Ketogenic diet alleviates neuronal ferroptosis in epilepsy via HDAC4/TFRC signalling

Keywords
ketogenic diet, epilepsy, ferroptosis, HDAC4, TFRC

Abstract

Introduction

Ketogenic diets (KD) recapitulate certain metabolic aspects of dietary restriction such as reliance on fatty acid metabolism and production of ketone bodies. This study aimed to investigate whether a KD might, like dietary restriction, affect brain functions in epilepsy.

Material and methods

Kainic acid (KA) injection was used to establish epilepsy model in vivo. Histone deacetylation 4 (HDAC4) mRNA expression was determined using RT-qPCR. Protein expression was detected using western blot. Gene expression was determined using immunofluorescence. The release of malondialdehyde (MDA), ferrous iron, and glutathione (GSH) was detected using corresponding commercial kits. The interaction between HDAC4 and transferrin receptor (TFRC) was verified using co-immunoprecipitation assay. Neuronal viability was detected using cell counting kit 8 (CCK-8) assay. Neuronal death was detected using propidium iodide (PI) staining.

Results

Epilepsy mediated iron accumulation- and lipid peroxidation-induced neuronal ferroptosis. Interestingly, KD treatment alleviated epilepsy as well as the accumulation of ferrous iron and lipid peroxidation, resulting in the inhibition of neuronal ferroptosis in epileptic models in vivo and in vitro. Mechanically, KD promoted the upregulation of HDAC4, which inhibited the acetylation of TFRC and suppressed its protein expression. However, downregulation of HDAC4 by its specific inhibitor LMK235 promoted the ferroptosis of neurons.

Conclusions

Collectively, KD protect against the ferroptosis of neurons in epilepsy via promoting HDAC4-mediated deacetylation and downregulation of TFRC. Therefore, KD may be a promising strategy for epilepsy.

Ketogenic diet alleviates neuronal ferroptosis in epilepsy via HDAC4/TFRC signalling

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Shot title: the effects of KD on epilepsy.

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Introduction

Epilepsy ranks as the second most common neurological disorder worldwide, affecting over 65 million patients globally [1]. Temporal lobe epilepsy (TLE) is the most common type of epilepsy [2,3]. The incidence of TLE is 32 to 82 cases per 10,000. Status epilepticus induces damages of nervous system and hippocampal neurons [4]. The progression of epilepsy promotes the increase in lipid peroxidation, ischemia, inflammation, and oedema, which are the key factors for the loss of hippocampal neurons, and hippocampal sclerosis [1,5]. Although great advances have been made in the treatment of epilepsy, such as anticonvulsant drugs (AEDs), long-term use of AEDs may induce drug-resistant epilepsy [6,7]. Therefore, to develop a novel strategy for epilepsy is urgently needed.

Ketogenic diets (KD) (a high-fat, adequate-protein, and low-carbohydrate diet) are a non-pharmacological treatment designed to mimic the effects of starvation in drug-resistant individuals [8]. The benefits of a KD in paediatric patients include improved seizure control, weight loss, as well as improved insulin sensitivity [9]. Ketones may confer neurologic protection. Ketone bodies exert anti-oxidant, anti-inflammatory, as well as induce cellular, epigenetic, and gut-microbiome alterations. KD show a positive impact on behavioral and cognitive functions [10]. However, the underlying mechanisms are still unknown.

Ferroptosis is a form of programmed cell death, characterized by lipid peroxidation and iron overload [11]. Ferroptosis is regulated by following three pathways: oxidative stress, lipid peroxidation, and iron metabolism [12,13]. The activation of ferroptosis-related pathways is collectively involved in the pathogenesis of epilepsy. For instance, hypoxia inducible factor-1α (HIF-1α)/ Heme oxygenase-1 (HO-1) pathway suppresses neuronal ferroptosis in epilepsy via inhibiting oxidant stress [14]. Neurotoxic A1-mediated lipid peroxidation promotes the ferroptosis of neurons in epilepsy via downregulating solute carrier family 7a member 11 (SLC7A11) [15]. Mitochondrial ferritin, which functions as an iron transporter, suppresses ferroptosis of neurons through maintaining iron homeostasis [16].

This study aimed to investigate the effects of KD on the neuron functions in the

progression of epilepsy. The effects of KD on iron homeostasis and neuron functions may open a new light on the progression of epilepsy.

Materials and methods

Cell culture

Mouse hippocampal neuronal cells (HT22) were provided by Procell (Wuhan, China). Cells were incubated with Dulbecco's modified eagle medium (DMEM) supplemented and less sugar DMEM with 10% fetal bovine serum (FBS) at 37℃ in an incubator with 5% CO_2.

Cells were treated with Mg^{2+}, Erastin, RSL3, and Fer-1, as well as the inhibitor of apoptosis (z-VAD-fmk), pyroptosis (PBzyme) and necroptosis (Nec-1s).

RT-qPCR

Cells were lysed using TRIzol buffer (R0016; Byyotime, China) and the total RNA was collected. RNA concentration and purity was detected using UV spectrophotometer (Bio-Rad, USA). Then RNA was reversely transcribed into cDNA using a reverse transcription kit (RR037B; Takara, Japan). Relative mRNA expression was determined using a SYBR® Premix Ex Taq™ kit (DRR041A; Takara, Japan) and calculated using a $2^{-\Delta\Delta Cq}$ method. The sequences of the primers used in PCR were as followed: histone deacetylase 4 (HDAC4), F: 5'-CTGCAAGTGGCCCCTACAG-3' and R: 5'-CTGCTCATGTTGACGCTGGA-3'; and glyceraldehyde-3-phosphate dehydrogenase, F: 5'-AGGTCGGTGTGAACGGATTTG-3' and R: 5'-GGGGTCGTTGATGGCAACA-3'.

Western blot analysis

Proteins were extracted from HT22 cells. Following concentration and denaturation, the proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and moved onto polyvinylidene fluoride membranes, which were blocked with 5% skimmed milk. Then membranes were incubated with primary antibodies, such as anti- HDAC4 (ab240643; 1: 1000, Abcam, UK), anti-acetyl H3K9

(ab190479; 1: 1000, Abcam, UK), and anti-transferrin receptor (TFRC) (ab214039; 1: 1000, Abcam, UK), and followed by secondary antibody (ab205718; 1: 10000, Abcam, UK). Finally, the immunoreactive bands were captured by using the enhanced chemiluminescence reagent. β-actin (ab227387; 1: 10000, Abcam, UK) served as loading control.

Co-immunoprecipitation (Co-IP) assay

Cells were lysed with radio immunoprecipitation assay lysis buffer (20-188; Millipore, USA). Afterwards, the protein lysates were collected and incubated with Protein A/G magnetic beads containing antibodies against HDAC4 (ab240643; 1: 30, Abcam, UK) and TFRC (ab214039; 1: 30, Abcam, UK). Subsequently, the beads were boiled and subjected to western blot assay.

Determination of malondialdehyde (MDA)

The MDA levels were detected using in cell lysates and measured using an MDA Kit (MAK568; Sigma-Aldrich, USA). Briefly, cells were lysed and centrifuged. Then the lysates were added with thiobarbituric acid. Finally, MDA levels were determined at wavelength of 532 nm.

Determination of intracellular iron levels

The intracellular iron levels were determined using a Colorimetric Iron Assay Kit (ab83366; Abcam, UK).

Glutathione (GSH) assay

GSH levels were determined using a GSH Assay Kit (ab112132; Abcam, UK). Briefly, cells were plated in a 24-well plate. After supplemented with Magnesium ion (Mg^{2+})-free solution, less sugar and Erastin, cells were lysed with GSH assay buffer. GSH was determined using a kinetic assay and calculated at the wavelength of 412 nm.

Cell counting kit (CCK-8) assay

After transfection, cells were collected and plated in a 24-well plate. Following the culture for 48h, the cells were supplanted with CCK-8 solutions (96992-100TESTS-F; Sigma-Aldrich, Germany) and mixed for another 90 min at 37℃. Subsequently, cell viability was quantified using a microplate reader at optic value of 450 nm.

Propidium iodide (PI) staining

After fixation in 4% paraformaldehyde and permeabilization with 0.2% Triton X-100, cells were blocked with 5% bovine serum. Then cells were stained with PI. The image was pictured by a microscope (M205 FA; Leica, Germany).

Animal experiment

Male Sprague Dawley (SD) rats (80–100 g) were purchased from Changsheng Biology (Liaoning, China). Rats were with free access to food and water. This study was approved by the Animal Care Board of The First Affiliated Hospital of Harbin Medical University.

Rats were randomly divided into three groups: sham group, kainite acid (KA) group, and KA+KD group. Rats in KA group were hypodermically injected with 10 mg/kg KA. Rats in KA+KD group were hypodermic injected with 10 mg/kg KA and administrated with KD formula (76% fat, 16% protein, 3% carbohydrate, and 5% dietary fibre in kcal) (Kuibuqianli Biology, Xuancheng, China). Rats in sham group were hypodermic injected with saline.

Immunofluorescence assay

After anaesthesia, the rats were sacrificed and the brain tissues were collected. The slides were fixed in paraffin, deparaffinized and immersed in ethylenediaminetetra-acetic acid. buffer After blocked with 1% bovine serum albumin regents, the sections were incubated with primary antibodies against NeuN (ab177487; 1: 100, Abcam, UK) and HDAC4 (ab313474; 1: 200, Abcam, UK), and then with secondary antibody (ab150077; 1: 200, Abcam, UK). Nuclei was counterstained with 4',6-diamidino-2-phenylindole. Finally, the images were captured using a microscope (M205 FA; Leica,

Germany).

Statistical analysis

Data were presented as mean ± standard deviation. Two-group comparison was analysed using Student *t* test. Multi-group comparison was analysed using analysis of variance. $P<0.05$ deemed as statistically significant.

Results

Epilepsy-induced ferroptosis-related neuronal loss

Ferroptosis-mediated neuron loss is involved in the progression of epilepsy. To further confirm this, neurons were treated with ferroptosis inducer (Erastin) as well as the inhibitor (Fer-1) after exposed to Magnesium ion (Mg^{2+})-free solution. We found that Erastin treatment significantly enhanced the effects of Mg^{2+} treatment and suppressed cell viability of neurons, suggesting that the progression of epilepsy may induce neuronal ferroptosis. However, epilepsy can induce other forms of cell death, such as apoptosis, pyroptosis and necroptosis. To further confirm this, cells were treated with the inhibitor of apoptosis (z-VAD-fmk), pyroptosis (PBzyme) and necroptosis (Nec-1s). As shown in Figure 1B, the decrease in the cell viability induced by Mg^{2+} treatment was significantly alleviated by Fer-1, which showed no significant alteration by the inhibitors of apoptosis, pyroptosis, and necroptosis. Mg^{2+} treatment significantly increased the release of MDA and ferrous iron (Fe^{2+}), and decreased GSH (Figure 1C-E), which was significantly enhanced by Erastin, but reversed by Fer-1. Moreover, the increase in the percentages of PI positive cells was significantly enhanced by Erastin (Figure 1F-G), whereas reversed by Fer-1. These findings confirmed that the progression of epilepsy is accompanied with the ferroptosis of neurons.

KD suppresses the ferroptosis of neurons induced by epilepsy in vitro

KD is an effective strategy for epilepsy. To conform this, HT22 cells were treated with KD after Mg^{2+} treatment. We found that the cell viability was significantly decreased by Mg^{2+} treatment, which was reversed KD treatment. KD treatment also significantly

antagonized the effects of Mg^{2+} treatment and decreased the release of MDA and ferrous iron (Fe^{2+}), and increased GSH (Figure 2B-D). KD treatment significantly reduced the percentages of PI positive cells induced by Mg^{2+} treatment (Figure 2E-F). These findings suggested that KD treatment alleviate neuronal ferroptosis in epilepsy.

HDAC4 is downregulated in epilepsy

GDS954 was used to analyze the genes that differentially expressed in epilepsy. We found that HDAC4 was significantly decreased (not presented in this study). HDAC4 participates in the progression of epilepsy. Then we determined HDAC4 expression in epilepsy in vitro models. We found that HDAC4 mRNA expression was significantly reduced after Mg^{2+} treatment (Figure 3A). This was paralleled with the results from western blot assay. As shown in Figure 3B-C, Mg^{2+} treatment significantly suppressed HDAC4 protein expression.

HDAC4 deficiency promotes the ferroptosis of neurons

To further confirm the roles of HDAC4 in epilepsy, neurons were treated with HDAC4 specific inhibitor LMK235. As shown in Figure 4A, KD treatment-mediated restoration of neuronal viability was significantly alleviated by LMK235. LMK235 also significantly promoted the release of MDA and Fe^{2+} (Figure 4B-C), whereas decreased GSH (Figure 4D). Moreover, LMK235 treatment significantly antagonized the effects of KD treatment and increased the percentages of PI positive cells (Figure 4E-F). These findings suggested that KD inhibits the ferroptosis of neurons in epilepsy via upregulating HDAC4.

HDAC4 promotes the histone deacetylation of TFRC

Ferroptosis is characterized by iron overload induced lipid peroxidation [17]. Therefore, we hypothesized that iron metabolism may be involved in the progression of epilepsy. TFRC, as an iron uptaker, promotes the ferroptosis of neurons during the pathogenesis of epilepsy. We found that the protein expression as well as the global acetylation of TFRC was significantly increased after Mg^{2+} treatment (Figure 5A-B). HDAC4, as a

member of histone deacetylase, regulates gene expression via exerting its histone deacetylase activity. Therefore, we hypothesized that KD may regulate ferroptosis via regulating HDAC4/TFRC pathway. Endogenous IP assay showed that TFRC can interact with HDAC4 (Figure 5C). IP experiments results showed that overexpression of HDAC4 could significantly reduce the acetylation of TFRC (Figure 5D).

KD alleviates epilepsy progression in vivo

To further confirm the effects of KD on epilepsy, rats were administrated with KA and/or KD. We found that KD treatment significantly reduced the total number of seizures induced by KA (Figure 6A). Moreover, KD treatment also significantly decreased the number of mean seizures/week since week2 (Figure 6B). We further found that KD treatment significantly improved motor skill learning (Figure 6C) as well as hippocampal associative memory (Figure 6D).

As shown in Figure 7A-C, KA treatment significantly increased the release of MDA and Fe^{2+}, whereas decreased GSH; however, this was alleviated by KD administration. KA injection significantly promoted the loss of neurons, which was alleviated by KD administration (Figure 7D). Moreover, we also found that KD administration reversed the effects of KA treatment and increased the percentages of HDAC4+NeuN+ cells (Figure 7E). This further confirm that KD alleviated epilepsy-mediated ferroptosis of neuron via upregulating HDAC4.

Discussion

Neuronal loss is key factor for epilepsy [18,19]. In this study, KD alleviated epilepsy-induced neuronal ferroptosis. Moreover, KD induced the upregulation of HDAC4. However, HDAC4 deficiency alleviated the effects of KD and promoted neuronal ferroptosis. Additionally, HDAC4 promoted histone deacetylation of TFRC, inducing the downregulation of TFRC. Therefore, KD may protect against epilepsy-mediated ferroptosis of neurons via regulating HDAC4/TFRC signalling.

Increasing studies have demonstrated that KD is an effective treatment for epilepsy [7,20,21]. KD alleviates the drug resistance. Olson et al.[22] report that KD protects

against host metabolism and seizure susceptibility. KD alleviates neurodegeneration and psychiatric disorders [23]. These findings suggest that KD may exerted protective effects via modulating metabolisms and neuron functions. This study focuses on the effects of KD on cellular functions of neurons. We found that KD suppressed iron overload and lipid peroxidation in epilepsy, resulting in restoration neuron functions as well as inhibition of the ferroptosis-related death of neurons. Epilepsy is accompanied with the loss of neurons. Therefore, KD-mediated inhibition of neuronal ferroptosis can be promising strategy for epilepsy.

HDAC4 a key factor underlying brain developmental alterations [24,25]. For instance, PP1-mediated dephosphorylation and nuclear accumulation of HDAC4 contributes to the neurotoxic effect [26]. Phosphorylation of HDAC4 increases sleep need [27]. Nuclear accumulation of HDAC4 exacerbates cerebral ischemia-reperfusion-induced neuron damages [28]. Interestingly, cytoplasmic HDAC4 recovers synaptic function in Alzheimer's disease [29]. These findings suggested that the roles of HDAC4 may vary with its location in neurons. In this study, the cytoplasmic expression of HDAC4 was reduced in epilepsy. Moreover, HDAC4 deficiency abated the effects of KD and promoted iron overload and lipid peroxidation, conferring to neuronal ferroptosis. Therefore, KD may inhibit neuronal ferroptosis via regulating HDAC4.

HDAC4 participates in regulating biological processes via targeting its downstream. For instance, nuclear HDAC4 interacts with HDAC5 to promote histone deacetylation of Na+/Ca+ exchanger isoform 3 promoter, contributing to neuronal stroke damage [30]. Overexpressed HDAC4 drives histone deacetylation of myocyte enhancer factor 2 and suppresses neural activity in amyotrophic lateral sclerosis [31]. In this study, HDAC4 and TFRC colocalized in cytoplasm of neurons. Moreover, HDAC4-mediated histone deacetylation of TFRC downregulated the latter expression. TFRC is a key regulator in iron metabolism [32]. However, TFRC is frequently overexpressed in brain disorders, such as stroke, amyotrophic lateral sclerosis, hypoxic-ischemic brain damage, as well as epilepsy [33-36]. Overexpression of TFRC contributes to the continuous accumulation of ferrous iron and autophagic lysosome-mediated degradation of ferritin, and promotes lipid peroxidation and cell collapse, resulting in ferroptosis-related cell

death [37,38]. In this study, TFRC was upregulated in epilepsy. However, KD-mediated overexpression of HDAC4 suppressed TFRC expression as well as neuronal ferroptosis. Although KD may protect against neural loss via inhibiting neuronal apoptosis [23,39], ferroptosis is differentiated from apoptosis: apoptosis is characterized by the accumulation of apoptosome and executed by caspase cascades, while ferroptosis is characterized by iron overload-induced lipid peroxidation [40,41]. In this study, increased ferrous iron and lipid peroxidation indicated that the death of neuron in epilepsy was in the form of ferroptosis. Moreover, inhibition of apoptosis, pyroptosis, and necroptosis by its specific inhibitor showed no effects on restoring neuronal function, which confirm the above findings, which further confirmed that epilepsy-mediated neuronal death is in the form of ferroptosis.

Numerous studies have demonstrated the efficacy of the KD in reducing seizure frequency in patients with refractory epilepsy. For instance, 62.5% of children with refractory epilepsy experience a significant reduction in seizures within three days of starting the KD [42]. Noviawaty et al.[43] also demonstrate that KD leads to a >50% reduction in seizure frequency in 45% of adult patients. These findings highlight the potential of the KD as a therapeutic option for epilepsy.

Future clinical trials should aim to further investigate the long-term effects of the KD on seizure control and quality of life in patients with epilepsy. These studies should also explore the potential benefits of the KD in different epilepsy subtypes and patient populations. Mechanistic studies should focus on the detailed pathways involved in KD-induced modulation of neuronal ferroptosis and TFRC signalling. This could involve the use of advanced molecular and cellular techniques to study the effects of the KD on iron metabolism, oxidative stress, and neuronal survival. Research should also explore the potential of combining the KD with other therapeutic interventions, such as antiepileptic drugs or antioxidants, to enhance seizure control and neuroprotection. This could provide a more comprehensive approach to managing epilepsy and improving patient outcomes.

Conclusions

In conclusion, KD protect against epilepsy. KD suppress neuronal ferroptosis in epilepsy via promoting HDAC4-mediated histone deacetylation of TFRC. This may broaden the view of KD on the treatment of epilepsy.

Funding

None.

Data availability

The data generated in the present study may be requested from the corresponding author.

Ethics statement

This study was approved by the Animal Care Aboard of The First Affiliated Hospital of Harbin Medical University.

Competing interest

The authors have declared that no competing interest exists.

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Figure legends

Figure 1. Epilepsy-induced ferroptosis-related neuronal loss

(A-B) Cell viability was detected using CCK-8 assay. (C) The release of MDA was determined using MDA assay. (D) The release of ferrous iron was detected using ELISA assay. (E) The release of GSH was detected using GSH assay. (F-G) The death of neurons was determined using PI staining. MDA: malondialdehyde (MDA); GSH: glutathione; CCK-8: cell counting kit PI: Propidium iodide. $^{**}P<0.01$, $^{***}P<0.001$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$.

Figure 2. KD suppresses the ferroptosis of neurons induced by epilepsy in vitro

(A) Cell viability was detected using CCK-8 assay. (B) The release of MDA was determined using MDA assay. (C) The release of ferrous iron was detected using ELISA assay. (D) The release of GSH was detected using GSH assay. (E-F) The death of neurons was determined using PI staining. KD: ketogenic diet; MDA: malondialdehyde (MDA); GSH: glutathione; CCK-8: cell counting kit PI: Propidium iodide. $^{***}P<0.001$,

$^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$.

Figure 3. HDAC4 is downregulated in epilepsy

(A) HDAC4 mRNA expression was detected using RT-qPCR. (B-C) HDAC4 protein expression was detected using western blot. HDAC4: histone deacetylase 4. $^{***}P<0.001$.

Figure 4. HDAC4 deficiency promotes the ferroptosis of neurons

(A) Cell viability was detected using CCK-8 assay. (B) The release of MDA was determined using MDA assay. (C) The release of ferrous iron was detected using ELISA assay. (D) The release of GSH was detected using GSH assay. (E-F) The death of neurons was determined using PI staining. KD: ketogenic diet; MDA: malondialdehyde; GSH: glutathione; CCK-8: cell counting kit PI: Propidium iodide; HDAC4: histone deacetylase 4. $^{**}P<0.01$, $^{***}P<0.001$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, $^{\&\&}P<0.01$.

Figure 5. HDAC4 promotes the histone deacetylation of TFRC

(A-B) Protein expression was detected using western blot. (C-D) The interaction between HDAC4 and TFRC was detected using Co-IP assay. HDAC4: histone deacetylase 4; TFRC: transferrin receptor.

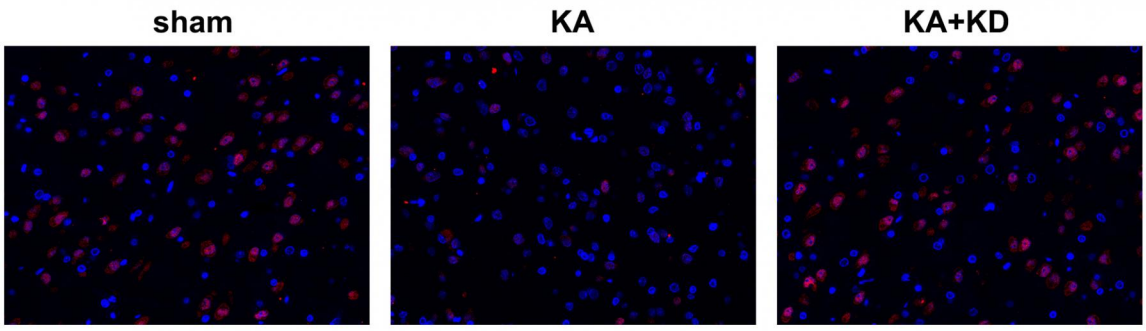
Figure 6. alleviates epilepsy progression in vivo

(A) Total number of seizures after KA and/or KD treatment. (B) Mean seizures per week after KA and/or KD treatment. (C) Motor learning skill test after KA and/or KD treatment. (D) Contextual fear conditioning testing after KA and/or KD treatment. KA: kainite acid; KD: ketogenic diet. $^{**}P<0.01$, $^{***}P<0.001$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$.

Figure 7. KD inhibits ferroptosis in vivo

(A) The release of MDA was determined using MDA assay. (B) The release of ferrous iron was detected using ELISA assay. (C) The release of GSH was detected using GSH assay. (D) NeuN expression in brain tissues was determined using immunofluorescence.

(E) NeuN and HDAC4 expression in brain tissues was determined using immunofluorescence. KA: kainite acid; KD: ketogenic diet; MDA: malondialdehyde (MDA); GSH: glutathione; HDAC4: histone deacetylase 4. ***P<0.001, $^{\#\#}P$<0.01.



KD suppresses the ferroptosis of neurons

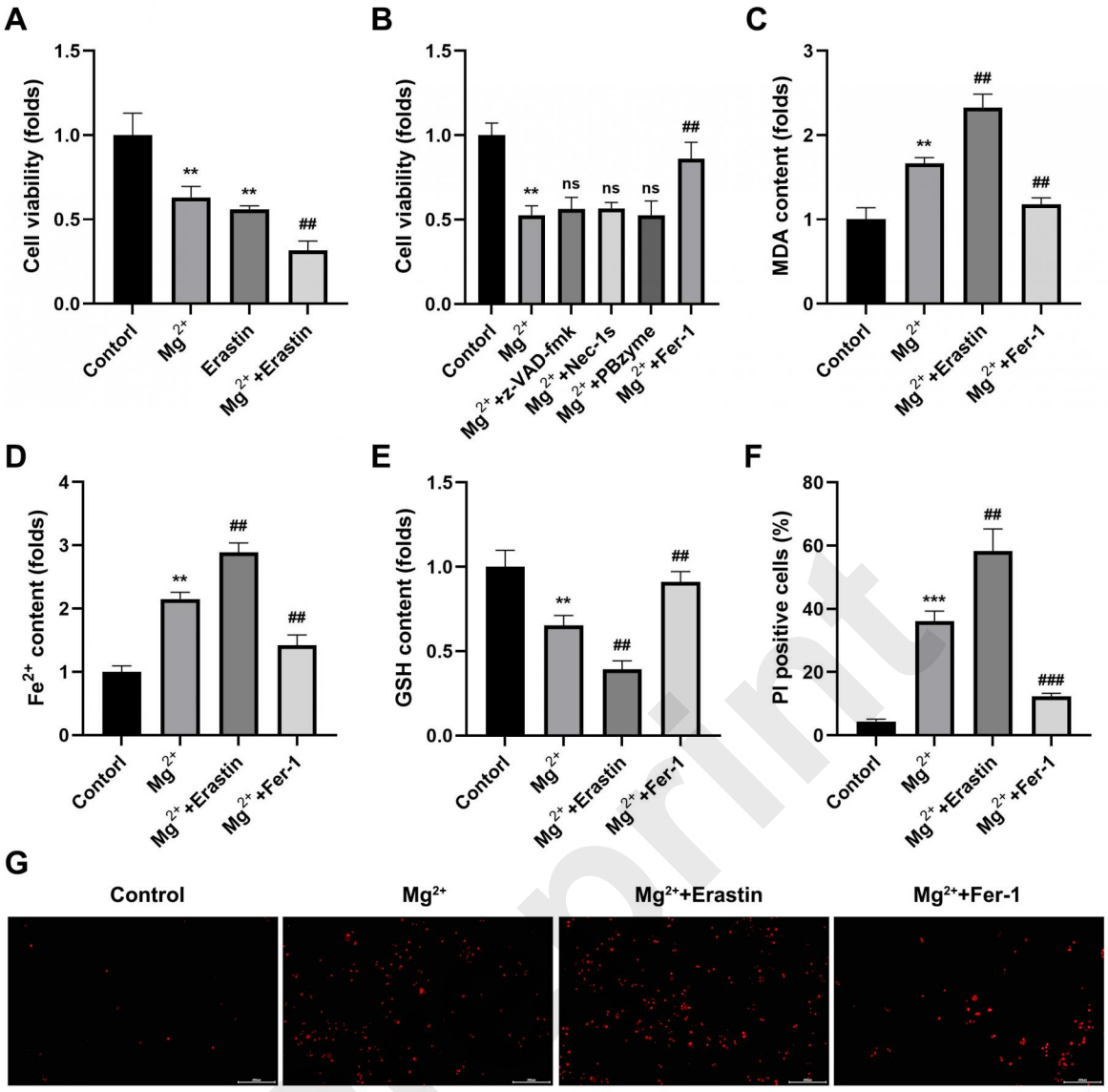


Figure 1. Epilepsy-induced ferroptosis-related neuronal loss
(A-B) Cell viability was detected using CCK-8 assay. (C) The release of MDA was determined using MDA assay. (D) The release of ferrous iron was detected using ELISA assay. (E) The release of GSH was detected using GSH assay. (F-G) The death of neurons was determined using PI staining. MDA: malondialdehyde (MDA); GSH: glutathione; CCK-8: cell counting kit PI: Propidium iodide. **P<0.01, ***P<0.001, ##P<0.01, ###P<0.001.

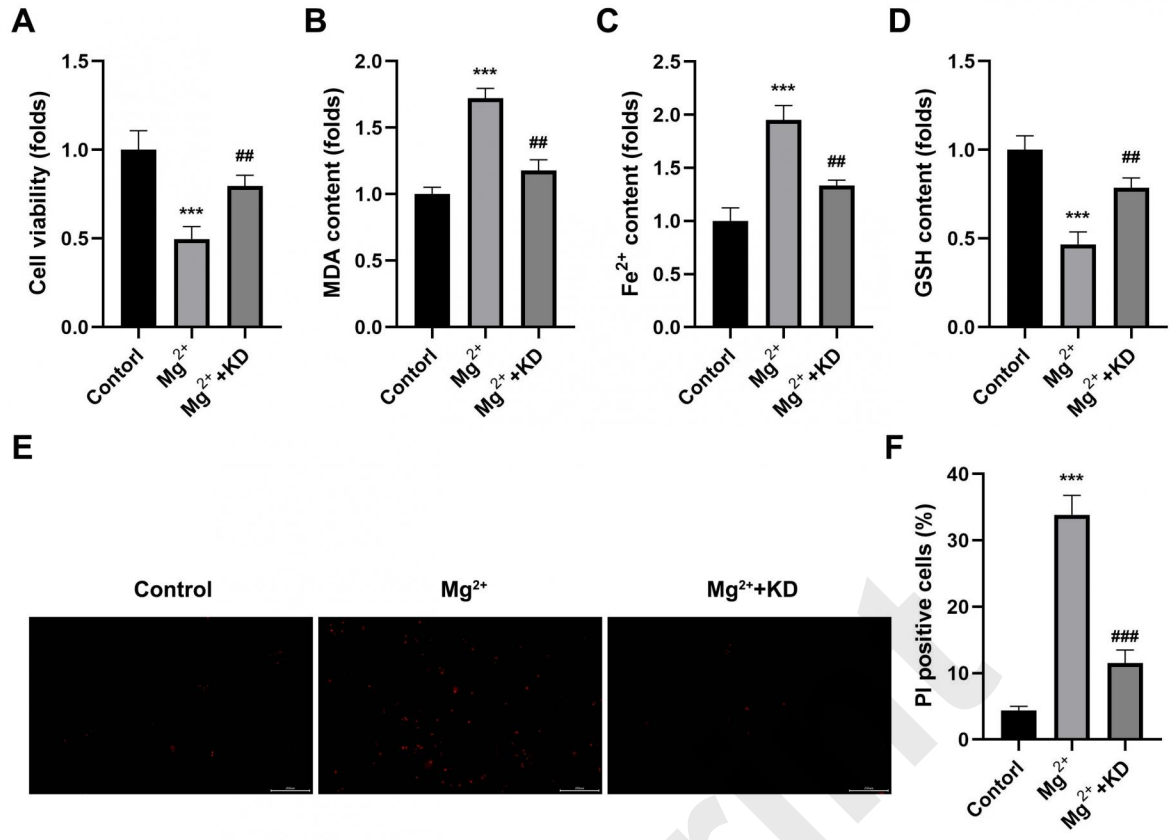


Figure 2. KD suppresses the ferroptosis of neurons induced by epilepsy in vitro
(A) Cell viability was detected using CCK-8 assay. (B) The release of MDA was determined using MDA assay. (C) The release of ferrous iron was detected using ELISA assay. (D) The release of GSH was detected using GSH assay. (E-F) The death of neurons was determined using PI staining. KD: ketogenic diet; MDA: malondialdehyde (MDA); GSH: glutathione; CCK-8: cell counting kit PI: Propidium iodide. ***P<0.001, ##P<0.01, ###P<0.001.

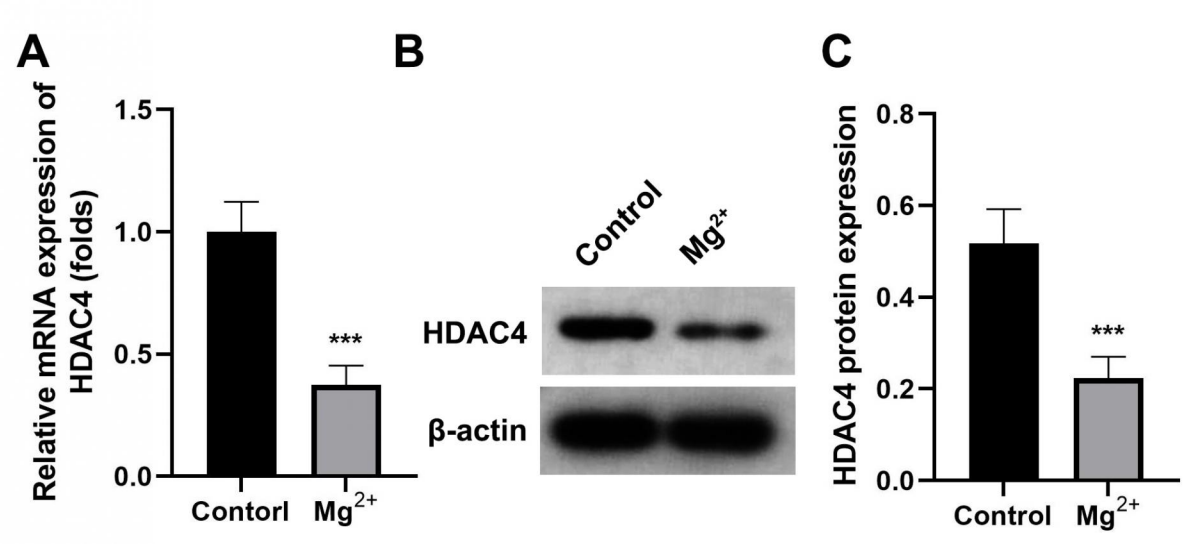
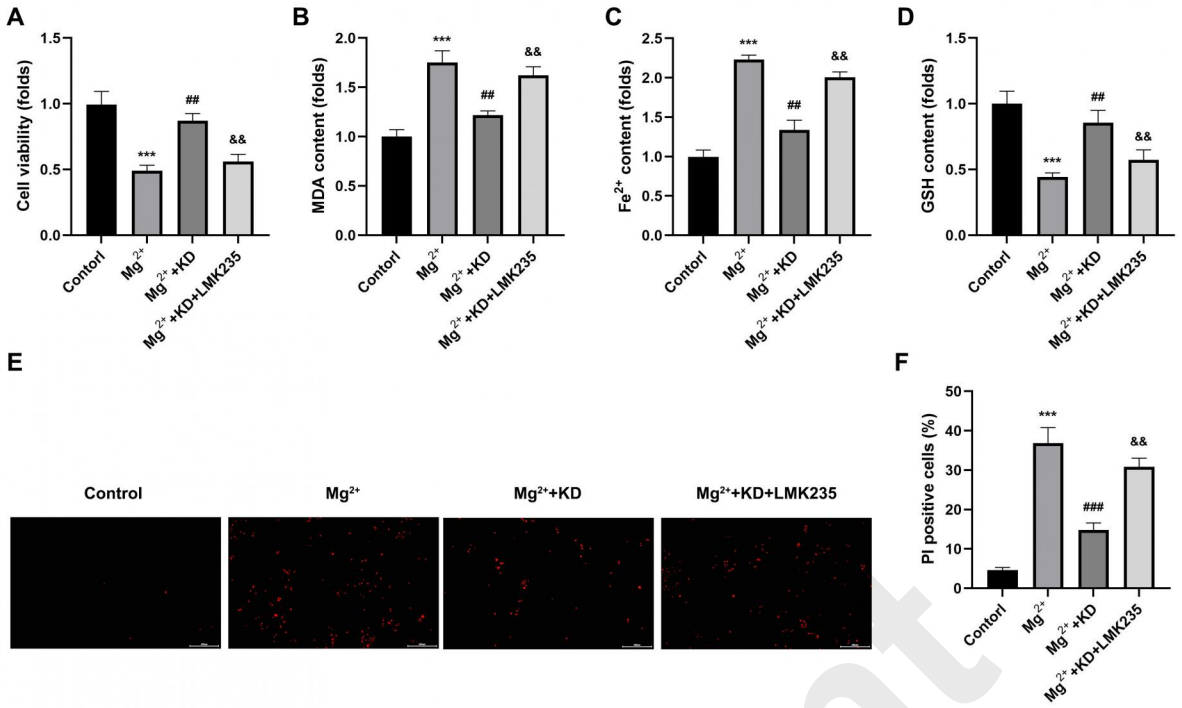


Figure 3. HDAC4 is downregulated in epilepsy
(A) HDAC4 mRNA expression was detected using RT-qPCR. (B-C) HDAC4 protein expression was detected using western blot. HDAC4: histone deacetylase 4. ***P<0.001.



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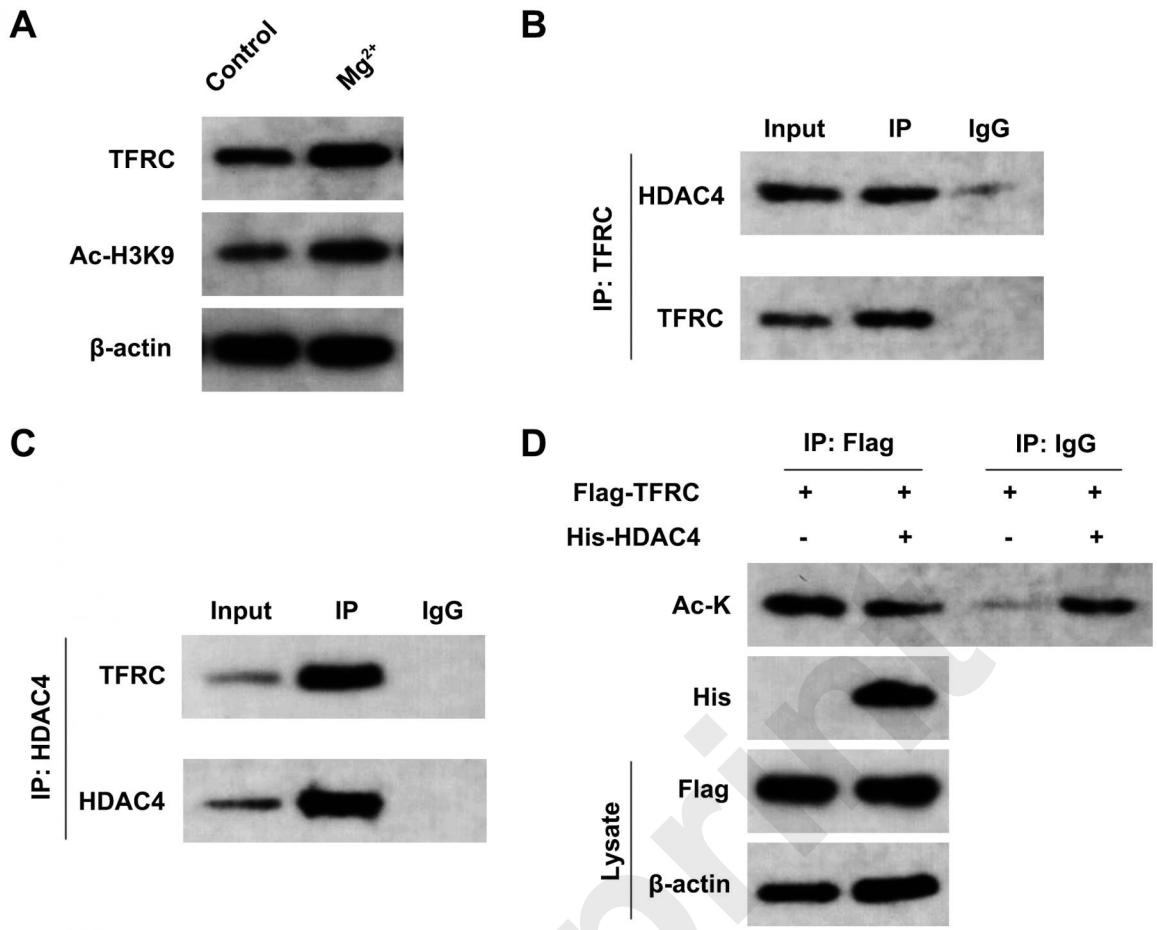

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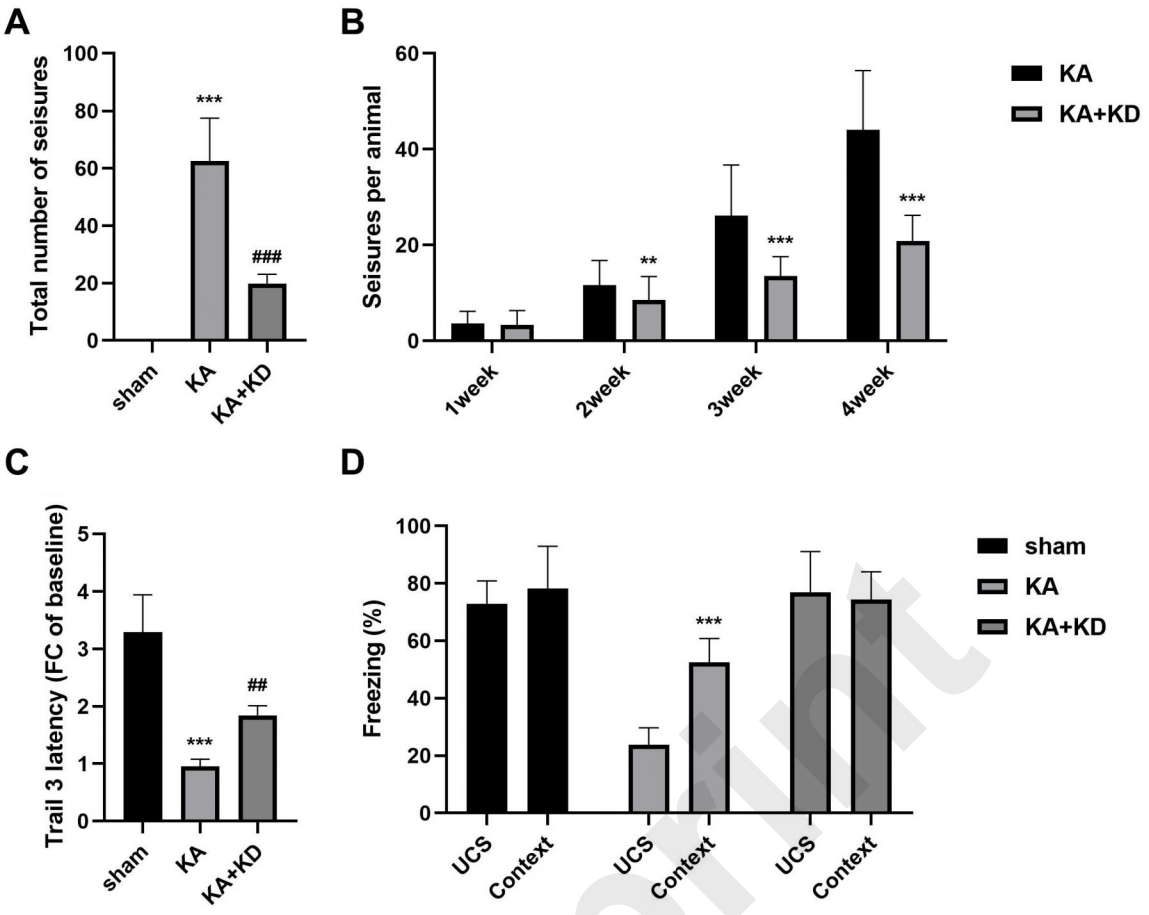

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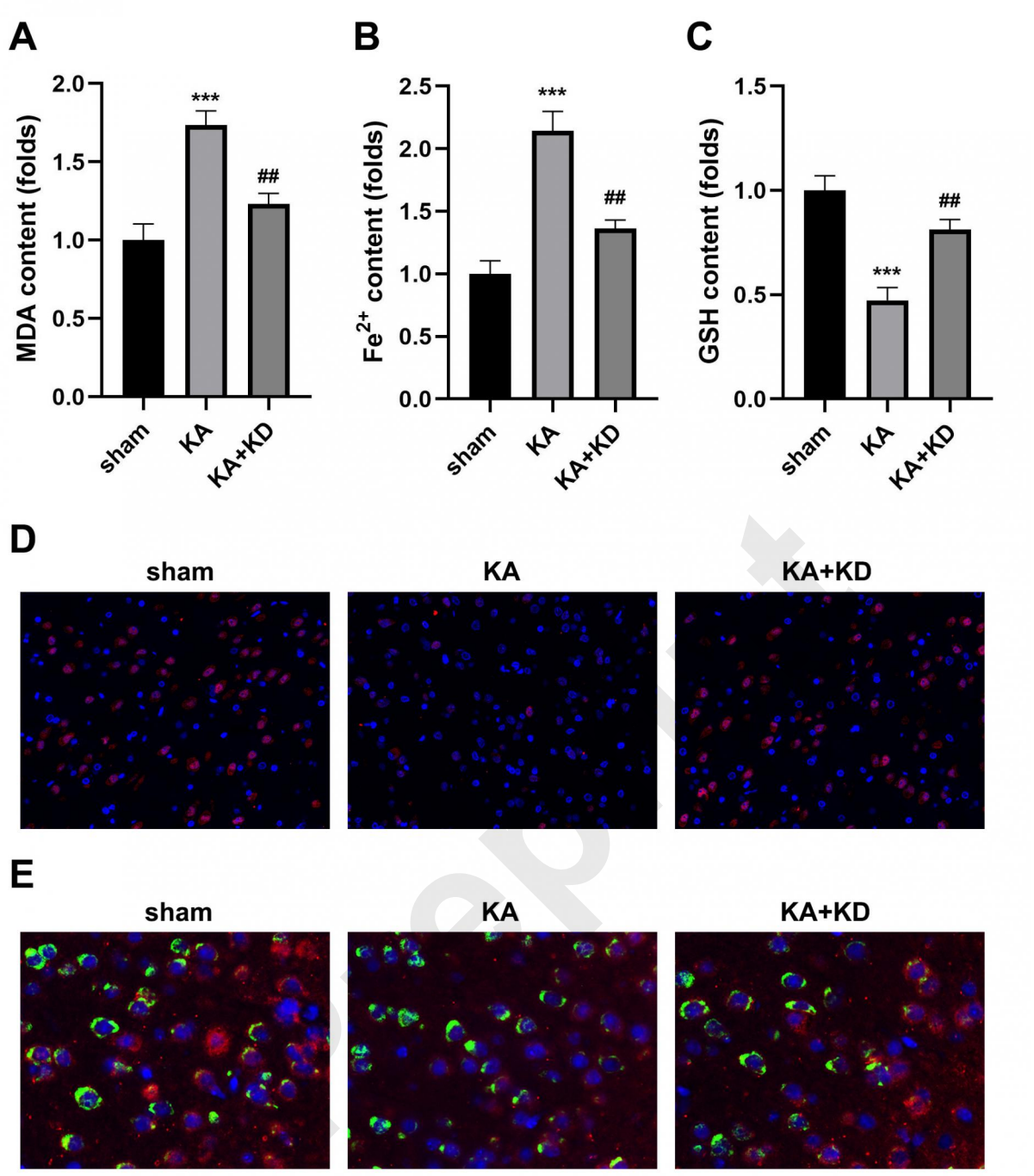

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