HDAC6 Inhibition Rescues Oxidative Stress-derived Neuronal Apoptosis Following Intracerebral Hemorrhage Via MDH1 Acetylation

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Research Article

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Abstract

Oxidative stress is implicated in functional recovery after brain damage caused by intracerebral hemorrhage (ICH). Histone deacetylase 6 (HDAC6) plays an important role in initiation of oxidative stress. However, studies have not explored the role and mechanism of HDAC6 in mediating oxidative stress in ICH. Our study revealed HDAC6 knockout mice are resistant to ICH-induced oxidative stress and the resultant neuronal apoptosis, as analyses performed through MDA and NADPH/NADP+ assays, ROS detection and determination of expression levels apoptosis-related protein through western blot. Further mechanistic studies showed that HDAC6 binds to Malate dehydrogenase 1 (MDH1) and mediates MDH1 acetylation on lysine residues at position 121 and 298. ICH associated damaging agents reduce the level of MDH1 acetylation by promoting the interplay between HDAC6 and MDH1. Whereas, acetylation of MDH1 increases when HDAC6 is inhibited. Functional verification of acetylation MDH1 demonstrates that non-acetylation mutant MDH1, rather than its acetylation resistant mutant, protects neuron from oxidative injury. In addition, HDAC6 inhibition failed to mediate brain damage alleviation when MDH1 was knockdown. Overall, the findings of the present study indicated the protect effect of HDAC6 inhibition resistance to brain damage during ICH requires MDH1 acetylation.

Introduction

Intracerebral hemorrhage (ICH), is a type of acute cerebrovascular disease caused by ruptured blood vessels within the brain, and it is associated with high morbidity and mortality rates (1–3). However, there is currently no effective treatment for ICH. Accumulating evidence suggests that oxidative stress is an important inducer of brain injury following ICH (4–7). Additionally, neurons are sensitive and susceptible to oxidative stress injury and predisposed to apoptosis when oxidative stress occurs (8, 9), Therefore, targeting the oxidative stress response is an important therapeutic strategy for ICH.

Histone deacetylase 6 (HDAC6), a member of the histone deacetylase family, was found to play an important role in oxidative stress response (10, 11). Tubastatin A (TubA), a selective inhibitor of HDAC6, has been shown to inhibit oxidative stress and apoptosis in acute kidney injury (AKI) mice (12). Additionally, tubacin, an HDAC6 inhibitor, can ameliorate the oxidative stress injury caused by low-density lipoprotein in endothelial cells (13). The role of HDAC6 in the central nervous system had been confirmed in recent years. For example, treatment with TubA or interference with HDAC6 expression by siRNA can significantly alleviate oxidative stress, reduce neuro-apoptosis and cerebral infarction volume in mice with experimental stroke (14, 15). Studies have demonstrated that histone deacetylase inhibitors (HDACi) improve the prognosis of central nervous system disorders such as Parkinson's disease (16) and Alzheimer's disease (17). These findings suggested that HDAC6 was involved in oxidative stress. Therefore, this study aimed to determine whether HDAC6 plays a role in the pathogenesis of ICH, and more specifically, the mechanism by which HDAC6 mediates oxidative stress and subsequent neuro-apoptosis during ICH.
Malate dehydrogenase 1 (MDH1) is one of the key enzymes in biological glucose metabolism and plays an important role in oxidative stress by participating in the malate-aspartic acid shuttle, coordinating glycolysis and mitochondrial respiration, and thereby regulating reduced coenzyme II (NADPH) / oxidized coenzyme (NADP+) ratio and cellular reactive oxygen species (ROS) activity (18, 19). There is evidence that stress oxidation can be enhanced when MDH1 expression or activity is decreased. MDH1 activity can be upregulated via posttranslational acetylation. It has been observed that acetylated MDH1 increases its activity and promotes fatty acid synthesis via increased acetyl CoA and NADPH generation (18). This indicates that MDH1 may act as a negative regulator of the oxidative stress response via acetylation.

In the present study, we demonstrated that HDAC6 knockout alleviated brain damage by protecting against oxidative stress-induced neuronal apoptosis following ICH. Further examination of the mechanism of action revealed that HDAC6 binds to and mediates MDH1 deacetylation at lysine 121 and 298. MDH1 acetylation promoted NADPH generation and decreased ROS levels, hence protecting neuron from oxidative damage. TubA treatment failed to rescue brain damage following ICH in MDH1 knockdown mice. These results implied that the mechanism by which neuronal protection conferred by HDAC6 inhibition is acetylation MDH1 dependent.

**Materials And Methods**

**Animals**

Dr. YAO (Duke University) provided the HDAC6 knockout mice (HDAC6 -/-) on a C57 BL/6J background. The animal used in this experiment were male wild-type mice (C57 BL/6J) and HDAC6 -/- mice at the age of 2-3 months, which were sex and age matched. The experiment procedures were approved by the Institutional Animal Use and Care Committee of Xuzhou Medical University. Mice had access to water and food ad libitum.

**Cell culture**

HT22 or HEK293T cells were obtained from the China type culture collection. Both of the cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 120 U/ml penicillin, and 100 mg/l streptomycin in a 37°C incubator containing 5% CO2.

**ICH model**

ICH model was established as previously described (20). Briefly, mice were anesthetized intraperitoneally with pentobarbital sodium (50 mg/kg), and collagenase VII was injected into the right striatum, which is located at 0.2 mm anterior, 2.5 mm lateral, and 3.5 mm deep relative to the bregma. The sham group received similar procedures with an injection of the corresponding solvent instead of collagenase VII.

**In vitro ICH model**
To mimic ICH-like conditions *in vitro*, hemin at the concentration of 10 μM according to our previous study (20) was applied to stimulate HT22 cells for 24 hours.

**Expression vectors and plasmid transfection**

The plasmids used in the study were purchased from Sangon Bioengineering (Shanghai, China), and the plasmids were as follows: Myc-tagged MDH1 and Flag-tagged HDAC6 vectors generated using the corresponding full-length sequences of wild-type MDH1 and HDAC6, respectively. Myc-MDH1<sup>K118R</sup>, Myc-MDH1<sup>K121R</sup>, Myc-MDH1<sup>K298R</sup>, and MDH<sup>3KR</sup> mutant vectors were generated by replacing lysine with arginine at K118, K121, and K298 sites individually or simultaneously. Myc-MDH1<sup>K121Q</sup> and Myc-MDH1<sup>K298Q</sup> mutant vectors were generated by replacing lysine with glutamine at K121 and K298 sites individually. Non-acetylate MDH1<sup>2KR</sup> (K to R) and acetyl-mimetic MDH1<sup>2KQ</sup> (K to Q) constructs were generated via mutations at both the K121 and K298 sites. The plasmid was transiently transfected using the reagent lipofectamine 2000 according to the manufacturer’s instructions. The primers used for the indicated mutant plasmids was shown in Table 1 (the capital letters denote the mutation sites).

**siRNA and transfection**

The small interfering RNA (siRNA) targeting HDAC6 used in this experiment was purchased from GenePharma (suzhou, China). The siRNA target sense chain was 5’-GCUUCUAACUGGUCCACUATT -3’ and the antisense chain 5’-UAGUGGACCAGUUAGAAGCTT-3’, the transfection into cells was conducted using lipofectamine 2000 according to the manufacturer’s protocols.

**AAV9, lentiviral and administration**

Flag-MDH1<sup>WT</sup>/Flag-MDH1<sup>2KR</sup> Adeno-Associated virus 9 (AAV9) and MDH1 lentiviral were obtained from Sangon Bioengineering (Shanghai, China), the primer applied for shRNA against MDH1 was shown in Table 1.

AAV9 or lentiviral were administered into the brain through intracerebroventricular injection. The administration site was located at 0.1 mm anterior, 1.0 mm lateral, and 3.0 mm deep relative to the bregma.

**Behavior analyses**

Behavior dysfunction induced by ICH was explored by determination of modified Neurological Severity Score (mNSS) and corner-turning test at day 3 post ICH, which was conducted as described previously (20).

**Determination of brain water content**

Analysis of brain water content was conducted as previously described (20).
**Nissl and TUNEL staining**

Brains were harvested after successive transcardial perfusion with PBS and 4% paraformaldehyde. Brain tissues were fixed with 4% paraformaldehyde at 4°C overnight. Further, brain specimens were coronally sectioned into 40-50-μm-thick slices from the frontal lobe to the visual cortex for Nissl and TUNEL staining.

Nissl staining was conducted as described previously (20). A total of seven representative injury areas of hematoma from frontal lobe to the visual cortex were analyzed using ImageJ software and calculated according to previous reported (21).

TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche, Germany) according to its manufacturers’ instructions. For neuron apoptosis analysis, the slices were then co-incubated with rabbit anti-NeuN antibody (ab177487, 1:500, Abcam, UK) overnight at 4°C following TUNEL operation and followed by incubation with an Alexa Fluor 488 secondary antibody (1:500, abcam, USA) at 37 °C for 2 h. Finally, the positive TUNEL+/NeuN cells from three different areas of tissue around the hematoma in each slice were analysed by Image J software.

**Western Blot analysis**

Protein isolated from peri-hematoma tissues and hemin-induced HT22 cells were employed for western blot analysis. The major steps of WB were carried out according to our previous report (21). The primary antibodies applied were listed in **Table 2**. Then, exposed to enhanced chemiluminescence kit (ECL) for image development after incubated with the corresponding second antibody at room temperature.

**Co-immunoprecipitation and immunoblotting assays**

Brain tissues or HT22 cells or 293T cells were lysed with Nonidet P-40 lysis buffer for 10 min and then centrifuged at 13, 000g and 4°C for 30 min to obtain the soluble proteins. A total of 1mg protein was precipitated using 1μg of indicated primary antibodies overnight. Samples were further incubated with 30μl of protein G agarose beads (Sigma Technology) for 4 h. Rabbit IgG was used as control. Subsequently, the mixture was centrifuged at 13, 000g and 4°C to obtain immunoprecipitants. Immunoprecipitants were washed thrice with ice-cold PBS buffer, eluted and boiled in 30μl of loading buffer. Immunocomplexes were then subjected to WB assay. The primary antibodies used were listed in **Table 2**. A total of 100μg of the lysates without precipitation with antibodies were analyzed directly by WB assay following normalization of total protein content.

**Cell immunofluorescence staining**

Cells were plated on coverslips and fixed with 4% paraformaldehyde for 20 min at room temperature. Further, cells were permeabilized and blocked with a mixture of 0.5% Triton X-100 and 10% goat serum for 1h at room temperature. Cells were then incubated with a rabbit anti-Myc antibody (ab32, 1:500, Abcam) or mouse anti-Flag antibody (ab18230, 1:500, Abcam) at 4°C overnight. Cells were washed and incubated
with secondary antibodies conjugated with anti-rabbit Alexa 488 (ab150077, 1:500, Abcam) or anti-mouse Alexa 594 (ab150080, 1:500, Abcam) at room temperature for 1 h. Images of the cells were acquired using Leica confocal microscope.

Malondialdehyde (MDA) and NADPH/NADP+ assay

Peri-hemorrhage tissues were isolated and washed with cold PBS on day 3 post-ICH, followed by homogenization with 1ml/0.1g pre-cooled lysis buffer. Homogenized samples were centrifuged at 13,000 g and 4°C for 10 minutes. The supernatant was obtained for determination of the concentration of MDA and NAPDH/NADP+ analysis using the corresponding MDA and NADPH/NADP+ assay kits (Beyotime Biotechnology) following the manufacturers’ instructions.

ROS analysis

HT22 cells were cultured in 96-well plates, and HDAC6 siRNA or the indicated MDH1 plasmids were transfected into the cells when cell density reached 70-80%. The cells were then subjected to heme 12h later and followed by oxidant-sensitive probe DCFH-DA labelled ROS detection, using a relative assay kit (Beyotime Biotechnology) according to the manufacturer’s instructions.

Statistical analysis

All data were presented in the form of mean ± standard deviation (mean ± SD). Graphpad Prism 8.0 was performed to statistical analysis of the data in our study. Comparison among multiple groups was analysed by One-way ANOVA with Tukey’s post hoc test. The T-test was used to compare differences between the two groups. The difference will be statistically significant is P < 0.05.

Results

HDAC6 knockout alleviates brain damage following ICH

To investigate the role of HDAC6 in ICH-induced brain damage, we used HDAC6 knockout (HDAC6−/−) mice in comparison to WT control mice. It was revealed that the HDAC6−/− mice had no functional HDAC6 gene as indicated by gene identification assay (Figure 1A) and no HDAC6 protein as detected by western blot (WB) analysis (Figure 1B), indicating that the HDAC6 gene had been entirely knocked out. Using Nissl’s staining, the injury volume was compared between WT and HDAC6−/− mice following ICH. As shown in Figure 1C and 1D, when HDAC6 was knocked out, the injury volume caused by ICH was significantly decreased compared with WT mice. In addition, our findings indicated that HDAC6 knockout greatly ameliorates neurological dysfunction following ICH, as seen by a reduction in the mNSS score and right turn percentage compared with WT mice (Figure 1E and 1F). Similarly, when HDAC6 was knocked out, the resulting cerebral edema caused by ICH was significantly reduced compared to WT mice (Figure 1G). Taken together, our findings indicated that HDAC6 knockout protected against ICH-induced brain damage.

HDAC6 knockout alleviates oxidative stress and neuro-apoptosis following ICH
We investigated the involvement of HDAC6 in oxidative stress. At 3d after collagenase VII injection, WT and HDAC6\(^{-/-}\) mice were subjected to oxidative stress tests (Figure 2A). Our findings confirmed that when WT mice were exposed to ICH, their Malondialdehyde (MDA) levels increased significantly, but markedly decreased in HDAC6\(^{-/-}\) mice (Figure 2B). In comparison with WT mice, the ratio of NADPH/NADP\(^+\) was markedly elevated in HDAC6\(^{-/-}\) mice following ICH (Figure 2C). We further assessed intracellular ROS generation in HT22 cells using the oxidant-sensitive probe 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA). We first transfected HDAC6 siRNA into HT22 cells for 12 h to knockdown HDAC6, which was confirmed by WB analysis (Figure 2D), and then exposed the cells to hemin for 24h before collecting the samples for ROS tests. The results showed that when HT22 cells were challenged with hemin, the level of ROS increased considerably, but decreased when HDAC6 was knocked down using HDAC6 siRNA (Figure 2E and 2F). Taken together, these findings indicate that HDAC6 knockdown could ameliorate the oxidative stress response during ICH.

Given that oxidative stress contributes to neuronal apoptosis, we further investigated the role of HDAC6 in neuronal apoptosis following ICH. In vivo, TUNEL staining showed the number of dead/dying neurons was considerably lower in HDAC6\(^{-/-}\) mice compared with WT mice, the total number of neuron between WT and HDAC6\(^{-/-}\) mice showed no significant difference (Figure 2G and 2H). Moreover, the apoptosis-associated proteins, including cleaved-caspase-3 and Bax were significantly increased in WT mice 3 d after ICH, however, there was a marked reduction in the above proteins when HDAC6 was knockout (Figure 2I). In vitro, immunoblotting analysis showed a significant increase in Bax and cleaved-caspase-3 expression in response to hemin, but a significant decrease after HDAC6 knockdown by HDAD6 siRNA (Figure 2J). Taken together, our study demonstrated that knocking out HDAC6 can alleviate neuro-apoptosis induced by ICH.

**Interaction between HDAC6 and MDH1**

Given that HDAC6 is a deacetylase, we used mass spectrometry to determine the potential substrate for HDAC6 to investigate the potential mechanism by which oxidative stress occurs. Among the 11 substrates identified by mass spectrometry, MDH1 was chosen for this study due to its reported role in oxidative stress. The co-immunoprecipitation (coIP) technology in vitro showed that Myc-MDH1 protein can be precipitated by anti-Flag antibody in immunoprecipitation assays of 293T cells with both Flag-HDAC6 and Myc-MDH1 transfection. Flag-HDAC6 can similarly be precipitated by anti-Myc antibody (Figure 3A). Additionally, we observed an in vivo interaction between endogenous HDAC6 and MDH1. Figure 3B shows that the MDH1 protein existed in the anti-HDAC6 antibody specific immunoprecipitates from WT mice brain extracts, but was absent in the control IgG immunoprecipitates. Similarly, we found that HDAC6 protein was also present in the anti-MDH1 antibody specific immunoprecipitates. In addition, we co-transfected Flag-HDAC6 and Myc-MDH1 plasmids into both 293T and HT22 cells and immunofluorescence confocal assay found that HDAC6 co-localized with MDH1 (Figure 3C). Therefore, our findings suggested that HDAC6 can interact with MDH1.
Next, we investigated whether HDAC6 deacetylates MDH1. We found that when 293T cells were co-treated with MDH1 and HDAC6, the level of MDH1 acetylation was significantly decreased compared to when 293T cells were treated with MDH1 alone (Figure 3D). Secondly, as shown in Figure 3E, when 293T cells were treated with TubA rather than DMSO, there was a considerable increase in MDH1 acetylation. We further conducted validation experiments in vivo. Immunoprecipitation with anti-acetyl-lysine antibody showed that the level of MDH1 acetylation was significantly upregulated in HDAC6 knockout mice compared to WT mice (Figure 3F). Therefore, we concluded that HDAC6 is capable of deacetylating MDH1.

**HDAC6 negatively regulates MDH1 acetylation in response to ICH**

Since the enzymatic activity of MDH1 can be enhanced by acetylation, we first investigated whether the damaging agents associated with ICH affect MDH1 acetylation. As shown in Figure 4A-C, the damaging agents (Hemin, Hemoglobin, Thrombin) significantly decreased MDH1 acetylation in HT22 cells. We further examined whether HDAC6 altered the degree of MDH1 acetylation during ICH. Immunoprecipitation analysis showed that the level of acetylated MDH1 was significantly decreased following hemin induction compared to the control group, but significantly increased following HDAC6 siRNA knockdown (Figure 4D). Additionally, we examined the effect of TubA on the level of acetylated MDH1 following ICH in mice. As shown in Figure 4E, TubA treatment can significantly increase the level of acetylated MDH1 during ICH when compared to the DMSO-treated group. Consistently, the level of MDH1 acetylation was significantly increased in HDAC6-/- mice compared to WT mice in the presence of ICH (Figure 4F). In vivo and in vitro findings, revealed that HDAC6 inhibition significantly increased the level of acetylated MDH1 following ICH.

To obtain additional insight into the role of HDAC6 in the ICH-induced decrease in MDH1 acetylation, we explored whether the ICH state affects the interaction between HDAC6 and MDH1. CoIP assay revealed that when mice were subjected to ICH surgery (Figure 4G), the interaction between HDAC6 and MDH1 was enhanced, whereas it was decreased upon TubA treatment. Therefore, this study's findings suggested that the decreased interaction between HDAC6 and MDH1 resulted in an increase in MDH1 acetylation when HDAC6 was inhibited.

**HDAC6 deacetylates MDH1 at the K121 and K298 residues**

To identify the specific lysine residues in MDH1 that may be acetylated by HDAC6, we first used the lysine residues prediction site (http://www.uniprot.org/) and a relevant published article, which showed the potential acetylation sites of MDH1 may be in Lys 118 (K), Lys 121 (K), and Lys 298 (K). To examine if HDAC6 acts on these MDH1 residues, we generated Lys-to-arginine mutants in these sites either separately or simultaneously. Our findings indicated that mutations in K121, K298 rather than K118 could significantly reduce MDH1 acetylation, and that acetylation was completely lost due to mutations in the three lysine residues (3KR) (Figure 5A). Additionally, the coIP assay showed that the interaction between HDAC6 and MDH1 was significantly impaired or hardly detectable when lysine residues 121 or 298 were
mutated alone or in combination with the three 118, 121, 298 lysines compared with wild-type MDH1, but not when lysine 118 was mutated alone (Figure 5B). Therefore, we concluded that HDAC6 may deacetylate lysines 121 and 298 of MDH1. Levels of acetylated MDH1 were indeed markedly decreased in 293T cells transfected with the acetyl-mimetic mutants (Myc-MDH1\textsuperscript{K121Q}, Myc-MDH1\textsuperscript{K298Q}) and non-acetylatable mutants (Myc-MDH1\textsuperscript{K121R}, Myc-MDH1\textsuperscript{K298R}), further confirming the acetylation sites of MDH1 (Figure 5C). We next generated Myc tagged MDH1 acetyl-mimetic mutants (Myc-MDH1 2KQ) and non-acetylatable mutants (Myc-MDH1 2KR), in which the mutations in the two lysines K121 and K298 were substituted with glutamine (Q) or arginine (R), respectively. The IP assay showed that the acetylation of MDH1 was lost when the lysines in 121 and 298 sites were replaced with arginine or glutamine (Figure 5D and 5E). Our findings collectively revealed that HDAC6 deacetylated MDH1 at the K121 and K298 residues.

**Acetylated MDH1 improves oxidative stress and neuro-apoptosis**

We examined whether acetylated MDH1 affected oxidative stress during ICH. First, we conducted a study in vitro. Our results showed that ROS was overproduced when HT22 cells were stimulated with hemin, however, the ROS level was significantly reduced when wild-type MDH1 or MDH1\textsuperscript{2KQ} were overproduced. In contrast, the MDH1\textsuperscript{2KR} failed to exhibit a protective effect against ROS production by hemin (Figure 6A). This finding revealed that acetylation of MDH1 plays a critical role in decreasing ROS generation in vitro. We further established model with MDH1 protein overexpression in WT mice by injecting MDH1 AAV9 virus (Flag-MDH1\textsuperscript{WT}, Flag-MDH1\textsuperscript{2KR}) with or without the corresponding green-fluorescent protein (GFP) into brain, autofluorescence without primary antibody labeling suggested MDH1 AAV9 could be successfully injected into brain (Figure 6B), and western blot analysis suggested that WT-MDH1\textsuperscript{WT} and MDH1\textsuperscript{2KR} overexpression could be detected in the intracranial cavity (Figure 6C and 6D). As shown in Figure 6E, when WT mice were subjected to ICH surgery, the level of MDA significantly increased, whereas it markedly decreased when Flag-MDH1\textsuperscript{WT} was overexpressed rather than Flag-MDH1\textsuperscript{2KR}. The ratio of NADPH/NADP+, which significantly decreased following ICH, was found to be markedly increased when treated with wild-type MDH1, but not with MDH1\textsuperscript{2KR} (Figure 6F). Overall, our findings revealed that acetylation of MDH1 played an important role in alleviating oxidative stress following ICH.

We further investigated the role of acetylated MDH1 in neuro-apoptosis during ICH in vivo and vitro. Immunoblotting of samples from the in vitro indicated groups revealed that the hemin-induced group had significantly higher Bax and cleaved-caspase-3 levels than the control group. However, following hemin exposure, the MDH1\textsuperscript{WT} or MDH1\textsuperscript{2KQ} treated group, but not the MDH1\textsuperscript{2KR} treated group, displayed significant decrease in Bax and cleaved-caspase-3 protein levels (Figure 6G). In vivo, intracranial targeted overexpression of Flag-MDH1\textsuperscript{WT} (wild-type MDH1-AAV9 vector) significantly reduced the increased expression of Bax and cleaved-caspase-3 caused by ICH in mice, while, Flag-MDH1\textsuperscript{2KR-AAV9} treatment failed to exert a protective effect (Figure 6H). In addition, neuronal death (NeuN/TUNEL+) was significantly reduced in WT mice with ICH following Flag-MDH1\textsuperscript{WT} treatment, but not Flag-MDH1\textsuperscript{2KR}
treatment (Figure 6I-K). Taken together, our findings indicated that acetylation MDH1 may help protect against neuro-apoptosis.

**Protective function of HDAC6 inhibition is acetylated MDH1 dependent**

In order to elucidate the underlying mechanism of the protective role of HDAC6 inhibition, we established MDH1 knockdown mice by injecting their brains with MDH1 lentivirus for 2 weeks and then subjected them to ICH surgery (Figure 7A). Autofluorescence and WB analysis revealed that 2 weeks after intraventricular injection, the MDH1 lentivirus significantly reduced intracranial MDH1 protein (Figure 7B and 7C). Following that, we used TubA to determine if TubA's effect on brain protection was acetylated MDH1 dependent. Nissl’s staining revealed that TubA treatment significantly reduced hematoma volume following ICH in WT mice. However, TubA exhibited a weakened protective effect on hematoma volume reduction when MDH1 protein was knocked down using lentivirus (Figure 7D and 7E). In addition, administration of TubA significantly alleviated brain edema and improved neurological dysfunction, but failed to exhibit this protective effect when MDH1 knockdown (Figure 7F-H). Therefore, our findings indicated that HDAC6 inhibition exerted its protective effect in an acetylated MDH1 dependent manner.

**Discussion**

The identification of HDAC6 as a regulator of oxidative stress sheds light not only on the possible mechanism underlying ICH-induced damage but also on the development of novel ICH therapies. In this study, we found that HDAC6 depletion protects against ICH-induced brain damage via oxidative stress and neuronal apoptosis defense. HDAC6 was found to interact with and deacetylate MDH1 at lysines 121 and 298. Depletion of HDAC6 contributed to an increase in MDH1 acetylation, which was found to protect neurons from oxidative stress damage in this study. As a result, the protective effect of the HDAC6 inhibitor TubA against ICH-induced brain damage was weakened with MDH1 knockdown. Thus, HDAC6 inhibition may exert its protective effect during ICH via MDH1 acetylation (Figure 7I). In this regard, targeting HDAC6/MDH1 signaling may provide a potential therapeutic approach for ICH treatment.

The conclusion that HDAC6 was involved in ICH and mediated oxidative stress-induced neuro-apoptosis came to the following observation. First, our study found that HDAC6 knockout reduced oxidative stress response induced by ICH. This was consistent with previous studies that HDAC6 blockade protects against oxidative stress (22–25). Second, we demonstrated that neuronal apoptosis was significantly increased upon ICH injury, however, this alteration can be overcome by HDAC6 knockout. These findings corroborate Butler et al(26) and Yuan et al(27) found that the specific HDAC6 inhibitor treatment can improve neuron survival in an oxidative stress-induced model or a rat cortical neuron model of oxygen-glucose deprivation. Third, HDAC6 knockout mice showed less brain damage compared to WT mice, including injury volume, brain edema and neurological dysfunction.

How does HDAC6 contribute to oxidative stress-induced brain injury following ICH? Since HDAC6 has been shown to regulate a variety of critical biological processes, such as ciliary disassembly (28), DNA repair (29), and axon growth (30) by deacetylating of its specific substrates. We hypothesized that
HDAC6 may exert its oxidative stress effect by mediating the deacetylation of some certain substrates associated with oxidative stress. Our current study established an interaction between HDAC6 and Malate dehydrogenase 1 (MDH1), and found that HDAC6 can modify the level of MDH1 acetylation during ICH. ICH-associated-damaging agents (hemin, hemoglobin and thrombin) reduced MDH1 acetylation in an HDAC6 dependent manner. MDH1, a dehydrogenase subtype, predominantly expressed in tissues with high aerobic metabolic demands, including brain(31–33), was reported involved in oxidative stress by regulating the NAPDH/NADP+ ratio and cellular ROS activity(34). Additionally, a reduction in MDH1 activity may induce cell death via enhanced oxidative stress and the corresponding damaged mitochondria (18, 35). More importantly, it is accepted that posttranslational can be used to modulate MDH1 function(18). We speculate that the relationship between HDAC6 and MDH1 will aid in elucidating the potential anti-oxidative mechanism by which HDAC6 inhibition may function during ICH.

Emerging evidence established MDH1’s critical involvement in the central nervous system diseases, where abnormal expression or activity of MDH1 has been associated with energy metabolism disorders, such as aging (36), somatic retardation, epilepsy, and progressive microcephaly (19), and is no longer limited to studies of tumor hypermetabolism (34, 37). However, the involvement of MDH1, particularly its acetylation in the development of oxidative stress during intracerebral hemorrhage remains unknown. In this study, we first determined that acetylation of lysine residues in MDH1 was K121 and K298 (2K), which mutants alter MDH1 acetylation and the association between HDAC6 and MDH1. We further demonstrated that the wild-type or the acetylation-mimetic 2KQ mutant, but not the acetylation-resistant 2KR mutant treatment significantly decreased the ROS levels and apoptosis proteins (Cleaved-caspase-3 and Bax) expression in HT22 cells challenged with hemin. Additionally, we demonstrated in vivo that AAV-mediated overexpression of non-acetylation mutant MDH1 rather than acetylation resistant MDH1 can significantly reduce oxidative stress response and neuronal apoptosis following intracerebral hemorrhage. Yi-Ping Wang et al. reported similar findings, that arginine methylation of MDH1 by CARM1 (another post-translational modification of MDH1) negatively regulated MDH1 activity contributing to a decrease in antioxidant stress capacity (35). Therefore, our findings established that MDH1 acetylation plays a critical role in cellular ROS defense and neuron protection during ICH.

Our further study demonstrated that HDAC6 inhibition protects against brain damage is through an increase in the level of acetylated MDH1. This conclusion is based on the following observation. TubA treatment significantly alleviated brain damage during ICH, as measured by decreased hematoma volume, cerebral edema, and neurological dysfunction score. However, the protective effect of TubA against brain damage was significantly weakened in the presence of MDH1 lentiviral knockdown. TubA’s inability to execute its protective role was hypothesized to be due to its failure to enhance the MDH1 acetylation level in ICH when MDH1 was knocked down. However, a specific knockdown of the MDH1 acetylation site in mice to investigate the effect of MDH1 acetylation on oxidative stress levels will be a more effective approach.

In summary, this study established for the first time that HDAC6 inhibition plays an important protective role in the oxidative stress response and neuron apoptosis during ICH, with enhanced MDH1 acetylation.
as a potential mechanism. This study may provide a novel therapeutic target for the treatment of ICH.

**Abbreviations**

Bax: Bcl-2 associated X protein;

Caspase-3: Cysteiny aspartate specific proteinase-3;

Co-IP: co-immunoprecipitation;

IP: Immunoprecipitation;

DMSO: Dimethyl sulfoxide;

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

HDACs: Histone deacetylases;

HDAC6: Histone deacetylases 6;

ICH: Intracerebral haemorrhage;

MDH1: Malate dehydrogenase 1;

MDA: Malondialdehyde;

mNSS: Modified neurological severity score;

NAD: Nicotinamide Adenine Dinucleotide;

NADPH: Nicotinamide Adenine Dinucleotide Phosphate;

ROS: Reactive oxygen species;

siRNA: Small interfering RNA;

TubA: Tubastatin A;

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling.

**Declarations**

**Study approval**

All animal experiments carried out were approved by the Jiangsu Provincial Animal Care, and all mice procedures were approved by the institutional Animal Use and Care Committee of XuZhou Medical
University.

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Authors' contributions

GYC and MW contributed to research design and manuscript writing. MW executed most experiments. CZ contributed to data acquirement and analyzation. LY assisted experiments accomplishment. WJM, BCL helped with most of the mouse experiments. YW assisted with protein immunoprecipitation. WFW, MYZ assisted with protein extraction and animal experiments. All authors read and approved the final manuscript.

Conflict of interest The authors declare no competing financial interests.

Consent for publication All authors have read and approved the manuscript.

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Data Sharing Statement

The datasets used and/or analysed during the present study are available from the corresponding author upon reasonable request.

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Tables

Table 1. List of oligonucleotides used in this study. Primers used for the mutant MDH1 vectors and shRNAs against MDH1 are listed.
Table 2. List of primary antibodies used in this study. Primary antibodies used for WB assay are listed.

| antibody                  | company                  | lot number | dilution ratio |
|---------------------------|--------------------------|------------|----------------|
| rabbit anti-BAX           | cell signaling Technology | #14796     | 1:1000         |
| rabbit anti-cleaved-caspase-3 | cell signaling Technology      | #9661     | 1:1000         |
| rabbit anti-HDAC6 antibody | cell signaling Technology      | #7558     | 1:1000         |
| rabbit anti-MDH1 antibody | Abcam                    | ab180152   | 1:20000        |
| anti-Myc                  | Abcam                    | ab32       | 1:20000        |
| rabbit anti-Flag          | Abcam                    | ab205606   | 1:20000        |
| mouse anti-GAPDH antibody | cell signaling Technology      | #51332    | 1:5000         |
| rabbit anti-Tubulin antibody | cell signaling Technology      | #2128     | 1:10000        |
Knockout of HDAC6 alleviates brain damage following ICH. (A) HDAC6⁻/⁻ mice had defects in HDAC6 gene amplification compared with WT mice; (B) HDAC6 protein was expressed in normal WT mice, but
was not expressed in HDAC6\textsuperscript{−/−} mice; (C) Representative Nissl’s staining images of hematoma volume in the indicated group; (D) Quantitative analysis of injury volume in the indicated groups (n=6/group); (E-F) Neurological dysfunction as detected by mNSS score and corner turn test in the indicated groups (n=10/group); (G) Level of cerebral edema in the indicated groups (n=6/group). *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 2

**Knockout of HDAC6 alleviated oxidative stress following ICH.** (A) In vivo experimental design for results presented in Figure 2B-C; (B-C) Malondialdehyde (MDA) level and NADPH/NADP\(^+\) ration in HDAC6\textsuperscript{−/−} and control WT mice at day 3 for the sham or ICH surgery groups (n=6/group); (D) The effect of HDAC6 siRNA was verified by WB assay (n=3/group); (E) Representative fluorescence images showing DCFH-DA labelled ROS levels in the indicated groups; (F) Quantitation of ROS content (n=6/group); (G) Representative images of neuron death in the peri-hemorrhage zone of HDAC6\textsuperscript{−/−} and control WT mice (neuron: green and TUNEL: red). Magnified images of NeuN/TUNEL\(^+\) staining were indicated with white dashed line squares. Scale bars: 50 \(\mu\)m; (H) Quantitative analysis of total NeuN cells, total TUNEL\(^+\) cells and NeuN/TUNEL\(^+\) cells in the indicated groups (n=6/group); (I) Representative western blot (WB) images and quantitative analysis of cleaved caspase-3 and Bax proteins in the indicated groups in vivo (n=3/group); (J) Representative WB images and quantitative analysis of cleaved caspase-3 and Bax protein in the indicated groups in vitro (n=3/group); *P < 0.05, ***P < 0.001.
Figure 3

Interaction between HDAC6 and MDH1 in vitro and in vivo. (A) 293T cells were single-transfected with Flag-HDAC6 plasmid or co-transfected with Myc-MDH1 plasmid. A total of 1 mg cell samples were immunoprecipitated by 1μg of anti-Flag or anti-Myc antibody 24h after transfection. Immunoprecipitants were then subjected to WB analysis with the indicated antibodies; (B) Brain samples of WT mice were subjected to IP assays with control anti-IgG or anti-HDAC6 or anti-MDH1 antibody, followed by WB analysis with the indicated antibodies; (C) Typical confocal images for HDAC6 (red) and MDH1 (green) in 293T and HT22 cells co-transfected with Flag-HDAC6 and Myc-MDH1 plasmids; (D) Cell lysates of 293T cells transfected with Myc-MDH1 plasmid alone or combined with Flag-HDAC6 plasmid were precipitated by anti-acetyl-lysine antibody. WB analysis of acetylated MDH1 level was then conducted; (E) 293T cells transfected with Flag-HDAC6 were treated with 10μM TubA. Cells were further subjected to IP assay to determine ac-MDH1 level; (F) Immunoprecipitation of brain tissue lysates of WT and HDAC6−/− mice using anti-acetyl-lysine antibody, followed by WB analysis of ac-MDH1 level.

Figure 4

HDAC6 negatively regulates MDH1 acetylation in response to ICH. (A-C) IP and WB analysis of the ac-MDH1 in HT22 cells treated with (A) Hemin, (B) Hemoglobin, and (C) Thrombin; (D) IP assay performed to determine ac-MDH1 level in the indicated group (n=3/group); (E) IP assay determined to examine
acetylation level of MDH1 in the indicated group (n=3/group); (F) IP and WB analysis of the acetylation level of MDH1 in HDAC6−/− and WT mice after subjection to ICH surgery (n=3/group); (G) colIP assay of the interaction between HDAC6 and MDH1 after ICH surgery or under TubA treatment in WT mice. *P < 0.05, ***P < 0.001.

Figure 5

**HDAC6 deacetylates lysine residues of MDH1 at position 121 and 298.** (A) 293T cells were transfected with wild-type and mutated plasmids of Myc-MDH1 (K118R, K121R, K298R, 3KR) for 24h, and then subjected to IP and immunoblotting assay with the indicated antibodies; (B) 293T cells were co-transfected with Flag-HDAC6 and Myc-MDH1 (WT, K118R, K121R, K298R, 3KR) plasmids for 24h, and then subjected to IP and immunoblotting assay with the indicated antibodies; (C) 293T cells were transfected with Myc-MDH1 (WT, K121R, K121Q, K298R, K298Q) plasmids for 24h, and then subjected to IP and immunoblotting assay with the indicated antibodies; (D) 293T or (E) HT22 cells were transfected with Myc-MDH1 plasmids (WT, 2KR, 2KQ) for 24h and then subjected to IP and immunoblotting assay with the indicated antibodies.

Figure 6

**Acetylated MDH1 alleviated oxidative stress and neuro-apoptosis following ICH.** (A) Representative fluorescence images showing DCFH-DA labelled ROS levels in the indicated groups; (B-C) Results for green-fluorescent protein (GFP)-MDH1 AAV9 virus (Flag-MDH1, Flag-MDH12KR) in the brain as identified by (B) autofluorescence without primary antibody staining and (C) WB assay; (D) WB results of MDH1 AAV9 virus without GFP labelling in the brain; (E-F) MDA level and NADPH/NADP+ ratio was assayed
using relative assay kit (n=6/group). (G) HT22 cells were transfected with WT-MDH1 and its mutant MDH1 (2KR, 2KQ) respectively for 12h and then subjected to hemin for 24h. Expression of cleaved caspase-3 and Bax proteins was assayed by WB (n=4/group); (H) WT mice were transfected with Flag-MDH1-AAV9 and Flag-MDH1<sup>2KR</sup>-AAV9 for 21d, and then subjected to ICH surgery. Expression of cleaved caspase-3 and Bax level was assayed by WB (n=4/group); (I) Representative images and quantitative analysis of neuron death in the peri-hemorrhage zone of indicated groups (neuron: green and TUNEL: red). Magnified images of NeuN/TUNEL<sup>+</sup> staining indicated by white dashed line squares. Scale bar: 50 μm; (J-L) Quantitative analysis of (J) total NeuN cells, (K) total TUNEL<sup>+</sup> cells and (L) NeuN/TUNEL<sup>+</sup> cells in the indicated groups (n=6/group). *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 7**

**Protective effect of HDAC6 inhibition depends on acetylation level of MDH1.** (A) Experimental design for results presented in Figure 9B-H; (B-C) Results of green-fluorescent protein (GFP)-MDH1 lentivirus in the brain as identified by (B) autofluorescence without primary antibody staining and (C) WB assay (n=3/group); (D) Representative images of brain injury assayed by Nissl's staining in the indicated group; (E) Quantitative analysis of injury volume in the indicated groups (n=6/group); (F) Cerebral edema was determined by the brain water content in the indicated groups (n=6/group); (G-H) Neurological function was assayed by mNSS score and corner-turning test in the indicated groups (n=10/group); (I) Model illustration of mechanism by which HDAC6/MDH1 signaling mediates oxidative stress-induced neuron apoptosis following ICH. Upon ICH injury, the interaction between HDAC6 and MDH1 was enhanced, which promoted HDAC6 mediated-MDH1 deacetylation at K121 and K298, thereby inhibiting NADP+ shift to NADPH, consequently, ROS was overproduced, thus contributing to neuron apoptosis. However, Administration of TubA (a specific HDAC6 inhibitor) alleviates the oxidative stress response by disrupting HDAC6 and MDH1 association, thus recovers neuron apoptosis. **P < 0.01, ***P < 0.001.