INTRODUCTION

Epilepsy is a disease characterized by an enduring predisposition to generate epileptic seizures and by the neurobiological, cognitive, psychological, and social consequences of this condition (1, 2). It is more prevalent in children. Neuronal damage and brain function abnormalities, including cognitive decline, behavioral abnormalities, depression, and other brain function abnormalities, as well as corresponding psychological and social changes, result from prolonged seizures. Therefore, early epilepsy control and brain protection treatment are of utmost importance. Due to its complexity, the pathogenesis of epilepsy remains unknown, and current research focused primarily on neurotransmitters (such as glutamate excitatory toxicity), ion channels (such as calcium overload), and genetic and immune abnormalities (3). In clinical practice, antiseizure medications (ASMs) remain the conventional treatment for epilepsy. With the deepening of research and the advancement of medical technology, surgery, nerve stimulation, and gene therapy have also been applied, but there are still few specific treatment methods for epilepsy diseases, and it is of great significance to explore new drug treatment methods. In recent years, multiple studies focusing on oxidative stress (OS) have suggested a close relationship between epilepsy and OS. OS is an easily observable pathophysiological process in both experimental epilepsy models and human epilepsy, yet Keap1/Nrf2/ARE signaling pathway is the most widely studied pathway for anti-OS in epilepsy. The antioxidant therapy can significantly improve the progression and prognosis of epilepsy (4). At present, several antioxidants, including resveratrol, astaxanthin, vitamin E, beta-carotene and their compounds, have made certain progress in the treatment of epilepsy (5-8). A large number of antioxidant compounds targeting mitochondria have been developed for diseases that impair mitochondrial function by countering mitochondrial oxidative stress, such as N-acetylcysteine and sulforaphane (4, 9). These antioxidant drugs can significantly improve the course of epilepsy and reduce the burden of seizure.

ANTIOXIDANT EFFECT OF DIMETHYL FUMARATE IN PENTYLENETETRAZOLE-KINDLED EPILEPSY MICE AND IS ACTIVATED BY NUCLEAR FACTOR ERYTHROID 2-RELATED FACTOR 2 PATHWAY

This study was designed to examine the anti-oxidative stress effect of dimethyl fumarate (DMF) on pentylenetetrazole (PTZ)-induced epileptic mice, and to evaluate the correlation of its mechanism with the nuclear factor E2-related factor 2 (Nrf2)-mediated signaling pathway. The experimental mice were separated into three groups: control, model, and DMF groups. Mice in the model group were administered PTZ to establish an epilepsy model, mice in the DMF group were administered DMF concurrently when modeling, and mice in the control group were administered a 0.9% NaCl solution. The latency, severity, and frequency of epileptic seizures in mice after each treatment were recorded, and the modeling success rate was computed at the conclusion of the experiment. The mice were euthanized, their levels of malondialdehyde (MDA), reactive oxygen species (ROS), superoxide dismutase (SOD), 8-hydroxy-deoxyguanosine (8-OHdG), and Nrf2 were measured, and the electron microscope was used to examine the mitochondrial damage of brain tissue. The latency of epileptic seizures was longer in the DMF group compared to the model group (P<0.05). The levels of MDA and ROS in the DMF group were lower than those in the model group (P<0.0001), and the activity of SOD in the DMF group was higher than that in the model group (P<0.0001); however, the levels of MDA and ROS were elevated and the activity of SOD was lower in both groups relative to the control group. The levels of 8-OHdG were lower in the DMF group than the model group (P<0.0001), however, the levels were higher in both groups compared to the control group. Mitochondrial abnormalities were more prevalent in the model group than in the DMF group, and more prevalent in both groups compared to the control group. The DMF group contained more Nrf2 content than the model group (P<0.0001), and both groups contained more Nrf2 than the control group. We concluded that the mechanism by which DMF reduced the level of oxidative stress in epileptic mice might involve the Nrf2-mediated signaling pathway.

Key words: dimethyl fumarate, epilepsy, mitochondria, nuclear factor E2-related factor 2, oxidative stress, pentylenetetrazole
These studies provide a new idea for further research on the pathogenesis of epilepsy and a new entry point for its effective treatment.

Dimethyl fumarate (DMF), a new synthetic drug, exerts a cell-protection and plays an anti-inflammatory role in the treatment of various diseases of the nervous system (10-12); it is currently used primarily in the treatment of multiple sclerosis, although, its mechanism of action is not entirely clear. However, relevant studies have demonstrated that its metabolite - monomethyl fumarate - is a niacin receptor agonist, and existing experimental study has demonstrated that DMF can activate the nuclear factor E2 related factor 2 (Nrf2) pathway in vivo and in vitro in animals and humans, by activating the downstream antioxidant enzymes to play the role of antioxidant stress, thereby alleviating the oxidative stress response of demyelination (13). Relevant studies have demonstrated that DMF can significantly reduce malondialdehyde (MDA) and reactive oxygen species (ROS) levels and increase superoxide dismutase (SOD) activity, thereby reducing oxidative stress and cell damage in the treatment of renal disease and cardiovascular disease (14, 15). It has been confirmed that this effect is primarily associated with the Nrf2 signaling pathway (12). No studies have been conducted on the use of DMF in the treatment of epilepsy; however, DMF has demonstrated good antioxidant stress ability in the treatment of a variety of diseases according to results from previous experiments, and it is expected to become a new treatment option for epilepsy. This study will investigate the protective effect of DMF against oxidative stress in epileptic diseases by inducing seizures in mice with pentylenetetrazole (PTZ). Among the existing methods, PTZ-mediated chemical ignition method is a good, repeatable and inexpensive method for inducing epilepsy in animal models, and the most widely accepted animal model for repeatable and inexpensive method for inducing epilepsy in mice with pentylenetetrazole (PTZ). Among the existing methods, PTZ-mediated chemical ignition method is a good, repeatable and inexpensive method for inducing epilepsy in animal models, and the most widely accepted animal model for studying the effects of novel antiepileptic molecules (16).

MATERIALS AND METHODS

Ethics approval

All experiments were evaluated and approved by the Ethics Committee of Qingdao Municipal Hospital. Principles of Laboratory Animal Care (NIH Publication Vol 25, No. 28 revised 1996; http://grants.nih.gov/grants/guide/notice-files/not96-208.html) were followed, as well as specific national laws (e.g. the current version of the German Law on the Protection of Animals) where applicable.

Experimental animals

Thirty healthy and clean Balb/C male mice, aged 2 weeks and weighing 10–15 g ±5 g, were purchased from Jinan Pengyue Laboratory Animal Care’ (NIH Publication Vol 25, No. 28 revised 1996; http://grants.nih.gov/grants/guide/notice-files/not96-208.html) were followed, as well as specific national laws (e.g. the current version of the German Law on the Protection of Animals) where applicable.

Experimental drugs and reagents

DMF (MedChemExpress); pentylenetetrazole (PTZ), and ROS stain (Sigma-Aldrich, St. Louis, MO, USA; Elisa kits for SOD, MDA (MedChemExpress, Monmouth Junction, NJ, USA; E-BC-K019-M, E-EL-0060c); Elisa kit for 8-hydroxy deoxyguanosine (8-OHdG) (Meimian, Jangsu, China MM-0211M2); as well as OCT embedding agent, PBS buffer, and electron microscope fixation solution (Servicebio, Wuhan, China).

Main experimental instruments

Multifunctional microplate reader (Mitutoyo, Kanagawa, Japan) and imaging system for positive fluorescence microscopy (Nikon, Tokyo, Japan).

Grouping of experimental mice

After 1 week of laboratory quarantine and acclimation, 30 Balb/c male mice aged 2 weeks were randomly divided into 3 groups of 10 animals each (labeled the control group, the epilepsy group, and the dimethyl fumarate intervention group (DMF group).

Establishment of epilepsy model

DMF treatment, and behavioral observation. PTZ was used to establish epilepsy models in both the model group and the intervention group by intraperitoneal injection of 35 mg/kg PTZ solution (5 mg/ml, solvent 0.9%NaCl) at 9:00–10:00 a.m. daily for 4 weeks (17). Mice in the model group were administered 0.9% NaCl solution intragastrically 30 min prior to intraperitoneal injection of PTZ; mice in the intervention group were administered 25 mg/kg DMF solution (5 mg/ml, solvent 0.9%NaCl) intragastrically 30 min prior to intraperitoneal injection of PTZ (18-20) and mice in the control group were administered 0.9% NaCl solution intragastrically and intraperitoneally as the control. During modeling, the latency, severity, and frequency of seizures within 30 to 60 minutes after intraperitoneal administration of PTZ were monitored in mice. In cases of severe convulsions, intraperitoneal chloral hydration was administered until the seizures ceased. The severity of seizures was measured using the Racine scale, which is frequently used to evaluate seizure activity in rodents.

The classification is as follows: Grade 0 - no seizure response; Grade 1 - immobility, eye closure, ear twitching, and facial clonus; Grade 2 - head nodding, associated with more severe facial clonus; Grade 3 - unilateral forelimb clonus and rearing, or bilateral forelimb clonus without rearing; Grade 4 - bilateral forelimb clonus with rearing, or falling on the side without rearing, but not generalized tonic-clonic seizures; Grade 5 - limb rearing, and falling on the back, with generalized tonic-clonic seizures.

Successful modeling of epileptic immature mice refers to a grade 4 or higher Racine score three consecutive times and favorable conditions following cessation of seizures (21). At 4 weeks post-modeling, the success rate of epilepsy modeling in the model group and the intervention group was determined, and mice were sacrificed for biochemical immune detection using blood and brain tissue.

Detection of mice serum samples

The eyeballs of mice were removed for blood collection, followed by blood centrifugation, and serum was collected and stored at −80°C in the refrigerator for later use. Elisa kits were used to detect the presence of SOD, MDA, and 8-OHdG, and absorbance at 450 nm was measured using a microplate reader in accordance with the Elisa kit’s instructions.

Detection of reactive oxygen species in mouse brain tissue

The frozen brain tissue was sectioned, rewarmed at room temperature, dried, followed by Pap Pen circle drawing and tissue autofluorescence quenching. After adding ROS dye solution, the section was incubated for 30 minutes at 37°C in a dark incubator. The nuclei were counterstained with DAPI, decolored in PBS
(pH 7.4), and the sections were slightly dried and sealed with an anti-fluorescence sealant. The fluorescence microscope was used to observe sections and acquire images, the imagine J software is used to analyze the average optical density values.

Detection of Nrf-2 in mouse brain tissue

After the brain tissue was paraffin-embedded, sliced, dewaxed, and antigenically repaired, a circle was drawn with a Pap Pen and serum was deposited to seal the tissue. Then, successive primary and secondary antibodies were added, the cell nucleus was counterstained with DAPI, the tissue autofluorescence was quenched, and the section was sealed for microscopy and photography (22). The image J software (NIH, Bethesda, MD, USA) used to analyze the average optical density values.

Observation of mitochondrial injury under transmission electron microscope

The brain tissue was placed in the fixation solution for the electron microscope, fixed, and stored at 4°C. Through fixation after AGAR pre-embedding, permeation, and embedding after dehydration at room temperature, the embedded plate was placed in a 60°C oven for polymerization for 48 hours. Then, the resin block was removed and cut into 60–80 nm ultra-thin sections, which were placed in the 150-mesh Formvar film for staining, and then observed under transmission electron microscopy for image collection and analysis.

Statistical analysis

SPSS25.0 software was used for data analysis and processing. All normally distributed measurement data are expressed as mean ± standard deviation. For intergroup comparison, one-way ANOVA was used, and the Bonferroni method was used to correct for multiple comparisons between groups. P<0.05 was considered statistically significant. GraphPad Prism (GraphPad Software, Boston, USA) and Adobe Illustrator were the plotting software utilized in this study.

RESULTS

Success rate of epileptic modeling and seizure of immature mice in each group

There were no abnormal behavior observed in the Control group, and modeling was successful in nine mice in both the Model group and the DMF group, with no significant differences between the two groups in the modeling success rate. The seizure latency of mice in the DMF group was significantly prolonged than Model group (P<0.05). However, there were no statistically significant differences between modle group and DMF group in terms of mean seizure grade and mean seizure frequency (P>0.05), as shown in Table 1.

Comparison of superoxide dismutase activity and malondialdehyde content in serum of mice in each group

The SOD activity level of mice in the DMF group was greater than that in the Model group (P<0.0001), whereas the MDA content of mice in the DMF group was less than that in the Model group (P<0.05). As shown in Fig. 1, the SOD activity level decreased, and the MDA level increased in both Model group and DMF group compared to the Control group. Thus, DMF could effectively enhance the antioxidant capacity of

Table 1. Seizures of mice in the Model group and the dimethyl fumarate (DMF) group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean latency (s)</th>
<th>Mean seizure grade</th>
<th>Total number of seizures (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model group</td>
<td>99.90 ± 22.25</td>
<td>3.43 ± 0.44</td>
<td>17.40 ± 2.01</td>
</tr>
<tr>
<td>DMF group</td>
<td>140.70 ± 45.06</td>
<td>3.10 ± 0.79</td>
<td>16.60 ± 2.06</td>
</tr>
<tr>
<td>P value</td>
<td>P&lt;0.05</td>
<td>P&gt;0.05</td>
<td>P&gt;0.05</td>
</tr>
</tbody>
</table>

Fig. 1. Serum malondialdehyde (MDA) content and superoxide dismutase (SOD) activity of mice in each group (** ** ** ** P<0.0001).
tissue cells, thereby reducing the production of oxidative products and tissue cell damage.

**Detection of reactive oxygen species content in brain cells of mice in each group**

*Fig. 2* depicts the immunofluorescence image of ROS in the brain tissue of mice from each group, with the red light denoting the ROS positive part, the higher the red light density, the higher the ROS content. Based on the analysis of the immunofluorescence map's mean optical density, the positive optical density of the Model group and DMF group is greater than that of the Control group, and the average optical density of fluorescence images of the Model group is greater than that of the DMF group (*P*<0.0001). The results indicate that DMF treatment was able to effectively enhance the anti-oxidative stress response and decrease the production of oxidative factors in epileptic immature mice.

**Detection of serum 8-hydroxy-deoxyguanosine content of mice in each group**

As shown in *Fig. 3*, the content of 8-OHdG in the DMF group and Model group was significantly higher than that in the Control group, and the content of 8-OHdG in the DMF group was significantly lower than that in the model group (*P*<0.0001). Thus, it is evident that DMF could significantly reduce mitochondrial damage in epilepsy model mice.

![Fig. 2. Reactive oxygen species (ROS) immunofluorescence image (×500) of the Control group (A), the Model group (B) and the Dimethyl fumarate (DMF) group (C), and histogram of the average optical density of each group. Mean optical density of each group (****P<0.0001).](image1)

![Fig. 3. Serum content of 8-OHdG in the Control group, the Model group and the Dimethyl fumarate (DMF) group (****P<0.0001).](image2)
Comparison of mitochondrial damage in the hippocampal tissue of mice in each group under electron microscope

Fig. 4 is an electron microscope image of the mitochondria of mice from the three groups. In each high-power field of view, it is evident that the number of mitochondria in the Model group had been drastically reduced, accompanied by irregular size, swelling, disordered cristae arrangement, vacuolation, and even collapse of some mitochondria. The number of mitochondria in the DMF group was also reduced, and the mitochondria intumesced slightly, but their size and morphology were essentially regular, and their ridge arrangement was essentially visible, with some cavitation. Mitochondria were abundant in the hippocampal tissue of immature mice in the Control group, with uniform size and morphology, clearly visible cristae and capsule, and no obvious edema or vacuolization. The electron microscopy image of mitochondria directly reflects the specific situation of mitochondrial damage in terms of number, morphology, and arrangement, indicating that DMF intervention could effectively mitigate mitochondrial damage in the hippocampus of epileptic mice.

Quantitative comparison of Nrf2 in each group

As depicted in Fig. 5, an immunofluorescence assay was used to measure the level of Nrf2 in the hippocampal tissue cells of the mouse brain, where green fluorescence indicated positive Nrf2, the higher the fluorescence density, the higher the Nrf2 content. The results showed that the average optical density of DMF group was higher than that of Model group (P<0.0001), while the average optical density of DMF group and Model group was lower than that of Control group, indicating that DMF
could increase the Nrf2 level in the hippocampal tissue of epileptic mice, thereby improving the antioxidant stress ability of Nrf2 signaling pathway and alleviate brain cell injury.

**DISCUSSION**

Epilepsy is a form of chronic brain disorder characterized by recurrent seizures (23). Therefore, it is crucial to correctly comprehend epilepsy and establish a reasonable and standardized diagnosis and treatment scheme in clinical practice. The ultimate objective of epilepsy treatment is not only to control seizures, but also to reduce nerve damage caused by each seizure in order to improve brain function and the quality of life of patients. ASMs are the most important clinical treatment option for epilepsy, and they are frequently chosen. ASMs are effective in most patients, but there is a lengthy transitional period before the drug achieves a relieving or controlling effect, during which the negative effects of epileptic seizures on brain function become noticeable. In addition, the therapeutic efficacy of ASMs is poor in some patients with refractory epilepsy, and prolonged and frequent seizures negatively impact the prognosis for brain damage (24). Therefore, with respect to the treatment of epilepsy, although, ASMs are required to control the frequency and severity of seizures, brain damage can be mitigated by reducing oxidative stress damage during seizures, which is of utmost importance for patients who do not respond to ASMs or respond inadequately (25). At present, there are cutting-edge research for the drug treatment of epilepsy has brought different new hopes, such as *Cicuta virosa, Nux vomica* and other different from the traditional anti-epileptic drugs, all help to improve the symptoms of epilepsy in experimental animals and improve various physiological conditions, so for the treatment of epilepsy, to find a new drug way out is very necessary (26, 27).

In this study, PTZ was used to induce epilepsy in mice, making the results credible. Behavioral observations of mice in the model group and DMF group revealed that, despite no statistically significant differences in the control of seizure severity, DMF treatment could significantly prolong the latency of seizures in mice, demonstrating a positive effect on the treatment of epilepsy and providing a reference for the treatment of epilepsy in the future.

ROS, MDA, and SOD are frequently employed indexes for measuring oxidative stress. A higher level of ROS indicates more severe oxidative injury to brain tissue cells. MDA, the end product of lipid peroxidation, is cytotoxic, and a higher level of MDA indicates more severe cell membrane oxidation damage. SOD, one of the main antioxidant enzymes in tissues, can eliminate ROS, thereby decreasing MDA production. The oxidative stress indexes of mice in the PTZ-induced epilepsy model group and the DMF group were significantly higher than those of mice in the control group, indicating that a high level of oxidative stress is involved in epilepsy, exceeding the body’s antioxidant ability, which is consistent with the findings of previous studies on oxidative stress and epilepsy, thereby providing a theoretical basis for antioxidant therapy for epilepsy.

The results of this study demonstrated that, in terms of the oxidative stress index, the DMF treatment in the DMF group effectively reduced the production of ROS and oxidative product MDA in epileptic mice, and increased the activity value of SOD, indicating that DMF could effectively reduce the oxidative stress level in epileptic mice, and increase the antioxidant ability of the body, thereby providing a certain protective effect to tissue cells. The reason the brain is especially susceptible to oxidative stress is because brain cells require more oxygen and have a higher metabolic rate, and cell membranes are rich in unsaturated fatty acids, providing a foundation for lipid peroxidation (28). Extensive evidence suggests that the overexcitation of neurons and oxidative damage resulting from the excessive production of free radicals play a significant role in the occurrence and development of epilepsy. Both the cause and effect of epileptic seizures are excessive oxygen free radicals. Extended epileptic seizures cause a redox imbalance, forming a vicious cycle. Controlling the level of oxidative stress in the body, on the other hand, may aid in the effective management of epileptic activity progression.

Mitochondria are the primary source of intracellular ROS during the oxidative stress process. Numerous studies support the notion that mitochondrial dysfunction is an important factor in the development of neurological diseases, particularly epilepsy (29-31). With the occurrence of pathological conditions, such as epilepsy, there is an excess of ROS in brain mitochondria (32). Mitochondrial DNA is more susceptible to oxidative damage than nuclear DNA due to the absence of histone protection and proximity to the electron transport chain. This results in the production of a large amount of 8-OHdG, which causes mitochondrial dysfunction, alters cellular energy metabolism and signal transduction, and ultimately leads to a variety of pathological reactions (33). In this study, the serum 8-OHdG content of mice in the DMF group was significantly lower than that of mice in the Model group, indicating that mtDNA damage of mice in the DMF group was less severe than that of mice in the Model group. Under an electron microscope, the morphology of mitochondria in mouse brain tissue was visible. In the Model group, mitochondrial swelling was evident, with the cristae disappearing and predominantly in a disruption pattern while in the DMF group, abnormal mitochondrial morphology was rare and predominantly manifested as swelling. Based on the content of serum 8-OHdG of mice and the observation of brain mitochondria under an electron microscopic, we found that DMF treatment effectively mitigated mitochondrial damage in epileptic mice and contributed to the maintenance of the normal energy metabolism of mitochondria, namely signal transduction, thereby protecting brain function.

Nrf2, a cellular antioxidant regulatory factor, can regulate the basic expression and induced expression of a series of antioxidant response element-dependent genes and participate in the control of a variety of programmed functions by regulating oxidation levels and oxidation signaling pathways (34). Under normal physiological conditions, Nrf2 is only expressed in the cytoplasm in an inactive state. The interaction between the endogenous inhibitor keap-1 and Nrf2 can promote the proteasome degradation of Nrf2 (34, 35). When an abundance of ROS is produced by tissue cells due to physical and chemical injury, ROS stimulation alters the conformation of Keap-1, releasing the Nrf2 transcription factor, which then enters the nucleus and binds with the sequence elements of antioxidant reaction (ARE). This stimulates the expression of downstream target genes and improves antioxidant activities such as SOD, thereby enhancing the antioxidant capacity of the body (35). According to the results of this study, the content of Nrf2 in the brain tissue of mice in the DMF group was greater than that in the Model group, and the levels in both groups were greater than the control group, suggesting that its antioxidant mechanism in epilepsy may be an Nrf2-mediated pathway, which is consistent with the findings of DMF in previous experimental studies (36-38).

DMF, an antioxidant, can significantly enhance the resistance of tissue cells to oxidative stress and reduce mitochondrial damage (39) in patients with neurodegenerative diseases (40) and ischemia-reperfusion injury (41).

According to the results of this study, we found that the intervention of DMF in an epileptic model significantly reduced the level of oxidative stress in epileptic immature mice, and the
mechanism of action may involve an enhancement of the signaling pathway transduction of antioxidant damage by increasing the level of Nrf2 in brain cells, and reduction of the damage of oxygen-derived free radicals to the mitochondria of brain cells, thereby maintaining the normal energy metabolism of cells, and enhancing the antioxidant ability of hippocampal tissue cells in epileptic immature mice. This study can serve as a positive reference for a variety of epilepsy treatment methods, however, additional research is necessary to evaluate the efficacy and safety of DMF at various doses.

**Author’s contribution:** Dr. Yong Chang, Dr. Mei Zhou and Dr. Rui-Yun Zhang contributed equally to this study.

**Funding:** This study was supported by a grant from Qingdao Shinan District Science and Technology Bureau project (2018-4-023-YY).

**Conflict of interests:** None declared.

**REFERENCES**


Received: April 15, 2023
Accepted: February 29, 2024

Author’s address: Dr. Rui-Yun Zhang, Department of Pediatrics, Qingdao Hospital, University of Health and Rehabilitation Sciences (Qingdao Municipal Hospital), No.1 of Jiaozhou Road, Qingdao 266011, China.
E-mail: ruiyunzhangdoc@126.com