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Original Article

Effects of alteplase on neurological deficits and expression of GFAP and GAP-43 in brain tissue of rats with acute cerebral infarction

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Abstract: Objective: To investigate the effects of alteplase on neurological deficits, as well as on the expressions of glial fibrillary acidic protein (GFAP) and growth-associated protein-43 (GAP-43) in brain tissues of rats with acute cerebral infarction (ACI). Methods: Sprague Dawley (SD) rats (n = 50) were enrolled in a trial to establish a ACI rat model; of these, 48 rats were succeeefully modeled and were randomized into either the model or alteplase group, whereas another 24 SD rats were included in the sham-operated group. Findings: No significant difference in scores was observed between the model and alteplase groups at T1 ($P > 0.05$); however, rats in the alteplase group demonstrated lower scores than those in the model group at T2, T3, and T4 ($P < 0.05$). Rats in the model group showed a larger cerebral infarction volume than those in the alteplase group ($P < 0.05$), and the infarction volume on day 1, 3, 6, and 9 was higher in rats in the alteplase group than those in the sham-operated group ($P < 0.05$). Conclusion: Treatment with alteplase can be effective in reducing cerebral infarction volume and moderating neurological deficits in ACI modeled rats within a 6-h time window, which may be correlated with the regulation of GFAP and GAP-43 expressions by alteplase.

Keywords: Alteplase, acute cerebral infarction, GFAP, GAP-43

Introduction

Stroke is the second leading cause of death and the main cause of disabilitie, worldwide [1]. Globally, 15 million individuals annually suffer stroke, resulting in 5.8 million deaths as well as permanent disabilities in approximately 5 million individuals [2]. Acute cerebral infarction (ACI) is a brain lesion that causes serious damage to the central nervous system, accounting for approximately 70% of all strokes and exhibiting high morbidity, mortality, and recurrence rates [3]. In addition to high treatment costs, ACI results in restricted social function, long-term disability, and premature death, resulting in serious implications for patients with ACI and their families. Therefore, curing ACI in its early stage needs to be urgently addressed.

Among the treatment options for ACI, thrombolytic therapy is currently recognized as an effective way to rapidly reopen the occluded portion of the vascular system, restoring blood flow, and reducing the ischemic area or cerebral infarction volume, which is the most important measure for restoring blood flow [4]. Intravenous thrombolysis with recombinant tissue plasminogen activator (r-tPA) [5] is the sole approved drug class for ACI and includes alteplase-a second-generation thrombolytic agent-that selectively activates fibrin-bound plasminogen, thereby promoting thrombolysis and dredging the occluded blood vessels [6]. Timely treatment with alteplase in patients with ACI can improve neurological function within 3-6 months [7]. Studies have demonstrated that alteplase is effective for patients with ACI via clinical improvement and subsequent prognosis management, thereby lowering the disability rate of these ACI patients without a higher risk of death caused by intracranial hemorrhage [8]. Furthermore, intravenous alteplase administration alleviates cerebral perfusion and neurological damage in patients with ACI [9].

Glial fibrillary acidic protein (GFAP), commonly found in astrocytes, ependymal cells, and radial glial cells (molecular weight: 40-50 kDa), is the most frequently used marker in diagnostic neuro-oncology. Growth-associated protein-43 (GAP-43) is a presynaptic protein located on the inner surface of the plasma membrane at the axon terminal. It is involved in the signal transduction of nerve endings and is highly expressed in neuronal development and synaptogenesis [10, 11]. Numerous studies have suggested abnormal GFAP and GAP-43 expressions in neurological disorders, including an increasingly higher level of GFAP expression which has been reported in studies conducted in rats with hypoxia [12]. The evaluation of whether melatonin regulates *N*-methyl-D-aspartate receptor (NMDAR) postsynaptic density-95 (PSD-95), GAP-43, and matrix metalloproteinase-9 (MMP-9) in cultured neurons exposed to glutamate excitotoxicity and in stroke modeled rats has demonstrated that melatonin is a modulator of neural plasticity via the upregulation of GAP-43, PSD-95, and MMP-9 proteins [13]. Melatonin upregulates GAP-43 and PSD-95 expression and improves dendritic regulation in cultured neurons exposed to glutamate excitotoxicity. In stroke modeled rats, melatonin administration resulted in GAP-43 and PSD-95 expression and MMP-9 inhibition.

Through the establishment of ACI rat models, the present study focused on the effects of alteplase on neurological deficits as well as on GFAP and GAP-43 expression in the brain tissue of ACI modeled rats.

Materials and methods

Materials, main drugs, and laboratory supplies

Specific pathogen-free Sprague Dawley (SD) rats aged 8-12 months and weighing 320-360 g were supplied by the Experimental Animal Center of the Affiliated Longyan First Hospital of Fujian Medical University, China. They were placed in an animal room at 21°C-24°C and a relative humidity of 52%-65%; the rats were fed *ad libitum* for 2 weeks under natural light prior to the experiments. The present study was approved by the ethics committee of the the Affiliated Longyan First Hospital of Fujian Medical University (approval number [2021]-(015)) where it was completed and strictly complied with the 2016 guidelines of the American Society of Mammologists for the use of wild mammals in research and education [14].

Main drugs and laboratory supplies were as follows: alteplase (Actilyse®, Germany; batch #: RK20180329n), thrombin (Beijing Solarbio Science & Technology Co., Ltd., China; T8021), 2,3,5-triphenyltetrazolium chloride (TTC) solution (BioRike, China; RK0004), GFAP monoclonal antibody (Abnova, China; MAB21013), GAP-43 monoclonal antibody (Elabscience, China; E-AB-22109), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) polyclonal antibody (Shanghai Yuduo Biological Technology Co., Ltd., China; YDQ694), and horseradish peroxidase goat anti-rabbit (immunoglobulin G) secondary antibody (BioVision, Inc., USA; 6401-05). A BCA (bicinchoninic acid) protein assay kit was obtained from LEAGEN (China; PT0001).

Preparation of the ACI rat model

We established an ACI model using 50 SD rats. Chloral hydrate (3.5 $mg \cdot kg^{-1}$ body weight) was intraperitoneally injected to anesthetize the rats before separating the peripheral artery and its branch arteries using a scalpel along the right center of the rats' necks. Thereafter, for embolizing the artery, thrombin-containing clotted blood was injected into intravenous indwelling catheters to form an embolus approximately 1 cm long. The indwelling needle was applied to puncture through the right common carotid artery to the internal carotid artery, a length of approximately 10 mm. After the indwelling needle was pulled out, the prepared embolus was quickly inserted into the internal carotid artery, and following which the right common carotid artery was ligated. The ACI rat model was considered established if the rats recovered from the anesthetic with symptoms such as failure to stably stand, circling toward the left or being skewed toward the left, and not walking spontaneously or loss of consciousness.

Grouping of experimental animals and drug intervention

Of these 50 ACI rats, 48 were equally randomized into either the model or alteplase group, excluding 2 who failed in modeling. The sham-operated group included another 24 SD rats. The rats in the alteplase group were injected

with alteplase (5 mg·kg^{-1} body weight) via the femoral vein within 6 h after establishing the model, whereas the rats in both the sham-operated and model groups were administered the same volume of normal saline.

Neurological deficits scoring

The neurological deficits in the modeled rats were evaluated using the modified Longa Score Scale for neurological function when the ACI models were established (T1) as well as at 6 (T2), 12 (T3), and 24 (T4) h after drug administration. The scoring criteria were as follows; 0: no neurological deficits, 1: failure to completely extend the left forepaw, 2: failure to extend the left forepaw, 3: slight circling toward the side of hemiparalysis, 4: severe circling toward the side of hemiparalysis, and 5: falling to the left.

Preparation of brain tissue sections

Six rats from all groups were randomly selected to prepare brain slices on day 1, 3, 6, and 9 after Longa Score Scaling. They were anesthetized with 10% chloral hydrate, placed on ice, and decapitated to remove their brain issues, which were immediately soaked in a 4% paraformaldehyde solution for 1 day. Thereafter, the brains were divided along the coronal plane using a vibratome into roughly 2-mm thick slices and immediately stored at -80°C in 0.1 mol/L phosphate-buffered saline (PBS) until further analysis, according to *The Stereotactic Atlas of The Rat Brain*.

Measurement of cerebral infarction volume

The brain slices were stained with 2% TTC solution and incubated at 37°C for 0.5 h in the dark before fixing in 10% paraformaldehyde solution overnight. Finally, they were removed and photographed using a digital camera. In ACI modeled rats, the cerebral infarction was grayish white, whereas the normal brain tissues remained red. The area of infarction was measured by subtracting the area of the nonlesioned ipsilateral hemisphere from that of the contralateral hemisphere, based on the Swanson's method. In addition to the absolute volume, the infarction volume was expressed as a percentage of the total brain volume.

Detection of GFAP and GAP-43 expressions in all rats using western blotting

For performing the western blot assay, the brain slices obtained from all groups were weighed and pulverized, following which the proteins from the brain slices were extracted by adding tissue lysate. Subsequently, 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on the extract to separate the proteins. The separated proteins were transferred from the gel onto a polyvinylidene difluoride (PVDF) membrane. Next, the membrane was blocked with 5% skim milk powder for 1 h and incubated overnight with GAP-43 primary antibody (1:10000) and GAPDH primary antibody (1:10000) in a refrigerator at 4°C. The following day, after washing thrice in PBS with Tween-20, the membrane was incubated with rabbit secondary antibody (1:5000) at room temperature for 1 h. The incubated membrane was re-washed thrice before imaging using electrogenerated chemiluminescence. GFAP protein concentration could be detected using a similar procedure to that of GAP-43, except that the dilution ratio was 1:20,000 for the GFAP primary antibody.

Statistical analysis

SPSS 21.0 (IBM Corp, Armonk, USA) software was used for statistical analysis, and GraphPad Prism 7 software was used for drawing based on the analyzed data. Enumeration data were expressed as [n (%)] in this trial, and rates were tested using chi-square test for comparison, whereas measurement data were presented as means ± standard deviations ($\bar{x}$ ± SD), among which multiple mean values were compared after single factor analysis of variance. The between-group differences were assessed using one-way analysis of variance. $P < 0.05$ indicated statistical significance.

Results

Comparison of the longa scores of rats in each group at different times

According to the Longa Score Scale, rats in the model and alteplase groups scored higher than those in the sham-operated group at T1, T2, T3, and T4 ($P < 0.05$). Rats in the alteplase group demonstrated lower scores than those in the model group at T2, T3, and T4 ($P < 0.05$). In the alteplase group, the rats showed lower neurological deficit scores at T2, T3, and T4 than at T1 ($P < 0.05$); at T3 and T4 than at T2 ($P < 0.05$); and at T4 than at T3 ($P < 0.05$) (**Table 1** and **Figure 1**).

Table 1. Comparison of longa scores of all groups at different times (n = 24, $\bar{x}$ ± SD, score)

Group	T1	T2	T3	T4	F	P
Sham-operated group	0.57 ± 0.11	0.56 ± 0.09	0.56 ± 0.15	0.57 ± 0.13	0.054	0.984
Model group	4.41 ± 0.74*	4.52 ± 0.51*	4.63 ± 0.62*	4.72 ± 0.57*	1.143	0.336
Alteplase group	4.47 ± 0.66*	3.68 ± 0.62*,#,a	2.34 ± 0.56*,#,a,b	1.21 ± 0.45*,#,a,b,c	149.462	< 0.001
F	361.208	461.351	416.001	660.346	-	-
P	< 0.001	< 0.001	< 0.001	< 0.001	-	-

Note: The Longa Scores in the alteplase group: *P < 0.05 compared with the sham-operated group and #P < 0.05 compared with the model group; aP < 0.05 at T2, T3, and T4 compared with T1; bP < 0.05 at T1, T3, and T4 compared with T2; cP < 0.05 at T1, T2, and T4 compared with T3.

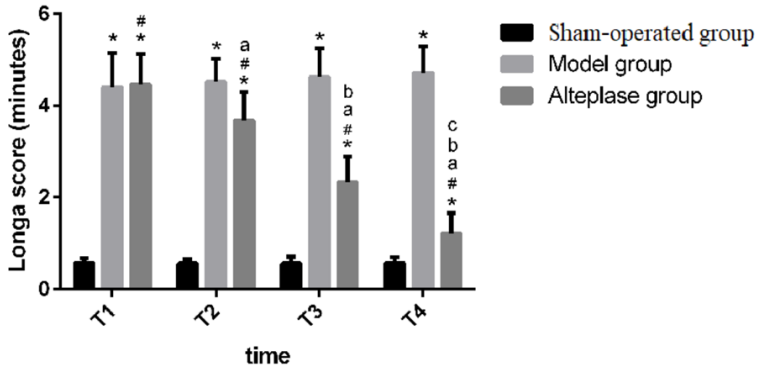

Figure 1. Comparison of longa scores of rats in all groups at different times. Note: The Longa Scores in the alteplase group: *P < 0.05 compared with the sham-operated group and #P < 0.05 compared with the model group; aP < 0.05 at T2, T3, and T4 compared with T1; bP < 0.05 at T1, T3, and T4 compared with T2; cP < 0.05 at T1, T2, and T4 compared with T3.

Comparison of cerebral infarction volumes in rats in all groups at different times

In all groups, the cerebral infarction appeared as a white region due to insufficient blood supply, adjacent to the surrounding red area, with a relatively normal blood supply; the model group showed the largest infarct area followed by the alteplase group and the sham-operated group. Cerebral infarction volume in rats in the model group was larger than those in the alteplase group (P < 0.05), in which an increase in the infarct area was observed compared with that in the sham-operated group at day 1, 3, 6, and 9 (P < 0.05). Moreover, at day 1, 3, 6, and 9, no significant changes in cerebral infarction volume was observed in both the sham-operated and model groups (P > 0.05). In the alteplase group, less infarction volume was observed on day 3, 6, and 9 (P < 0.05) than on day 1; on day 6 and 9 than day 3 (P < 0.05); and on day 9 than day 6 (P < 0.05) (**Table 2** and **Figure 2**).

Comparison of GFAP expression in all groups at different times

In the western blot assay, GFAP expression in the brain tissues in the model and alteplase groups was higher than that in the brain tissues of the sham-operated group on day 1, 3, 6, and 9 (P < 0.05), whereas the GFAP expression in the alteplase group was lower than that in the model group (P < 0.05). GFAP expression in the sham-operated group showed no significant changes on day 1, 3, 6, and 9 (P > 0.05). However, GFAP expression in both the model and alteplase groups increased on day 3 and 6 compared with that on day 1 (P < 0.05) and notably decreased on day 9 (P < 0.05). Compared with day 3, day 6 and 9 revealed a considerable decrease in GFAP expression in the model and alteplase groups (P < 0.05). GFAP expression was less in the brain tissues of rats in the model and alteplase groups on day 9 compared with that on day 6 (P < 0.05) (**Table 3** and **Figure 3A**).

Comparison of GAP-43 expression in all groups at different times

According to the western blot assay, GAP-43 expression in the brain tissues in the model and alteplase groups was higher than that in the brain tissues in the sham-operated group on day 1, 3, 6, and 9 (P < 0.05), whereas GFAP expression in the alteplase group was lower than that in the model group (P < 0.05). GAP-43 expression in the sham-operated group showed no significant changes on day 1, 3, 6, and 9 (P

Table 2. Comparison of cerebral infarction volume of rats at different times (n = 6, $\bar{x} \pm SD$, %)

Group	Day 1	Day 3	Day 6	Day 9	F	P
Sham-operated group	4.89 ± 0.75	4.56 ± 0.67	4.53 ± 0.56	4.23 ± 0.71	0.956	0.433
Model group	41.89 ± 3.07*	42.13 ± 5.22*	44.29 ± 4.45*	45.89 ± 3.78*	1.220	0.328
Alteplase group	34.89 ± 5.07*,#	27.89 ± 4.34*,#,a	21.89 ± 3.87*,#,a,b	14.89 ± 2.67*,#,a,b,c	26.168	< 0.001
F	194.832	129.164	203.800	384.582	-	-
P	< 0.001	< 0.001	< 0.001	< 0.001	-	-

Note: Cerebral infarction volume in the alteplase group: *P < 0.05 compared with the sham-operated group and #P < 0.05 compared with the model group; aP < 0.05 at T2, T3, and T4 compared with T1; bP < 0.05 at T1, T3, and T4 compared with T2; cP < 0.05 at T1, T2, and T4 compared with T3.

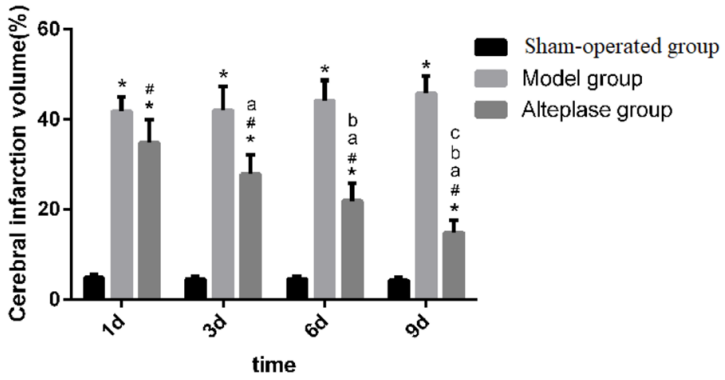

Figure 2. Comparison of cerebral infarction volume in rats in all groups at different times. Note: Cerebral infarction volume in the alteplase group: *P < 0.05 compared with the sham-operated group and #P < 0.05 compared with the model group; aP < 0.05 at T2, T3, and T4 compared with T1; bP < 0.05 at T1, T3, and T4 compared with T2; cP < 0.05 at T1, T2, and T4 compared with T3.

> 0.05). However, GAP-43 expression in both the model and alteplase groups increased on day 3, 6, and 9 compared with that on day 1 (P < 0.05). Compared with day 3, day 6 and 9 revealed a substantial decrease in GAP-43 expression in the model and alteplase groups (P < 0.05). GAP-43 expression was less in the brain tissues of rats in the model and alteplase groups on day 9 compared with that on day 6 (P < 0.05) (**Table 4** and **Figure 3B**).

Discussion

ACI is a medical emergency that affects 795,000 individuals annually in the United States. The incidence of ACI continues to increase because of the growing aging population, thereby exerting a serious adverse impact and a burden on human health and society [15]. ACI is caused by a severe reduction of blood or oxygen supply, typically occurring due to a blood clot that blocks the major blood vessels [16]. No effective and safe management strategy has yet been well established for the general recognition of patients with ACI [17]. Therefore, establishing safe and effective treatment that is generally accepted by patients with ACI is crucial for patients, their families, and society.

Currently, research on the pathophysiological mechanisms of ACI has shown progress mainly by treating ACI rats with r-tPA and then considering this as a simulated procedure for patients with ACI. Simplified modeling based on studies of Chen *et al*. was applied in the present study [18]. The model and alteplase group scored 4.41 ± 0.24 and 4.47 ± 0.26, respectively, at T1 on the Longa Score Scale, both indicating a failure to stand stably, circling toward the left or being skewed toward the left, and not walking spontaneously. Only 2/50 rats showed modeling failure, leading to a success rate of 96.00%. Therefore, this modeling method is simple and convenient and has demonstrated a high success rate.

Intravenous infusion of r-tPA within 3 h after modeling is considered the most effective therapy for ACI [19]. However, the narrow time window requires patients with ACI to visit an emergency room within 3 h of ACI onset, which could only be met by approximately 21% of patients with ACI in the United States, among which approximately 8% were eligible for thrombolytic therapy [20]. Expansion of the time window to receive a mechanical thrombectomy for patients with ACI is of great significance with respect to life safety. Reportedly, r-tPA improves the neurological deficits of patients with ACI within 6 h of onset in several studies [21], and

Table 3. Comparison of GFAP expression in all groups at different times (n = 6, $\bar{x} \pm SD$)

Group	Day 1	Day 3	Day 6	Day 9	F	P
Sham-operated group	0.89 ± 0.15	0.95 ± 0.12	0.91 ± 0.09	0.98 ± 0.10	0.709	0.558
Model group	2.12 ± 0.32*	3.48 ± 0.27*	2.65 ± 0·36*	1.69 ± 0.31*	35.461	< 0.001
Alteplase group	1.52 ± 0.18*,#	2.45 ± 0.18*,#,a	1.75 ± 0.22*,#,a,b	1.03 ± 0.16*,#,a,b,c	74.091	< 0.001
F	43.289	243.404	73.238	21.462	-	-
P	< 0.001	< 0.001	< 0.001	< 0.001	-	-

Note: GFAP expression in the alteplase group: *P < 0.05 compared with the sham-operated group and #P < 0.05 compared with the model group; aP < 0.05 at T2, T3, and T4 compared with T1; bP < 0.05 at T1, T3, and T4 compared with T2; cP < 0.05 at T1, T2, and T4 compared with T3.

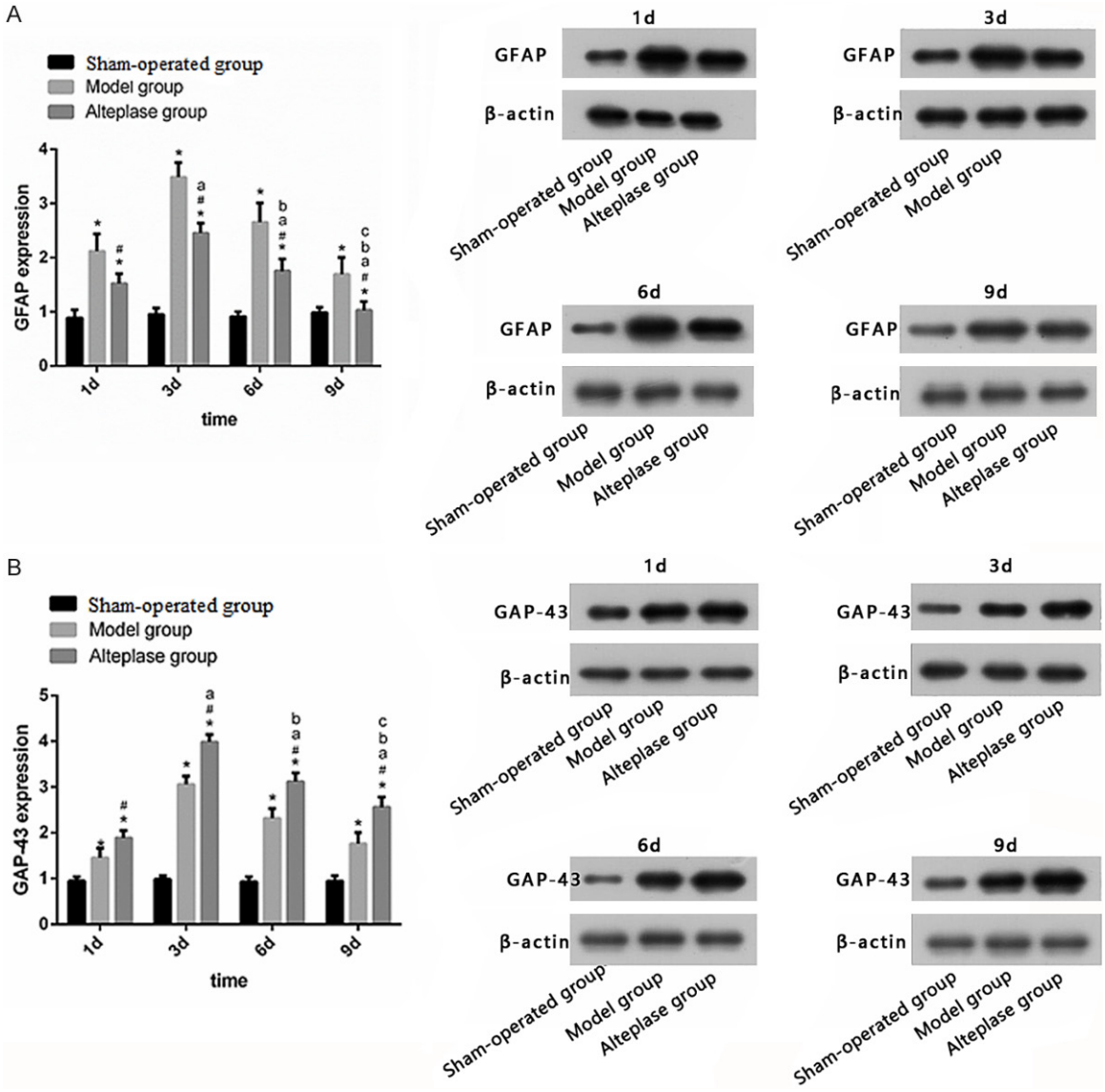

Figure 3. Comparison of GFAP and GAP-43 expression in all groups at different times. A: GFAP expression in all groups at different times, B: GAP-43 expression in all groups at different times *P < 0.05 compared with the sham-operated group and #P < 0.05 compared with the model group; aP < 0.05 at T2, T3, and T4 compared with T1; bP < 0.05 at T1, T3, and T4 compared with T2; cP < 0.05 at T1, T2, and T4 compared with T3.

Table 4. Comparison of GAP-43 expression in all groups at different times (n = 6, $\bar{x}$ ± SD)

Group	Day 1	Day 3	Day 6	Day 9	F	P
Sham-operated group	0.75 ± 0.09	0.78 ± 0.08	0.73 ± 0.11	0.74 ± 0.12	0.273	0.844
Model group	1.35 ± 0.21*	3.06 ± 0.18*	2.32 ± 0.21*	1.76 ± 0.24*	73.898	< 0.001
Alteplase group	1.89 ± 0.16$^{*,\#}$	3.98 ± 0.17$^{*,\#,a}$	3.12 ± 0.19$^{*,\#,a,b}$	2.56 ± 0.22$^{*,\#,a,b,c}$	135.245	< 0.001
F	75.239	721.631	288.631	124.405	-	-
P	< 0.001	< 0.001	< 0.001	< 0.001	-	-

Note: GAP-43 expression in the alteplase group: *P < 0.05 compared with the sham-operated group and $^{\#}$P < 0.05 compared with the model group; ^{a}P < 0.05 at T2, T3, and T4 compared with T1; ^{b}P < 0.05 at T1, T3, and T4 compared with T2; ^{c}P < 0.05 at T1, T2, and T4 compared with T3.

other studies on the effects of alteplase in these patients within the 3-6-h time window suggested the effective inhibition of alteplase on infarct growth [22]. Further, Si et al. [23] reported that r-tPA could reduce infarct volume within 6 h after ACI onset in numerous middle cerebral artery occlusion (MCAO) rat models. Our study suggested that compared with the model group, rats in the alteplase group exhibited a smaller cerebral infarction volume, which is consistent with the above findings. Moreover, rats in the alteplase group scored less than those in the model group at T2, T3, and T4 using the modified Longa Score Scale. This reflected the reduced infarction volume that was observed as well as the relief of neurological deficits within the expanded time window of 6 h.

GFAP is a landmark intermediate filament protein in astrocytes, almost all of which are found in the central nervous system [24]. Reactive gliosis refers to a wide range of glial reactions induced by brain damage, which is characterized by the increased expression of specific markers, including GFAP of astrocytes [25]. García-Álvarez et al. [26] showed that GFAP was highly expressed in rat models with spinal cord injuries. Moreover, a synthetic glycolipid, IG20, inhibited GFAP expression, a marker of astrocytes, thereby suppressing astrocyte proliferation to reduce glial scar formation. According to study by Petro et al. [27], GFAP expression was upregulated in modeled rats with cerebral infarction and decreased with alteplase administration. The detection of GFAP expression in the brain tissues in the three groups of the present study showed higher GFAP expression in the model group within 9 days after modeling compared with that in the alteplase group. Consistent with the above research results, this finding indicated that alteplase administration may reduce glial scar formation by suppressing GFAP expression.

GAP-43 is an axonal membrane protein that is crucial for nerve fiber growth, development, and regeneration; synaptic function maintenance; and transmitter release [28]. Caspase 3 (CASP3) and GAP-43 rapidly increase following ischemic injury, mainly in neurons in modeled mice. It was suggested that GAP-43 and CASP3 are part of a common molecular pathway involved in the early response to ischemic events occurring after stroke onset [29]. Li et al. investigated whether exogenous retinoic acid (RA) could upregulate GAP-43 mRNA and protein expression and found that initially following ischemia, GAP-43 expression considerably increased and then decreased in modeled rats with MCAO. RA administration upregulated GAP-43 expression, reduced infarction volume, and promoted functional neurological recovery. It was concluded that the neuroprotective mechanism of RA was responsible for the upregulation of GAP-43 mRNA and protein [30]. The results of the present study showed that GAP-43 expression first increased and then decreased after the model was well established in the model group, whereas GAP-43 expression markedly increased following alteplase administration in the alteplase group compared with the model group. These findings are almost consistent with the above findings and indicate that alteplase promotes functional nerve recovery and nerve fiber regeneration by upregulating GAP-43 expression.

Our study confirmed that it was beneficial to treat ACI modeled rats with alteplase within an expanded time window of 6 h, coupled with a proved effect of alteplase on both GFAP and GAP-43 expression. However, there were some limitations to our study; we did not focus on the

optimal dosage of alteplase for ACI or on the regulatory mechanisms of alteplase on GFAP and GAP-43. These deficiencies will be addressed in future studies.

In conclusion, alteplase treatment resulted in considerable reductions in cerebral infarction volume and ameliorated neurological deficits in ACI rats within an extended time window of 6 h, which may be related to the regulation of GFAP and GAP-43 expressions by alteplase.

Acknowledgments

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Disclosure of conflict of interest

None.

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