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journal homepage: <https://www.sciopen.com/journal/2097-0765>Dimethyl fumarate attenuates isoflurane-induced cognitive dysfunction and neurodevelopmental injury in juvenile mice *via* the Keap1-Nrf2 signaling pathway

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## ABSTRACT

Isoflurane is frequently employed as an inhalation anesthetic in pediatric medicine. The research found that repeated exposure to isoflurane had adverse effects on neurodevelopment. Despite the elusive nature of the underlying mechanisms, dimethyl fumarate (DMF) has been recognized as a biologically active compound with neuroprotective properties. This study examines the protective effects and underlying mechanisms of DMF both *in vivo* and *in vitro* against cognitive dysfunction in mice induced by repeated exposure to isoflurane. DMF treatment ameliorated cognitive dysfunction in mice subjected to isoflurane, alleviating neuronal and myelin injury and abnormal astrocyte death. *In vitro* studies demonstrated that DMF enhanced antioxidant enzyme activities, mitigated cellular oxidative stress, and improved mitochondrial function in cells exposed to isoflurane. Overall, we found that DMF alleviates cognitive impairment resulting from repeated isoflurane exposure. This effect is mediated through the hydrolytic metabolism of DMF, which enhances cellular energy production, activates the kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, reduces intracellular oxidative stress, and increases cellular antioxidant levels.

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## 1. Introduction

Each year, millions of children undergo surgical procedures necessitating anesthesia and sedative medication<sup>[1]</sup>. It is imperative to underscore that the growing use of anesthesia in pediatric patients has sparked mounting public health concerns regarding its safety. In 2016, the U.S. Food and Drug Administration (FDA) cautioned that repeated or prolonged administration of general anesthetics and sedatives during surgical procedures in children under 3 years of age or pregnant women in late gestation may imply the neurological development of children<sup>[2]</sup>. Numerous clinical studies have indicated

that children undergoing anesthesia face a heightened risk of cognitive dysfunction and structural brain alterations. General anesthetics have been documented to induce acute structural neurological injury in juvenile animals, encompassing non-human primates and rodents, leading to neurotoxicity resulting in learning and behavioral deficits in adulthood. Consequently, prudence is warranted in the administration of general anesthetics to young animals<sup>[3-7]</sup>.

Isoflurane is frequently employed as an anesthetic in pediatric patients. Research indicates that repeated exposure to isoflurane at concentrations of 1%–1.5% can induce neurotoxicity, resulting in cognitive dysfunction<sup>[4]</sup>. This neurotoxic effect is attributed to isoflurane's impact on both neuronal and non-neuronal cells, such as astrocytes. Astrocytes, the most abundant cells in the human brain, are pivotal in providing neural support, facilitating synaptogenesis, and regulating transmitter processes<sup>[8-9]</sup>. Exposure to isoflurane compromises the neural support capacity of astrocytes<sup>[10]</sup>, selectively

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disrupts calcium signaling pathways<sup>[11]</sup>, induces oxidative stress<sup>[12]</sup>, and inhibits mitochondrial complex I, thereby impeding adenosine triphosphate (ATP) synthesis<sup>[13]</sup>. These effects precipitate aberrant cell death<sup>[14–15]</sup>, subsequently eliciting neurological abnormalities and nerve damage. While antioxidants are commonly employed for neuroprotection against oxidative stress<sup>[12]</sup>, further research is needed to elucidate the mechanisms by which anesthetics induce astrocyte damage and exert neuroprotective effects.

Dimethyl fumarate (DMF), a derivative of fumaric acid, undergoes hydrolysis to fumaric acid catalyzed by intestinal esterase following oral administration, thereby supplying energy for cellular tricarboxylic acid cycle metabolism<sup>[16–18]</sup>. DMF has been proven to possess antioxidant, anti-inflammatory, and neuroprotective effects across various neural cell types. Additionally, DMF has demonstrated significant therapeutic efficacy in animal models of neurodegenerative diseases, including Parkinson's disease (PD) and Alzheimer's disease (AD)<sup>[19–20]</sup>. Moreover, DMF exhibits potent immunomodulatory properties and has been utilized in the treatment of psoriasis and multiple sclerosis. Nevertheless, its mechanism of neuroprotection remains incompletely understood<sup>[21–22]</sup>.

This study established a model of repeated isoflurane exposure-induced cognitive dysfunction in young mice to evaluate the neuroprotective effects of DMF. The objectives were to investigate: 1) the neuroprotective effects of DMF on memory and motor dysfunction induced by repeated isoflurane exposure in young mice; 2) the protective effects of DMF on abnormal astrocyte death induced by isoflurane exposure; and 3) the effects of DMF on increasing cellular energy supply through hydrolytic metabolism *via* the kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2) pathway and enhancing cellular antioxidant capacity to maintain astrocyte neural support and synaptogenesis during isoflurane exposure.

## 2. Material and methods

### 2.1 Reagents and materials

Dimethyl fumarate (DMF; D106460) was purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Reactive oxygen species (ROS) assay kit was obtained from Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China). ATP content assay kit (JN24734) was provided by Jining Biotechnology Co., Ltd. (Shanghai, China). Glutathione (GSH) and glutathione disulfide (GSSG) assay kit (S0053) was purchased from Beyotime Biological Co., Ltd. (Shanghai, China). Cell counting kit-8 (CCK8; G4103) was obtained from Servicebio (Wuhan, China). Fumaric acid enzyme-linked immunosorbent assay (ELISA) kit (SHQ4441) was purchased from Shuhua Biotechnology Co., Ltd. (Shanghai, China).

### 2.2 Solution preparation

A total of 2 minimal alveolar concentration (MAC) isoflurane solution, a 10 mmol/L isoflurane solution was prepared by injecting 130  $\mu$ L of isoflurane into 100 mL of PBS in the volumetric flask. The volumetric flask was wrapped in aluminium foil and stirred for 24 h to dissolve the isoflurane. Before use, pour 30 mL of stock solution into a 50 mL centrifuge tube and vortex for 5–10 s to make a working

stock solution. For cell treatment, the working stock solution can be diluted using Dulbecco's modified Eagle's medium (DMEM) to achieve a concentration of 0.45 mmol/L isoflurane in solution, corresponding to 2.5% atmospheric isoflurane (approximately 2 MAC) in equilibrium with an aqueous medium<sup>[23]</sup>.

DMF solution, take 0.5 g powder of Sodium carboxymethyl cellulose (CMC-Na) and dissolve in 100 mL ddH<sub>2</sub>O to form 0.5% CMC-Na solution. Then take 0.3 g DMF powder and dissolve in 100 mL CMC-Na.

### 2.3 Animal and treatments

This protocol was approved by the Ethics Committee for Laboratory Animals and Laboratory Animal Welfare of Nanjing Agricultural University. The studies conformed to National Institutes of Health (NIH) guidelines, and we wrote this article in accordance with the Guidelines for Applicable Animal Research: guidelines for *in vivo* experiments (ARRIVE). We endeavoured to reduce the number of animals used in the studies. Specific pathogen free (SPF), 14-day gestation C57BL/6 mice were supplied by Shanghai Slac Animal Co., Ltd. (Shanghai, China). A total of 32 mice, all of which were all maintain in SPF-grade rooms under controlled conditions (12 h light/dark cycle, humidity of  $(50 \pm 15)\%$ , temperature of  $(22 \pm 2)^\circ\text{C}$ ) and provided with laboratory standard food (AIN-93M) and water. When the mice were 3 days before delivery, the females were housed in separate cages. The mice were randomly divided into 4 groups ( $n = 8$ ):

1) Control group (CON): mice received continuous intragastric administration of saline (50 mg/kg) for 14 days, commencing on the 7<sup>th</sup> day postpartum.

2) Isoflurane group (ISO): starting from the seventh day after parturition, anesthesia was sustained for 4 h over 3 consecutive days utilizing an anesthesia box, delivering 2 MAC of isoflurane at a rate of 0.3 L/min per day, with gas flow and concentration monitored *via* an anesthesia meter.

3) Dimethyl fumarate + isoflurane group (ISO + DMF): commencing on the 7<sup>th</sup> day after parturition, mice in this group underwent anesthesia for 4 h on 3 consecutive days using an anesthesia box, delivering 2 MAC of isoflurane at a rate of 0.3 L/min per day. Gas flow and concentration were monitored with an anesthesiometer. Additionally, DMF (50 mg/kg) was intragastrically administered post-anesthesia daily for 14 consecutive days.

4) Blank fumaric acid group (CON + DMF): DFM (50 mg/kg) was administered intragastrically every day for 14 consecutive days starting from the 7<sup>th</sup> day after birth.

The timeline of study was shown in Fig. 1A.

### 2.4 Barnes maze test

According to previous studies<sup>[24]</sup>. The maze comprises a white circular platform with a diameter of 90 cm, featuring 20 holes measuring 5 cm in diameter, with one hole linked to a black escape box. A total of 4 colored and shaped markers were positioned in 4 directions of the maze to serve as visual cues for the mice. Throughout the experiment, the mice were motivated to enter the escape box by exposure to bright light and noise. Prior to the start of the experiment, the mice were introduced to both the platform and the escape box



to familiarise them with the environment. In the initial training session, a plastic box was inverted and positioned at the center of the platform to constrain the mice's movement and as a starting point. After a 10 s interval, the plastic box was removed, permitting the mice to find the escape box. If a mouse failed to locate the escape box within 4 min, it was gently guided to enter it and remain inside for 1 min. This training was repeated 3 times each day for 3 consecutive days. The formal experiment procedure involved placing the mice at the center of the platform, removing the starter box, and initiating the timer to assess its memory retention. Subsequently, the number of incorrect probes and the paths explored by the mice within a 3 min interval were recorded and assessed using the Barnes Maze Scale over 5 consecutive days. Barnes Maze Scale is shown in Table S1.

## 2.5 Y-maze test

The maze comprises three arms spaced at 120-degree intervals<sup>[25]</sup>. Mice were placed in the maze 1 day before the start of the experiment to acclimatize them to the environment. For the formal experiment, the mice were placed at the end of the starting arm and timed, and the number of times the mice travelled through each arm within 3 min was recorded. Each route of a mouse traversing through each arm within a 3 min span was logged. Successive entry into 3 different arms was deemed as one correct alternation, and the percentage of such correct alternations was computed using the formula:

$$\text{Percentage of correct alternations (\%)} = \frac{\text{Number of correct alternations}}{\text{Total number of alternations}} \times 100$$

## 2.6 Pole test

Initially, mice were positioned atop a rubber ball situated at the summit of a 70 cm wooden pole. Subsequently, the mice were released and the time taken to turn at the top of the pole and the total time taken to descend to the bottom were recorded. Prior to each trial, the wooden pole underwent sanitization with 75% alcohol and underwent testing over a span of 5 days<sup>[26]</sup>.

## 2.7 Golgi staining

Following anesthesia, mice underwent cardiac perfusion with 4% paraformaldehyde, after which brain tissues were dissected out of the skull, fixed in 4% paraformaldehyde for 24 h, and subsequently immersed in Golgi staining solution under light-free conditions for a duration of 14 days. During this period, the Golgi staining solution was changed every day. Throughout this period, the Golgi staining solution was changed daily. After the 14-day period, the brain tissue underwent sectioning. These sections were then encapsulated in glycerine gelatine and captured images through a microscope.

## 2.8 Luxol fast blue (LFB) staining

Brain tissues were immersed in a 0.1% LFB staining solution and incubated overnight at 28 °C in a temperature-controlled incubator. Subsequently, the tissues underwent differentiation in a solution comprising 95% ethanol and 0.05% lithium carbonate, followed by dehydration, transparency enhancement, and sealing.

## 2.9 RNA extraction and quantitative real-time the polymerase chain reaction (PCR) analysis

RNA was extracted from brain tissues or cells using the Trizol (G3013, Servicebio, Wuhan, China), followed by reverse transcription of the total RNA into cDNA using the Transcription Kit (G3332, Servicebio, Wuhan, China). Fluorescent quantitative real-time PCR kit (G3325, Servicebio, Wuhan, China) was employed to detect the mRNA expression levels. The Ct values were normalized to  $\beta$ -actin. The primer sequences are shown in Table S2.

## 2.10 Immunofluorescence staining

Brain tissues or cells were fixed with paraformaldehyde, incubated at room temperature after adding 1 mL of 0.5% TritonX-100 (GC204003-100 mL, Servicebio, Wuhan, China) clear, closed with bovine serum albumin (BSA) (GC305010-5 g, Servicebio, Wuhan, China) and incubated with primary antibody at 4 °C overnight and fluorescent secondary antibody at room temperature for 1 h, then 4',6-diamidino-2-phenylindole (DAPI) (G1012-10 mL, Servicebio, Wuhan, China) re-stained and images were collected by fluorescence microscope. Antibody information is shown in Table S3.

## 2.11 Transmission electron microscopy

Brain tissues were taken and fixed in 2.5% glutaraldehyde, dehydrated in graded concentrations (30%-50%-70%-80%-95%-100%-100%) of ethanol, embedded in acetone-epoxy resin and incubated in an oven overnight at 37 °C. Sections were made using an ultra-microtome, and images were captured using a transmission electron microscope.

## 2.12 Cell culture

### 2.12.1 Primary astrocyte isolation and culture

Mouse subsequent to decapitation, mice' cortices were aseptically dissected in Petri dishes containing ice-cold Hank's Balanced Salt Solution (HBSS) (G4204, Servicebio, Wuhan, China) supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (10 mmol/L) and gentamicin sulfate (5 µg/mL). The dissected tissue was then cut into pieces and dissociated through trypsin treatment (10 µL/mL), followed by trituration using sterile glass pipettes. Cultures were maintained in DMEM medium (G4515, Servicebio, Wuhan, China) supplemented with 10% fetal bovine serum (FBS) (G8003, Servicebio, Wuhan, China) and incubated at 37 °C in a controlled atmosphere of 5% O<sub>2</sub> and 95% CO<sub>2</sub><sup>[27]</sup>. This procedure yields astrocyte-enriched cultures, as validated by immunostaining against the astrocytic marker glial fibrillary acidic protein (GFAP). Cells were seeded in 6-well plates with DMEM medium supplemented with 10% FBS and cultured at 37 °C in a 5% CO<sub>2</sub> environment. Upon reaching 70% confluency, cells were grouped and subjected to a 12-h treatment regimen, including: CON group (conventional medium), ISO group (2 MAC ISO medium), ISO + DMF group (2 MAC ISO medium + 20 µmol/L DMF), and CON + DMF group (containing 20 µmol/L DMF medium).

### 2.12.2 C8-D1A cells culture

C8-D1A(SNL-275) cell was purchased from Wuhan Sunncell Biotechno-logy Co., Ltd. (Wuhan, China).

### 2.13 Cell viability assay

Cells were cultured in 96-well plates and subjected to treatment with varying concentrations of DMF (0, 10, 20, 30, and 40  $\mu\text{mol/L}$ ) for a duration of 12 h. Cell viability was assessed and quantified utilizing the CCK8.

### 2.14 ELISA

Fumaric acid levels in brain and cells were measured by ELISA kits according to the instructions.

### 2.15 ROS assay

According to the kit instructions, the cell or brain precipitates were resuspended in a single-cell suspension containing DCFH-DA as the provided instructions. Subsequently, the suspension was incubated with a reactive oxygen donor for 30 min within a cell incubator. Afterward, the suspension was centrifuged, the supernatant was removed, and the precipitates were washed 1–2 times with PBS. Finally, the washed precipitates were collected and subjected to measurement of optical density (OD) values using a fluorescent microplate reader in 520 nm laser.

### 2.16 Determination of ATP content

Following the provided instructions, acidic extract was added to brain tissue or cell samples. The resulting mixture was then centrifuged to obtain the supernatant, to which an equal amount of alkaline extract was added. After a subsequent centrifugation step to remove any debris, an appropriate amount of color developer was added to the supernatant. Finally, the ATP content was determined and calculated using a microplate reader.

### 2.17 Determination of GSH and GSSG content

Following the instructions provided with the kit, the standard curve was generated. The treated samples were then added to a 96-well plate and incubated appropriately. The OD values at 405 nm were measured using a microplate reader, and the sample concentrations were calculated based on the standard curve.

### 2.18 Western blot analysis

Add radioimmunoprecipitation assay lysate containing 1% phenylmethylsulfonyl fluoride (PMSF) (G2008, Servicebio, Wuhan, China) to the brain tissue or cells, mix and lysed for 15 min, then centrifuged at 4 °C, 12 000 r/min for 15 min. Take the supernatant and test the protein concentration with bicinchoninic acid assay (BCA) protein assay kit (G2026, Servicebio, Wuhan, China), then add 5 $\times$  loading buffer (G2075, Servicebio, Wuhan, China). Samples

were separated in sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, and the polyvinylidene difluoride (PVDF) membrane (WGPVDF45, Servicebio, Wuhan, China) was activated using methanol and transferred. After 30 min of blockage with protein free rapid blocking buffer (G2052, Servicebio, Wuhan, China), the primary antibody was incubated overnight at 4°C and the secondary antibody was incubated at room temperature for 2 h. The proteins were detected using a special ultrasensitive enhanced chemiluminescence luminescent (ECL) solution (G2161, Servicebio, Wuhan, China). Antibody information is shown in Table S3.

### 2.19 Chromatin immunoprecipitation (ChIP) and qPCR analysis

Cells were digested with trypsin, and  $2 \times 10^7$  cells were collected. The cells were crosslinked with 1% formaldehyde for 10 min, and the reaction was quenched with 0.125 mol/L glycine. Chromatin was extracted and sonicated to produce DNA fragments of 200–1 000 bp. The fragmented chromatin was immunoprecipitated using antibodies against Nrf2 (80593-1-RR, Proteintech, Wuhan, China) or IgG (negative control) (GB111739-100, Servicebio, Wuhan, China). After reverse crosslinking, the DNA was purified, and specific primers were designed to target the promoter regions of *HO-1* and *NQO1* genes, spanning the ARE motif and Nrf2 binding sites, for qPCR analysis. The *SOD* gene was used as a positive control in the qPCR analysis. The primer sequences were shown in Table S2. Fold enrichment was calculated using the  $2^{-\Delta\Delta Ct}$  method, where  $\Delta Ct = Ct_{IP} - Ct_{input}$ , to quantify the relative expression of the ARE fragment at the target gene loci. All reactions were performed in triplicate.

### 2.20 Statistical analysis

The data were expressed as mean  $\pm$  standard deviations (SDs). The statistical analyses were conducted using IBM SPSS Statistics Version 20 (International Business Machine Co.) and GraphPad Prism 8 (GraphPad Software Co.). The Shapiro-Wilk test was employed to assess the Gaussian distribution of the data. Variations in the learning curves across groups in the Barnes tests were examined using two-way analysis of variance (ANOVA) with variance measurements. The model included the main effects of ISO and DMF treatments, along with the interactions between these effects. Bonferroni was applied to correct for multiple comparisons of behavioral test data and molecular biology test indicators. A two-way ANOVA was used to evaluate the correlation between DMF and ISO-induced anesthesia, aiming to test the hypothesis that DMF could mitigate ISO-induced neurological damage. The statistical significance of the main effects was assessed using Bonferroni post hoc tests. *P* value < 0.05 was considered statistically significant.

The statistical power of this study was analyzed as described previously<sup>[28–29]</sup>. The sample size was determined through experimental design, pre-experimentation, and conventional numerical settings using PASS 15 (NCSS Software Co.) (a type 1 error (*P* = 0.05), 90% power). To reduce the error rate, each sample was analyzed in three technical replicates.

### 3. Results

#### 3.1 Effect of DMF on basal physiological values in isoflurane-exposed mice

The experimental procedure is shown in Fig. 1A. Mice were randomly allocated into four groups starting on postnatal day 7 (P7), with their body weights assessed triweekly. On P19, the mice were housed in separate cages, with a single cage accommodating 3 mice. The investigation monitored dietary and water consumption changes in each cage at 3-day intervals. The findings revealed no significant differences in body weight or in the consumption of dietary and water among the 4 groups, indicating the absence of significant toxicity within any groups. (Figs. 1B-D).

#### 3.2 Effects of DMF on cognitive function in isoflurane-exposed mice

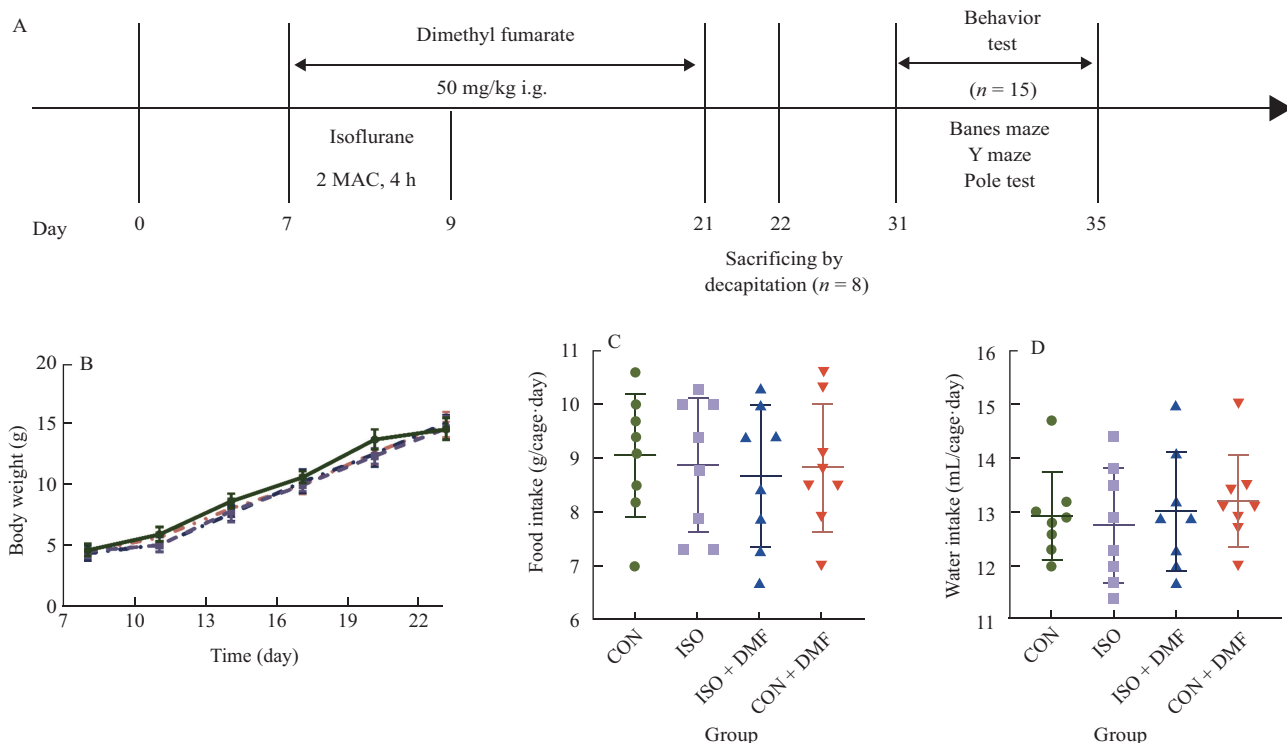
The Barnes maze serves as a dependable method for behavioral tests, probing the learning and memory ability of mice. Figs. 1E presents the experimental results, revealing that mice exposed to repeated isoflurane at a young age exhibited significantly longer total latency and lower search strategy scores compared to control mice.

Notably, DMF supplementation significantly ameliorated this change. It is noteworthy that DMF alone (CON + DMF) did not result in significant differences compared to CON.

The pole test serves as a widely adopted method in behavioral tests to assess fine motor function in mice. Experimental outcomes, as depicted in Fig. 1F, reveal that mice exposed to repeated isoflurane at a young age exhibited significantly prolonged latencies to reach both the top and bottom compared to control mice. Notably, treatment with DMF significantly mitigated this alteration and no significant changes were observed with DMF alone compared to CON (Figs. 1F).

Y-maze spontaneous alternation is a behavioral test method used to measure the willingness of rodents to explore new environments. As shown in Fig. 1G, the spontaneous alternation rate of repetitive isoflurane-exposed mice at a young age was significantly lower than that of control mice, and treatment with DMF significantly ameliorated this change. There was no significant change with DMF alone compared with controls.

These behavioral test results indicate that repeated exposure to isoflurane during early development induces cognitive dysfunction in mice. DMF demonstrates significant efficacy in mitigating the isoflurane-induced attenuation of learning and memory, fine motor skill impairment, and decreased exploratory behavior in novel environments.



**Fig. 1** Timeline and the effects of DMF supplementation on body weight, food intake and water intake of mice. Behavioral experiment results of mice in different groups. (A) Experimental scheme for treatment and behavior tests. The effects of DMF treatment on (B) body weight, (C) food intake, and (D) water intake. (E) Results of Barnes maze tests. Schematic representation of the mice before and after Barnes maze training in each group. (E<sub>1</sub>) As compared to the CON group, ISO group had the significant difference in escape latency time. (E<sub>2</sub>) As compared to the CON group, ISO group had the significant difference in strategies scores (E<sub>3</sub>). As compared to the ISO group, ISO + DMF group had the significant difference in escape latency time. (E<sub>4</sub>) As compared to the ISO group, ISO + DMF group had the significant difference in strategies scores. (E<sub>5</sub>) As compared to the CON group, CON + DMF group had the no significant difference in escape latency time. (E<sub>6</sub>) As compared to the CON group, CON + DMF group had the significant difference in strategies scores. (F) Results of Pole tests. (F<sub>1</sub>, F<sub>2</sub>) As compared to CON group, ISO group had the significant difference in latency to descent and turn. There was significant difference between DMF + ISO group and ISO group. There was no significant difference between CON group and CON + DMF group. (G) Results of Y-Maze tests. There was significant difference between CON group and ISO group in Spontaneous alternations. There was significant difference between ISO group and ISO + DMF group. There was no difference between CON group and CON + DMF group. Data presented as mean  $\pm$  standard error,  $n = 8$  in each group (body weight  $n = 8$  mice in each group, food intake  $n = 8$  cages in each group, water intake  $n = 8$  in each group),  $n = 15$  mice in each group (Barnes maze, Pole test, Y maze); ns means no significant difference,  $*P < 0.05$ .

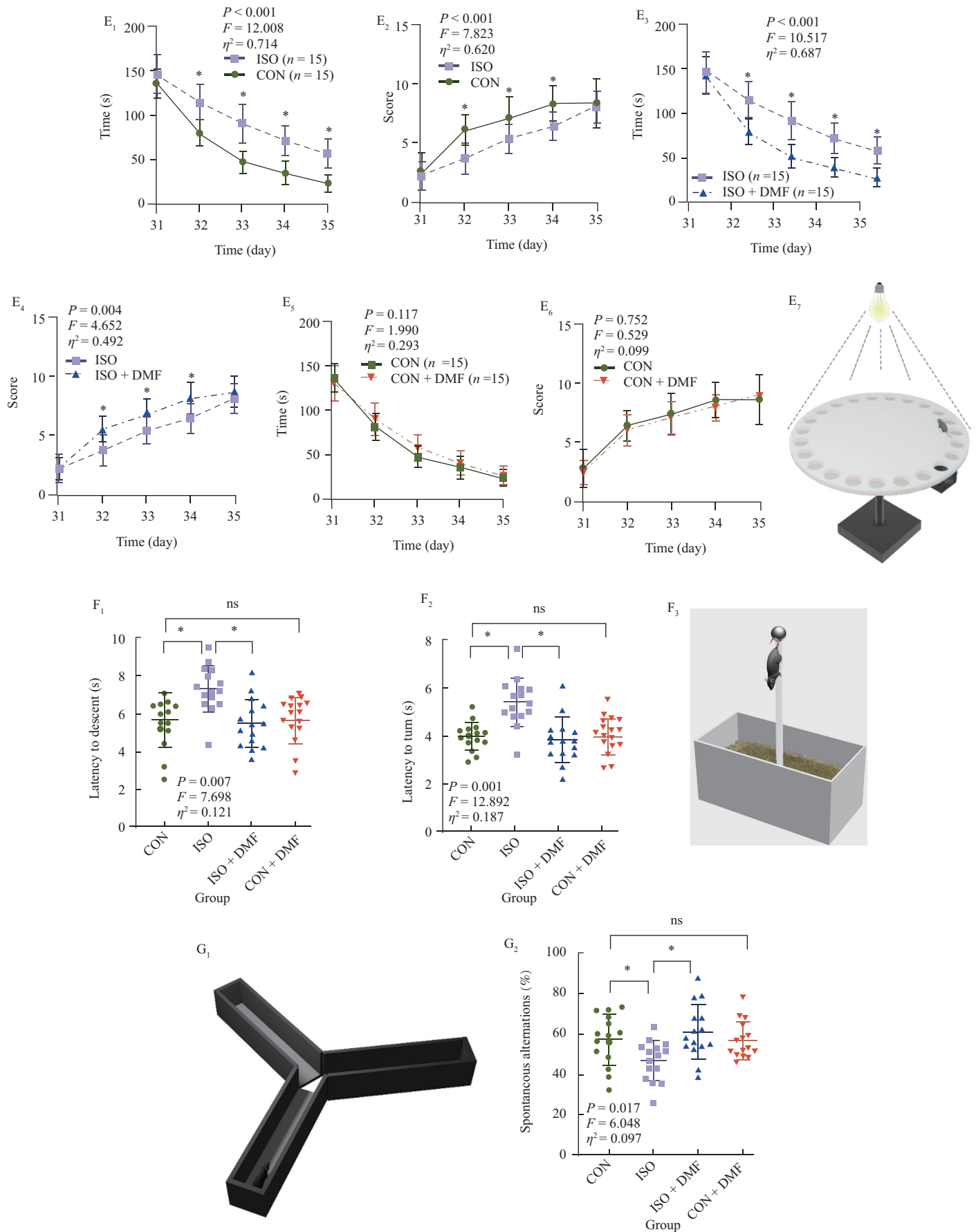


Fig. 1 (Continued)



### 3.3 Effect of DMF on brain histological changes in isoflurane-exposed mice

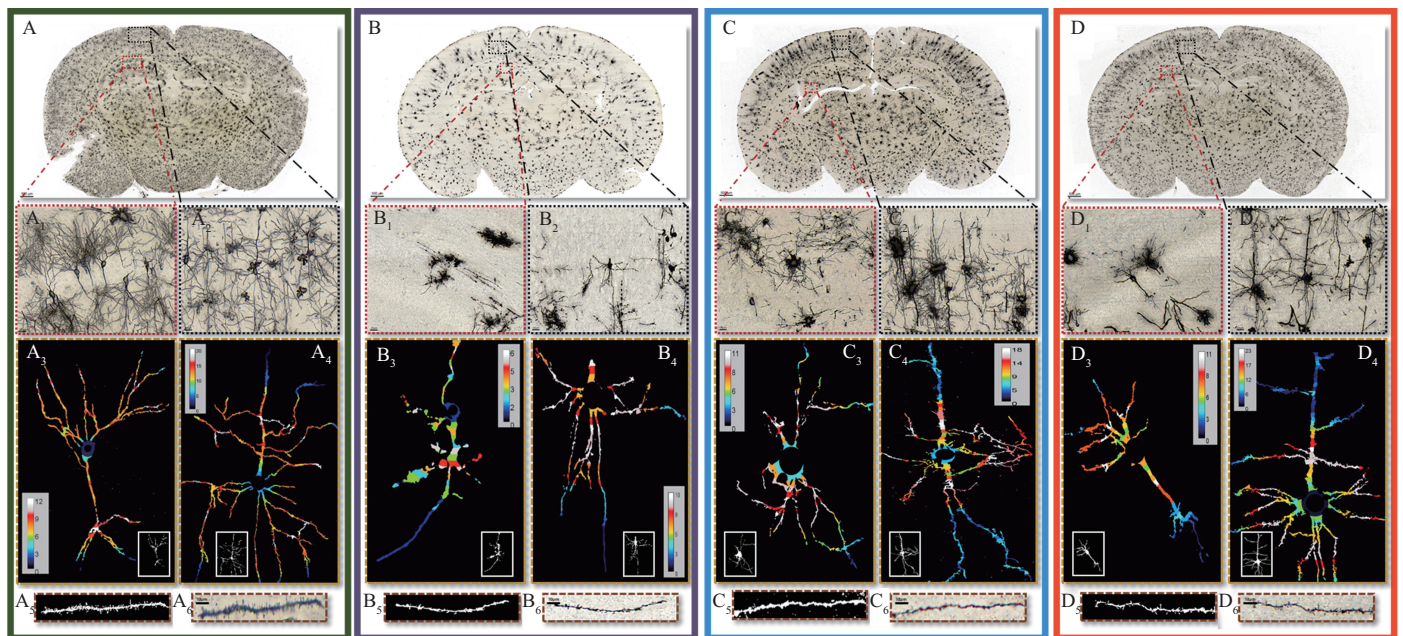
Golgi staining serves as a dependable method for examining the structure of brain cells and observation of individual neuron changes. Brain tissues were collected from different groups on P9. The experimental (Fig. 2) were analyzed using Sholl analysis, revealing a significant reduction in the number of neuronal branches in the parietal and hippocampal regions of the cerebral cortex, as well as a decreased density of neuronal spines in mice repeatedly exposed to isoflurane during the juvenile stage, compared to CON (Figs. 2A-D). Notably, DMF treatment significantly mitigated this alteration. There was no significant change with DMF alone compared to the CON (Figs. 2E-K).

LFB staining is commonly employed to visualize myelin changes in the central nervous system (CNS). The experimental outcomes, depicted in Figs. 2V<sub>1</sub>-V<sub>4</sub>, revealed a significant reduction in the mean OD values of LFB staining in the cerebral cortex and hippocampal regions of the mice repeatedly exposed to isoflurane during the juvenile stage compared to the control group, indicating myelin damage. However, DMF treatment significantly alleviated this damage (Figs. 2V<sub>5</sub>-V<sub>6</sub>).

GFAP is an intermediate filament protein predominantly expressed in astrocytes. NeuN, serves as a typical and specific neuronal marker for brain tissue. Results of immunofluorescence, depicted in Figs. 3A-D, revealed reduced expression levels of GFAP and NeuN in the cerebral cortex of the mice repeatedly exposed to isoflurane during early development, indicative of injury. Remarkably, DMF treatment significantly mitigated this injury, with no significant changes observed with DMF alone compared to CON (Figs. 3E and F).

### 3.4 Effect of DMF on ultrastructural changes in brain tissue of isoflurane-exposed mice

To further investigate the impact of DMF on ultrastructural alterations in brain tissues of isoflurane-exposed mice, transmission electron microscopy was utilized to examine cellular substructures. As shown in Figs. 2U, swollen and ruptured mitochondria, as well as blurred mitochondrial membranes, were observed in the brains of juvenile mice subjected to repeated isoflurane exposure. The treatment with DMF significantly ameliorated the mitochondrial damage and no significant alterations were observed with DMF treatment alone compared to CON.



**Fig. 2** (A-D) Golgi staining results of 4 groups. (A<sub>1</sub>, B<sub>1</sub>, C<sub>1</sub>, D<sub>1</sub>) Neuron of hippocampal regions. (A<sub>2</sub>, B<sub>2</sub>, C<sub>2</sub>, D<sub>2</sub>) Neuron of cerebral parietal lobe regions. (A<sub>3</sub>, A<sub>4</sub>, B<sub>3</sub>, B<sub>4</sub>, C<sub>3</sub>, C<sub>4</sub>, D<sub>3</sub>, D<sub>4</sub>) Sholl analysis of individual neuron corresponds to the Figure above. (A<sub>5</sub>, A<sub>6</sub>, B<sub>5</sub>, B<sub>6</sub>, C<sub>5</sub>, C<sub>6</sub>, D<sub>5</sub>, D<sub>6</sub>) Dendrite spine of the group.

(E, F) As compared to the CON group, ISO group had the significant difference in neuronal branches in both cerebral parietal lobe and hippocampal regions. (G, H) As compared to the ISO group, ISO + DMF group had the significant difference in both cerebral parietal lobe and hippocampal regions. (I, J) As compared to the CON group, CON + DMF group had no significant difference in both cerebral parietal lobe and hippocampal regions. (K) Results of neuronal spine density.

As compared to CON group, ISO group had the significant difference in spines density. There was significant difference between DMF + ISO group and ISO group. (L) Results of ROS generation. As compared to CON group, ISO group had the significant difference in ROS levels. There was no difference between CON group and CON + DMF group. (M, N, O, P, Q, R, S, T) Western blot analysis of Nrf2, Keap1, HO1, NQO1, GFAP, SIRT3 and SOD protein expression.

(Y) Results of the content of GSH, GSSG and the ratio of GSSG to GSH. As compared to CON group, ISO group had significant difference in content of GSH and the ratio of GSSG to GSH. As compared to ISO group, ISO + DMF group had significant difference in content of GSH and the ratio of GSSG to GSH. There was no difference between CON group and CON + DMF group. There was no difference among the groups. (U) Results of transmission electron microscopy. (V) Results of LFB staining. (V<sub>1a</sub>, V<sub>2a</sub>, V<sub>3a</sub>, V<sub>4a</sub>, V<sub>5</sub>, V<sub>6</sub>) LFB staining of parietal lobe of cerebral cortex. (V<sub>1b</sub>, V<sub>2b</sub>, V<sub>3b</sub>, V<sub>4b</sub>) LFB staining of cerebral cortex and hippocampus.

Quantification of LFB staining of cerebral parietal lobe and hippocampal regions. As compared to CON group, ISO group had the significant difference in the mean OD values. As compared to ISO group, ISO + DMF group had significant difference in the mean OD values. There was no difference between CON group and CON + DMF group. (W, X) Results of levels of ATP and fumaric acid. As compared to CON group, ISO group had the significant difference in the levels of ATP fumaric acid. As compared to ISO group, ISO + DMF group had significant difference in the levels of ATP fumaric acid. There was significant difference between CON group and CON + DMF group. Data presented as mean  $\pm$  standard error,  $n = 8$  mice in each group. Green frame represents CON group, purple frame represents ISO group, blue frame represents ISO + DMF group, orange frame represents CON + DMF group; ns means no significant difference,  $*P < 0.05$ .

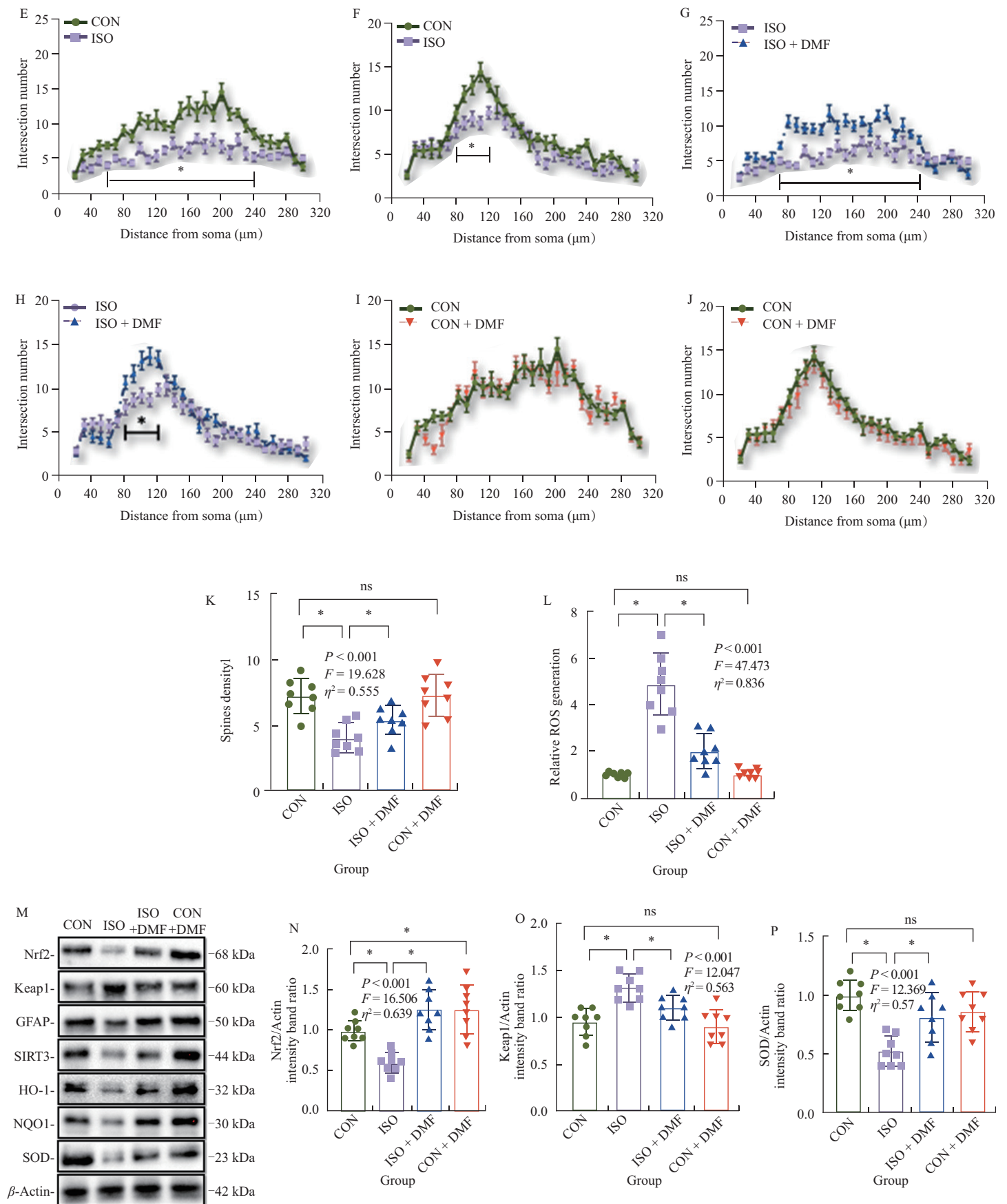


Fig. 2 (Continued)

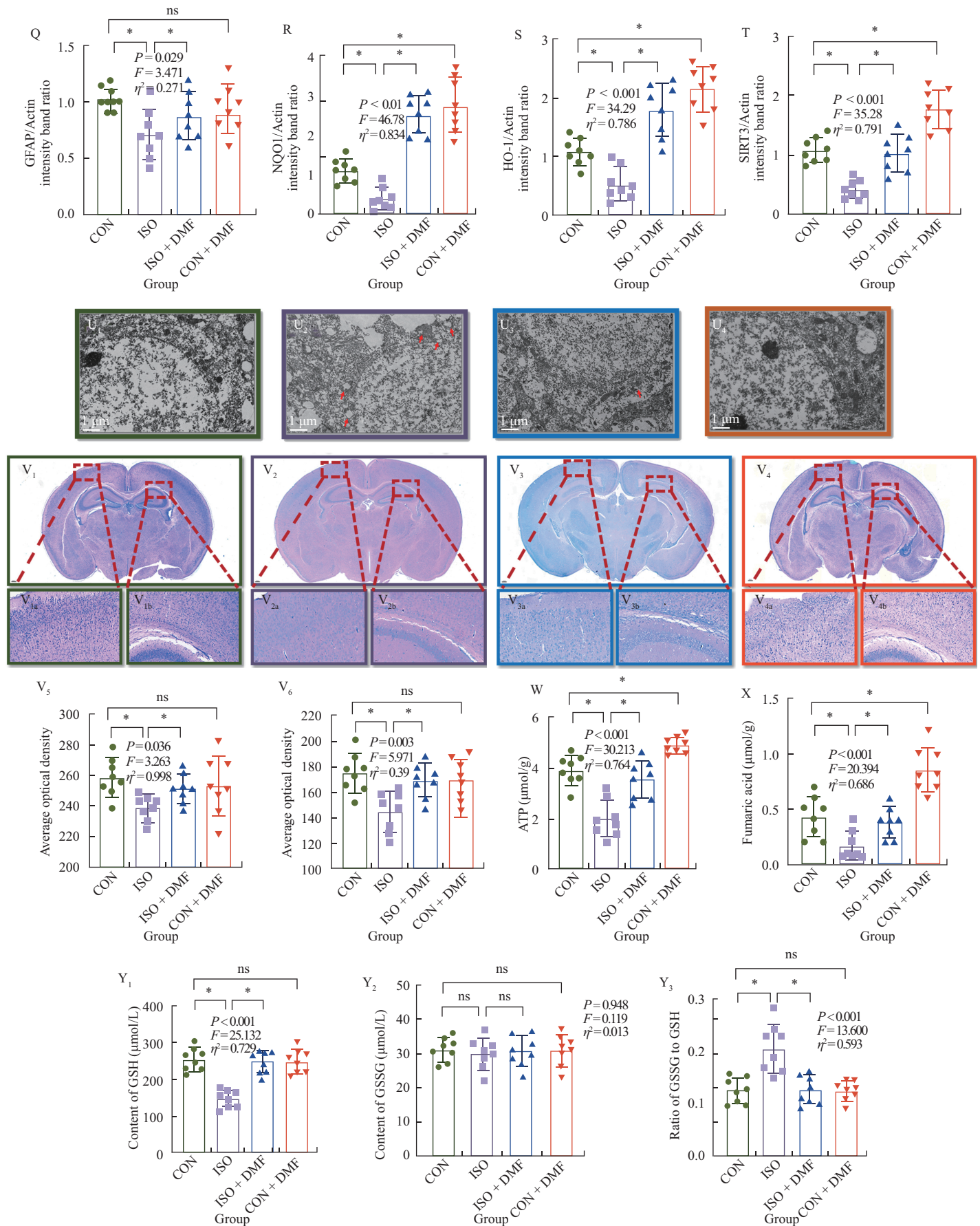
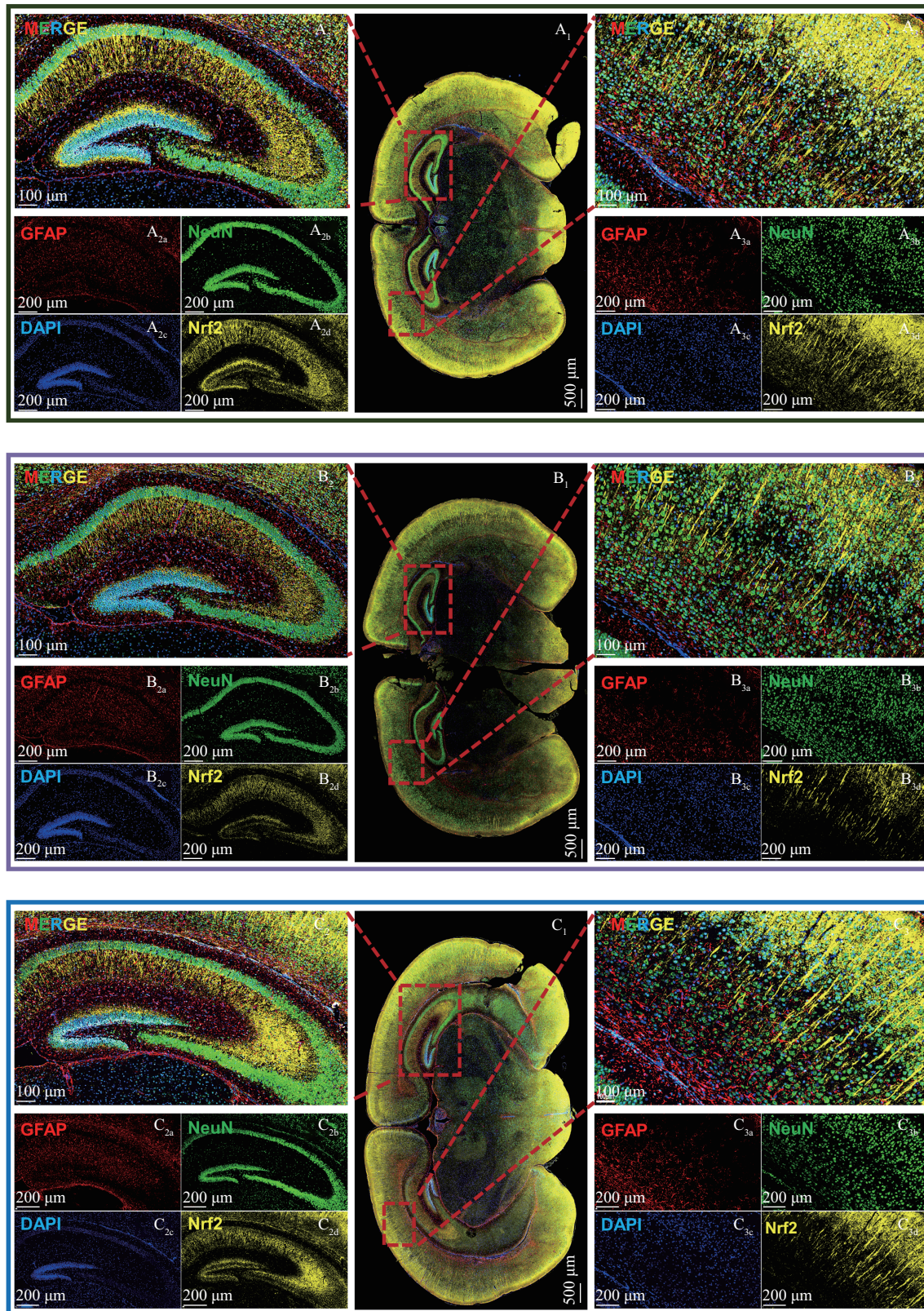


Fig. 2 (Continued)





**Fig. 3** (A-D) Results of immunofluorescence staining. Red represents GFAP, green represents NeuN, blue represents DAPI, yellow represents Nrf2.

(A<sub>2</sub>, B<sub>2</sub>, C<sub>2</sub>, D<sub>2</sub>) Immunofluorescence staining of hippocampal regions. (A<sub>3</sub>, B<sub>3</sub>, C<sub>3</sub>, D<sub>3</sub>) Immunofluorescence staining of cerebral parietal lobe. (E, F) Quantification of immunofluorescence staining in cerebral parietal lobe and hippocampal regions. As compared to CON group, ISO group had the significant difference in expression of NeuN, GFAP and Nrf2. There was significant difference in expression of Nrf2 between CON group and CON + DMF group. There was no significant difference in expression of NeuN and GFAP between CON group and CON + DMF group. Data presented as mean  $\pm$  standard error,  $n = 8$  mice in each group. Green frame represents CON group, purple frame represents ISO group, blue frame represents ISO + DMF group, orange frame represents CON + DMF group; ns means no significant difference,  $*P < 0.05$ .



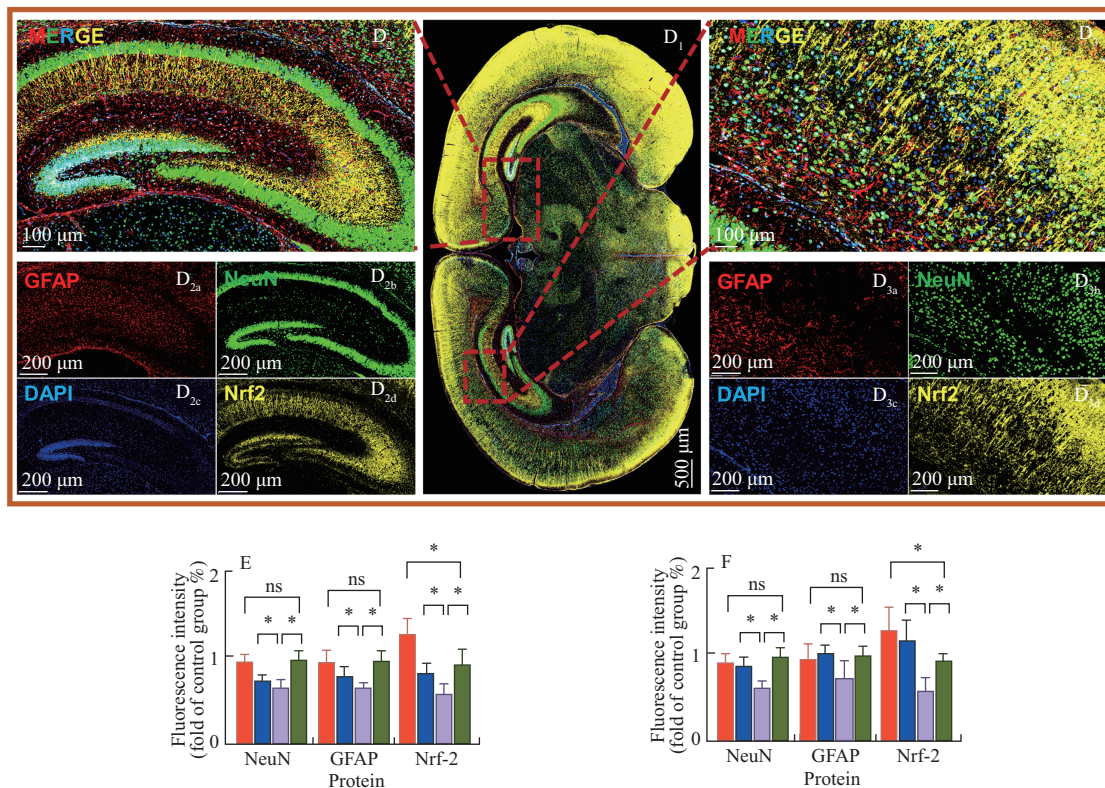


Fig. 3 (Continued)

### 3.5 Effect of DMF on oxidative phosphorylation products of citric acid cycle in brain tissue of isoflurane-exposed mice

As shown in Figs. 2W and X, we observed a significant reduction in the levels of fumaric acid and ATP, both pivotal components of the oxidative phosphorylation process, in the brain tissues of juvenile mice repeatedly exposed to isoflurane. However, supplementation with DMF resulted in a significant increase in both fumaric acid and ATP levels compared to ISO. DMF alone also significantly increased the levels of fumaric acid and ATP compared to CON.

### 3.6 Effect of DMF on antioxidant indexes of brain tissue in isoflurane-exposed mice

Compared to CON, the protein expression of SOD (Figs. 2M, P) and the content of glutathione (GSH) (Fig. 2Y<sub>1</sub>) were significantly decreased in mice exposed to repeated isoflurane during early developmental stages. Notably, GSSG (Fig. 2Y<sub>2</sub>) represents the oxidized form of GSH, and the ratio of GSSG to GSH (GSSG/GSH) (Fig. 2Y<sub>3</sub>) serves as an indicator of oxidative stress levels. ROS levels showed an inverse trend (Fig. 2L). Therefore, it was demonstrated that DMF could significantly mitigate the oxidative stress induced by isoflurane.

### 3.7 Effect of DMF on Keap1-Nrf2 pathway in brain tissues of isoflurane-exposed mice

Research has demonstrated that activation of the Nrf2-Keap1 signaling pathway can effectively combat inflammation and inhibit

oxidative stress<sup>[30]</sup>. As depicted in Fig. 2, the administration of DMF significantly enhances the expression of Nrf2, while notably reducing the expression of Keap1. This alteration significantly mitigates the isoflurane-induced changes in the expression of related genes, as well as its downstream proteins, heme oxygenase 1 (HO1) and NAD(P)H quinone oxidoreductase 1 (NQO1) (Figs. 2M, R and S). Moreover, the activation of Nrf2 led to increased expression of Sirtuin-3 (SIRT3) and SOD1 (Figs. 2M, P and T). The corresponding changes in mRNA expression were shown in Fig. S1.

### 3.8 Effect of DMF on the viability of primary astrocytes and C8-D1A in isoflurane-treated mice

In this study, astrocytes, primarily involved in providing energy and support within the nervous system, were selected as the focal point for *in vitro* analysis. To ensure the comprehensiveness and validity of our study, we utilized not only the immortalized cell line C8-D1A but also isolated primary mouse astrocytes for experimentation. Based upon our prior research, a 12 h treatment with 2 MAC isoflurane was selected. Various concentrations of DMF were concurrently administered to the cells, (Figs. 4A, H, M and 5A, H, M). Subsequently, a concentration of 20  $\mu\text{mol/L}$  DMF was determined as the optimal experimental dosage.

### 3.9 Effect of DMF on Keap1-Nrf2 signaling pathway in primary astrocytes and C8-D1A of isoflurane-treated mice

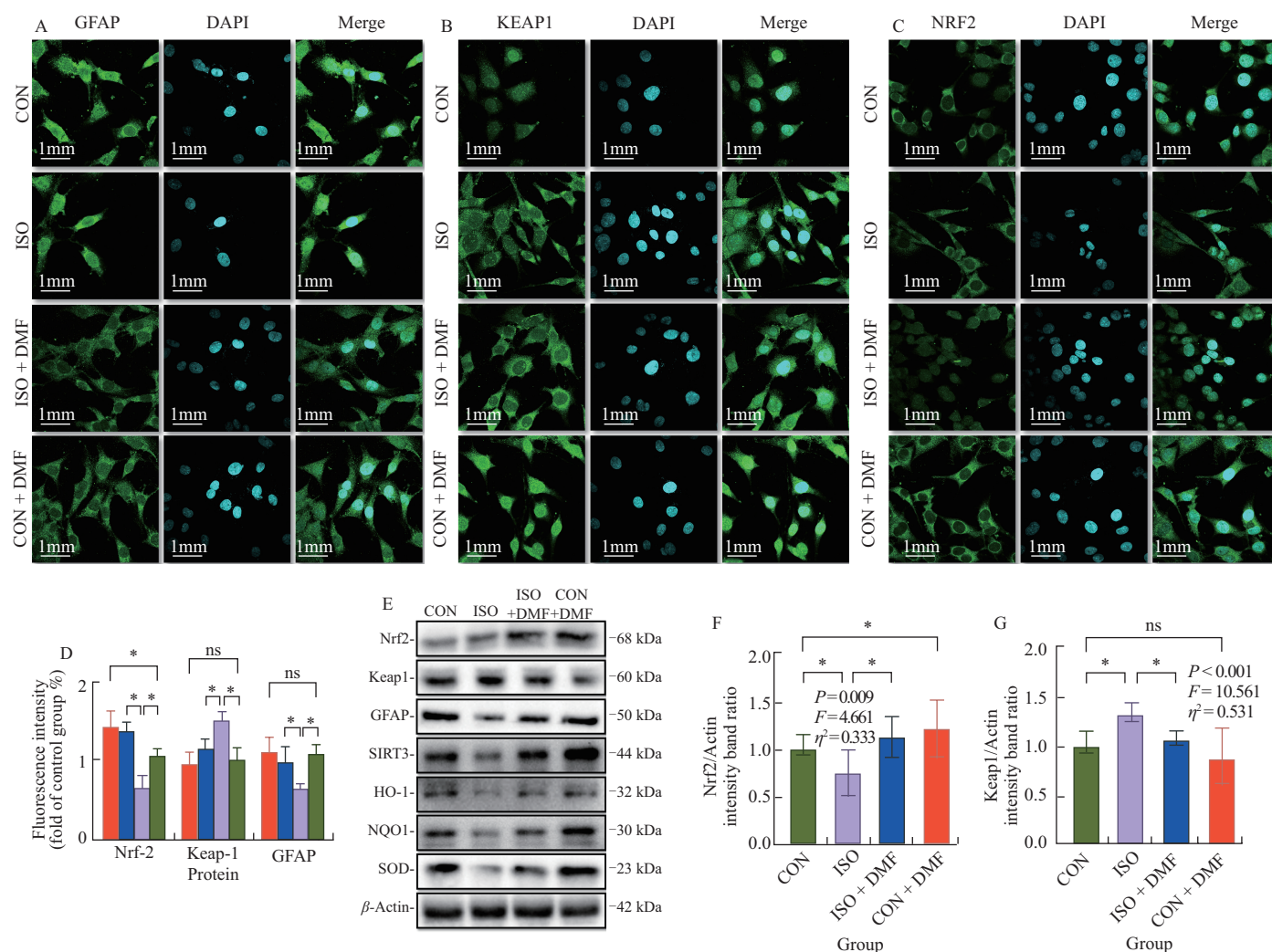
The experimental outcomes, illustrated in Figs. 4 and 5, demonstrate that the treatment of DMF significantly increased the



expression of Nrf2 while decreasing the expression of Keap1. This intervention effectively modifies the alterations in protein expression of related genes induced by isoflurane exposure. Additionally, the immunofluorescence and immunoblotting results showed in Figs. 4L and 5B-K revealed a decrease in the expression of Nrf2 in cells following isoflurane treatment, coupled with an elevation in the expression of Keap1. And isoflurane could significantly reduce the expression of downstream HO-1, NQO1 and Sirtuin3 (SIRT3). However, DMF could increase the expression of Nrf2 while significantly decreasing the expression of Keap1. And significantly increased the expression of downstream HO-1, NQO1 and SIRT3.

Additionally, we investigated the changes in antioxidant response element (ARE) fragment expression in this study by indirectly measuring the mRNA expression levels of downstream molecules HO-1 and NQO1, and directly measuring the Ct values of motif containing Nrf2 binding sites in the promoter regions of HO-1 and

NQO1 through ChIP-qPCR. Furthermore, we explored the molecular relationship between DMF and the Keap1-Nrf2 signaling pathway. The primer sequences for amplifying and measuring *HO-1* and *NQO1* mRNA expression, as well as the promoter regions spanning the Nrf2 binding sites, were provided in Table S2. As shown in Figs. 4S and 5T, SOD1/2 was used as a positive control and IgG as a negative control. Compared to the CON group, isoflurane treatment significantly reduced the expression of *HO-1* and *NQO1* genes containing the ARE region in primary mouse astrocytes. The addition of DMF significantly increased the expression of *HO-1* and *NQO1* genes in the ARE region. As shown in the Supplementary Figure, isoflurane treatment significantly reduced *HO-1* and *NQO1* mRNA expression in mouse brain tissue, primary astrocytes, and C8-D1A cells compared to the CON group. However, DMF supplementation significantly increased the mRNA levels of these 2 genes.



**Fig. 4** Effect of DMF in mice primary astrocytes. (A-C) Results of immunofluorescence staining. (D) Quantification of immunofluorescence staining. (E-L) Western blot analysis of Nrf2, Keap1, HO1, NQO1, GFAP, SIRT3 and SOD protein expression. (M) Results of cell viability assay. (N, O) Results of levels of ATP and fumaric acid. As compared to CON group, ISO group had the significant difference in the levels of ATP fumaric acid. As compared to ISO group, ISO + DMF group had significant difference in the levels of ATP fumaric acid. There was significant difference between CON group and CON + DMF group. (P, Q, R) Results of the content of GSH, GSSG and the ratio of GSSG to GSH. As compared to CON group, ISO group had significant difference in content of GSH and the ratio of GSSG to GSH. As compared to ISO group, ISO + DMF group had significant difference in content of GSH and the ratio of GSSG to GSH. There was no difference between CON group and CON + DMF group. (S) ChIP-qPCR results for the HO-1 promoter region containing the ARE fragment. (T) ChIP-qPCR results for the NQO1 promoter region containing the ARE fragment. Data presented as mean  $\pm$  standard error,  $n = 8$  mice in each group. Green represents CON group, purple represents ISO group, blue represents ISO + DMF group, orange represents CON + DMF group; ns means no significant difference,  $*P < 0.05$ .

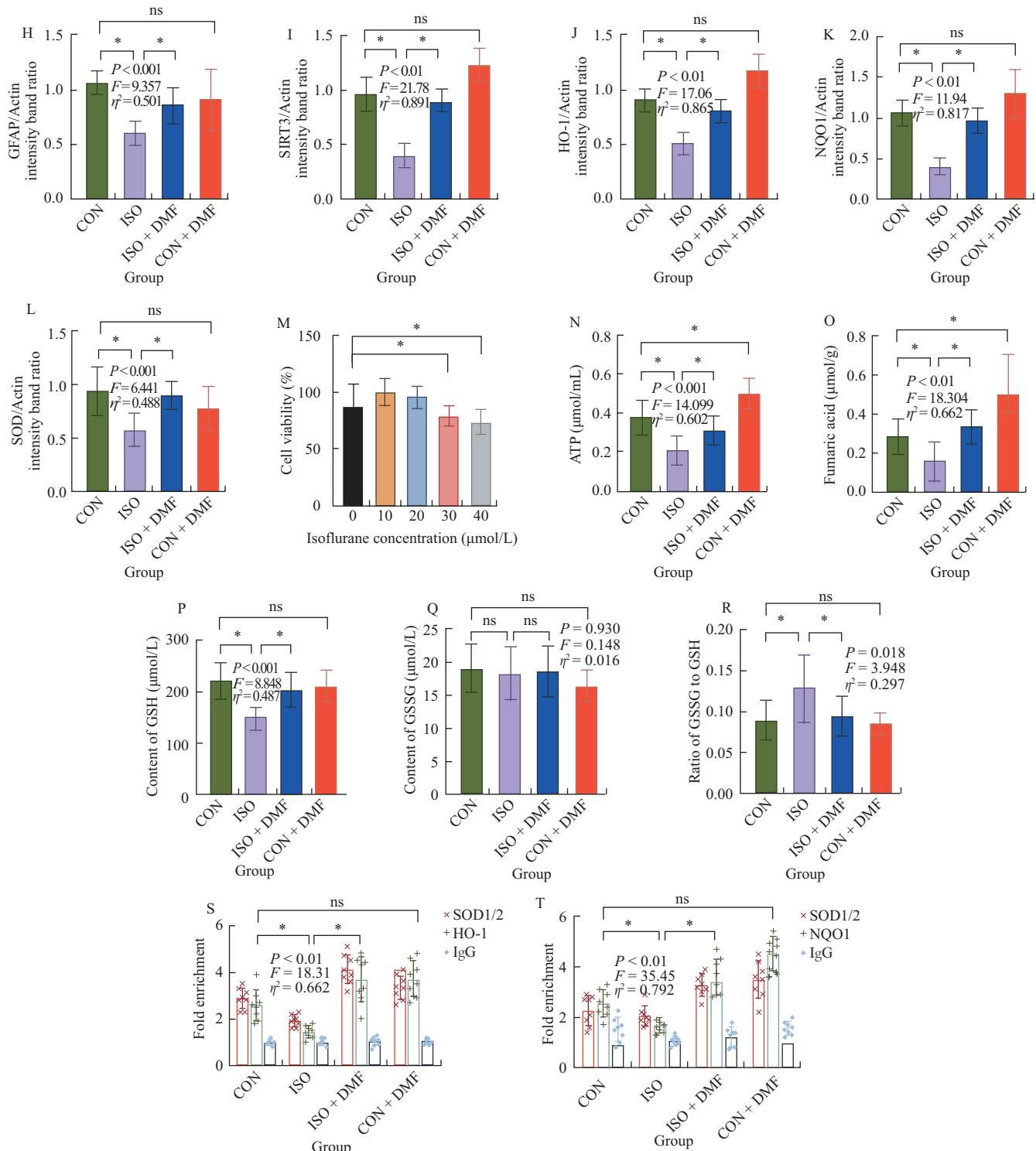


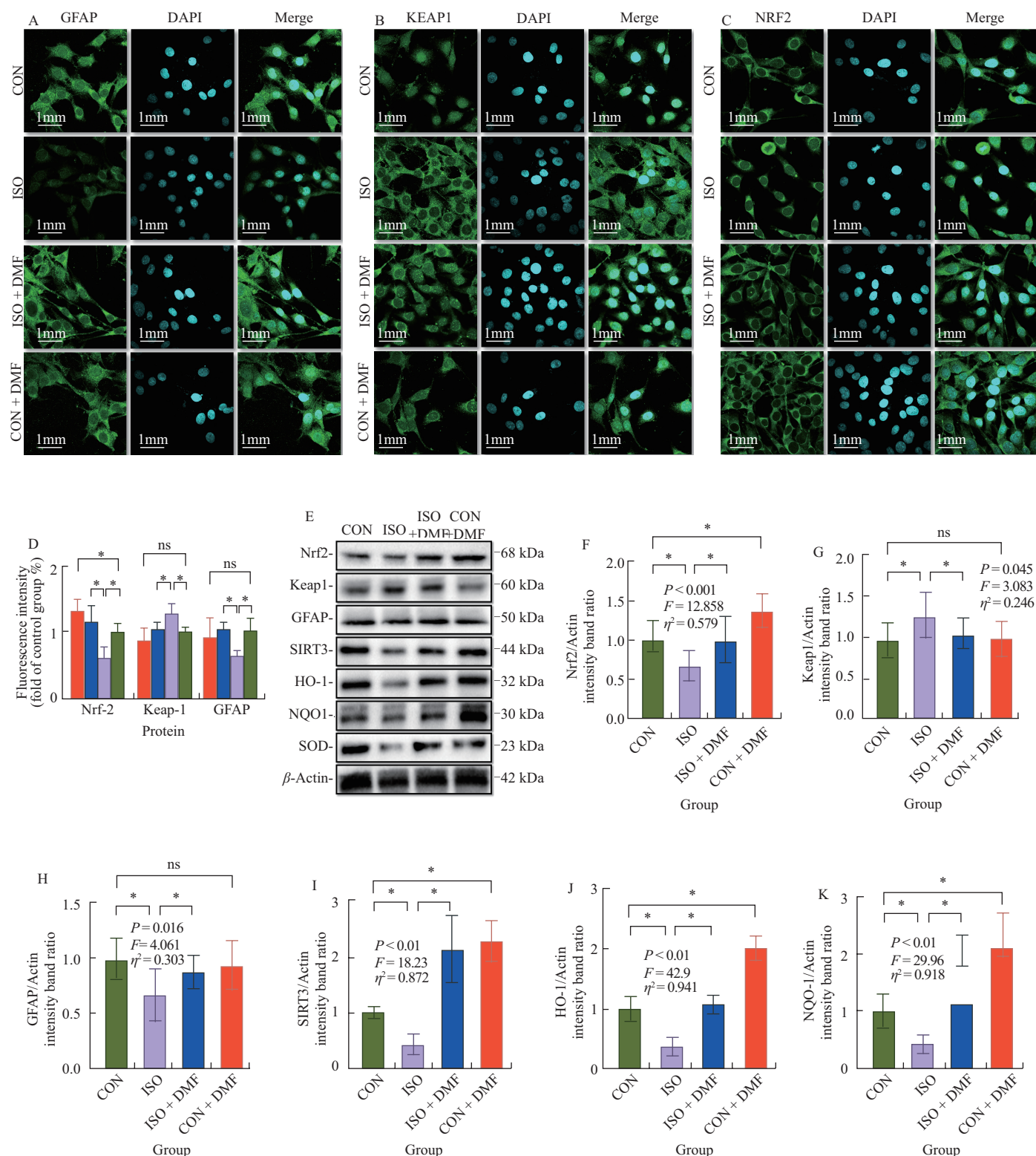
Fig. 4 (Continued)

### 3.10 Effect of DMF on oxidative indices in primary astrocytes and C8-D1A of isoflurane-treated mice

The results presented in Figs. 4 and 5 demonstrated a noteworthy decrease in the expression of SOD (Figs. 4E and L) and the ratio of GSSG/GSH (Figs. 4P-R) in the cells of the isoflurane-treated group when compared to the control group. However, the treatment of DMF significantly increased the expression of SOD and GSH.

### 3.11 Effect of DMF on oxidative phosphorylation products of citric acid cycle in primary astrocytes and C8-D1A of isoflurane-treated mice

The results of the experiment, as shown in Figs. 4K, L and 5K, L, indicated a significant decrease in the cellular levels of fumaric acid and ATP following isoflurane treatment. Furthermore, DMF alone markedly elevated intracellular fumaric acid and ATP content compared to the control group.



**Fig. 5** Effect of DMF in C8-D1A. (A-C) Results of immunofluorescence staining. (D) Quantification of immunofluorescence staining. (E, F, G, H, I, J, K, L) Western blot analysis of Nrf2, Keap1, HO1, NQO1, GFAP, SIRT3 and SOD protein expression. (M) Results of cell viability assay. (N, O) Results of levels of ATP and fumaric acid. As compared to CON group, ISO group had the significant difference in the levels of ATP fumaric acid. As compared to ISO group, ISO + DMF group had significant difference in the levels of ATP fumaric acid. There was significant difference between CON group and CON + DMF group. (P, Q, R) Results of the content of GSH, GSSG and the ratio of GSSG to GSH. As compared to CON group, ISO group had significant difference in content of GSH and the ratio of GSSG to GSH. As compared to ISO group, ISO + DMF group had significant difference in content of GSH and the ratio of GSSG to GSH. There was no difference between CON group and CON + DMF group. There was no difference among the groups. Data presented as mean  $\pm$  standard error,  $n = 8$  mice in each group. Green represents CON group, purple represents ISO group, blue represents ISO + DMF group, Orange represents CON + DMF group; ns means no significant difference,  $*P < 0.05$ .



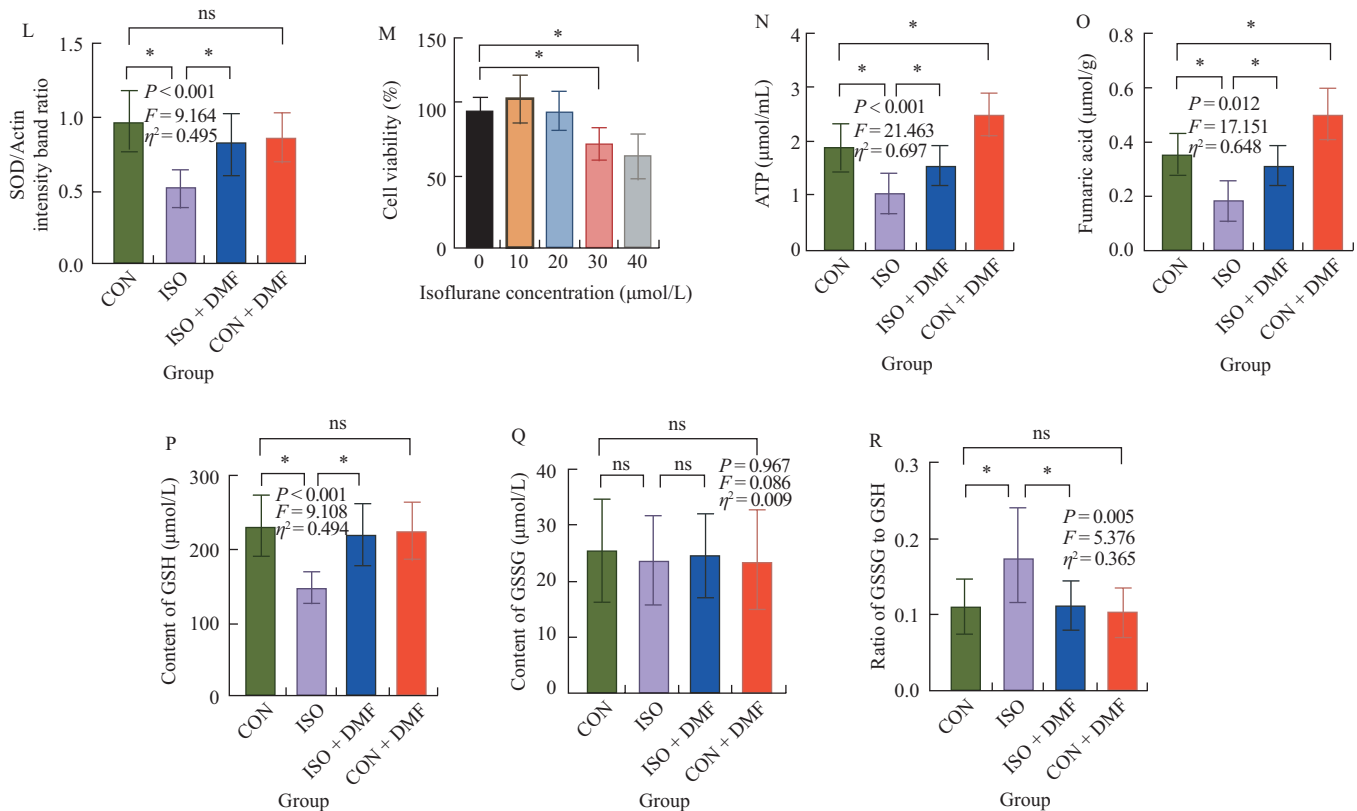


Fig. 5 (Continued)

#### 4. Discussion

The safety of anesthetic exposure to the developing brain is a significant health concern. Clinical studies have identified an association between repeated and extended exposures to both anesthesia and surgery in early life, and the occurrence of postoperative cognitive deficits. These deficits may result from abnormal cell death, neuroinflammation, or synaptic dysfunction<sup>[31]</sup>. The findings of this study align with previous research indicating that exposure to isoflurane during early neurodevelopment impairs the learning and memory capacity phenotype in mice<sup>[32]</sup>. Furthermore, our study illustrates that DMF can effectively mitigate the neurological damage induced by isoflurane exposure.

DMF, which can be hydrolysed to fumaric acid, exhibits various biological effects and is typically synthesized through chemical methods. It had found widespread application in preserving and preventing corrosion in foodstuffs, fruits, vegetables, and leather products owing to its potent bacteriostatic properties and stability<sup>[33]</sup>. Since 1994, DMF has been authorized as an oral medication for psoriasis treatment<sup>[34]</sup>. Early research postulated a connection between disruptions in the tricarboxylic acid cycle and psoriasis development, suggesting that DMF supplementation could effectively mitigate this pathological process<sup>[35]</sup>. Subsequent investigations further uncovered DMF's potential in treating multiple sclerosis (MS), leading to its approval by the FDA and the European Medicines Agency (EMA) in 2013 for MS treatment<sup>[36]</sup>. As research advanced, DMF exhibited diverse biological activities, providing robust evidence for its extensive application in medicine. Furthermore, DMF can activate helper T-lymphocytes (Th) and stimulate T-cells to produce cytokines,

including interleukin (IL)-4 and IL-5, to enhance immune responses<sup>[37]</sup>. Additionally, DMF binds to Keap1, inducing a conformational change that facilitates the nuclear translocation of Nrf2, thereby eliciting neuroprotection against oxidative stress<sup>[38]</sup>. While numerous studies have explored DMF's role in neurodegenerative diseases, there is a scarcity of reports on its mechanism in neurodevelopmental processes. This study aims to examine the preventive impact of DMF on cognitive dysfunction induced by isoflurane exposure in young mice, as well as its underlying mechanism. Our study revealed that DMF exhibits antioxidant effects, and its metabolite, fumaric acid, resulting from further hydrolysis, serves as a substrate in the tricarboxylic acid cycle to bolster cellular energy metabolism, thus exerting a neuroprotective role during brain development.

Pharmacokinetic investigations have demonstrated that DMF undergoes rapid and spontaneous hydrolysis to monomethyl fumarate (MMF) owing to the high chemical reactivity of its ester group. It has been observed that MMF constitutes the principal metabolite of DMF following oral administration<sup>[39]</sup>. The ester groups of DMF and MMF are both susceptible to hydrolysis and undergo Michael reactions to bind sulfhydryl groups (-SH). However, due to the neutral charge of DMF, its hydrolysis rate is significantly higher compared to MMF<sup>[40]</sup>. MMF can undergo further hydrolysis to form fumaric acid, which promotes the tricarboxylic acid cycle. Nevertheless, this reaction proceeds at a relatively low rate, leading to a gradual change in fumaric acid content. In current clinical practice, treatments involving DMF with a longer duration exhibit greater efficacy. The average peak plasma concentration of MMF reached 1.87 mg/L following twice-daily oral administration of 240 mg DMF<sup>[41]</sup>. Studies have compared the equivalence of daily oral administration of 75 mg/kg DMF in mice with

the recommended human dose of 480 mg/day, indicating similarity in drug exposure levels<sup>[42]</sup>. However, when the DMF dose was increased to 160 mg/kg, it exhibited toxicity in mice<sup>[43]</sup>. Additionally, studies have demonstrated that a gavage dose of 50 mg/kg DMF for 7 days yielded neuroprotective effects in mice<sup>[44]</sup>. Considering these findings, the gavage dose for mice in this study was set at 50 mg/kg for 14 consecutive days to assess both the neuroprotective effect and safety profile of DMF.

Behavioral tests indicated that isoflurane exposure led to impaired spatial learning, memory, and motor coordination in young mice, consistent with previous findings<sup>[32]</sup>. However, continuous gavage of DMF significantly ameliorated these dysfunctions. Additionally, Golgi staining and LFB staining demonstrated substantial loss of myelin sheath in the mouse brains following isoflurane exposure, along with reduced numbers and branches of neurons and decreased branch length. Conversely, DMF treatment effectively mitigated these pathological changes. Transmission electron microscopy observations further revealed isoflurane-induced mitochondrial damage, characterized by swollen and deformed mitochondria and blurred inner mitochondrial membranes. We hypothesize that exposure to isoflurane in young mice damages organelles, including mitochondria, and disrupts ATP and fumaric acid synthesis, thereby interfering with cellular energy metabolism and resulting in abnormal cell death. The findings of ELISA experiments supported our hypothesis, revealing a significant reduction in the levels of ATP and fumaric acid in the brain tissues of young mice following isoflurane exposure. Furthermore, DMF effectively mitigated inner mitochondrial membrane damage and elevated fumaric acid levels in the brain tissues, thereby enhancing energy metabolism. However, there was no significant difference between the CON and CON + DMF groups, which could be attributed to the following mechanism. DMF, a derivative of fumarate, functions as a metabolic intermediate in the TCA cycle and is positioned downstream of glycolysis within the cellular energy production pathway. Additionally, several rate-limiting enzymes in the TCA cycle play key roles in regulating metabolic processes. In the isoflurane-treated experimental group, cognitive dysfunction was primarily driven by the depletion of ATP and fumarate. DMF ameliorates this damage by enhancing the TCA cycle through the replenishment of ATP and fumarate. However, in the CON + DMF group, where ATP and fumarate levels were already adequate under normal physiological conditions, additional DMF supplementation did not exert a noticeable effect on the experimental outcomes. Hayes demonstrated that DMF activates the Keap1-Nrf2 pathway, leading to Nrf2 dissociation and translocation to the nucleus, where it binds to the antioxidant response element (ARE), thereby enhancing cellular antioxidant capacity<sup>[45]</sup>. This binding regulates ARE-mediated gene expression and induces the production of antioxidant enzymes, thereby enhancing cellular antioxidant capacity in response to various stimuli<sup>[46]</sup>. As demonstrated by our experimental results, DMF treatment activated Nrf2, while simultaneously initiating ARE and its downstream antioxidant genes. Additionally, Nrf2 activation has been linked to increased ATP and GSH synthesis<sup>[47]</sup>. Our study corroborates these findings, revealing significantly decreased levels of the *Nrf2* gene and protein expression and increased levels of the *Keap1* gene and protein expression in the brain tissue of mice following isoflurane anesthesia. Additionally, downstream proteins of the Keap1-Nrf2 signaling pathway, including HO1 and

NQO1, exhibited corresponding alterations. Continuous gavage of DMF resulted in elevated Nrf2 expression and decreased Keap1 expression. Concurrently, the level of ROS in the brain tissue of mice markedly rose following isoflurane anesthesia. In contrast, ROS level exhibited a significant reduction in the DMF group, accompanied by a noteworthy increase in the expression of HO1, NQO1, SIRT3 and SOD. This indicated an enhancement in mitochondrial energy metabolism and cellular antioxidant capacity. Nrf2 and SIRT3 interact and work together to regulate oxidative stress, mitochondrial function, and cellular metabolic homeostasis<sup>[48]</sup>. SIRT3 enhances mitochondrial antioxidant capacity through its deacetylase activity, reducing ROS production and indirectly activating Nrf2<sup>[49]</sup>. In turn, Nrf2 activates antioxidant genes and promotes SIRT3 expression, further strengthening mitochondrial antioxidant defenses. Together, they play a critical role in maintaining cellular health<sup>[50-51]</sup>.

Astrocytes, the most abundant cell type in the brain, are pivotal in learning and memory processes owing to their morphological diversity and structural plasticity in synaptic activity<sup>[52]</sup>. Furthermore, astrocytes serve as vital energy-supplying cells in the brain and possess the capability to sense and interconnect with synaptic activity<sup>[53-54]</sup>. Previous studies had portrayed the function of astrocytes solely as supportive for neurons. With the advancement of research, it has become evident that astrocytes can respond to various neurotransmitters, cytokines, and other substances during neural conduction. Additionally, they can express neurotransmitter-like receptors through exocytosis, thereby regulating ionic homeostasis within the brain through feedback mechanisms<sup>[55]</sup>. These discoveries underscore the intricate and indispensable roles of astrocytes in the nervous system. Glutamate serves as a primary excitatory neurotransmitter in the brain, and isoflurane, a commonly used inhalational anaesthetic, inhibits this neurotransmitter in the central nervous system to induce clinical effects like sedation and loss of consciousness. Nonetheless, during anesthesia, inhaled anesthetics like isoflurane activate *N*-methyl-*D*-aspartic acid receptors and voltage-dependent calcium channels, causing a significant influx of  $\text{Ca}^{2+}$  into cells, which ultimately leads to cell death<sup>[56]</sup>. As outlined in the study by Simard et al., astrocytes have the capability to uptake glutamate and convert it into glutamine to sustain synaptic activity and support neuronal metabolic functions<sup>[57]</sup>. Additionally, astrocytes can produce ATP during glutamatergic neuronal activity, which is utilized to modulate synaptic activity<sup>[58]</sup>. Research has delved into the mechanisms underlying glutamate release from astrocytes, revealing a complex interaction between astrocytes and neuronal activity<sup>[59]</sup>. Furthermore, astrocytes play a crucial role in the blood-brain barrier (BBB) and can secrete cytokines such as vascular endothelial growth factor and transforming growth factor- $\beta$  (TGF- $\beta$ ) to facilitate the formation and repair of the BBB<sup>[60]</sup>. To deeply investigate the molecular mechanism of DMF neuroprotection in the developing nervous system, we opted for the C8-D1A cell line and successfully isolated primary mouse astrocytes for detailed cellular experiments. GFAP serves as a major component of the astrocyte cytoskeleton and is widely acknowledged as a marker protein for astrocytes. Elevated expression of GFAP typically signifies heightened astrocytic responsiveness during neurological injury or pathological conditions<sup>[61]</sup>. Interestingly, our study observed a significant decrease in GFAP expression following isoflurane treatment. One potential explanation is that isoflurane induces aberrant death in a substantial

number of astrocytes. The expression of GFAP was markedly elevated in mice treated with DMF *via* gavage. Furthermore, our investigation revealed that DMF effectively mitigated abnormal cell death induced by decreased ATP and fumaric acid levels in astrocytes following isoflurane exposure. Subsequent studies demonstrated that DMF treatment activated the Keap1-Nrf2 signaling pathway, augmenting the antioxidant capacity of astrocytes, and thereby conferring its neuroprotective effects. These discoveries furnish a crucial experimental foundation for the utilization of DMF in the realm of neuroprotection.

In summary, based on the results from *in vivo* and *in vitro* experiments, we propose that DMF supplementation effectively mitigates cognitive dysfunction, learning and memory deficits, and motor coordination dysfunctions in young mice exposed to repeated isoflurane anesthesia. Specifically, DMF enhances astrocytic energy production through hydrolytic metabolic processes, thereby preserving their neural support and synaptic function. Additionally, DMF activates the Keap1-Nrf2 pathway and enhances antioxidant enzyme activity, thereby ameliorating oxidative stress in mouse brain cells. These findings not only offer valuable insights into the protective effects of DMF against isoflurane anesthesia-induced cognitive dysfunction and motor coordination deficits but also present a novel avenue for investigating potential neuroprotective agents.

### Conflict of interests

The authors declare there were no conflicts of interest in past 36 months before the manuscript was submitted.

### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://doi.org/10.26599/FSHW.2024.9250435>.

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