarct volume and functional outcome, verapamil IA[22] alone demonstrated greater benefit compared to nitroglycerin IA[22,23] and current combination IA, but the effect was negligible.

Although overall positive, this study did have limitations. The sample sizes for this exploratory study were small, and would require larger numbers of animals in future confirmatory studies. Verapamil and lubeluzole need to be administered IA individually and compared to an IA combination. If an IA combination is found to be of greater benefit than individual compounds, a dose response study would be required to optimize the combination. Finally, although blinded and randomized, this exploratory study evaluated single-sex (male) young animals. Future confirmatory studies should examine both sexes, as well as aged and diseased animals to more closely mimic the stroke population.

It was the goal of this study to combine two potentially neuroprotective compounds to determine safety, feasibility, and neuroprotection. This was accomplished by delivering our compounds of IA immediately following recanalization targeting stroke-affected tissue directly, and demonstrates this approach may be more broadly applicable to other potential neuroprotective agents.

CONCLUSION

This study successfully demonstrated safety, feasibility, and neuroprotective potential of combination therapeutics administered IA as an adjunct to ELVO recanalization. Given the growing utilization of mechanical thrombectomy, these pharmacotherapeutic approaches warrant further exploration.

CONFLICT OF INTEREST

The authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

M.E.M., J.M.R., G.J.B., and J.F.F. wrote the manuscript. M.E.M., G.J.B., and J.F.F. designed the research. M.E.M., J.M.R., and A.G. performed the research. M.E.M. and J.M.R. analyzed the data.

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