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Mechanism of Secondary Brain Injury after Intracerebral Hemorrhage Based on DNA Demethylase TET1 and the Therapeutic Role of Vitamin C

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Acknowledgments.

Authors' contributions

Author Contributions Statement

M.X., Q.T., and L.H. contributed equally to this work, including experimental design, data collection, and manuscript drafting. H.Y., H.F., and L.L. participated in the establishment of the animal model (intracerebral hemorrhage induction), performed neurobehavioral assessments (modified Neurological Severity Score, corner test), and conducted sample processing (brain tissue collection, RNA/protein extraction). J.Z. assisted in molecular experiments, including reverse transcription quantitative polymerase chain reaction (RT-qPCR) and Western blotting, and performed preliminary data analysis. Q.L. and J.C. provided guidance on experimental methodology, supervised data validation, and revised the manuscript for important intellectual content. W.Y. conceived and designed the overall study, obtained funding support, coordinated the research team, finalized the manuscript, and takes responsibility for the integrity of the work. All authors reviewed and approved the final manuscript.

Competing interests

The authors declare no competing interests

Ethics declaration

This study was conducted in strict compliance with the Regulations for the Administration of Experimental Animals in China (1988). All animal care procedures, experimental protocols, and study designs were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) and the Office of Laboratory Animal Resources of Zhengzhou University.

Clinical trial number

Not applicable.

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Data availability

All the data in this study are available from the corresponding author on reasonable request.

Abstract

Background: Secondary brain injury (SBI) is the primary driver of progressive neurological decline following intracerebral hemorrhage (ICH), yet clinically effective interventions remain severely limited. Dynamic DNA methylation remodeling, orchestrated by the ten-eleven translocation (TET) family of dioxygenases, is increasingly recognized as vital for neurological recovery. Vitamin C (VitC), a highly translatable neuroprotective antioxidant and obligate TET cofactor, mitigates ischemic brain injury. However, its therapeutic potential in ICH and the specific role of the VitC-TET1 epigenetic axis in post-ICH SBI remain uncharacterized.

Methods: Utilizing a collagenase-induced murine ICH model, we evaluated the temporal expression dynamics of TET1 (1-3 days post-ICH) and its correlation with neurological deficits and cerebral edema. We employed lentivirus-mediated TET1 overexpression and systemic VitC administration to investigate the regulation of the Bcl-2/Bax/Caspase-3 apoptotic pathway and 5-hydroxymethylcytosine (5hmC) modifications. Crucially, TET1-knockout mice were utilized to definitively confirm the TET1-dependency of VitC's neuroprotective efficacy.

Results: Neuron-specific TET1 expression and global 5hmC levels were significantly downregulated during the acute phase of ICH, robustly correlating with the severity of neurological impairment. Targeted TET1 overexpression attenuated neuronal apoptosis and significantly alleviated neurodeficits and edema. Strikingly, VitC intervention (optimally at 500 mg/kg) restored TET1 enzymatic activity, enhanced 5hmC modifications, and exerted robust anti-apoptotic neuroprotection. Importantly, these protective effects were completely abrogated in TET1-knockdown mice, confirming a highly specific TET1-dependent mechanism.

Conclusion: The TET1-neuronal apoptosis axis is a critical epigenetic determinant of ICH pathophysiology. Enhancing TET1 activity via VitC represents a highly promising, epigenetically targeted translational strategy to combat post-ICH SBI.

Keywords: TET1; DNA demethylation; intracerebral hemorrhage; neuronal apoptosis; Vitamin C;

1. Introduction

Intracerebral hemorrhage (ICH) is a devastating subtype of stroke characterized by disproportionately high mortality and severe long-term disability, with no effective pharmacological treatments currently available^[1-3]. The pathological sequelae of ICH extend far beyond the primary mechanical injury caused by hematoma expansion^[4-6]. It encompasses secondary brain injury (SBI)—a progressive cascade of neuroinflammation, oxidative stress, and programmed neuronal cell death driven by the breakdown products of extravasated blood^[7-10]. Given the prolonged and destructive nature of SBI, elucidating its molecular underpinnings is a critical priority for identifying actionable therapeutic targets to halt neurological deterioration.

Recent evidence suggests that epigenetic dysregulation is a major contributor to the vulnerability of the brain following acute vascular injury^[11, 12]. Among these mechanisms, dynamic DNA methylation remodeling, orchestrated by the ten-eleven translocation (TET) family of dioxygenases, is increasingly recognized as central to neurological homeostasis^[13, 14]. TET proteins catalyze the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), initiating active DNA demethylation and enabling context-dependent gene transcription^[15-17]. While previous studies in ischemic stroke have implicated TET2 and TET3 in modulating neuroinflammation and angiogenesis^[18, 19], the specific epigenetic landscape in hemorrhagic stroke remains poorly defined. Of the three TET paralogs, TET1 exhibits the highest expression in neurons and plays an indispensable role in maintaining synaptic plasticity and neuronal survival^[20-22]. Importantly, TET1 deficiency has been linked to the suppression of neuroprotective genes and the exacerbation of neuronal apoptosis^[23, 24]. However, whether TET1 specifically dictates the progression of SBI following ICH—and whether its activity can be therapeutically targeted—remains unresolved.

Vitamin C (ascorbate; VitC) represents a highly promising candidate to bridge this translational gap. Beyond its well-characterized ability to scavenge reactive oxygen species and suppress classical inflammatory pathways^[25-27], VitC functions as an

obligate enzymatic cofactor for TET proteins, directly maximizing 5hmC generation and epigenetic reprogramming^[28, 29]. In recent models of ischemic stroke, high-dose VitC has been shown to attenuate secondary brain damage specifically via TET1 activation^[30]. Despite its excellent safety profile, blood-brain barrier permeability, and immense potential for clinical repurposing, the therapeutic efficacy of the VitC-TET1 epigenetic axis in the context of ICH has not been investigated.

In the present study, using a collagenase-induced murine model of ICH, we hypothesized that TET1-mediated epigenetic remodeling is a critical determinant of neuronal survival post-ICH, and that VitC exerts robust neuroprotection by directly augmenting TET1 activity. By investigating the TET1-5hmC axis and its downstream regulation of the Bcl-2/Bax/Caspase-3 apoptotic pathway, our findings aim to provide a solid mechanistic rationale for ascorbate-based, epigenetically targeted neuroprotective strategies in hemorrhagic stroke.

2. Materials and Methods

2.1 Experimental Animals and Ethics Statement

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Zhengzhou University. The study was conducted and reported in compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Adult male C57BL/6J mice (8–10 weeks old, 22–25 g) were obtained from the SPF (Beijing) Biotechnology Co, Ltd. Only male mice were utilized to exclude the potential neuroprotective confounding effects of endogenous estrogen. Mice were housed in a specific pathogen-free (SPF) facility ($22 \pm 1^{\circ}\text{C}$, $50 \pm 5\%$ humidity, 12-h light/dark cycle) with ad libitum access to standard chow and water.

2.2 ICH Model and VitC Dosing

Following a one-week acclimation period, mice were anesthetized with an isoflurane/oxygen mixture and mounted on a stereotaxic frame. A burr hole was drilled 0.5 mm anterior and 2.0 mm lateral to the bregma. Using a microinjector, we infused 200 nL of 0.03 U type IV collagenase (Sigma) dissolved in saline into the right striatum

(depth: 4.0 mm) at a rate of 100 nL/min. The needle was left in place for 10 minutes post-injection to prevent backflow^[31]. Sham controls received an equal volume of sterile saline. Postoperative mice were maintained on heating pads until full recovery. To determine the optimal therapeutic dose, vitamin C (VitC) was administered intraperitoneally based on the protocol described by Kondo et al.^[32]. Mice were assigned to three dosage groups (n = 3 per group): L-VitC (200 mg/kg), M-VitC (500 mg/kg), and H-VitC (1,000 mg/kg), all dissolved in saline. We then evaluated which dose most effectively increased TET1 expression in the perihematomal tissues. After identifying the optimal dose, we applied this specific VitC treatment in subsequent experiments using mice subjected to TET1 knockdown prior to ICH induction.

2.3 Neurobehavioral Assessments

To comprehensively evaluate post-injury neurological function, we performed the modified Neurological Severity Score (mNSS) and the corner test at 24-hour intervals^[33, 34]. The mNSS assesses motor, sensory, reflex, and balance deficits on a scale of 0 to 18, where a higher score reflects more severe impairment. For the corner test, each mouse was placed in a 30°-angled apparatus for 10 trials to determine lateralized sensorimotor dysfunction. The percentage of left turns (left turns/10 × 100%) was recorded; a reduced frequency of left turns indicates a more severe right striatal injury.

2.4 Brain Water Content

Brains (excluding brainstem)^[35] were weighed wet, dried at 80°C for 72 h, and reweighed. Edema was calculated as: $[(\text{wet weight} - \text{dry weight}) / \text{wet weight}] \times 100\%$.

2.5 Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted using TRIzol, reverse-transcribed (PrimeScript RT Kit, Takara), and amplified with SYBR Green (TB Green Premix, Takara) on a QuantStudio Flex. Primers:

Gene	Forward (5'→3')	Reverse (5'→3')
TET1	GACCCTCATAAGCAGAGAGGAAAA	GACACCAGAGAAAGGACGCC
TET2	CTGAGCCAAAAGAGGAAGAGAG	TTCCTTCAGACCCAAACAGCA
TET3	CAGTCCGAGTTCCTACCTG	TTCCCCTTTTCTCCATACCGAT
GAPDH	TCGGTGTGAAGGGATTTGGC	CCTTCAGGTGAGCCCCAGC

Data were analyzed via the $2^{-\Delta\Delta C_t}$ method with GAPDH normalization.

2.6 Western Blotting

To prepare cytoplasmic and nuclear fractions, we utilized a commercial extraction kit (Beyotime, China). Equal amounts of protein samples (10–20 μ g) were resolved on 12% SDS-PAGE gels and transferred onto PVDF membranes. After blocking with 5% non-fat milk, the membranes were incubated overnight at 4°C with primary antibodies against TET1, TET2, TET3 (1:1000, Abcam), Bcl-2, Bax, cleaved-Caspase3 (1:800, Cell Signaling Technology), or GAPDH (1:5000, Proteintech). Band intensities were subsequently visualized and quantified using ImageJ software.

2.7 Immunofluorescence (IF)

Following transcardial perfusion with 4% paraformaldehyde, the harvested brains were embedded in paraffin and cut into 4- μ m sections. Slices were subjected to antigen retrieval and blocked with 5% BSA. For double immunolabeling, the sections were co-incubated overnight at 4°C with primary antibodies against TET1 (1:200) and specific cellular markers (NeuN for neurons, GFAP for astrocytes, or IBA1 for microglia). The following day, sections were washed and incubated with Alexa Fluor-conjugated secondary antibodies (1:500). Nuclei were counterstained with DAPI, and images were captured using a Zeiss LSM 900 confocal microscope.

2.8 Dot Blot Assay

Extracted genomic DNA was denatured and spotted onto nitrocellulose membranes at

a concentration of 35 ng/μL. After UV cross-linking, the membranes were blocked with 5% BSA and incubated with an anti-5hmC primary antibody (1:1000, Active Motif). Signal intensities were then quantified by densitometry using ImageJ.

2.9 TET Hydroxylase Activity Assay

Nuclear protein fractions were extracted from perihematomal tissues, and protein concentrations were determined using a BCA protein assay kit (BioTek Instruments, Winooski, VT, USA). TET hydroxylase activity was then quantified using a specific colorimetric kit (ab156912, Abcam) according to the manufacturer's instructions. The absorbance, corresponding to the hydroxymethylation of 5mC substrates, was measured at 450 nm using a microplate reader (specify brand/model, e.g., Bio-Rad Laboratories). Final TET activity was normalized to the total nuclear protein concentration.

2.10 Statistical Analysis

All statistical analyses were conducted using SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Continuous variables are presented as the mean ± standard deviation (SD). The normality of data distribution was evaluated using the Shapiro-Wilk test, and homogeneity of variance was assessed via Levene's test. For normally distributed data with equal variances, multiple comparisons among groups were performed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. If the data failed to meet the assumptions of normality or homoscedasticity, the non-parametric Kruskal-Wallis test was applied, followed by Dunn's post hoc test. A two-sided *P*-value < 0.05 was considered statistically significant.

3. Results

3.1 Temporal Profile of Hematoma Resolution, Edema, and Neurological Deficits Following ICH

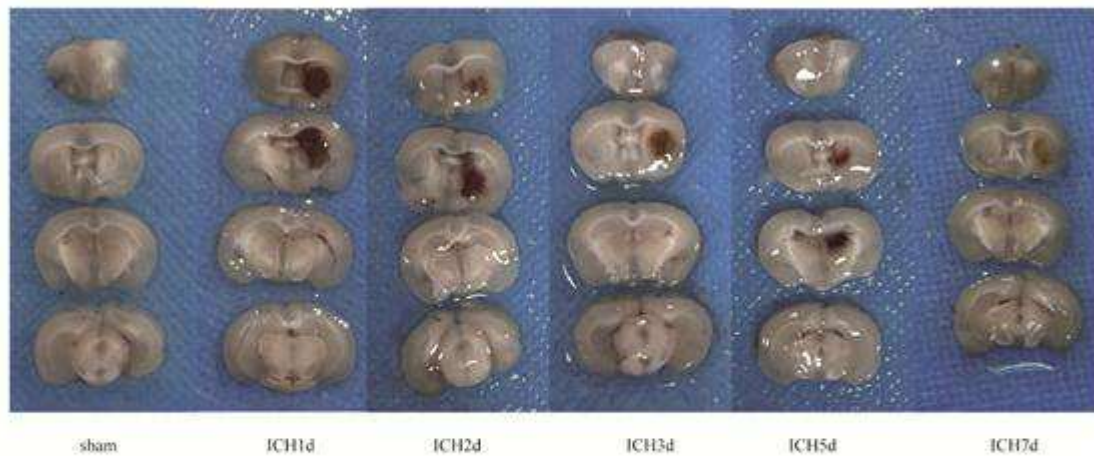
To establish the baseline progression of SBI, we subjected mice to a stereotactic

injection of type IV collagenase into the right striatum. Macroscopic analysis confirmed prominent hematoma formation at 1 day post-ICH. The hematoma subsequently underwent progressive resolution and was nearly completely absorbed by day 7 (Fig. 1A).

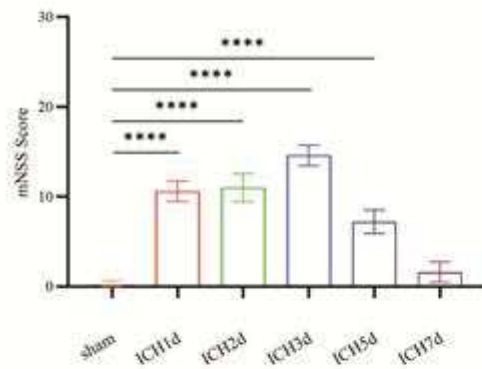
Behavioral assessments tightly correlated with these pathological changes. Compared to sham-operated controls, ICH mice exhibited severe neurological impairment, reflected by significantly elevated mNSS during the acute phase (days 1 through 5 post-injury). Spontaneous functional recovery was observed thereafter, with scores approaching baseline levels by day 7 (Fig. 1B). Similarly, the corner turn test revealed a pronounced sensorimotor asymmetry, indicated by a drastically reduced percentage of left turns from days 1 to 5, which also normalized by day 7 (Fig. 1C).

Consistent with the observed neurobehavioral deficits, brain water content in the ipsilateral hemisphere—a direct indicator of cerebral edema—peaked acutely and remained significantly elevated through day 5 ($P < 0.05$ vs. Sham). By day 7, the edema had largely subsided (Fig. 1D), temporally aligning with the resolution of both the hematoma and the behavioral dysfunction.

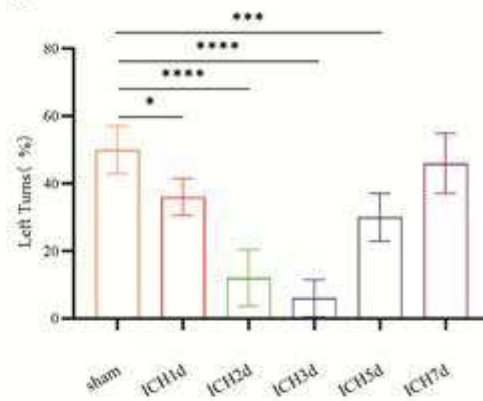
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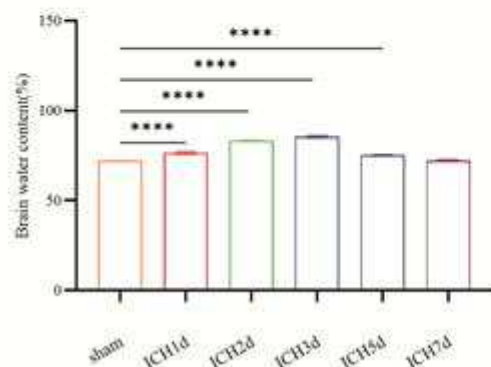


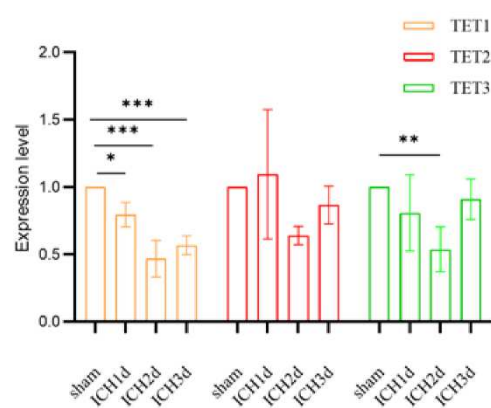
Figure 1. Temporal profile of hematoma resolution, neurological deficits, and cerebral edema following ICH. (A) Representative macroscopic images of hematoma formation and its progressive absorption in the striatum over 7 days post-injury. (B, C) Neurobehavioral assessments evaluating sensorimotor functional recovery using the mNSS (B) and the corner turn test (C) (n = 8 per group). (D) Quantification of brain water content in the ipsilateral hemisphere as an indicator of cerebral edema (n = 5-6

per group). Data are expressed as mean $\pm$ SD. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. $*p < 0.05$, $****p < 0.0001$ vs. the Sham group.

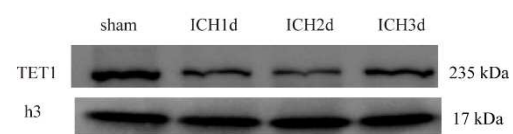
3.2 Temporal Expression Profile of TET Paralogues Following ICH

To investigate the epigenetic landscape during the acute phase of ICH, we first quantified the mRNA expression levels of the TET family in perihematomal tissues. RT-qPCR analysis demonstrated a robust and rapid suppression of Tet1 and Tet3 transcripts at 1 and 2 days post-injury (Fig. 2A). Notably, while Tet3 expression returned to baseline by day 3, Tet1 levels remained significantly lower than those in sham controls, despite a partial recovery. In contrast, Tet2 mRNA abundance exhibited no significant fluctuations throughout the 3-day observation period (Fig. 2A). Consistent with the transcriptional data, Western blot analysis confirmed a significant reduction in TET1 protein levels within the striatum during the initial 48 hours post-ICH (Fig. 2B, D). To pinpoint the cellular source of this decline, we performed double-immunofluorescence labeling. Our results revealed that TET1 was exclusively localized in NeuN-positive neurons (Fig. 2F). No co-localization was observed with GFAP-positive astrocytes or IBA-1-positive microglia, indicating that the ICH-induced loss of TET1 is a neuron-specific event that temporally coincides with the onset of neurological deficits.

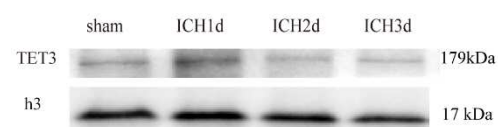
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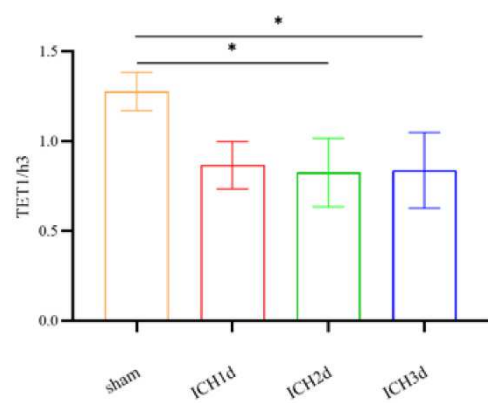
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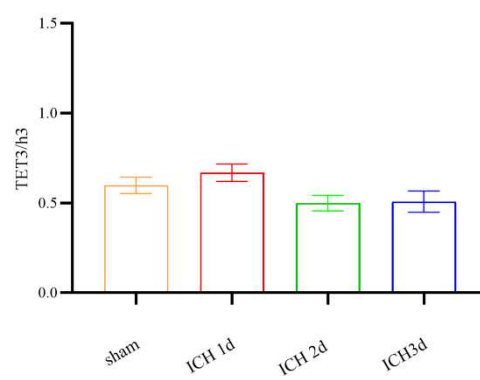
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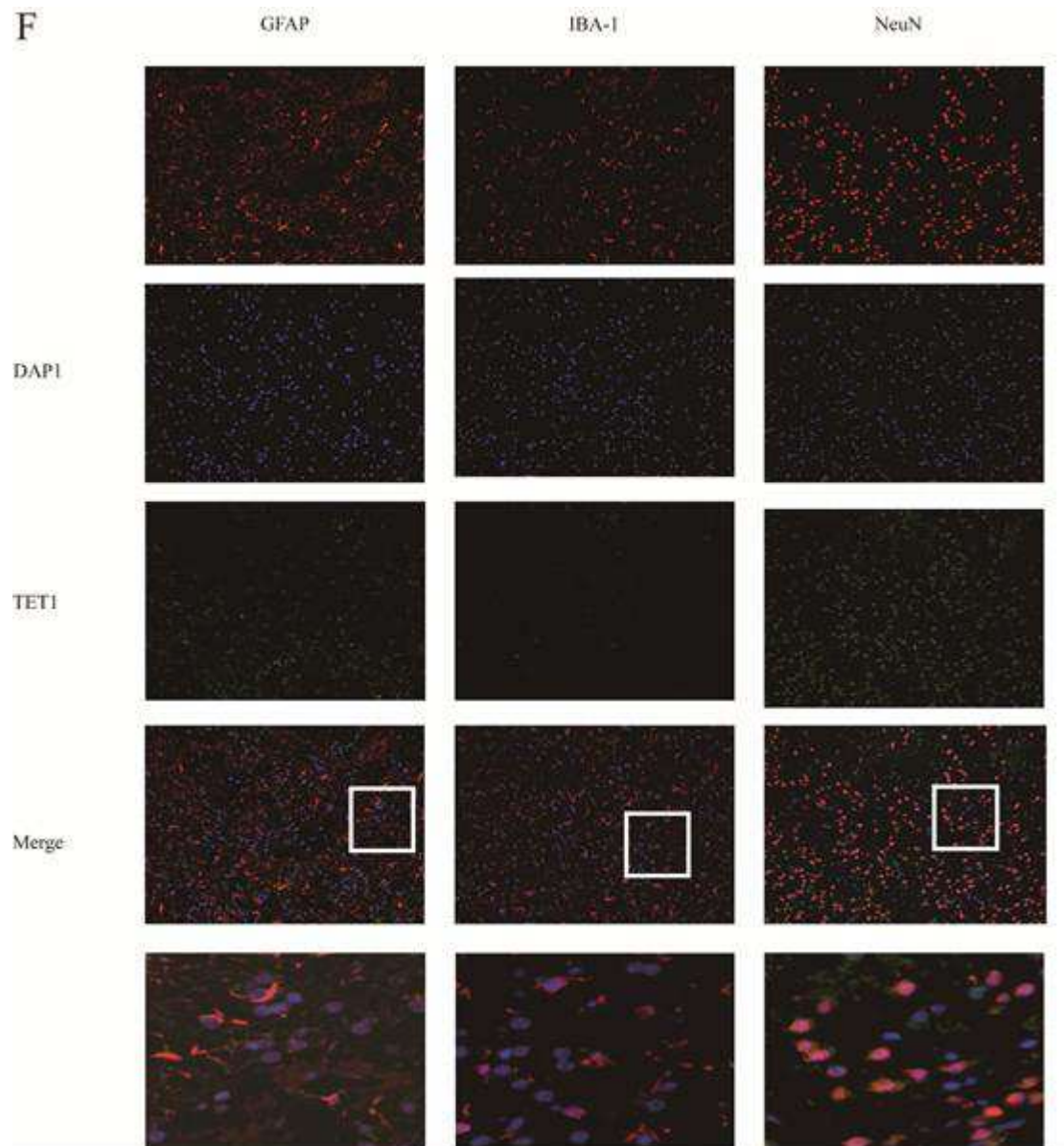


Figure.2 Temporal dynamics of TET paralog expression and neuronal localization of TET1 following ICH. (A) mRNA expression profiles of *Tet1*, *Tet2*, and *Tet3* in the perihematomal striatum from 1 to 3 days post-ICH, quantified by RT-qPCR (n = 3 per group). (B, C) Representative Western blots showing the protein levels of TET1 (B) and TET3 (C) during the acute phase (n = 3 per group). (D, E) Densitometric quantification of TET1 (D) and TET3 (E) protein levels relative to GAPDH (n = 3 per group). (F) Double immunofluorescence labeling showing the neuronal localization of TET1 in the striatal perihematomal region. Scale bar = 100 μ m. (n = 3). Data are presented as mean $\pm$ SD. Statistical significance was determined by one-way ANOVA followed by *Tukey's post hoc* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the Sham

group.

3.3 Overexpression of TET1 ameliorates SBI after ICH

We found that TET1 expression dropped sharply during the acute phase of ICH and was mainly detected in neurons (Fig. 2). This led us to ask whether TET1 might protect against ICH-induced neurological impairment. To test this, we overexpressed TET1 in the striatum using a lentiviral vector before inducing ICH.

Compared to ICH controls, mice with TET1 overexpression showed better neurological function, with lower mNSS scores on days 1, 2 and 3 post-injury (Fig. 3A). The corner turn test gave similar results: TET1-overexpressing mice turned left more often than ICH controls (Fig. 3B). In line with these behavioral gains, TET1 overexpression also reduced cerebral edema, as measured by lower brain water content (Fig. 3C). Of note, the empty vector group did not show any of these improvements, confirming that the observed effects were specific to TET1.

Taken together, these data indicate that TET1 overexpression can alleviate ICH-induced neurological dysfunction and brain edema, suggesting TET1 as a potential therapeutic target for secondary brain injury after ICH.

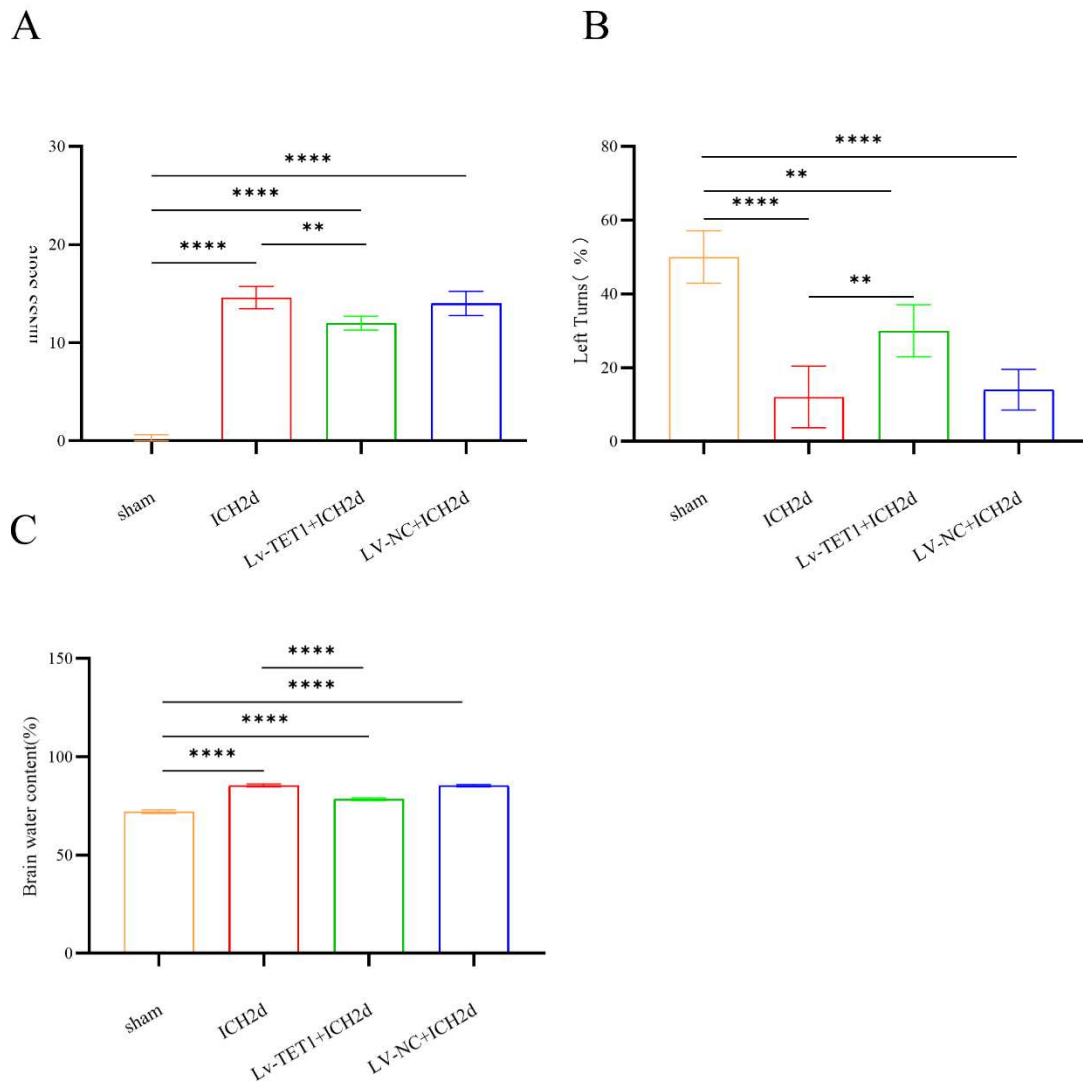


Figure 3. Neuronal TET1 overexpression mitigates neurological impairment and cerebral edema following ICH. (A, B) Time-course of neurological deficits assessed by the mNSS (A) and sensorimotor asymmetry evaluated via the corner turn test (B) (n = 8 per group). (C) Quantification of cerebral edema through brain water content analysis on day 3 post-injury (n = 3 per group). Data are expressed as mean ± SD. Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test. **p < 0.01, ****p < 0.0001 vs. the Empty Vector group.

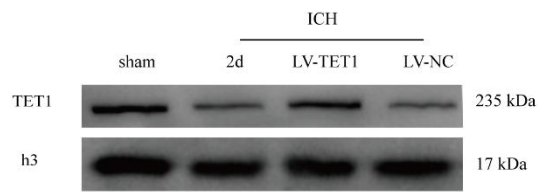
3.4 TET1 Augmentation Suppresses Neuronal Apoptosis via the Intrinsic Pathway

To explore whether TET1-mediated neuroprotection is linked to the inhibition of

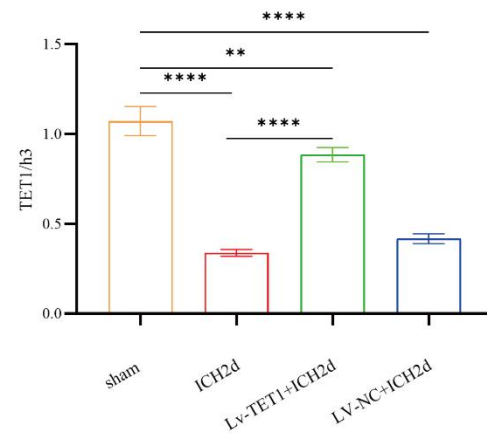
programmed cell death, we examined the expression of core apoptotic regulatory proteins in the perihematomal region. Western blot analysis demonstrated that the acute downregulation of TET1 following ICH was accompanied by a pro-apoptotic shift in the Bcl-2 family balance. Notably, lentivirus-mediated TET1 overexpression significantly antagonized this shift, characterized by a robust induction of the anti-apoptotic protein Bcl-2 and a concomitant suppression of Bax and cleaved-Caspase3 levels (Fig. 4A-F). These molecular changes suggest that TET1 exerts its protective effects by stabilizing the mitochondrial apoptotic gateway.

The anti-apoptotic efficacy of TET1 was further validated by in situ histological analysis. NeuN and TUNEL double-labeling revealed a dense population of apoptotic neurons in the vehicle-treated ICH group. However, consistent with the observed biochemical changes, mice receiving LV-TET1 exhibited a substantial reduction in the frequency of TUNEL-positive neurons within the striatal perihematomal zone (Fig. 4G-H). Collectively, these data indicate that restoring TET1 activity effectively preserves neuronal survival by attenuating the intrinsic apoptotic cascade initiated after ICH.

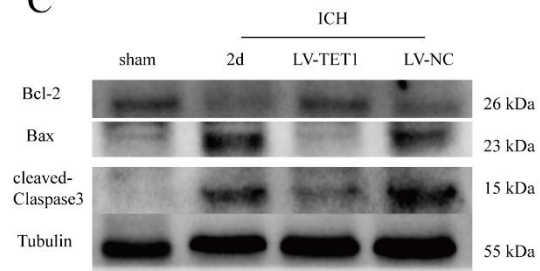
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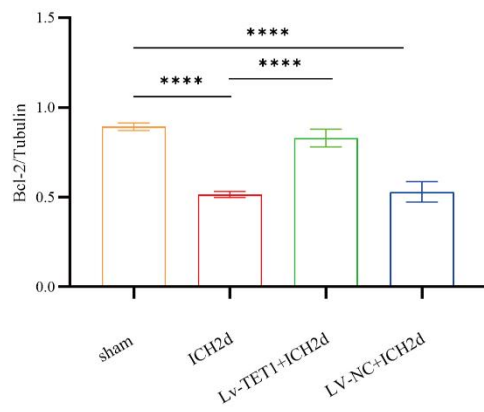
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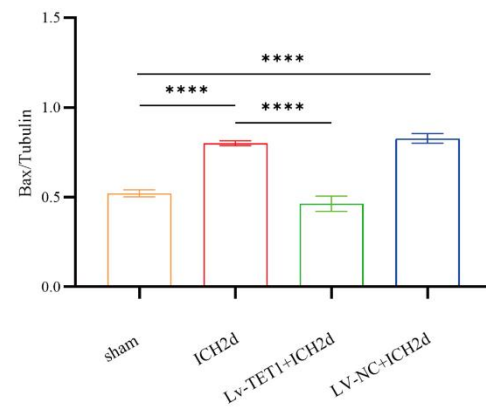
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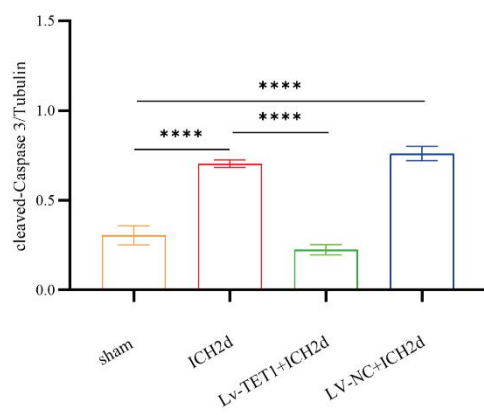
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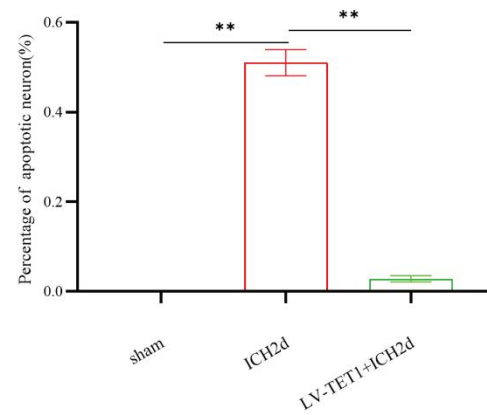
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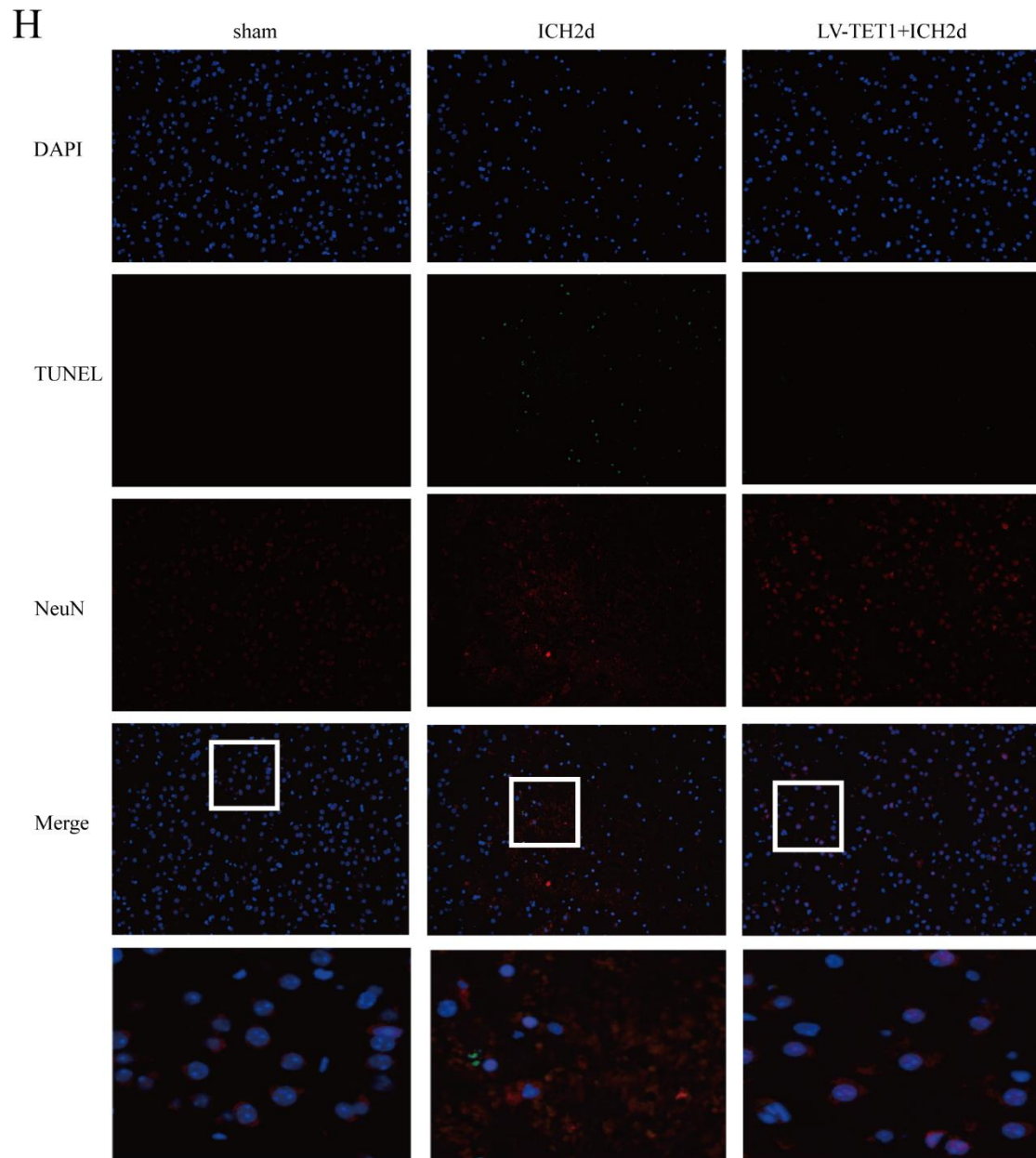


Figure 4. TET1 overexpression inhibits the intrinsic apoptotic pathway and reduces neuronal loss following ICH. (A) Representative Western blots of TET1 and apoptosis-related markers (Bcl-2, Bax, and cleaved-Caspase3) in perihematomal tissues. (B) Densitometric quantification of nuclear TET1 normalized to Histone H3 (n = 3 per group; *Note: N=5 is highly recommended for TSR*). (C-F) Quantitative analysis of Bcl-2, Bax, and cleaved-Caspase3 protein levels normalized to β -tubulin (n = 3 per group). (G) Quantification of apoptotic neurons expressed as the percentage of TUNEL+/NeuN+ cells relative to total NeuN+ cells (n = 3 per group). (H)

Representative micrographs showing co-localization of TUNEL (red) and NeuN (green) in the perihematomal region. Scale bar = 50 μ m. Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. ** $p < 0.01$, **** $p < 0.0001$ versus the ICH + Empty Vector group.

3.5 Vitamin C Confers TET1-Dependent Neuroprotection Following ICH

To establish the optimal therapeutic dose of VitC, we evaluated its impact on TET1 protein expression in the perihematomal region. Western blot analysis demonstrated a dose-dependent upregulation of TET1 across all tested concentrations (200, 500, and 1000 mg/kg) relative to vehicle-treated ICH mice. Notably, the 500 mg/kg dose proved most efficacious, effectively restoring TET1 to near-sham levels (Fig. 5A, B). Consequently, this optimized dose was utilized for all subsequent *in vivo* interventions. Systemic administration of VitC (500 mg/kg) yielded robust neuroprotection against ICH-induced injury. Compared to untreated ICH controls, VitC-treated mice displayed significantly accelerated recovery of sensorimotor function, reflected by lower mNSS scores (Fig. 5C) and a normalization of lateralized turning behavior in the corner test (Fig. 5D). Concurrently, VitC intervention effectively mitigated ICH-induced cerebral edema, markedly reducing brain water content in the ipsilateral hemisphere (Fig. 5E). These results collectively indicate that: 500 mg/kg VitC optimally restores TET1 expression after ICH; VitC enhances both TET1 expression and its catalytic activity; and the neuroprotective effects of VitC are TET1-dependent.

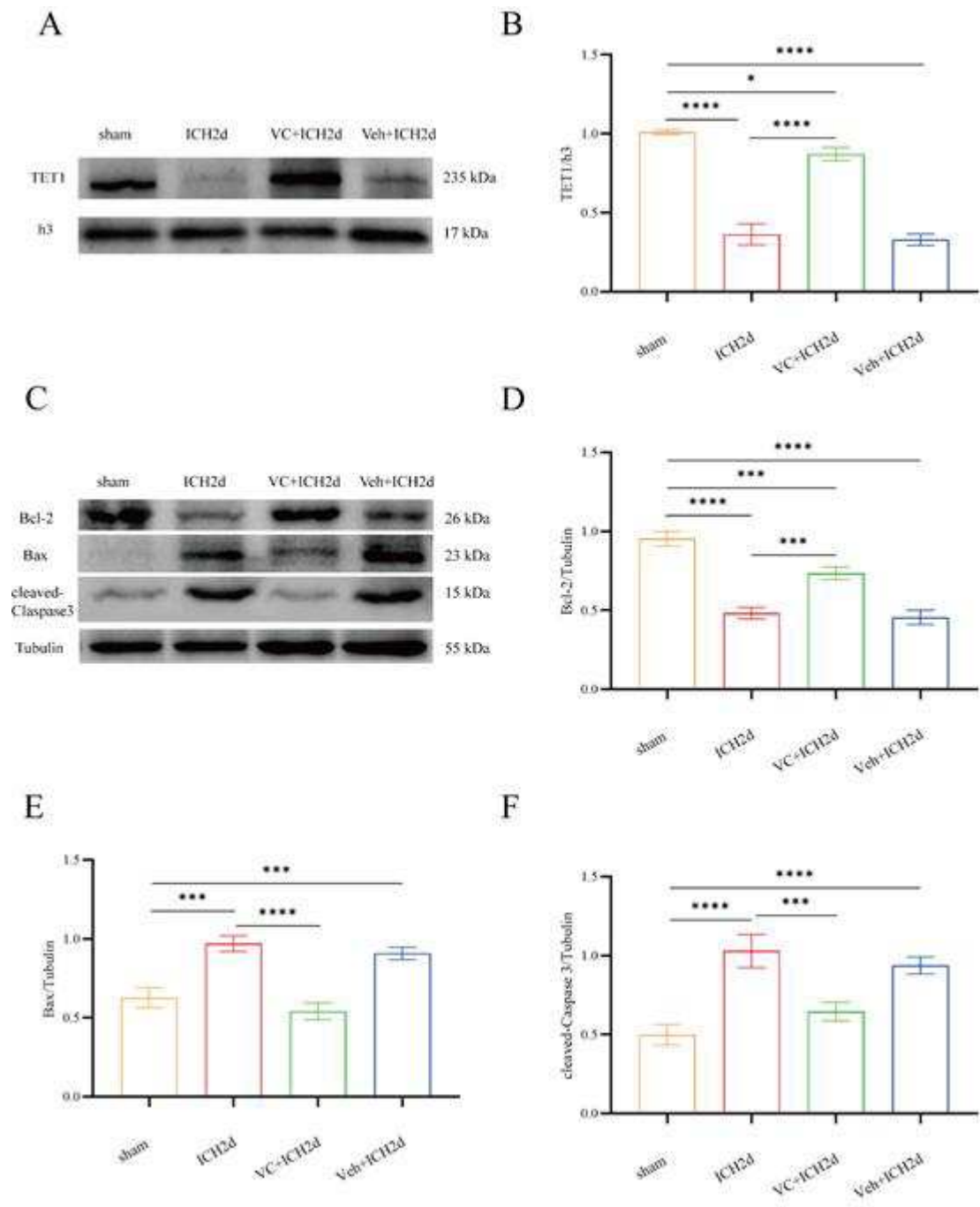


Figure 5. Optimization of Vitamin C dosage and its TET1-dependent neuroprotective efficacy. (A, B) Representative Western blots (A) and densitometric quantification (B) of nuclear TET1 expression in perihematomal tissues across various VitC doses (n = 3 per group). TET1 levels were normalized to Histone H3. (C, D) Functional recovery assessed via the mNSS (C) and sensorimotor asymmetry evaluated by the corner turn test (D) (n = 8 per group). (E) Measurement of brain water content in the ipsilateral hemisphere on day 3 post-ICH (n = 5 per group). Data are expressed as mean $\pm$ SD. Statistical comparisons were performed via one-way ANOVA followed

by Tukey's *post-hoc* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus the ICH + Vehicle group.

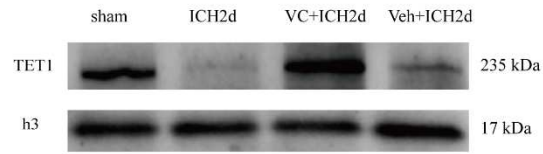
3.6 Vitamin C Attenuates Neuronal Apoptosis via a TET1-Dependent Epigenetic Pathway

Having established that VitC upregulates TET1 expression (Fig. 5), we next investigated its specific anti-apoptotic efficacy and the underlying epigenetic mechanisms. Western blot and dot blot analyses confirmed that systemic VitC administration not only elevated TET1 protein levels but also significantly enhanced its enzymatic activity, evidenced by a global increase in 5-hydroxymethylcytosine (5hmC) content in the perihematomal region compared to vehicle-treated ICH controls (Fig. 6A-B, G-I).

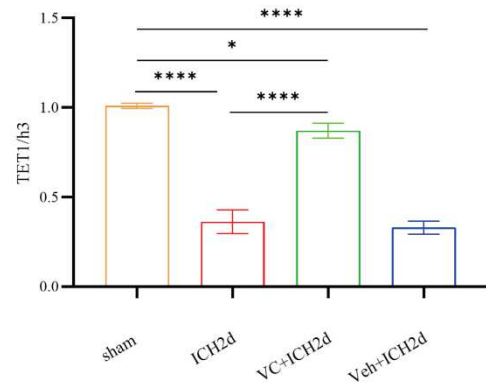
This epigenetic remodeling was accompanied by a pronounced shift in the intrinsic apoptotic balance. Specifically, VitC treatment markedly induced the expression of the anti-apoptotic protein Bcl-2 while suppressing the accumulation of pro-apoptotic Bax and cleaved-Caspase3 (Fig. 6C-F). Consistently, in situ histological evaluation via NeuN/TUNEL double-labeling demonstrated a substantial reduction in the frequency of apoptotic neurons following VitC intervention (Fig. 6J-K).

Crucially, the anti-apoptotic and epigenetic benefits of VitC were completely abolished in mice subjected to TET1 knockdown prior to ICH (sh-TET1+VitC group). In these TET1-deficient animals, both the Bcl-2/Bax balance and the neuronal apoptosis rates reverted to levels indistinguishable from the untreated ICH baseline (Fig. 6C-F, K). These reversal data confirm that VitC mitigates ICH-induced neuronal apoptosis specifically by augmenting TET1-dependent epigenetic regulation.

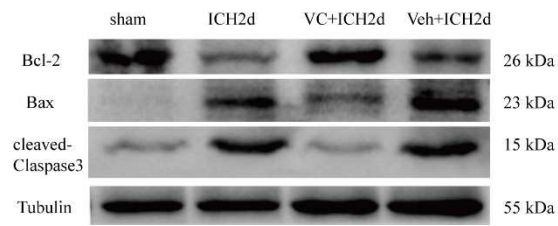
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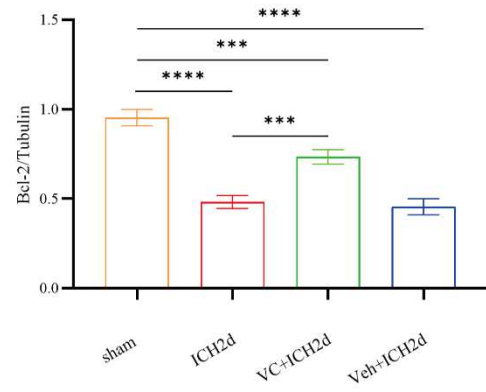
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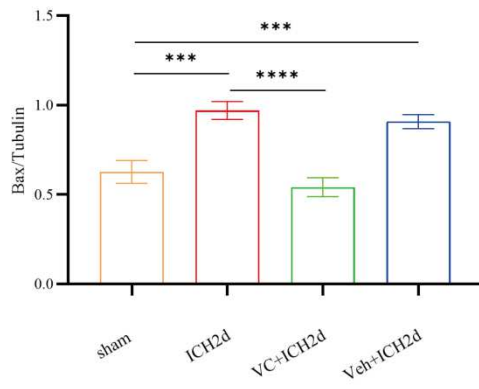
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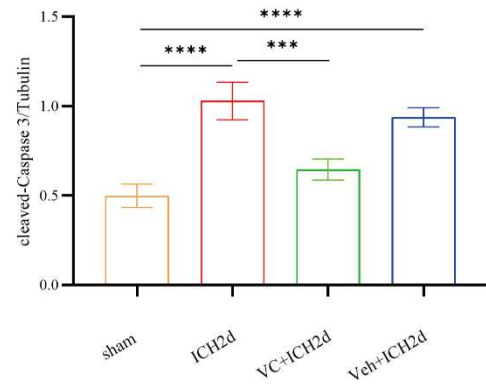
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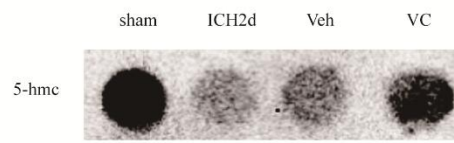
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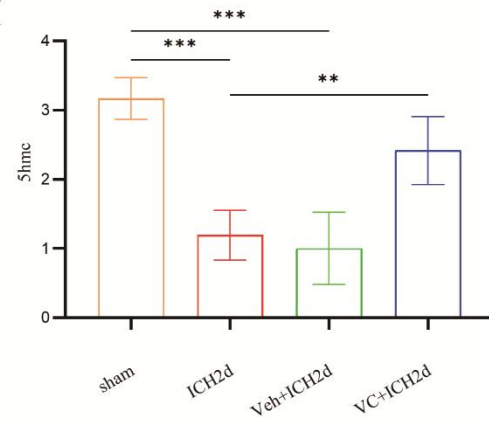
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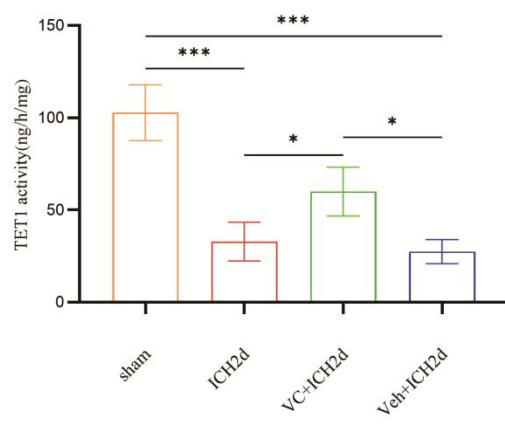
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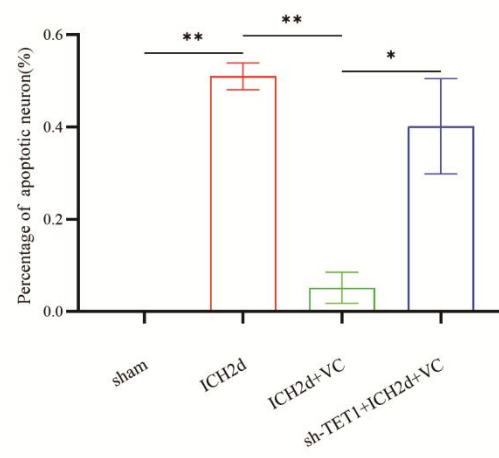
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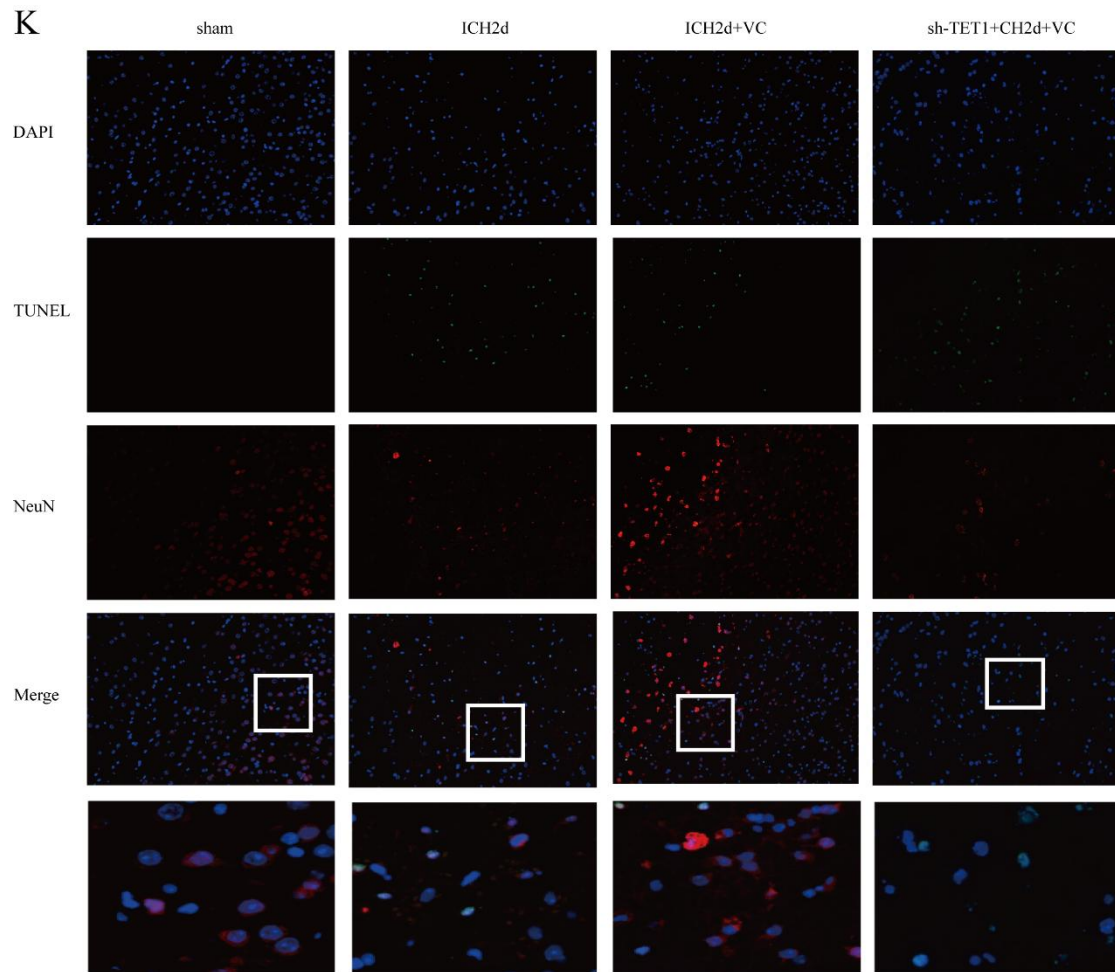


Figure 6. Vitamin C attenuates ICH-induced neuronal apoptosis via a TET1-dependent epigenetic mechanism. (A-F) Representative Western blots (A, C) and densitometric quantification (B, D-F) of nuclear TET1 (normalized to Histone H3) and cytoplasmic apoptosis-related proteins (Bcl-2, Bax, and cleaved Caspase-3, normalized to β -tubulin) in the perihematomal region ($n = 3$ per group). (G, H) Representative dot blot (G) and corresponding quantification (H) of global 5hmC levels ($n = 3$ per group). (I) Measurement of TET1 enzymatic activity in tissue extracts ($n = 5$ per group). (J) Quantification of neuronal apoptosis, expressed as the ratio of TUNEL-positive to NeuN-positive cells ($n = 3$ per group). (K) Representative TUNEL (red) and NeuN (green) co-immunofluorescence images of the perihematomal zone. Data are presented as mean $\pm$ SD. Statistical differences were evaluated using one-way ANOVA followed by Tukey's *post-hoc* test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ versus the ICH + Vehicle group; $*p < 0.05$, $**p < 0.01$ versus the VitC treatment alone group.

4. Discussion

In this study, we demonstrate that TET1, a highly neuron-enriched DNA demethylase, functions as a critical epigenetic safeguard against SBI following ICH. Our data reveal a rapid, injury-induced depletion of TET1 that temporally coincides with the onset of neurological deficits and profound neuronal apoptosis. Conversely, restoration of TET1 activity—either via genetic overexpression or systemic administration of high-dose VitC—robustly curbed the intrinsic apoptotic cascade and mitigated cerebral edema. Crucially, the neuroprotective efficacy of VitC was completely abrogated in TET1-deficient mice, cementing the indispensable role of the TET1-5hmC epigenetic axis in its therapeutic mechanism (**Figure 7**).

The pathological sequelae of ICH are driven by a complex interplay of oxidative stress, neuroinflammation, and programmed cell death^[36-38]. While aberrant DNA methylation remodeling is increasingly recognized in cerebrovascular diseases^[19, 39], the specific contributions of TET paralogues remain context-dependent. Interestingly, previous investigations in ischemic stroke models have predominantly highlighted TET3 upregulation as a compensatory neuroprotective mechanism^[40, 41]. In stark contrast, our ICH model unmasked a profound downregulation of TET1, whereas TET3 levels normalized rapidly and TET2 remained undisturbed. This discrepancy suggests that hemorrhagic and ischemic injuries trigger distinct epigenetic biosignatures, positioning TET1 as a unique and highly specific vulnerability in the ICH-afflicted brain.

Mechanistically, we link this ICH-induced TET1 loss directly to the destabilization of neuronal survival pathways. TET1 catalyzes the continuous oxidation of 5mC to 5hmC, a process known to maintain the transcriptional activation of neuroprotective and anti-apoptotic genes^[23, 24, 42]. Consistent with this, our biochemical analyses confirmed that TET1 depletion parallels the downregulation of Bcl-2 and the accumulation of pro-apoptotic Bax and cleaved-Caspase3. Restoring TET1, either genetically or pharmacologically, effectively reversed this pro-apoptotic shift. Thus, TET1 appears to exert its tissue-protective effects through a conserved epigenetic mechanism that fortifies the mitochondrial apoptotic gateway.

From a translational perspective, identifying a clinically viable strategy to rescue this epigenetic decline is imperative. VitC, beyond its classical role as a radical scavenger,

operates as an obligate enzymatic cofactor for TET proteins by maintaining the active Fe^{2+} state within their catalytic domain^[43-45]. Notably, we observed that the optimal neuroprotective dose (500 mg/kg) required to restore TET1 expression and global 5hmC levels is substantially higher than the doses typically utilized for basic antioxidant support^[46]. This high-dose requirement underscores that the robust neuroprotection observed here is not merely a byproduct of ROS neutralization, but rather hinges on the active reprogramming of the neuronal epigenome. Given its excellent safety profile, favorable blood-brain barrier penetrance, and current availability in critical care settings, high-dose intravenous VitC represents a highly translatable candidate for immediate repurposing in acute ICH management.

Despite these promising findings, several limitations warrant consideration. First, our study utilized exclusively young adult male mice; considering that ICH incidence and neurorepair capacity are profoundly influenced by biological sex and advancing age, future studies must validate this VitC-TET1 axis in female and aged ICH models. Second, our observation window was restricted to the acute phase (7 days post-injury). Longitudinal studies are required to determine whether TET1-mediated epigenetic remodeling confers enduring neurobehavioral recovery and long-term structural plasticity. Finally, the precise downstream gene loci uniquely demethylated by TET1 in the hemorrhagic striatum remain to be mapped via whole-genome bisulfite sequencing.

5. Conclusion

This study identifies the rapid loss of neuronal TET1 as a critical epigenetic driver of secondary brain injury following ICH. Systemic administration of high-dose Vitamin C rescues this epigenetic deficit, driving 5hmC-mediated suppression of neuronal apoptosis and facilitating robust functional recovery. The identification of this highly specific, translatable VitC-TET1 axis provides a novel and actionable therapeutic framework for the management of hemorrhagic stroke.

Figure of scientific hypothesis models

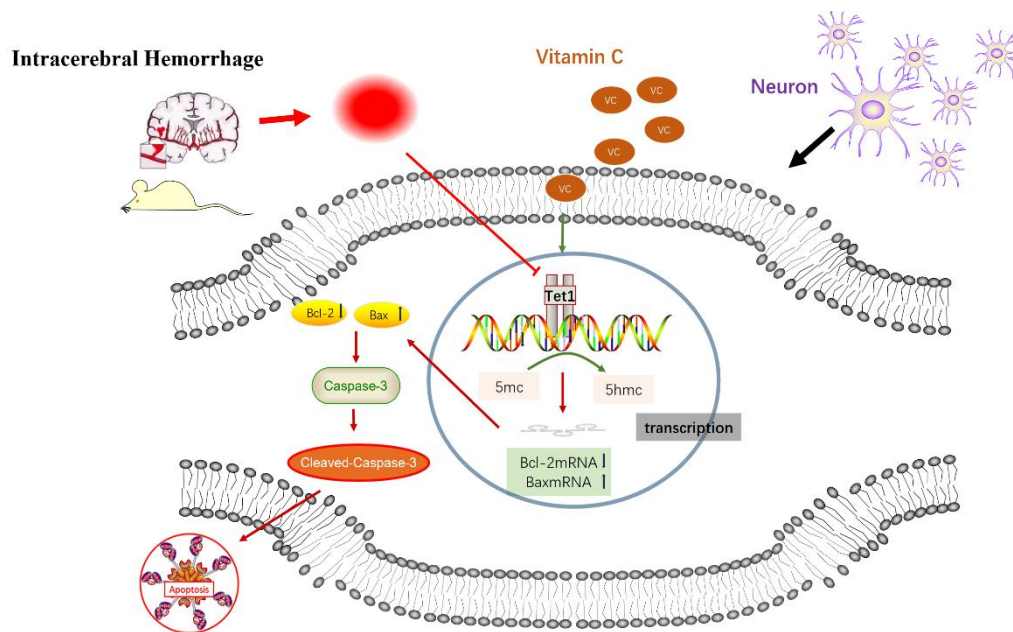


Fig. 7. Schematic summary illustrates that TET1 deficiency following ICH results in the suppression of 5hmC modification and overactivation of pro-apoptotic pathways (Bax/Caspase-3), which further downregulates the expression of the anti-apoptotic protein Bcl-2. This sequence of events leads to increased neuronal apoptosis and exacerbates neurological deficits. VitC treatment, by virtue of its cofactor activity, restores TET1 expression and 5hmC levels, thereby inhibiting the apoptotic cascade and facilitating neuronal survival.

Abbreviations

ICH: Intracerebral Hemorrhage;

SBI: Secondary Brain Injury;

TET1: Ten-eleven Translocation Methylcytosine Dioxygenase 1;

5mC: 5-Methylcytosine;

5hmC: 5-Hydroxymethylcytosine;

5fC: 5-Formylcytosine;

5caC: 5-Carboxycytosine;

VitC: Vitamin C;

mNSS: Modified Neurological Severity Score;

RT-qPCR: Reverse Transcription Quantitative Polymerase Chain Reaction;

IF: Immunofluorescence;
ANOVA: Analysis of Variance;
LSD: Least Significant Difference;
BSA: Bovine Serum Albumin;
PVDF: Polyvinylidene Fluoride;
SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis;
GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase;
Dnmt3a: DNA Methyltransferase 3a;
NLRP3: NOD-Like Receptor Pyrin Domain-Containing Protein 3;
LncRNA TUG1: Long Non-Coding RNA Taurine Upregulated Gene 1;
DOCK1: Dedicator of Cytokinesis 1;
NF- κ B: Nuclear Factor-Kappa B;
PGE2: Prostaglandin E2;
 α -KG: α -Ketoglutarate;
ESCs: Embryonic Stem Cells;
iPSCs: Induced Pluripotent Stem Cells;
NSCLC: Non-Small Cell Lung Cancer;
ROS: Reactive Oxygen Species;
SOD1/2: Superoxide Dismutase 1/2;
FKBP5: FK506 Binding Protein 5;
OCT4: Octamer-Binding Transcription Factor 4;
NANOG: NANOG Homeobox;
sh-TET1: TET1 Short Hairpin RNA (TET1 Knockdown);

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