Original Article

Cerebral Inhibition of the H3K9 Methylation Could Ameliorate Blood-Brain Barrier Dysfunction and Neural Damage in Vascular Dementia

Sehati, F[1], Ranjbaran, M[1], Nabavizadeh, F[1,2], Karimian, SM[1], Hosseindoost, S[2], Ashabi, G[1*]

1. *Department of Physiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*
2. *Electrophysiology Research Center, Neuroscience Institute, Tehran University of Medical Sciences, Tehran, Iran*

How to cite this article: Sehati F, Ranjbaran M, Nabavizadeh F, Karimian SM, Hosseindoost S, Ashabi G. Cerebral Inhibition of the H3K9 Methylation Could Ameliorate Blood-Brain Barrier Dysfunction and Neural Damage in Vascular Dementia. Archives of Razi Institute. 2024;79(5):989-995. DOI: 10.32592/ARI.2024.79.5.989

ABSTRACT

Dementia encompasses a broad category of brain diseases characterized by various degenerative or vascular components that lead to a long-term and often gradual decline in cognitive abilities, significantly affecting daily functioning. Literature indicates that the G9a/GLP enzyme, through the upregulation of histone 3 lysine 9 dimethylation (H3K9me2), plays a pivotal role in vascular dementia (VD). The increase in H3K9 methylation by G9a/GLP during VD inhibits the expression of neuroprotective proteins and diminishes the expression of proteins crucial for maintaining blood-brain barrier (BBB) integrity. Using a model of permanent common carotid artery (CCA) occlusion, we investigated the effects of a G9a/GLP inhibitor (BIX01294) on VD. Following CCA occlusion, BIX01294 (22.5 µg/kg) was administered intraperitoneally three times a week for one month. We assessed neuronal damage using Nissl staining, BBB permeability via the Evans blue test, and measured brain water content. Western blot analysis was employed to evaluate the hippocampal levels of Bax and Bcl2 proteins. Treatment with BIX01294 enhanced BBB stability ($P < 0.05$) and subsequently reduced brain edema compared to the VD group ($P < 0.05$ for both measures). Neuronal injury in the CA1 region of the hippocampus significantly decreased following BIX01294 administration compared to the VD group ($P < 0.05$). Furthermore, the Bax/Bcl2 ratio markedly decreased in the treatment group ($P < 0.0001$). In summary, our research demonstrates that inhibiting H3K9 methylation can prevent the progression of vascular dementia by reducing cerebral edema and neuronal apoptosis in the hippocampus following ischemic stroke.

Keywords: H3K9; Brain Edema; Blood-Brain Barrier; Vascular Dementia; Cerebral Ischemia

Article Info:
Received: 18 December 2023
Accepted: 20 February 2024
Published: 31 October 2024

Corresponding Author's E-Mail:
gh-ashabi@tums.ac.ir

1. Introduction

The term "dementia" refers to a broad category of neuropathologically distinct chronic brain disorders that share a progressive decline in cognitive functions. This decline inevitably results in memory loss, difficulties in problem-solving, disorientation, mood swings, and deterioration in language and communication abilities (1). Age-related neurological disorders, particularly vascular dementia (VD), link vascular conditions such as cerebral ischemia, hemorrhagic stroke, ischemic stroke, and hypoxia to cognitive deficits (2). Epigenetic alterations significantly impact the pathophysiology and functional recovery following ischemic brain injury. Histone methylation, a type of epigenetic modification, affects the transcriptional regulation of genes, often occurring on the N-terminal arginine or lysine residues of histones H3 and H4. Methylation of histone 3 at lysines 4 (H3K4), 36 (H3K36), and 79 (H3K79) typically associates with transcriptional activation, while methylation at lysines 9 (H3K9), 27 (H3K27), and 20 (H4K20) correlates with transcriptional repression (3–5).

Previous studies have shown that deacetylation of H3K9 and increased levels of H3K9me2 are linked to the suppression of the glutamate receptor 2 (GluR2) gene in the CA1 neuronal promoter during ischemic events (6). Furthermore, after internal carotid artery occlusion, molecular investigations reveal dysregulation of various histone methyltransferases (HMTs) and histone lysine demethylases (HDMs), alongside a significant global reduction in transcriptional repression induced by the H3K9me2 epigenetic modification in the striatum (7). Additionally, pharmacological and genetic inhibition of SUV39H1 and G9a markedly promotes neuronal survival following oxygen and glucose deprivation (OGD) (8). Wilson et al. discovered that G9a regulates axon growth by modulating the RhoA signaling pathway in both in vitro and in vivo studies (9). Genome-wide chromatin immunoprecipitation analyses using postmortem human brain tissues from the lateral temporal lobes indicated a significant reduction in H3K122ac and H3K4me1 markers in Alzheimer's disease (AD) samples. These epigenetic modifications correlate with an enrichment of transcriptional patterns associated with pro-disease pathways, such as inflammation and cell death (10). In sporadic AD patients, a modest study of postmortem temporal cortices revealed a relationship between H3K9me3 and aberrant heterochromatin structures. Notably, in AD patients, gene expression levels negatively correlate with H3K9me3 occupancy in genomic regions (11). Another investigation indicated that while H3K27me3, a marker of dormant genes, remained unchanged, AD brains exhibited decreased levels of H3K4me3, which is indicative of active gene transcription. Nevertheless, the specific examination of histone methylation changes in vascular dementia remains relatively scarce. Based on previous research, we hypothesized that inhibiting restrictive histone methyltransferases would lead to gene activation and neuroprotection in ischemic conditions. Thus, the objective of this study was to evaluate the advantages of pharmacologically inhibiting H3K9 methylation in VD.

2. Materials and Methods

2.1. Experimental Design

Fifty-six male Wistar rats weighing 250-270 g were randomly divided into four groups:

1-Sham Group (n=14)

After anesthesia with ketamine (70 mg/kg) and xylazine (10 mg/kg), the common carotid artery (CCA) was exposed, and the neck wound was closed without carotid ligation. Rats received 5 µl of normal saline, the solvent for BIX01294, via intraventricular (ICV) injection three times a week for one month.

2-BIX01294 Group (n=14)

Surgery was performed in the neck midline without carotid ligation, and animals received BIX01294 (22.5 µg/kg dissolved in normal saline) via ICV injection three times a week (12).

3-Vascular Dementia (VD) Group (n=14)

Animals underwent permanent occlusion of the bilateral common carotid arteries to induce the VD model. Rats received 5 µl of normal saline via ICV injection three times a week for one month. Laser Doppler flowmetry confirmed the induction of cerebral hypoperfusion.

4-VD + BIX01294 Group (n=14)

VD was induced as previously described, followed by ICV injection of BIX01294 for one month (three times a week at a dosage of 22.5 µg/kg dissolved in normal saline). The mortality rate across all groups was 10-15%, and ultimately, 14 surviving rats were assigned to each group.

2.2. Stereotaxy and Cannula Implantation

The animals were placed in a stereotaxic device and anesthetized with an intraperitoneal injection of ketamine hydrochloride and xylazine. A 23-gauge guide cannula was positioned 1 mm above the intended injection site according to Paxinos and Franklin's atlas (2001). The stereotaxic coordinates for the right lateral ventricle were DV: 2.9 mm from the skull surface, ML: 1.4 mm from the sagittal suture, and AP: -0.9 mm from the bregma. Dental acrylic was used to secure the cannula, and 27-gauge stainless steel stylets were inserted to keep the guiding cannula free of debris. Each animal was allowed one week to recover from the anesthesia and surgery (13).

2.3. Induction of VD model (common carotid artery occlusion)

One week following stereotaxic surgery, rats were anesthetized with xylazine (10 mg/kg) and ketamine hydrochloride (70 mg/kg). A ventral midline incision was made in the neck region to carefully separate the left and right CCAs from the adjacent vagus nerve, which were then permanently ligated with a 6-0 silk suture.

2.4. Nissl staining

Nissl staining was performed following the protocol described by Ooigawa et al. (2006) (14). Briefly, coronal

sections (7 microns thick) of paraffin-embedded fixed samples were prepared using a rotary microtome and placed on albumin-coated slides. Following hydration and clarification, the slides were stained with 1% cresyl violet, and sections were mounted with a cover slip. The number of dark neurons in the right hemisphere slices was counted to assess neuronal damage in the hippocampal region.

2.5. Evaluating the hippocampal Bax, and Bcl2 protein levels by Western Blotting

The prefrontal tissue was stored at -80°C after being frozen in liquid nitrogen. All samples were homogenized in the appropriate lysis solution and centrifuged at 15,000 rpm for 5 minutes to extract total protein. The protein concentration in the supernatants was measured using the Bradford method. Standardized lysates corresponding to 30 µg of protein were loaded onto SDS-PAGE (12.5% polyacrylamide gel) and transferred to a PVDF membrane (Abcam, UK). The membranes were then blocked with 2% electrochemiluminescence blocking reagent from the Advanced Kit (Sigma Aldrich, USA) and probed overnight with primary antibodies. Subsequently, membranes were treated with rabbit IgG-horseradish peroxidase-conjugated secondary antibodies, which were detected using the reagent from the chemiluminescence kit. For detection of β-actin as an internal control, blots were stripped with stripping buffer (pH 6.7) and then probed with an anti-β-actin antibody. All antibodies were sourced from Cell Signaling Technology, USA.

2.6. Evans Blue Test

To evaluate the permeability of the blood-brain barrier (BBB), the animals received an intravenous injection of Evans blue (2%, 2 ml/kg; Sigma 206334). Two hours later, the rats were anesthetized and perfused with saline through the left ventricle. Following euthanasia, the brains were removed, and the cerebellum was separated. The brain was weighed and homogenized in 2 ml of 50% trichloroacetic acid, then subjected to centrifugation at 10,000 rpm for 20 minutes. The supernatant was diluted 1:3 with ethanol, and the absorbance of Evans blue was measured using a spectrophotometer at 620 nm. The absorbance values were compared against a standard curve (Figure 2C), and data were reported as micrograms of Evans blue per gram of tissue.

2.7. Brain Water Content

On the thirtieth day, the animals were deeply anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) until death. Their brains were then removed and transferred to pre-weighed vials. The wet brain vials were measured meticulously before undergoing a controlled incubation period of 48 hours at 100°C. After incubation, the vials were reweighed to determine the dry weight of the brain. The brain water content was calculated using the following formula:

$$\frac{\text{Wet brain (g)} - \text{Dry brain (g)}}{\text{Wet brain weight (g)}} \times 100$$

2.8. Statistical Analysis

All data were evaluated using one-way analysis of variance (ANOVA), with multiple comparisons performed using Tukey's HSD test. Data are presented as mean ± S.E.M., with a p-value of less than 0.05 indicating a significant difference between groups.

3. Results

3.1. BIX01294 Injection Reduced Neural Damage in Vascular Dementia

Chronic vascular dementia (VD) induction significantly increased the rate of neuronal death in the hippocampus, as determined by Nissl staining ($P<0.001$). However, one month of BIX01294 treatment prevented hippocampal injury and decreased neuronal death, as shown in Figure 1 ($P<0.05$).

3.2. Treatment with BIX01294 Decreased Brain Edema in Vascular Dementia

As illustrated in Figure 2A, VD induction led to significant cerebellar edema. The brain water content in the VD group was significantly higher than in the sham group ($P < 0.01$). In contrast, BIX01294 treatment in the treatment group significantly reduced cerebral edema ($P<0.05$).

3.3. BIX01294 Treatment Recovered BBB Integrity in Vascular Dementia

We assessed BBB disruption during VD using Evans blue dye. Leakage of Evans blue, indicative of BBB injury, was significantly higher in the VD group than in the sham group ($P<0.01$). BIX01294 treatment significantly decreased Evans blue levels and improved BBB permeability ($P<0.05$) (Figure 2B).

3.4. The Bax/Bcl2 Ratio in Vascular Dementia Was Reduced by BIX01294 Treatment

Compared to the sham group, the expression of the pro-apoptotic protein Bax increased during the experimental period, while the anti-apoptotic protein Bcl2 significantly decreased in the VD group ($P<0.0001$). Consequently, the Bax/Bcl2 ratio was elevated in the VD group compared to the sham group, indicating the activation of apoptotic pathways. However, the administration of BIX01294 in the treatment group significantly lowered the Bax/Bcl2 ratio ($P<0.0001$) (Figure 3).

4. Discussion

Age-related diseases, including neurodegeneration, are more likely to develop as epigenetic machinery errors accumulate over time. Many brain diseases affecting the elderly lead to dementia by impairing synaptic plasticity, which in turn affects memory and learning. In this study, we demonstrate that inhibition of transcriptional repressors at the histone methylation level can effectively enhance neuronal survival in an in vivo model of vascular dementia. Previous studies have shown that histone methylation significantly impacts neuronal survival under various disease conditions.

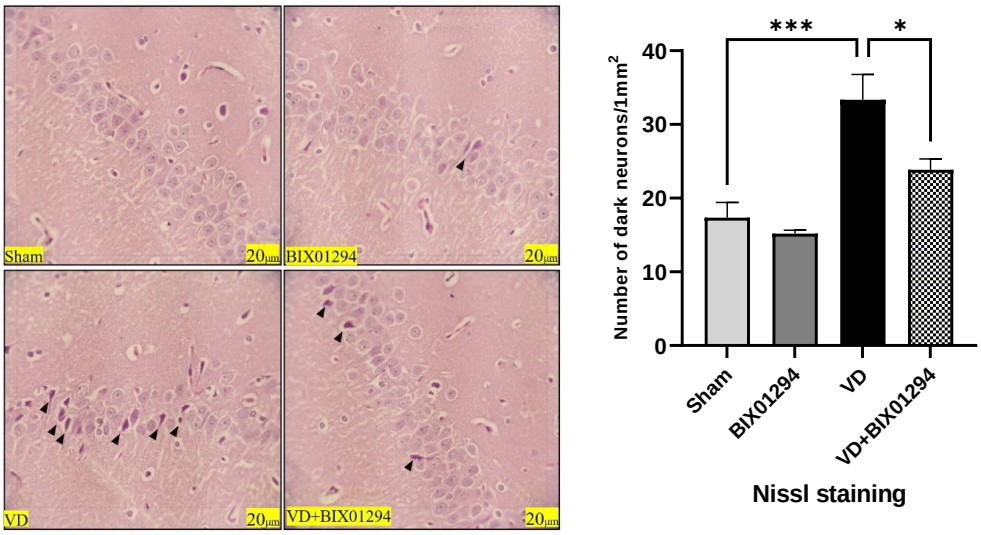

Figure. 1. Histological change of hippocampus CA1 area cells in Nissl staining. Data are presented as Mean ± S.E.M. *indicates significant differences with vascular dementia (VD) group. *P < 0.05 and ***P< 0.001.

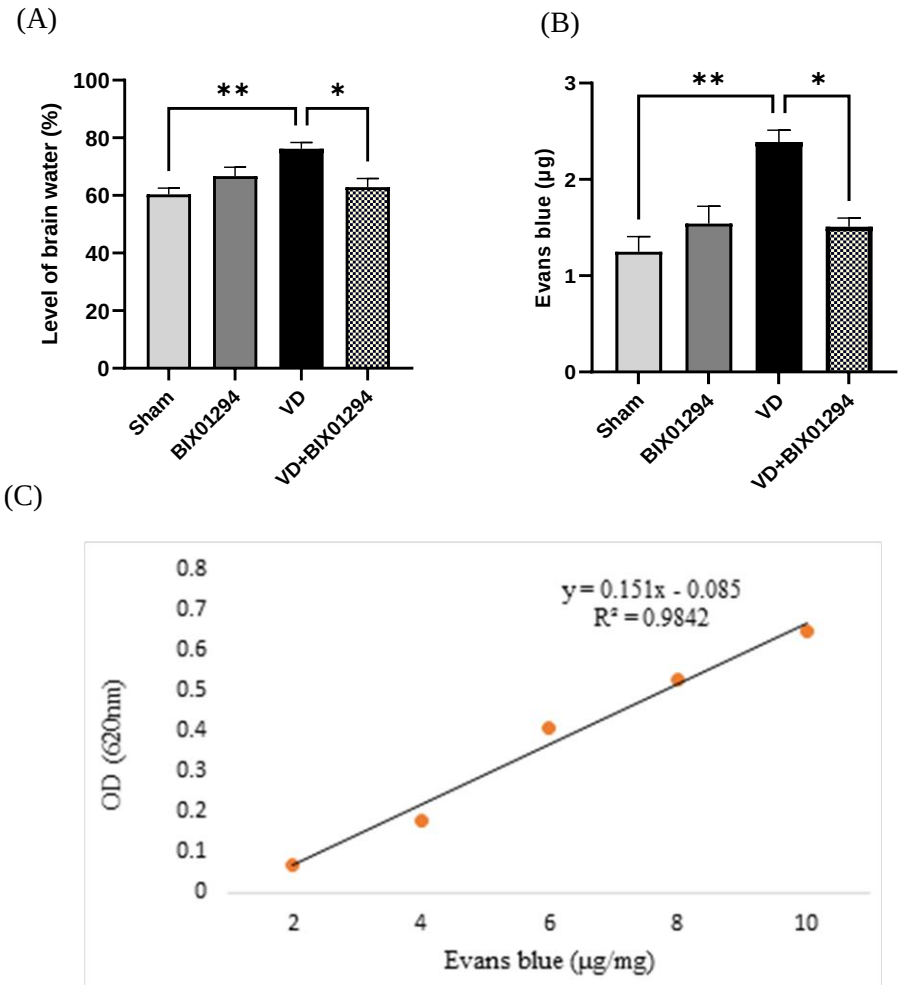

Figure. 2. A) Brain water content (n=3). **B**) Evans blue test (n=3). Data are presented as Mean ± S.E.M. * indicates significant differences with vascular dementia (VD) group. *P < 0.05, **P< 0.01. **C)** Evans blue standard curve, OD= optical density.

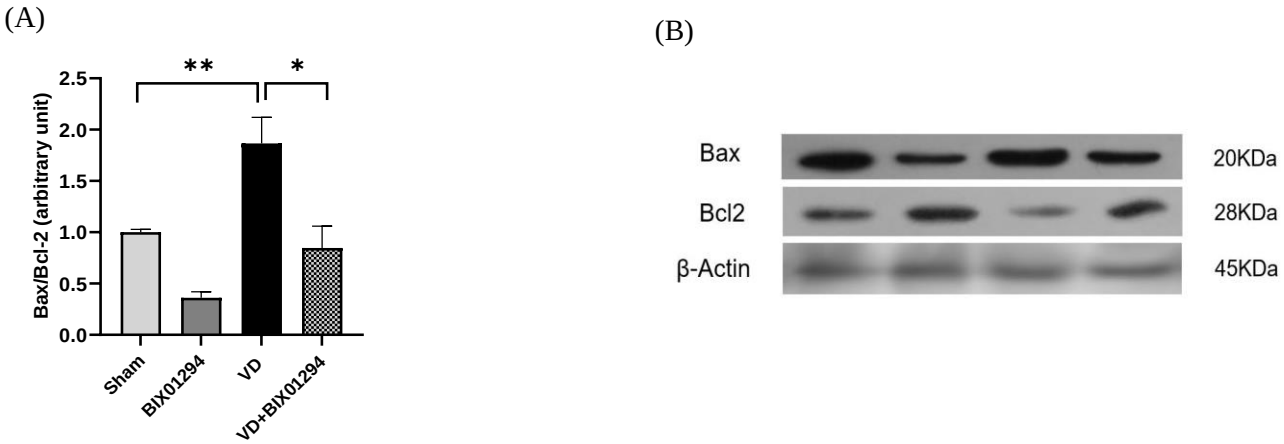

Figure. 3. A) Represented blot of Bax/Bcl2 (n=3). **B**) The density of Bax, Bcl2 and β-Actin proteins band which measured in the hippocampus area (n=3). Data are presented as Mean ± S.E.M. * indicates significant differences with vascular dementia (VD) group. **P<0.01.

For instance, G9a enzyme transfection in a rat model of hypoxia led to an increase in apoptotic cells, while sevoflurane treatment reduced apoptosis by inhibiting G9a expression. Elevated levels of two H3K9me2-specific enzymes, SUV39H1 and G9a, have been observed following strokes. Furthermore, G9a-specific inhibitors A-366 and BIX01294 have been reported to prevent the degeneration of neurons and astrocytes while decreasing the volume of brain infarcts caused by strokes. In another study, administration of docosahexaenoic acid (DHA) was found to increase H3 and Bcl2 acetylation while decreasing H3 methylation and caspase-3, suggesting that positive regulation of histone modifications can reduce neuronal damage. Conversely, some studies report that G9a enzyme activity decreases neural death in renal carcinoma cells, with its inhibition by BIX01294 preventing tumor growth through increased apoptosis. Our findings support the theory that G9a/GLP complex inhibition reduces Bax protein levels and increases Bcl2, thereby preventing neuronal death in vascular dementia. Nissl staining also indicates that BIX01294 significantly reduces brain injury compared to the VD group. Histone methylation and G9a enzyme activity are known to be beneficial, even essential, for numerous physiological processes. However, unlike neurological diseases, the pathological conditions underlying cancer are distinct, characterized by uncontrolled cell division, where upregulating G9a expression aids in tumor growth regulation. Additionally, our study demonstrates that BIX01294-inhibited H3K9 methylation enhances BBB integrity and reduces cerebral edema. The BBB regulates molecular exchange between the blood and brain, thus maintaining the brain's environment for essential processes. However, components of the BBB can be altered, and its function compromised in certain conditions, such as aging, hypertension, and cerebral ischemia. In recent years, numerous attempts have been made to repair epigenetic modifications to mitigate BBB damage. For example, a study utilizing valproic acid and sodium as histone deacetylase (HDAC) inhibitors in a rat model of ischemic stroke reported benefits in reducing tight junction (TJ) protein degradation, such as Claudin-5 and ZO-1. In another study, HDAC inhibitor treatment in rats subjected to cerebral ischemia/reperfusion injury resulted in increased expression of TJ proteins, including ZO-1, Occludin, and Claudin-5, thus stabilizing the BBB. Imakito et al. (2019) investigated cerebral edema and BBB dysfunction in a mouse model of viral encephalopathy, finding that the influenza virus caused BBB breakdown by degrading TJ proteins. Their assessment of 84 enzymes controlling histone structure during viral encephalopathy revealed a significant increase in the expression of the H3K9 methyltransferase Setdb2 compared to the control group. Moreover, immunoprecipitation on chromatin demonstrated that the Caveolin-1 promoter exhibited heightened H3K9 methylation, leading to decreased Caveolin-1 expression and compromised BBB integrity. In conclusion, H3K9me2 inhibitors may reduce neural damage in vascular dementia by improving BBB integrity and preventing cerebral edema formation.

Acknowledgment

The authors acknowledge the grant support of the Tehran University of Medical Sciences fund, Iran, for the current PhD thesis (grant no. 1401-3-101-62531), National Institute for Medical Research Development (NIMAD), Iran (Grant no. 4000041), and Cognitive Sciences and Technologies Council with follow-up code 12255.

Authors' Contribution

F.S contributed to acquisition of data, analysis and interpretation of data and drafting of the manuscript. M.R, F.N, and SM.K contributed to critical revision of the manuscript for important intellectual content and statistical analysis. S.H contributed to western blot test. G.A contributed to study concept and design and also administrative, technical, and material support.

Ethics

The Experimental procedure were confirmed by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran (Approval code: IR. TUMS.MEDICINE.REC.1400.651) which is in accordance with international guidelines for animal experiments (The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments)).

Conflict of Interest

The authors affirm that they have no conflict of interests. Furthermore,the authers hereby confirm that we have reviewed and complied with the relevant Instructions to Authors, the Ethics in Publishing policy, and Conflicts of Interest disclosure.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

References

1. DeTure MA, Dickson DW. The neuropathological diagnosis of Alzheimer's disease. Mol Neurodegener. 2019;14(1):1–18.
2. Yang Y, Zhao X, Zhu Z, Zhang L. Vascular dementia: A microglia's perspective. Ageing Res Rev. 2022;101734.
3. Karlić R, Chung HR, Lasserre J, Vlahoviček K, Vingron M. Histone modification levels are predictive for gene expression. Proc Natl Acad Sci. 2010;107(7):2926–31.
4. Dong X, Weng Z. The correlation between histone modifications and gene expression. Epigenomics. 2013;5(2):113–6.
5. Afonso J, Shim WJ, Boden M, Fortes MRS, da Silva Diniz WJ, de Lima AO, et al. Repressive epigenetic mechanisms, such as the H3K27me3 histone modification, were predicted to affect muscle gene expression and its mineral content in Nelore cattle. Biochem Biophys Reports. 2023;33:101420.
6. Jhelum P, C Karisetty B, Kumar A, Chakravarty S. Implications of epigenetic mechanisms and their targets in cerebral ischemia models. Curr Neuropharmacol. 2017;15(6):815–30.
7. Chakravarty S, Jhelum P, Bhat UA, Rajan WD, Maitra S, Pathak SS, et al. Insights into the epigenetic mechanisms involving histone lysine methylation and demethylation in ischemia induced damage and repair has therapeutic implication. Biochim Biophys Acta (BBA)-Molecular Basis Dis. 2017;1863(1):152–64.
8. Schweizer S, Harms C, Lerch H, Flynn J, Hecht J, Yildirim F, et al. Inhibition of histone methyltransferases SUV39H1 and G9a leads to neuroprotection in an in vitro model of cerebral ischemia. J Cereb Blood Flow Metab. 2015;35(10):1640–7.
9. Wilson C, Giono LE, Rozes-Salvador V, Fiszbein A, Kornblihtt AR, Caceres A. The histone methyltransferase G9a controls axon growth by targeting the RhoA signaling pathway. Cell Rep. 2020;31(6).
10. Nativio R, Lan Y, Donahue G, Sidoli S, Berson A, Srinivasan AR, et al. An integrated multi-omics approach identifies epigenetic alterations associated with Alzheimer's disease. Nat Genet. 2020;52(10):1024–35.
11. Lee MY, Lee J, Hyeon SJ, Cho H, Hwang YJ, Shin J, et al. Epigenome signatures landscaped by histone H3K9me3 are associated with the synaptic dysfunction in Alzheimer's disease. Aging Cell. 2020;19(6):e13153.
12. Gupta-Agarwal S, Franklin A V., DeRamus T, Wheelock M, Davis RL, McMahon LL, et al. G9a/GLP histone lysine dimethyltransferase complex activity in the hippocampus and the entorhinal cortex is required for gene activation and silencing during memory consolidation. J Neurosci. 2012;32(16):5440–53.
13. Zarrindast MR, Nasehi M, Piri M, Bina P. Anxiety-like behavior induced by histaminergic agents can be prevented by cannabinoidergic WIN55, 212-2 injected into the dorsal hippocampus in mice. Pharmacol Biochem Behav. 2010;94(3):387–96.
14. Ooigawa H, Nawashiro H, Fukui S, Otani N, Osumi A, Toyooka T, et al. The fate of Nissl-stained dark neurons following traumatic brain injury in rats: difference between neocortex and hippocampus regarding survival rate. Acta Neuropathol. 2006;112:471–81.
15. Farbood Y, Sarkaki A, Khalaj L, Khodagholi F, Badavi M, Ashabi G. Targeting Adenosine Monophosphate-Activated Protein Kinase by Metformin Adjusts Post-Ischemic Hyperemia and Extracellular Neuronal Discharge in Transient Global Cerebral Ischemia. Microcirculation. 2015;22(7):534–41.
16. Soltani N, Soltani Z, Khaksari M, Ebrahimi G, Hajmohammmadi M, Iranpour M. The changes of brain edema and neurological outcome, and the probable mechanisms in diffuse traumatic brain injury induced in rats with the history of exercise. Cell Mol Neurobiol. 2020;40(4):555–67.
17. Cacabelos R, Teijido O. Epigenetic drug discovery for Alzheimer's disease. Epigenetics aging Longev. 2018;453–95.
18. Shan W, Wu Y, Han X, Chen Q, Wu J. The mechanism of sevoflurane post-treatment alleviating hypoxic-ischemic encephalopathy by affecting histone methyltransferase G9a in rats. Bioengineered. 2021;12(2):9790–805.
19. Sharifulina S, Dzreyan V, Guzenko V, Demyanenko S. Histone methyltransferases SUV39H1 and G9a and DNA

methyltransferase DNMT1 in penumbra neurons and astrocytes after photothrombotic stroke. Int J Mol Sci. 2021;22(22):12483.
20. Sadli N, Ackland ML, De Mel D, Sinclair AJ, Suphioglu C. Effects of zinc and DHA on the epigenetic regulation of human neuronal cells. Cell Physiol Biochem. 2012;29(1–2):87–98.
21. Shi R, Zheng C bo, Wang H, Rao Q, Du T, Bai C, et al. Gastrodin alleviates vascular dementia in a 2-VO-vascular dementia rat model by altering amyloid and tau levels. Pharmacology. 2020;105(7–8):386–96.
22. Padurariu M, Ciobica A, Lefter R, Serban IL, Stefanescu C, Chirita R. The oxidative stress hypothesis in Alzheimer's disease. Psychiatr Danub. 2013;25(4):401–9.
23. Hudson LC, Bragg DC, Tompkins MB, Meeker RB. Astrocytes and microglia differentially regulate trafficking of lymphocyte subsets across brain endothelial cells. Brain Res. 2005;1058(1–2):148–60.
24. Montagne A, Barnes SR, Sweeney MD, Halliday MR, Sagare AP, Zhao Z, et al. Blood-brain barrier breakdown in the aging human hippocampus. Neuron. 2015;85(2):296–302.
25. Erickson MA, Banks WA. Age-associated changes in the immune system and blood–brain barrier functions. Int J Mol Sci. 2019;20(7):1632.
26. Park MJ, Sohrabji F. The histone deacetylase inhibitor, sodium butyrate, exhibits neuroprotective effects for ischemic stroke in middle-aged female rats. J Neuroinflammation. 2016;13:1–14.
27. Wang Z, Leng Y, Tsai LK, Leeds P, Chuang DM. Valproic acid attenuates blood–brain barrier disruption in a rat model of transient focal cerebral ischemia: the roles of HDAC and MMP-9 inhibition. J Cereb Blood Flow Metab. 2011;31(1):52–7.
28. Su L, Liang D, Kuang SY, Dong Q, Han X, Wang Z. Neuroprotective mechanism of TMP269, a selective class IIA histone deacetylase inhibitor, after cerebral ischemia/reperfusion injury. Neural Regen Res. 2020;15(2):277.
29. Imakita N, Kitabatake M, Ouji-Sageshima N, Hara A, Morita-Takemura S, Kasahara K, et al. Abrogated Caveolin-1 expression via histone modification enzyme Setdb2 regulates brain edema in a mouse model of influenza-associated encephalopathy. Sci Rep. 2019;9(1):284.