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Alpha 1-Antitrypsin Therapy Mitigated Ischemic Stroke Damage in Rats

Huong L. Moldthan^{1,*}, Aaron C. Hirko^{2,*}, Jeffrey S. Thinschmidt², Maria Grant², Zhimin Li³, Joanna Peris³, Yuanqing Lu¹, Ahmed Elshikha¹, Michael A. King^{2,4}, Jeffrey A. Hughes⁵, and Sihong Song^{1,#}

¹Department of Pharmaceutics, University of Florida College of Pharmacy, Gainesville, FL 32610, U.S.A.

²Department of Pharmacology & Therapeutics, University of Florida College of Medicine, Gainesville, FL 32610, U.S.A.

³Department of Pharmacodynamics, University of Florida College of Pharmacy, Gainesville, FL 32610, U.S.A.

⁴Department of Veterans Affairs Medical Center, Gainesville, FL 32602, U.S.A.

⁵Monsanto Co., Chemistry, Q2B, 800 N Lindbergh Blvd, St Louis, MO 63167, USA.

Abstract

Currently, the only effective therapy for acute ischemic stroke is the thrombolytic agent recombinant tissue plasminogen activator. α_1 -Antitrypsin, an endogenous inhibitor of serine proteinases and a primary acute phase protein with potent anti-inflammatory, anti-apoptotic, antimicrobial and cytoprotective activities, could be beneficial in stroke.. The goal of this study was to test whether α_1 -antitrypsin could improve ischemic stroke outcome in an established rat model. Middle cerebral artery occlusion was induced in male rats via intracranial microinjection of endothelin-1. Five to ten minutes following stroke induction rats received either intracranial or intravenous delivery of human α_1 -antitrypsin. Cylinder and vibrissae tests were used to evaluate sensorimotor function before and 72 hours after middle cerebral artery occlusion. Infarct volumes were examined via either 2,3,5-triphenyltetrazolium chloride assay or magnetic resonance imaging 72 hours after middle cerebral artery occlusion. Despite equivalent initial strokes, at 72 hours the infarct volumes of the human α_1 -antitrypsin treatment groups (local and systemic injection) were statistically significantly reduced by 83% and 63% ($p < 0.0001$ and $p < 0.05$ respectively) compared with control rats. Human α_1 -antitrypsin significantly limited sensory motor systems deficits. Human α_1 -antitrypsin could be a potential novel therapeutic drug for the protection against

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#Correspondence: shsong@ufl.edu.

*HLM and ACH contributed equally.

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neurodegeneration following ischemic stroke, but more studies are needed to investigate the protective mechanisms and efficacy in other animal models.

Introduction

World-wide, there are more than 50 million survivors of stroke and transient ischemic attack (TIA) (1), producing an immense burden on the healthcare infrastructure as well as national economies (2). At present, recombinant tissue plasminogen activator (rtPA) is the only FDA-approved therapeutic agent for ischemic stroke. The major functions of rtPA are dissolving blood clots and promoting reperfusion. In the United States, less than 2% of stroke patients are able to access rtPA treatment, most frequently due to delays in patient evaluation (2).

Ischemic stroke, the most prevalent type, initiates a series of events including cellular bioenergetic failure, excitotoxicity, oxidative stress, blood brain barrier (BBB) disruption, microvascular injury, homeostatic activation, and inflammation (3, 4). Although restoring perfusion via rtPA to the ischemic tissue is important, cascades of necrosis, apoptosis and inflammation commence within minutes of severe deprivation. Increasing evidence shows that genetically programmed cell death during post-ischemic tissue inflammation (which can last days to weeks) contributes significantly to the ultimate pathology (5–7). Therefore, therapies that may target these aspects of ischemia are needed in order to improve the outcome of stroke.

Alpha 1-antitrypsin (AAT) is a conserved serum proteinase inhibitor which has anti-inflammatory, anti-apoptotic and cytoprotective properties. AAT is primarily synthesized in the liver (8), secreted into the blood stream (1.0–2.5 mg/mL), and is relatively stable with a half-life of 4–5 days in humans (9, 10). Previous studies have demonstrated that AAT inhibits the production of pro-inflammatory cytokines (IL-1 α , IL-6, IL-8, and TNF- α), and enhances anti-inflammatory cytokine IL-10 production by increasing cellular cyclic adenosine monophosphate (cAMP) levels (11,12). AAT has shown anti-apoptotic and anti-inflammatory effects as early as 2 h and as late as 24 h following kidney ischemia/reperfusion in an animal model by inhibiting neutrophil superoxide production, TNF α production, and decreasing caspase-1 and -3-like activities (13,14). AAT-mediated interruption of IL-8 binding to its receptors limits neutrophil infiltration in the lungs (15). In addition to inhibiting serine proteases, AAT also inhibits cysteine proteinase activity (e.g., caspase-3). Coupled with the ability to enter cells, this has been shown to protect pancreatic α cells, lung, kidney and liver cells against caspase-dependent apoptosis (16). Based on these activities of AAT we hypothesized that it may hold therapeutic potential in limiting stroke pathology that results from intrinsic inflammatory and proteolytic processes. In the present study, we tested whether AAT may be protective in an ET-1-induced transient middle cerebral artery occlusion (tMCAO) model in rats.

Materials and methods

Reagents: Rat endothelin-1 (ET-1), MW = 2492.0, purchased from American Peptide Company, Inc, (CA, U.S.A.), was dissolved in sterile phosphate buffered saline (PBS) to

make a stock concentration of 80 μM . Artificial cerebrospinal fluid (aCSF) was obtained from Fisher Scientific, Inc. (PA, U.S.A.) Clinical grade human alpha 1 anti trypsin (hAAT,, Prolastin C, Grifols, Inc., NC, U.S.A.), was dissolved in sterile water followed the manufacture indtruction Other materials, such as phosphate buffered saline (PBS), saline, formalin and isoflurane were purchased from Fisher Scientific (NH, U.S.A.)

Animals: All procedures were performed with prior approval from the University of Florida *Institutional Animal Care and Use Committee (IACUC)*. Male Sprague-Dawley rats, 7–8 weeks in age, (weight 220–250 g) were purchased from Charles River Laboratories International, Inc. (MA, U.S.A.). The rats were acclimated for 3 or more days before the start of any experiments. Animals with incomplete data sets due to death and non-response to behavior tests were eliminated from the study data.

A model of transient/reversible middle cerebral artery occlusion (MCAO) using ET-1 was employed and generated as previously described (17). Briefly, under aseptic conditions, animal was anesthetized and the scalp was retracted and a midline incision was made in the skin from the point above bregma until the lambda point. A small hole (3 mm i.d.) was drilled in the cranium adjacent to the left MCA at 0.2 mm anterior, 5.2 mm lateral, and 1.0 mm from the ventral skull surface. A 27-gauge needle was used to inject ET-1 or hAAT at a rate of 1 $\mu\text{L}/\text{min}$. After ET-1 injection, the needle was left in place for an additional 3 min before being slowly withdrawn. The injection system was flushed by distilled water and loaded with subsequent treatment agents (intracranial delivery experiments only). The detailed schedule for treatments is described in the tables 1 and 2 Animals were examined daily during the acute post-operative period. No animals required treatment or euthanasia during the post-operative period.

Treatments: In this study, Human AAT was delivered by methods, i.e., intracranial (i.c) and intravenous (i.v) administration.. For the intracranial (i.c) injection, 3 μL of either hAAT (70 μg) or PBS (as a control) were injected into the parenchyma approximately 5–10 min following ET-1 injection. For intravenous (i.v), a dose of 40 mg/kg hAAT or PBS was injected via the tail vein 5–10 minutes following induction of stroke (ET-1 injection). Dosing was determined based on our previous experiments in other animal models (16–19).

Laser Doppler Perfusion Imaging

Laser Doppler Perfusion Imaging: A laser -Doppler imaging system ((MOORLDI, Moor Instrument Ltd, UK) was used to measure the relative flux/perfusion of the brain which is strongly associated with the spatial and temporal characteristics of the changes in cerebral blood flow (CBF) response (20–22). Imaging studies measuring sequential perfusion changes after ischemia often require the animals to be immobilized for long periods of time (at least 2.5 h). Isoflurane, a volatile anesthetic, has been shown to reduce early neuronal death in animal models of focal cerebral ischemia (23). Therefore, to avoid the influence of isoflurane, animals used in these studies, exposed to isoflurane for approximately 3 h, were not used for testing the therapeutic effects, and were sacrificed following the last measurement (survival time was about 4 h).

The treatment details were exactly the same as the treatments in the “Animal Model” and “Treatments” above, except that an aCSF group was not included. One animal died during imaging due to isoflurane overdose, and was replaced to provide 3 animals per treatment. The imaging was performed at 4 different time points: pre-injection, 10–20 min, 30–35 min, and 55–65 min after ET-1 injection. The pseudo-coloring of relative flux (number of red blood cells multiplied by speed) illustrates “cool” colors for relatively low flux and “warm” colors for relatively high flux. The 16-bit color scans were made with an arbitrarily assigned unit from 0 (lower limit) to 1,000 or more (upper limit). The scan speed was 10 ms/pixel, scan area was about 1.8 cm × 2.3 cm and the total scan duration per animal was approximately 10 min. Actual blood flow is highly correlated to flux except at supranormal pressures (24) not relevant to these experiments. Percent reductions of perfusion on the injected side of the brain were calculated using the following equation 1:

$$\text{Flux} \%, \text{Fi} - \text{Fc} \times 100 \quad (\text{Equation 1})$$

where Fi is the flux value of the ipsilateral side (injected side) and Fc is the flux value of the contralateral side (opposite or unaffected side).

Cylinder test

The cylinder test was performed as previously described with slight modification (17,25). Briefly, the test rat was placed inside a clear glass cylinder (40 cm height, 20 cm i.d.) to test the forelimb use behavior, which can reveal functional asymmetries resulted from different forms of brain injury, including cortical damage (26) and nigrostriatal neurodegeneration (27). Digital video recordings of vertical exploration during 3 min of trials were scored offline frame-by-frame.

Vibrissae test

Measurement of forelimb asymmetry was scored using the vibrissae test (evoked forelimb placing) as previously described (17, 28). An NTSC camera was used to record digital video of 10 iterations of vibrissae stimulation and the corresponding responses, for each side, for each animal. The delay of forelimb placing for each side was expressed by relative time response and calculated using equation 2:

$$\text{Relative time response} = \frac{\text{Seconds to place } (3 \text{ days after surgery})}{\text{Seconds to place } (before surgery)} \quad (\text{Equation 2})$$

Histopathology

The 2,3,5-triphenyltetrazolium chloride (TTC) staining assay is one of the most commonly used methods to measure ischemic lesions (29–32). Animals in control and treatment groups were sacrificed at day 3 post ET-1 injection. The brains were quickly isolated, placed in cold PBS (0–4°C) for 30 min and then sectioned into 2-mm-thick coronal slices using a brain slice matrix (Leica Microsystems, IL, U.S.A.). The tissue sections were held in cold PBS for 3 min before they were incubated in TTC solution (0.05% TTC in PBS) for 30 min at 37°C. The sections were washed 3 times (1 minute each) with PBS and fixed in 0.1 M phosphate

buffered formaldehyde (PBF). Calibrated digital images of tissue sections were made at 600-dpi scanner resolution with 48-bit color and saved as TIFF files.

Magnetic Resonance Imaging

Three days after ET-1-injection, brain damage was visualized in live animals from the groups ET-1 alone (N = 8) and ET-1+hAAT (i.v.) (N = 14) maintained under 1–2% isoflurane anesthesia in the Advanced Magnetic Resonance Imaging and Spectroscopy (AMRIS) facility at the McKnight Brain Institute of the University of Florida. An 11T/470 MHz MRI Spectrometer (Magnex Instruments, U.K.) using a Bruker Avance Console and Paravision software (Bruker BioSpin MRI, Inc, MA, U.S.A.) were used to generate a standard T2-weighted spin-echo sequence (rapid acquisition with relaxation enhancement) through continuous 1mm thick sections covering the entire brain region. Body temperature was monitored and maintained with heated ventilation at 35–37°C for the entire scan time. Total acquisition time for MR imaging was about 35 minutes.

Image Analysis

Infarct areas of brain slices from all experiments, including from TTC assay and MRI imaging were analyzed in a blinded manner using the NIH ImageJ version 5.0 (rsbweb.nih.gov/ij) and Jim version 5.0 respectively. Regions of interest (ROI) were created encompassing infarct areas of each section (in mm²), and total infarct sizes for each rat were operationally defined as the sum of infarct areas over all slices in which they appeared.

Quantification hAAT in the brain

Following MRI, a sample brains (n=4 for both hAAT i.v and saline groups) were extracted and frozen at –80 C. Brains were and homogenized 3mL lysis buffer. After centrifugation, hAAT concentrations were determined by human AAT specific ELISA as previously described (33, 34).

Statistical Analysis

Unless specified, all data are expressed as arithmetic means $\pm$ s.e.m. Two-way ANOVA (time and treatment) was followed by Bonferroni *post hoc* tests to compare behavioral dependent variables between groups. Comparisons of infarct area were made by 2-tailed Student's t-test; and p-values less than 0.05 were considered to be significant. Comparisons of brain hAAT concentrations were done by Mann Whitney test following F-test showing significant differences ($p < 0.0001$) between group variances.

Results

Human AAT Did not Affect the ET-1-Induced Ischemia

In order to test the effects of hAAT on middle cerebral artery occlusion (MCAO) induced by ET-1, cohorts (9 rats were divided randomly into 3 equal groups) of rats were stereotactically injected with ET-1 adjacent to the left MCA of the brain. About 10–15 min after ET-1 injection, hAAT or saline was intracranially injected at the same site in 2 groups (ET1+hAAT (i.c.) and ET-1+saline groups), and the third group (ET1+hAAT (i.v.) group)

received hAAT intravenously. The relative reduction in flux in rat brain was measured using a laser Doppler scanner. As shown in Figure 1A, calculations made using equation 1 showed that approximately 20 min following the ET-1 injection the flux statistic at the injection site dropped markedly from ~ 1,600 perfusion units (PU) to less than 900 PU in all groups (i.e. ET-1+saline group, ET-1+hAAT (i.c.), and ET-1+hAAT (i.v.) groups). The average relative flux values in all groups were reduced by 40–50% compared to the base line. The maximally reduced flux was maintained for up to 35 min, followed by the initiation of reperfusion (Figure 1B). No significant difference among the three groups was detected; illustrating that hAAT, either locally or systemically, did not alter the ischemic induction by ET-1.

Local Delivery of hAAT Mitigates ET-1-Induced Stroke Outcome

In a separate experiment, 3 rats were intracranially injected with ET-1 and hAAT as previously described. Data from the control groups, receiving aCSF or ET-1 alone, were retrieved from our previous study (17) as the set of experiments were performed during the same time period and using identical methodology. Three days after ET-1 injection, behavioral tests and brain damage evaluation were performed.

Cylinder test results showed that animal behavior was normal before treatment, i.e., both forelimbs were used for postural support in aCSF, ET-1 alone, and ET-1+hAAT (i.c.) groups $80.00 \pm 7.0\%$, $79.20 \pm 5.16\%$, and $82.50 \pm 4.15\%$ of the time, respectively; with no statistically significant difference among the 3 groups (Figure 2). Three days after ET-1 injection, the use of both forelimbs in the ET-1 alone group was significantly reduced ($22.6 \pm 6.3\%$), indicating profound impairment of contralateral forelimb function, whereas no impairment was observed in the group which received the aCSF injection ($76.67 \pm 4.08\%$). In the hAAT treatment group, ET-1+hAAT (i.c.), the use of forelimb was significantly higher than that in ET-1 alone group ($60.00 \pm 6.10\%$, $p < 0.05$). These results clearly demonstrate that local administration of hAAT mitigated the deficit of motor function caused by ischemic stroke in rats.

Vibrissae tests were performed to test the effect of hAAT on sensorimotor function. We measured forelimb pacing time before and 3 days after ET-1 injection and calculated relative time response that represented the time to place forelimb as a fold increase of pre-treatment tests (using equation 2). As shown in Figure 3, the ipsilateral relative time responses in aCSF, ET-1 alone and ET-1+hAAT groups were 1.53 ± 0.50 , 1.75 ± 0.19 , and 1.53 ± 0.16 respectively, and no statistically significant difference was observed among the groups. These data indicated that ipsilateral forelimb placing was not affected by the middle cerebral artery occlusion or AAT treatment in rats after 3 days. Intriguingly, contralateral forelimb relative time responses in ET-1+hAAT (i.c.) group were significantly smaller than those in the ET-1 alone group (7.61 ± 0.63 , 20.96 ± 4.70 , respectively) indicating that hAAT treatment can limit the loss of sensorimotor function induced by MCAO in the rat model. The contralateral forelimb relative time response in the aCSF group remained near 1 (1.27 ± 0.15), and significantly smaller compared to ET-1 alone group. These data confirmed that ET-1 injection induced profound sensorimotor deficits in contralateral forelimb placing responses that were not affected by the surgery procedure.

Infarct size results based on TTC assays are presented in Figure 4. The average of total infarct areas of the model group (receiving ET-1 alone) was significantly larger than that of the hAAT treatment group ($198.90 \pm 13.30 \text{ mm}^2$ and $33.70 \pm 2.80 \text{ mm}^2$, respectively, $p < 0.001$). These results indicate that the local hAAT delivery significantly reduced the infarct zone by ~83%. Typically, the infarct in the model group involved the rostro-central dorsolateral cortex, subcortex, and basal ganglia whereas in the treatment group the infarct area was more limited to the cortex region.

Systemic Delivery of hAAT Mitigated ET-1-Induced Stroke Outcome

Although direct local injection of hAAT clearly prevented brain damage from ET1-induced MCAO, this invasive approach is clinically unfeasible. In order to test the effect of systemic hAAT delivery on ischemic stroke, we performed experiments using separate groups of animals. Because we found no effects from aCSF in the previous studies, only control and hAAT treatment groups were utilized. Eight rats in the control group received ET-1 (i.c.) and 1 mL of sterile saline (hAAT vehicle) intravenously (i.v.). Fourteen rats in the hAAT treatment group were intracranially (i.c.) injected with ET1 and systemically (i.v.) injected with hAAT (40 mg/kg, i.v.) immediately after ET-1 injection.

Cylinder test results showed that animal behavior was normal before treatment, i.e. both forelimbs were used for postural support against cylinder walls of ET-1+saline and ET-1+hAAT (i.v.) groups on $84.5 \pm 1.2\%$ and $87.9 \pm 3.2\%$ of events, respectively; with no statistically significant difference between the 2 groups (Figure 5). Three days after the injections, both groups revealed significant deficits compared to pre-treatment. However, the hAAT treatment group showed significant deficit reduction in using 2 forelimbs for cylinder exploration compared to the controls ($72.6 \pm 6.2\%$ and $35.4 \pm 5.9\%$, respectively, $p < 0.001$), indicating that intravenous delivery of hAAT can ameliorate motor function deficit induced by ET-1-induced cerebral ischemia.

Results from vibrissae test are shown in Figure 6. The contralateral relative time response in ET1+hAAT (i.v.) groups was significantly smaller than that of the ET-1+saline group (2.39 ± 0.43 and 4.01 ± 0.87 , respectively). Consistent with unilateral motor system injury, the ipsilateral side to the injection side was not affected by the MCA occlusion (1.27 ± 0.23 and 0.99 ± 0.11 , respectively). These results indicated that intravenous delivery of hAAT can mitigate sensorimotor function deficit caused by ischemic stroke.

Effects of systemic hAAT on infarct size 3 days after ischemic stroke are shown in Figure 7A. The infarct areas for ET-1+saline and ET-1+hAAT (i.v.) were $141.8 \pm 37.6 \text{ mm}^2$ and $53.0 \pm 17.7 \text{ mm}^2$, respectively, $p < 0.05$, indicating that systemically administered hAAT significantly reduced the infarct zone by ~63%. A sample of these brains analyzed for hAAT concentration showed ET-1+saline injected controls had a calculated concentration of $11.21 \pm 0.21 \text{ ng}$ of hAAT per g of brain tissue (below the detectable range of the assay). The ET-1+hAAT (i.v.) group had a calculated concentration of $34.79 \pm 12.95 \text{ ng}$ of hAAT per g of brain tissue ($p = 0.029$, Figure 7B).

Discussion

Human alpha 1-antitrypsin has been used for more than two decades as augmentation therapy for chronic obstructive pulmonary disease (COPD) that is related to AAT deficiency. Our laboratory has been investigating its functions and has shown its beneficial effects in several different experimental models of diseases, including rheumatoid arthritis (35), diabetes (26, 36, 37), and bone loss (38). However, the effect of AAT on brain injury induced by stroke has never been reported. In this study, we demonstrated that hAAT therapy protected against brain tissue injury and improved stroke outcome. Human AAT has been used to treat AAT deficient patients for many years and has shown low or no side effects (39). Considering its excellent safety profile, the current results suggest potential for a new clinical application of hAAT in the treatment of stroke, and perhaps other brain diseases associated with inflammation and programmed cell death.

A growing body of literature shows that hAAT has anti-inflammatory and cytoprotective properties (40). In this study, we showed that local delivery of hAAT protected ET-1-induced brain injury and mitigated behavioral deficits. Although the mechanism of this protection remains to be further investigated, it is possible that hAAT inhibits ischemia- and reperfusion-induced neuron cell death by inhibiting caspase-1 and -3 (19). Human AAT may also inhibit local expression of ischemia- and reperfusion-induced inflammatory cytokines and activated astrocytes or microglia. Therefore, administration of hAAT to the site of ischemic stroke insult may mitigate cell death and inflammatory responses.

It is intriguing that systemic delivery of hAAT significantly improves stroke outcome and is detectable in the brain. Since AAT is a 52 kD glycoprotein thought to have limited ability to pass through an intact BBB (41), the following possible mechanisms may contribute to its protective effects. First, ET-1 is a known mediator that directly increases BBB permeability (42). Also, stroke increases BBB permeability to macromolecules, e.g., immunoglobulin M (IgM, 450 kd) (43). Second, ET-1-induced ischemia/reperfusion can lead to dysfunction of endothelial cells and phasic leaking of the BBB (44). Third, the injection of ET-1 possibly causes local mechanical compromise of the BBB. In all cases, circulating hAAT passes through the BBB, reaching the impaired area of the brain and displaying local protective functions. A fourth possibility is that hAAT may indirectly reduce brain tissue damage by inhibiting the infiltration and migration of lymphocytes to the penumbra of the ischemic region (45). By this mechanism, systemic administration of hAAT could be clinically beneficial even if it does not penetrate the brain itself. Although the beneficial effects of systemic administration of hAAT may be limited after the recovery of BBB, such treatment may also protect the brain from the recurrence of stroke, which occurs frequently. In addition, since BBB damage in stroke is partly induced by the inflammatory response and results in pathogenic vasogenic edema, hAAT, as an anti-inflammatory protein, may also promote maintenance and restoration of BBB integrity.

In the present study, approximately 20 min after ET-1 injection relative cerebral blood flow rapidly decreased to 40–50% of baseline in all animals and remained maximally decreased for at least 20–30 min. The result is consistent with the previous study that showed micro-injection of ET-1 near MCA induced a blood flow reduction of 30–75% in the region

supplied by the artery (46, 47). There was no significant difference among the 3 groups in CBF reduction, indicating that hAAT had no direct influence on ET-1 induced MCAO. Therefore, our results indicate that the protective effects of hAAT are mediated not through blocking the activity of ET-1, but rather by blocking the subsequent biochemical/inflammatory responses and limiting damage. We chose to use an ET-1 stroke model because the microinjection near the middle cerebral artery is simple and minimally invasive thus often resulting in a high animal survival rate, which is important for studies of neuroprotective drugs (48). However, the reperfusion process is ET-1-dose dependent (49). In our experiments utilizing laser-Doppler perfusion imaging, reperfusion began as early as 55 min to as long as more than two hours following ET-1 application, proving a source of possible variability. Another issue is that ET-1 can induce astrogliosis and facilitates axonal sprouting which may interfere with the interpretation of neural repair experiments (50). Also the use of ET-1 to induce a vasospasm stroke model does not result in a blood clot which is typical in thrombotic and embolic strokes in humans. Given the limitations of ET-1 mouse model, future studies using different animal models will provide a better understanding of the neuroprotective effects of AAT.

In the present study, we treated the animals with hAAT (i.c. and i.v.) 5–10 minutes after the ET-1 induction. While such interval is enough to avoid the interaction of hAAT with ET-1 as discussed above, we consider that the protective effect observed in these experiments are preventive, but not injury-reversing. Whether hAAT can be effective after the onset of the insult, and the effect of delay on therapeutic effect, will require further investigation.

Conclusions

Both local and systemic deliveries of hAAT resulted in neuroprotection and mitigation of behavioral deficits induced by ischemic stroke in rats. Systemic delivery resulted in detectable concentrations of hAAT in the brain 3 days after injection. Results from this study indicate a promising new application of hAAT as a therapy for ischemic stroke.

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List of abbreviations

AAT	Alpha 1-antitrypsin
aCSF	Artificial cerebrospinal fluid
AMRIS	Advanced magnetic resonance imaging and spectroscopy
ANOVA	Analysis of Variance
BBB	Blood brain barrier
CBF	Cerebral blood flow

CNS	Central nervous system
ET-1	Endothelin-1
FDA	Food and Drug Administration
hAAT	Human alpha 1-antitrypsin
ICAM-1	Intracellular cell adhesion molecule-1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
MMPs	Matrix metalloproteinases
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
PBF	Phosphate buffered formaldehyde
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
rtPA	Recombinant tissue plasminogen activator
s.e.m.	Standard error of the mean
TIA	Transient ischemic attack
tMCAO	Transient middle cerebral artery occlusion
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor-alpha
TTC	2,3,5-triphenyltetrazolium chloride
uPA	Urokinase plasminogen activator

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Figure 1.

Perfusion measurements of AAT study. (A). Color photographs of an animal brain recorded at different time points (pre-injection, 10–20 min, 30–35 min, and 60–65 min following ET-1 injection) by a digital camera of a laser Doppler system, corresponding closely with the blood flow image. Look-up table shows arbitrarily assigned perfusion unit (PU) from the lower limit of 0 to an upper limit of 1,000 and above. The skulls illustrate the holes and injection sites. The top, middle, and bottom panels are images of ET-1+saline, ET-1+hAAT (i.c.), and ET-1+hAAT (i.v.) groups, respectively. (B). Percentage perfusion reduction as a

function of time calculated using formula 3-1. All rats were injected with ET-1 (240 pmol in 3 μ L PBS) proximal to the left MCA. About 5–10 min after ET-1 injection, hAAT (1.35 nmol in 3 μ L saline for i.c.) or (192 nmol in 1 mL saline for i.v.) was administered at the same site in the two groups. The dotted, closed diamond solid and closed triangle solid lines represent the average levels in ET-1 alone, hAAT (i.c.), and hAAT (i.v.) groups, respectively. N = 3 for all groups. The differences at all time points of all groups were not statistically significant. Data are means $\pm$ s.e.m.

Figure 2.

Cylinder test of ET-1 alone vs. ET-1 and intracerebral hAAT delivery. Rats were placed in a transparent cylinder for 3 min. Forelimb use during exploration by the rats was scored. Each bar represents the average percentages $\pm$ s.e.m. of using both forelimbs in the indicated group. ET-1 group, N = 3, ET-1 + hAAT group, N = 3, *, **, ***: $p < 0.05$; 0.01; 0.001, respectively.

Figure 3.

Vibrissae test of ET-1 alone vs. ET-1 and intracerebral hAAT delivery. The relative time response (fold increase relative to pre-MCAO response time) was calculated using equation 2 and presented. ET-1 group, N = 3, ET-1 + hAAT, N = 3, *: $p < 0.05$, ***: $p < 0.0001$. Data are means $\pm$ s.e.m.

Figure 4.

Infarction area labeled for mitochondrial activity of AAT study (2,3,5-triphenyltetrazolium chloride, TTC) and calculated using ImageJ v5.0. Two TTC-staining slices from two animals representing each group, the left one for group receiving ET-1 alone, and the right one for groups receiving ET-1 and intracranial hAAT administration. Off-white color areas show dead tissues while red areas indicate vital tissues. ET-1-alone group N = 3, ET-1 and hAAT treatment group, N = 3. ***: $p < 0.001$. Data are means $\pm$ s.e.m.

Figure 5.

Cylinder test of ET-1 alone vs. ET-1 and intravenous hAAT delivery. Rats were placed in a transparent cylinder for 3 min. Forelimb use during exploration by the rats was scored. Each bar represents the average percentages $\pm$ s.e.m. of using both forelimbs in the indicated group. ET-1 alone group, N = 8, ET-1 + hAAT (i.v.), N = 14, *: $p < 0.05$, ***: $p < 0.001$.

Figure 6.

Vibrissae test of ET-1 alone vs. ET-1 and intravenous hAAT delivery. The relative time response (fold increase relative to pre-MCAO response time) was calculated using equation 2 and presented. ET-1 alone group, N = 8, ET-1 + hAAT (i.v.), N = 14. Data are means $\pm$ s.e.m. *: p < 0.05

Figure 7.

Systemic (i.v.) delivered hAAT. (A) Infarction area calculated from MRI images using ImageJ v5.0. Data are means $\pm$ s.e.m. ET-1 + saline group, N = 8, ET-1 + hAAT (i.v.), N = 14. *: $p < 0.05$ by t-test. (B) Measured concentration from whole brain lysates following MRI. Data are means $\pm$ s.e.m. ET-1 + saline group, N = 4, ET-1 + hAAT (i.v.), N = 4. *: $p < 0.05$ by Mann-Whitney test. Dashed line represents minimum level of detection for hAAT.

Table 1

Treatment summary of for ET-1 alone and ET-1+hAAT (i.c.) groups

Group	Treatment	Number of animals	Survival rate	Data collected
ET-1 alone	3 μ L(240 pmol) of ET-1 (i.c.)	3	3/3	3
ET-1+hAAT(i.c.)	3 μ L (240 pmol) of ET-1 (i.c.) 3 μ L (70 μ g) of hAAT (i.c.)	3	3/3	3

Table 2

Treatment summary for ET-1+saline and ET-1+hAAT (i.v.) groups

Group	Treatment	Number of animals	Survival rate	Data collected
ET-1+saline	3 μ L (240 pmol) of ET-1 (i.c.) 1 mL of saline (i.v.)	8	8/8	8
ET-1+hAAT(i.v.)	3 μ L (240 pmol) of ET-1 (i.c.) 40 mg/kg (i.v.)	14	14/14	14