

Tissue kallikrein attenuates early ischemic edema by facilitating glymphatic system kinetics in pMCAO mice

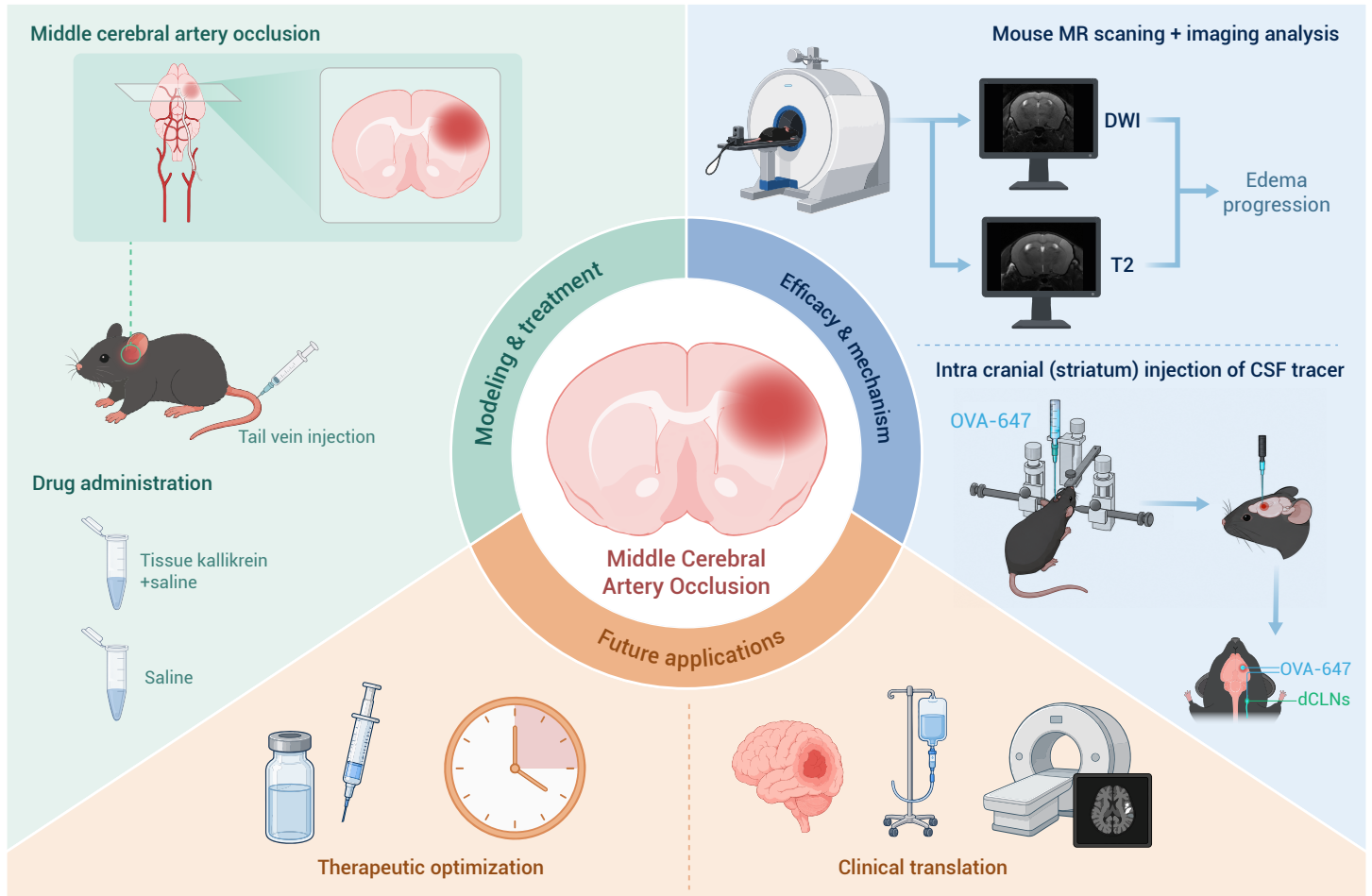
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Glymphatic kinetics maintain brain homeostasis via fluid influx-efflux and modulate cerebral edema.
- Our previous study confirmed impaired glymphatic transport in permanent middle cerebral artery occlusion mice.
- Tissue kallikrein induces cerebrovascular vasodilation and subsequently facilitates glymphatic fluid dynamics.
- This study underscores the need for clinical studies to explore TK's impact on ischemic edema.
- TK mitigated brain edema in pMCAO mice with prolonged DWI/T2 mismatch, probably by boosting glymphatic efflux.

Tissue kallikrein attenuates early ischemic edema by facilitating lymphatic system kinetics in pMCAO mice

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The glymphatic system (GS) maintains brain fluid homeostasis and is potentially associated with cerebral ischemic edema. Tissue kallikrein (TK) enhances local cerebrovascular vasodilation and facilitates the GS kinetics. Herein, this study hypothesized that TK could relieve early ischemic edema after vascular occlusion by altering glymphatic dynamics. In permanent middle cerebral artery occlusion (pMCAO) models, brain water content was measured to evaluate edema severity, diffusion-weighted imaging (DWI) and T2-weighted imaging (T2WI) mismatch—defined as an acute ischemic lesion observed in DWI not corresponding to a lesion in T2WI—was employed to assess edema progression. The fluorescent tracer OVA-647 was injected into the mouse striatum to quantify interstitial fluid (ISF) perfusion in the brain parenchyma and fluid outflow to the deep cervical lymph nodes (dCLNs). Immunohistochemistry was further applied to assess aquaporin-4 (AQP4) expression on the ventricular ependymal walls. Statistical results showed that TK-treated mice exhibited a significantly extended DWI/T2WI mismatch duration (98.0 ± 11.43 min vs. 63.0 ± 8.08 min; $P = 0.0286$) and lower ipsilateral brain water content at 24 hours ($P = 0.0159$). Meanwhile, the cerebrospinal fluid (CSF)/ISF tracer area fraction in the striatum was increased, and dCLN fluorescence intensity rose markedly ($P = 0.0349$ and $P = 0.0002$, respectively), alongside upregulated AQP4 expression in the ependymal wall ($P = 0.0016$). To conclude, TK effectively alleviates ischemic brain edema in pMCAO mice, as validated by reduced tissue water content and a prolonged DWI/T2WI mismatch. Its protective function is realized by upregulating ependymal AQP4 to boost glymphatic fluid perfusion and clearance.

INTRODUCTION

Cerebral infarction is featured by high incidence, mortality, and disability rates worldwide.¹ As is well documented, brain edema and secondary elevated intracranial pressure are severe complications of ischemic stroke that negatively affect stroke outcomes.² Despite several studies having attempted to improve clinical outcomes following edema, the available findings remain limited.

The glymphatic system (GS), a perivascular fluid pathway in the brain that facilitates the exchange of cerebrospinal fluid (CSF) and interstitial fluid (ISF) in the perivascular space (PVS), plays a crucial role in maintaining brain homeostasis and may participate in ischemic edema.^{3,4} Within the glymphatic network, subarachnoid CSF infiltrates the interstitium of the brain via perivascular astrocytic aquaporin-4 (AQP4) channels, renews the local ISF, and then drives the perivenous drainage of local ISF and its solute to achieve effective waste removal and preserve volume homeostasis.³ The dynamics of glymphatic fluid maintain homeostasis via the fluid movement into and out of the brain, which has been implicated in edema initiation,⁵ progression, and even elimination. Our previous study determined that glymphatic transport kinetics were impaired after the ischemic edema appeared in permanent middle cerebral artery occlusion (pMCAO) models, as quantified using contrast-enhanced MRI (11.7T) by employing an injection of gadobenate dimeglumine (BOPTA-Gd) into the cisterna magna of mice, were accompanied by depolarization of AQP4, in the cortex, striatum, and periventricular

zone.⁶

As a key component of the kallikrein/kinin system (KKS), tissue kallikrein (TK) releases kinins (bradykinin and kallidin) from kininogen,⁷ which in turn promotes local cerebrovascular vasodilation,⁸ thereby supporting its beneficial role in increasing vasodilatory activity and pulsation of the cerebral arteries, which can facilitate kinetics in GS as a driving force for the perivascular exchange of CSF-ISF.⁴ Consequently, we speculate that Tissue Kallikrein can mitigate early ischemic edema following arterial occlusion by altering the kinetics of the glymphatic fluid.

MATERIALS AND METHODS

Animals

C57BL/6 and AQP4 (-/-) male mice (25–30g, 8–10 weeks old) were utilized as experimental models. All animals were housed with a 12 h light/12 h dark cycle in a temperature-controlled room at 25 ± 2 °C and provided water and food ad libitum. Briefly, a total of 70 male C57BL/6 mice were randomly assigned to the following three groups using Excel-generated random numbers: Sham, pMCAO + vehicle, and pMCAO + Tissue Kallikrein. Additionally, 6 male AQP4 (-/-) mice were assigned to the AQP4 (-/-) group. The experimenters were blinded to data collection and analysis. The study was conducted in accordance with the principles of the National Science Council of the Republic of China. All animal experiments were approved by the Fudan University Huashan Hospital institutional review board (no. 2022JS-HSYY-279). Every effort was made to minimize the number of experimental animals and animal suffering.

Drug Administration

TK or saline solution was administered immediately and 22 h (for the 24 h pMCAO group) after pMCAO via tail vein injection. Mice were injected with 17.5×10^{-3} NAU/kg tissue kallikrein (Guangdong Tianpu Biochemical Medicine Co., Ltd., Guangdong, China) in saline solution or were given saline solution only.⁹

pMCAO model and 2,3,5-triphenyl tetrazolium chloride staining (TTC staining)

To explore the mechanism of ischemic brain edema without BBB interruption, a permanent MCA occlusion model wherein the integrity of the BBB was preserved was generated using the methodology outlined in a previously published article.¹⁰ Briefly, the mice were initially anesthetized with 4% isoflurane, which was subsequently adjusted to a maintenance range between 1.5 and 3% in 1 L/min oxygen flow via the nose cone. A laser Doppler probe was fixed on the skull 5 mm lateral to and 2 mm posterior to Bregma. Next, a coated filament (1622A4, Cinontech Co.Ltd., Beijing, China) was inserted into the left MCA under stereomicroscopic guidance. Concurrently, CBF recordings were obtained using laser Doppler to ensure that only mice presenting with CBF measurements below 25% of the baseline were enrolled.

Animals were anesthetized and perfused with 20 mL of ice-cold saline. After immediate decapitation, the brains were sliced into six 1.5 mm-thick coronal sections and placed in 2% 2,3,5-triphenyl tetrazolium chloride (TTC) (Sigma-Aldrich) solution at 37 °C for 10 min, respectively. The slices were

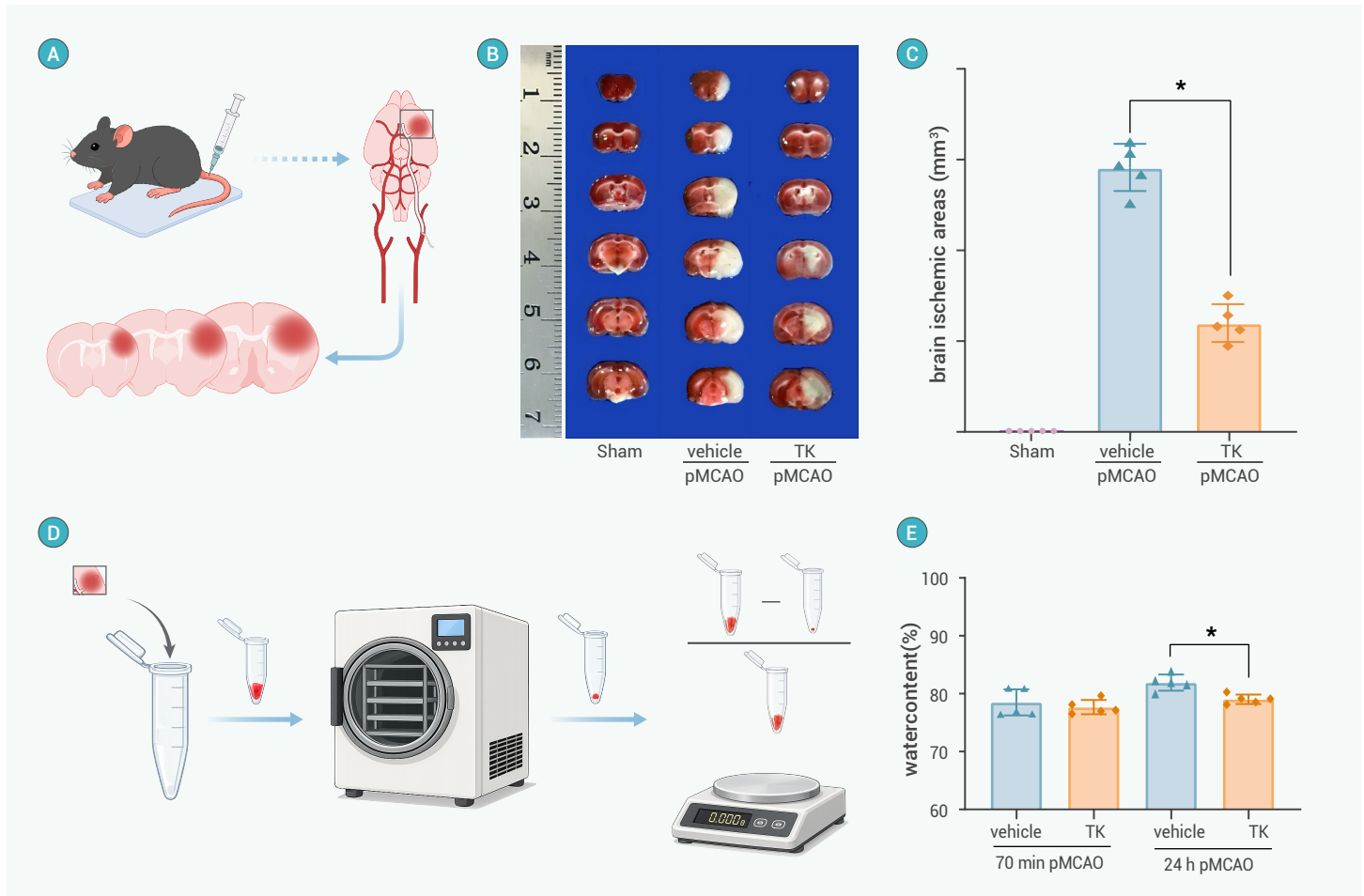


Figure 1. Treatment with TK following pMCAO reduced cerebral infarction volume and alleviated tissue edema (A) Schematic depiction of MCAO model. (B) Representative images of six corresponding coronal brain sections stained with 2,3,5-triphenyl tetrazolium chloride for sham, vehicle-treated mice, and TK-treated mice following 24 h of pMCAO. (C) Quantifying the infarction volume after 24 h of pMCAO (n = 5). (D) Schematic depiction of brain tissue water content assay. The boxed area of schematic images indicates the specific range of brain tissue used in the cerebral water content analysis. (E) Quantification of water content following euthanasia at 70 min and 24 h after pMCAO (n = 5).

flipped once every 2 min and then washed three times with ddH₂O. Images were collected using a digital camera, and the infarcted region (white) was quantified using ImageJ software.

Cerebral water content (edema assay)

To assess brain edema in each group, mice were euthanized 70 min and 24 h after pMCAO, respectively. The injured or uninjured contralateral hemisphere was divided along the midline and weighed to measure the wet weight (WW); Figure 1 illustrates the specific range. Subsequently, brain tissues were dried by incubating at 37 °C for 48 h to dry weight (DW). The water content (%) of each sample was calculated using the following formula: $(WW - DW) / WW \times 100\%$.¹¹

MRI experiments and imaging analysis

Following pMCAO surgery, animals were transferred to an MRI-compatible cradle. Afterward, 1.5% isoflurane in oxygen was consistently delivered to the animal through the nasal cone. The core body temperature of mice was maintained at 37 °C, and the respiratory rate was maintained between 70 and 100 breaths per minute. Each MRI was acquired on an 11.7T Pharmascan MRI system (Bruker, Germany) equipped with surface coils. T2-weighted imaging (T2WI) was performed with the following parameters: TR/TE 2500/30 ms, FOV = 16 mm × 16 mm, matrix = 256 × 256, ST = 0.5 mm, FA = 90°. Diffusion-weighted imaging (DWI) was performed with parameters set as follows: TR/TE 3200/23 ms, FOV = 16 mm × 16 mm, matrix = 180 × 180, ST = 0.3 mm, FA = 90°. The progression of ischemic edema was indicated by a mismatch between diffusion-weighted imaging (DWI) and T2-weighted imaging (T2WI), an acute ischemic lesion in DWI without a corresponding

lesion in T2WI, and analyzed by a blinded investigator. The apparent diffusion coefficient (ADC) values were automatically calculated using the 11.7T Pharmascan MRI system and displayed as a parametric map that indicated the degree of water molecule diffusion through different tissues. Consequently, ADC measurements were acquired for a given region by drawing regions of interest (ROIs) on the ADC map,¹² and the ROIs were mapped by selecting lesion areas with high signal intensity on DWI.

Intracranial injection of CSF tracer

In the other groups, 2 μL of CSF tracer was injected into the striatum with a microsyringe pump and a pulled glass micropipette 70 min after blood occlusion.⁶ This time point was selected owing to the absence of further progression of edema detected in the DW imaging at 70 min after pMCAO (14 min before MR plus 56 min in MR). Fluorescent CSF tracer-AF647 (ovalbumin-conjugated Alexa Fluor 647, 45 kDa; Invitrogen) was mixed with 1X phosphate buffer saline (PBS) to achieve a final concentration of 1%. Following injection into the striatum (coordinates from the Bregma 1.0 mm rostral, 2.0 mm lateral, and 3.2 mm deep), the mice were completely anesthetized. Then, the skull was exposed, a small burr hole was drilled using a dental drill. Subsequently, a pulled glass micropipette that was connected and sealed to a 10 μL syringe with hot melt glue was inserted into the brain using the stereotactic device. Intra-cranial injection was performed at a controlled flow rate of 0.1 μL/min for approximately 20 min. The micropipette was left in place for 10 additional min to allow the diffusion and perfusion of the tracer.

Penetration of ISF in the striatum

To evaluate the kinetics of GS by penetrating the fluorescent CSF tracer in

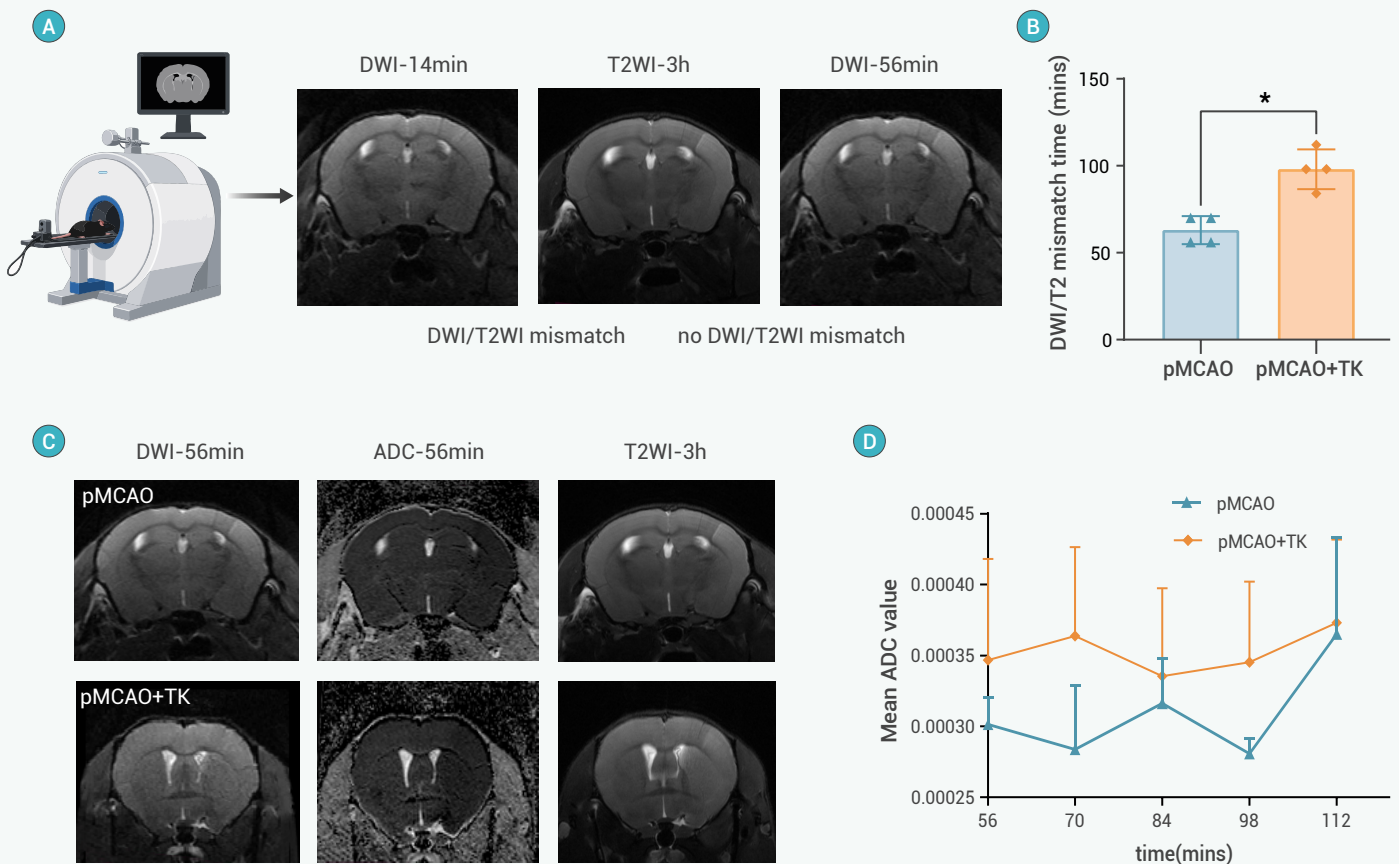


Figure 2. Treatment with TK after pMCAO attenuated the progression of brain edema (A) Representative coronal DWI/T2-weighted MR images from pMCAO mice delineating hyperintense areas indicative of edema at different levels of the striatum and cortex. (B) Quantification of DWI/T2 mismatch time following pMCAO ($n = 4$); TK treatment significantly prolonged DWI/T2 mismatch time (edema progression assay) after pMCAO in the ipsilateral side compared to mice treated with vehicle. (C) Representative coronal DWI/T2-weighted MR images and corresponding ADC map from TK-treated and vehicle-treated mice showing ADC values, indicating edema at the striatum and cortex. (D) Quantification of ADC values following pMCAO ($n = 4$); The ADC value (edema assay) was comparable between TK-treated pMCAO mice and those treated with vehicle in the ipsilateral side at the five pre-defined time points.

the brain parenchyma ex vivo, 30 min after starting tracer infusion, the anesthetized animals were transcardially perfused with ice-cold 1X PBS and 4% paraformaldehyde (PFA) for brain fixation. Thereafter, the brains were excised and postfixed overnight at 4 °C, then cut into 200 μ m thick slices using a vibratome. The time points in this section were identified in our previous study, wherein the penetration and elimination of glymphatic drainage were quantified using 11.7T contrast-enhanced magnetic resonance imaging (CEMRI) following intracisternal injections of gadobenate dimeglumine (BOPTA-Gd). The lateral ventricle perfusion by the tracers at 28 min postinjection and the efflux of the contrast agent in the cochleovestibular nerves, optic nerves, cribriform plate, and peritracheal lymph nodes were detected.⁶

Efflux of ISF into the deep cervical lymph nodes (dCLNs)

To evaluate GS kinetics via interstitial solute efflux, drainage of CSF tracer from brain parenchyma to dCLNs was observed using a method outlined in an earlier article, with minor modifications.¹³ After transcardially perfusing with ice-cold 1XPBS and 4% PFA 30 min for brain fixation, dCLNs were dissected and postfixed overnight at 4 °C.

Immunohistochemical analysis

After deparaffinization, hydration, and antigen retrieval, sections were washed and incubated with the 3% hydrogen peroxide (H₂O₂) at room temperature for 15 min to block endogenous peroxidase. Then tissues were washed and blocked with 3% BSA at room temperature for 30 min. Tissue sections were incubated with primary mouse antibody AQP4 (1:2, 000, #GB12529, Servicebio) at 4°C overnight. Following PBS wash, sections were

incubated with secondary antibody goat antimouse IgG (1:200, #GB23303, Servicebio) labeled with horseradish peroxidase, incubate at room temperature for 50 min. Then visualized with diaminobenzidine reagent (#G1211, Servicebio).

Immunofluorescence imaging

Sham, pMCAO, and pMCAO+TK mice ($n = 5$, each group) were used to evaluate GS. Six slices (bregma +0.38 to +1.10 mm, equivalent slices from each animal) and 5 dCLNs were selected for each animal. Brain sections and dCLNs were embedded in Fluoroshield mounting medium with DAPI (Abcam, ab104139) and covered with a coverslip. Images were acquired using fluorescent microscopy (Olympus VS120) under 10 $\times$ objective power and analyzed using ImageJ software by a blinded investigator. Gain and exposure levels were determined on control slices, then kept constant throughout the study.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 8. Data were presented as mean $\pm$ standard deviation (SD) or mean diff [95% confidence intervals]. The unpaired t-test was used for the comparisons between the two groups. One-way ANOVA was used to evaluate differences among multiple groups, followed by Tukey's multiple comparison test. Two-way ANOVA was used to evaluate serial MRI ADC data. A P -value < 0.05 was considered statistically significant. All data relevant to the study are included in the article. The remaining data are available upon reasonable request.

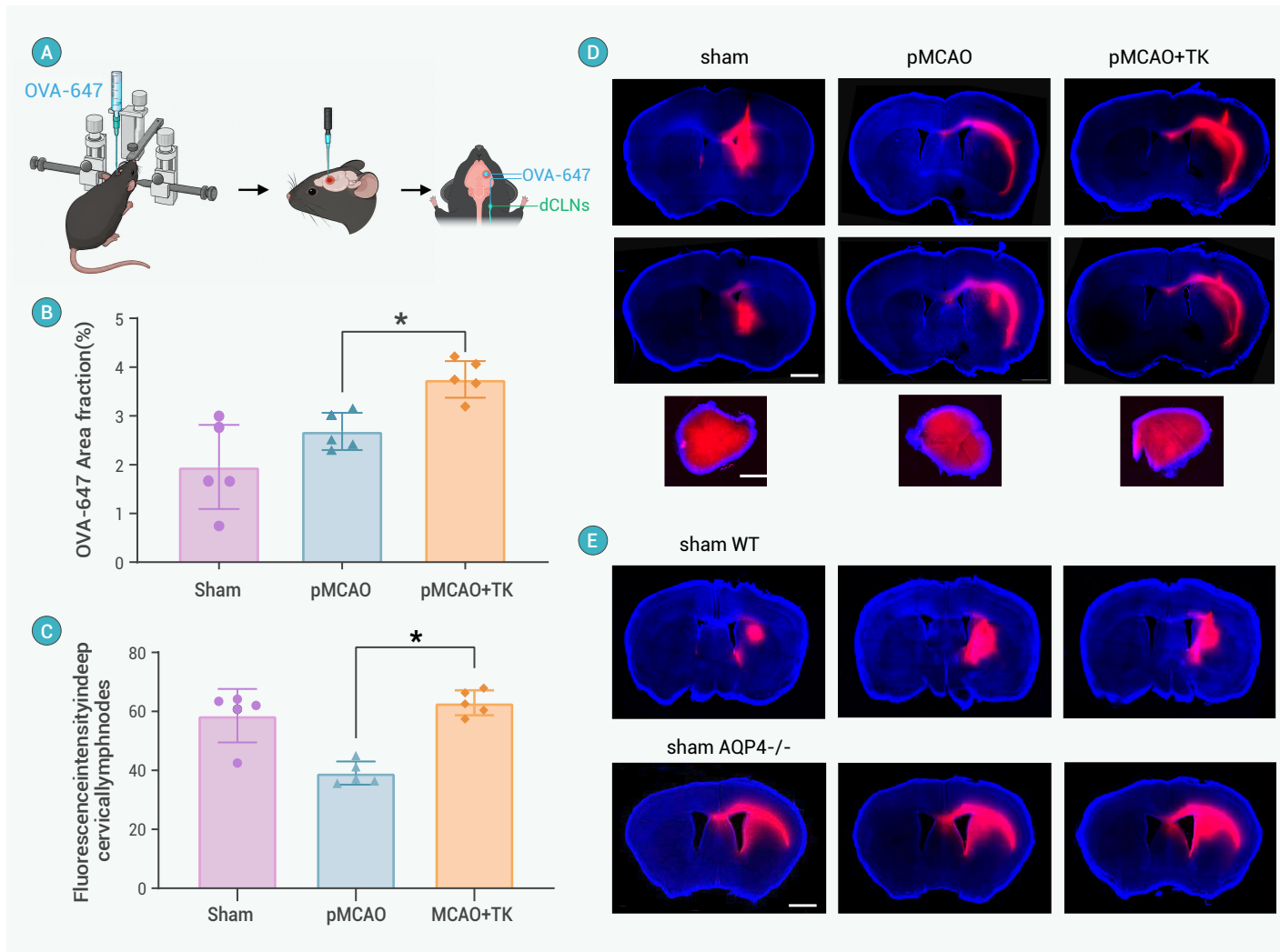


Figure 3. Interstitial fluid perfusion and drainage were immediately impaired following pMCAO and reversed by TK treatment (A) Schematic depiction of intrastratial injection of OVA-647 (+1.0 mm AP, +2.0 mm ML, and -3.2 mm DV from Bregma). (B) Quantitative analysis of the percentage area of fluorescence in brain sections ($n = 5$). (C) Quantitative analysis of fluorescence intensity in the dCLNs ($n = 5$). (D) Representative images (top) of OVA-647 distribution in the brain. Scale bar: 1000 μ m; Representative images (bottom) of the fluorescence intensity of OVA-647 drainage into dCLNs; scale bar: 500 μ m. (E) Representative images of OVA-647 dispersion in the sham group of WT and AQP4^{-/-} mice. Scale bar: 1000 μ m.

RESULTS

TK Reduced Cerebral Infarction and Edema After pMCAO

To determine the effects of TK on cerebral infarction and ischemic edema, 17.5×10^{-3} PNAU/kg of TK or vehicle were intravenously injected into the tail vein of mice. Infarct volume was evaluated 24 h post-pMCAO. TK-treated mice exhibited significantly smaller infarct areas (white, Figure 1B) and reduced infarct volume versus vehicle ($60.00 \pm 10.37 \text{ mm}^2$ vs. $145.8 \pm 13.01 \text{ mm}^2$; $p = 0.0079$, Figure 1C). Cerebral water content was analyzed in defined ipsilateral regions (Figure 1D). TK decreased water content in ipsilateral hemispheres at 24 h (0.82 ± 0.01 vs. 0.79 ± 0.01 ; $p = 0.0159$), but not at 70 min (0.79 ± 0.02 vs. 0.78 ± 0.01 ; $p = 0.6905$, Figure 1E). Our data revealed that TK treatment at a dose of 17.5×10^{-3} PNAU/kg attenuated cerebral infarction and edema following 24 h pMCAO.

TK Delayed Edema-to-Infarction Progression on MRI

DW-MRI at 11.7T revealed hyperintense regions (edema) in the ipsilateral hemisphere within 120 min post-pMCAO in vehicle- and TK-treated mice. T2W images at 3 h showed hyperintense infarct regions that remained stable thereafter. The DWI/T2 mismatch, indicating evolving edema-to-infarction, progressively resolved (Figure 2A). TK significantly prolonged mismatch duration versus vehicle ($98.0 \pm 11.43 \text{ min}$ vs. $63.0 \pm 8.08 \text{ min}$; $p = 0.0286$), confirming delayed edema progression (Figure 2B). Representative coronal DWI, T2W, and ADC maps are shown as Figure 2C. ADC values, reflecting restricted water diffusion in cytotoxic edema,^{14,15} were quantified within DWI-

hyperintense regions. The ADC value in the ipsilateral parenchyma was comparable between the TK-treated mice and vehicle-treated pMCAO mice ($p = 0.6374$) (Figure 2D). These results suggested that TK may alleviate edema through mechanisms unrelated to directly ameliorating cellular swelling.

TK Enhanced Interstitial Fluid Perfusion and Restored Deep Cervical Lymphatic Drainage After pMCAO

Our previous study has investigated the pattern of glymphatic flow that could account for the liquid drainage in the parenchyma and periventricular zone following induced acute ischemic brain edema in pMCAO, and explored the dysfunction of GS in the cortex, striatum, and periventricular zone.⁶ Therefore, this study assessed the kinetics of GS via imaging coronal brain sections and dCLNs by intra-cranial injection of OVA-647 as the CSF/ISF tracer in the striatum of sham and vehicle-, TK-treated mice to explore the effect of TK in boosting the perfusion and efflux of glymphatic fluid and in relieving ischemic edema. After 100 min of MCA occlusion and/or 30 min of OVA-647 injection into the striatum, OVA-647 perfusion and efflux were evaluated.

In sham mice, red fluorescent tracer (OVA-647) accumulated adjacent to the ipsilateral lateral ventricles, particularly in the inferior horn, indicating intact periventricular CSF/ISF clearance (Figure 3D, top panel). However, in pMCAO mice, tracer distribution shifted from ventricles toward the ipsilateral cortex and striatum, demonstrating impaired periventricular ISF efflux (Figure

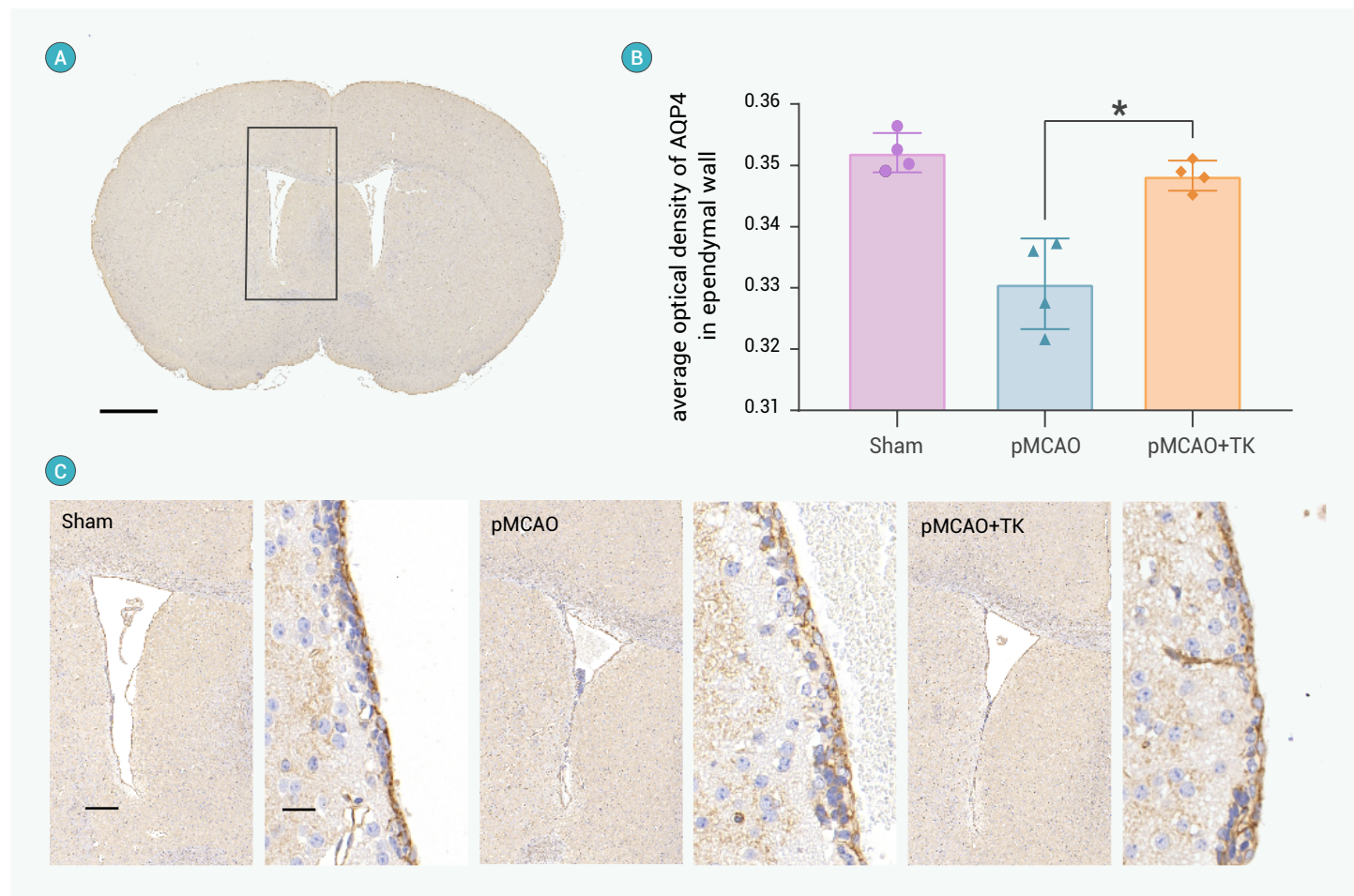


Figure 4. Immunohistochemical staining of AQP4 in ependymal wall (A, C) Representative images showing AQP4 immunohistochemical staining (brown) of coronal brain sections in the LV ependymal wall in 70 min post-pMCAO animals; these images show comparable morphology of the bilateral ependyma walls outlining LV; boxed areas are enlarged images; scale bar in Fig A: 1000 μ m; scale bar in Fig C: 200 μ m and 20 μ m. (B) quantification of AQP4 expression in the ependymal wall in the four brain sections ($n = 4$).

3D, top panel). TK treatment significantly increased OVA-647 area fraction in the ipsilateral striatum versus vehicle (1.066 [95%CI, 0.0755-2.056]; $p = 0.0349$, Figure 3B), indicating enhanced GS perfusion.

Parenchymal and ISF macromolecules are primarily cleared via dCLNs.¹⁶ In the sham group, intense OVA-647 fluorescence in dCLNs at 30 min post-striatal injection confirmed efficient tracer drainage (Fig. 3D, bottom panel). Vehicle-treated pMCAO mice exhibited significantly reduced dCLN fluorescence intensity compared with sham group (19.47 [95%CI, 8.961-29.98]; $p = 0.0009$), indicating impaired ISF efflux. While TK treatment reversed this deficit, increasing dCLN signal versus vehicle group (23.83 [95%CI, 13.32-34.35]; $p = 0.0002$, Figure 3C), indicating the suppression of ISF efflux in pMCAO brain edema tissue can be attenuated by TK.

TK Preserved Ependymal AQP4 Expression After pMCAO

To explore the effects of TK on AQP4 expression in the ependymal wall, immunohistochemical analysis was performed in sham, vehicle, and TK-treated mice. Representative images showing AQP4 immunohistochemical staining (brown) of coronal brain sections in the lateral ventricle (LV) ependymal wall revealed that AQP4 expression was decreased in the LV ependymal wall of ipsilateral pMCAO brain (Figure 4A and 4C). These images also revealed average optical density (AOD) values with inapparent abnormalities in ependymal architecture and decreased AQP4 expression in the ipsilateral ependymal wall happened as early as 70 min following pMCAO. TK treatment significantly increased AQP4 AOD versus vehicle (0.0176 [95%CI, 0.0273-0.008]; $p = 0.0016$, Figure 4B), indicating rescue of AQP4 expression.

Collectively, these results demonstrated that TK attenuated early ischemic edema by restoring glymphatic function after pMCAO. Within 70 minutes post-occlusion, pMCAO impaired ISF dynamics, evidenced by suppressed periventricular efflux, reduced parenchymal perfusion, and disabled deep cervical

lymph node drainage. TK treatment rescued ependymal AQP4 expression, redirected ISF to periventricular pathways, and enhanced dCLN efflux. This restoration of glymphatic clearance delayed edema-to-infarction progression, prolonging DWI/T2 mismatch time by nearly 50%.

DISCUSSION

This study assessed the effects of TK in mitigating the progression of edema by DWI/T2WI mismatch time in 11.7T MR. Part of the figures has been published as a conference abstract previously.¹⁷ Notably, the clinical outcome of cerebral infarction is highly contingent upon the presence of brain edema.²¹⁸ The DWI/T2WI mismatch area in this study may indicate the transformation of cytotoxic edema to vasogenic edema, as there exists a key time window between the onset of restricted diffusion (indicated by DWI) and the disruption of the blood brain barrier (BBB) (indicated by T2WI).¹⁹ Our previous studies has explored the outcome and safety of using DWI/T2WI mismatch to guide intravenous tissue plasminogen activator therapy for patients with acute ischemic stroke, in which we identified the varies of patients' potentially salvageable penumbra.^{19,20} DWI- fluid attenuated inversion recovery (FLAIR) mismatch has known as predictive of symptom onset within 4.5 h and the existence of salvageable tissue,²¹ the imaging time window or tissue window of recanalization application regarding infarction is essential. Therefore, it is clinically pivotal to extend the "therapeutic time window" (TTW) in patients with cerebral infarction.²² We postulate that TK treatment inhibits the progression of edema (indicated by prolonged DWI/T2WI mismatch time) may be the key to prolonging TTW in individual patients and subsequently enabling more targeted therapeutic interventions.

Kallikreins are secreted serine proteases widely expressed in various tissues and share an identical conserved domain.^{23,24} They play a vital role in

various physiological and pathophysiological processes, including cardiovascular, brain, tooth, respiratory, dermatological, and neoplastic diseases.²⁵ This study was carried out with TK serving as a replenishment therapy, initiating and maintaining vasodilation in the mammalian system, which may impact GS and contribute to fluid homeostasis, ultimately mitigating the degree and progression of edema. The infarction in the cortex of TK-treated group (17.5 × 10⁻³ NAU/kg) appeared to be smaller, potentially ascribed to the potential effect of TK on collateral circulation abundant in the cortex and rescue of peripheral ischemia.²⁶ In the nervous system, B2R is localized in the cerebral cortex, thalamus, hypothalamus, brain stem, basal nuclei, and the ependyma of the lateral and third ventricles.²⁷ Activated TK can synthesize Lys-bradykinin, an agonist of the kinin B2 receptor²⁸ that is conducive to a flow-dependent arterial dilation response by activating B2 receptors and releasing nitric oxide (NO).²⁹ Cerebral arterial pulsation is vital for the movement of glymphatic fluid, which is linked to cerebral fluid homeostasis and tissue edema.⁴ Herein, the proportion of water content in mice in the pMCAO and pMCAO+TK group was detected after 24 h of MCA occlusion to validate the protective role of TK in acute ischemic edema.

To investigate the relationship between the efficacy of TK for the treatment of edema and glymphatic kinetics, OVA-647 was intracranially injected into the striatum of each animal group. It is worthwhile emphasizing that our results exposed that the paracellular flow of fluorescent in the sham group was evenly distributed around the lateral ventricle, with a considerable outflow from dCLNs, highlighting the benign flow of glymphatic efflux under physiological conditions and the critical role of the lateral ventricle in brain fluid transport. As the drainage role of lateral ventricles to the contrast agent in cisterna magna, which was assessed in our previous study.⁶ Taken together, these data suggested the existing pathway content at the ependymal ventricular lining and governing CSF/ISF exchange and dynamics. Furthermore, this study demonstrated that CSF/ISF tracer OVA-647 area fraction (indicative of ISF perfusion) in the ipsilateral striatum and the fluorescence intensity in dCLNs (indicative of ISF efflux) were enhanced in the TK-treated mice than in the pMCAO group. These findings demonstrated that the perfusion of ISF in the brain parenchyma and its subsequent efflux, which is indicative of glymphatic kinetics, were instantly impaired following pMCAO and ameliorated by TK treatment. Improving glymphatic efflux provides an alternative approach to ischemic stroke to alleviate cerebral ischemic edema.

Herein, we found the paracellular flow of fluorescent in the sham WT group dispersed around the lateral ventricle, indicating the benign flow of glymphatic efflux in physiological conditions and the significant role of lateral ventricle in brain fluid transport. Compared to the sham WT group, the red fluorescence did not accumulate around the lateral ventricles in sham AQP4 (-/-) brain sections but distributed throughout the ipsilateral hemisphere. Accordingly, the effects of periventricular area on the perfusion of ISF may be associated with the AQP4 function in the ependymal wall.

Edema was associated with AQP4 depolarization in both the perivascular spaces of the parenchyma and the periventricular zone, which has been illustrated in the previous study as well.⁶ This specific distribution of AQP4 in the basolateral membrane of the ventricular ependyma suggests the dominant role in water transport.³⁰ To investigate whether TK efficacy in treating edema is related to dynamic AQP4 regulation, we performed the Immunohistochemical analysis of AQP4 expression in the ependymal wall. We found that AQP4 expression in the ipsilateral ependymal wall of pMCAO mice decreased compared to the sham group while increased in TK treatment, indicating that TK up-regulates the AQP4 expression in the ependymal wall and may enhance the restricted diffusion water by regulating AQP4 expression. Hydrocephalus is a central nervous system disorder characterized by defective CSF turnover in the brain ventricles,³¹ which may related to the ependyma denudation and decrease of AQP4 in the ventricular ependymal cells.

This study had certain limitations. The presented experiments are descriptive and lack a strong mechanistic description supported by data. The connection between aquaporin 4 and TK is proposed but not resolved with strong scientific evidence. Furthermore, to confirm that the protective effects of TK are AQP4-dependent, the authors should perform experiments in AQP4-/- mice subjected to pMCAO model and pMCAO + TK treatment (Figure 3E).

These findings highlighted a protective role of TK in ischemic edema, as evidenced by the reduced water content and prolonged DWI/T2 mismatch

time, which was likely mediated by rescuing ependymal AQP4 expression, redirecting ISF to periventricular perfusion pathways, and enhancing dCLN efflux. Improving glymphatic dynamics offers an alternative approach for addressing ischemic stroke and attenuating cerebral ischemic edema.

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J.Z., W.S., Y.T., C.S., and Y.X. performed the research and participated in data collection and revision of this manuscript. W.C. and Q.D. designed the research. H.Zhao and H.Zhang analyzed the data. J.Z. wrote this manuscript and plotted the figures. All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests for this work. Manuscript is approved by all authors for publication.

ETHICAL STATEMENT AND PATIENT CONSENT

All animal experiments were approved by the Fudan University Huashan Hospital institutional review board (no. 2022JS-HSY-279). No human participants included.

DATA AND CODE AVAILABILITY

Data used in this manuscript are available on reasonable request.