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Original Article

# Analysis of the Inhibitory Effect of Dihydroartemisinin on Inflammatory Responses in Mice with Neuropathic Pain Induced by the Japanese Encephalitis Virus NS1

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## ABSTRACT

**Objectives:** To elucidate the underlying mechanism through which Dihydroartemisinin (DHA) exerts its influence on mice with Neuropathic Pain (NP) induced by the NS1 protein of the Japanese Encephalitis Virus (JEV). **Materials and Methods:** 15 C57BL/6N mice were allocated into a control group (normal mice), a model group (NP model induced by NS1), and a DHA group (NP model induced by NS1 and treated with DHA by gavage). Following corresponding intervention treatments for each group, behavioral tests were carried out, and the levels of inflammatory factors and stress injury markers in the brain tissue were detected. Histopathological damage in the brain tissue was visualized using Hematoxylin-Eosin (HE) staining, and the fluorescence intensities of transmembrane protein 119 (TMEM119) and chemokine (C-C motif) ligand 3 (CCL3) were determined. Finally, the expression of the ALK5/SMAD3 pathway in the mouse brain tissue was examined. **Results:** Under DHA intervention, the struggling time in the Tail-Suspension Test (TST) and Forced-Swimming Test (FST), as well as escape latency, were notably shortened in the DHA group compared to the model group, while the staying time in the Open-Field Test (OFT) was prolonged ($p<0.05$). Additionally, both the levels of inflammatory cytokines and the degree of stress-induced injury in the DHA group were significantly lower than those in the control group ($p<0.05$). HE staining results indicated an improvement in the histopathological damage of the brain tissue in the DHA group, accompanied by a decrease in the fluorescence intensities of TMEM119 and CCL3. Furthermore, it was discovered that DHA activated the expression of the ALK5/SMAD3 signaling pathway. **Conclusion:** DHA alleviates NP in mice by inhibiting the neuro-inflammatory response, which is achieved through the activation of the ALK5/Smad3 signaling pathway.

**Keywords:** Neuropathic pain, Dihydroartemisinin, Inflammatory response, Oxidative Stress.

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## INTRODUCTION

Neuropathic Pain (NP) is the pain caused by the damage and dysfunction of the somatic nervous system and is also one of the major complications of various peripheral or central nervous system disorders.[1] Epidemiological data indicate that the prevalence of NP in the general population ranges from approximately 3% to 17%.[2] In contrast, among patients with nerve injury, the incidence of this condition can soar to as high as 95%.[3] Clinically, NP presents as persistent and uncontrollable pain. Meanwhile, patients may experience neurological symptoms such as nerve numbness and muscle weakness. These manifestations not only significantly disrupt patients' daily living activities but also carry the potential to precipitate negative emotional states, such as anxiety and depression.[4] Despite the availability of multiple therapeutic agents for NP in contemporary clinical practice, such as gabapentinoids and carbamazepine, these medications commonly encounter challenges, including suboptimal long-term efficacy and a high recurrence rate of NP.[5] Consequently, the exploration of a novel, safe, and effective treatment strategy for NP is of paramount importance in enhancing the prognosis of patients afflicted with this condition.

Dihydroartemisinin (DHA), a derivative and active metabolite of artemisinin, has been proven by research to exert anti-inflammatory effects by reducing the production of inflammatory mediators and regulating inflammation-related pathways, In light of this, DHA is widely applied in the treatment

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of various inflammatory diseases in clinical practice.[6] Regarding various neurological diseases, DHA has also demonstrated excellent application results. For instance, DHA can alleviate hypoxic-ischemic brain injury in neonatal rats by inhibiting oxidative stress damage.[7] Additionally, it can improve the cognitive function of mice afflicted with Tauopathies.[8] As for NP, as confirmed by Zhu CY's research, DHA intervention in mice with spinal cord ligation can lower the pain threshold of these mice.[9] Recently, a study by Liu Y confirmed that DHA combined with pregabalin can improve the inflammatory response in NP,[10] which lays the foundation for the use of DHA in NP. However, the study by Liu Y *et al*., is a Chinese-language study and lacks a reference mechanism for promoting the research and use of DHA internationally. In addition, Zhu C *et al*., published a study on NP mice in 2022, and they mentioned that DHA exerts analgesic and antidepressant effects by down-regulating HnRNPA1 in the spinal cord and hippocampus,[11] which provided us with ideas for the study. Of course, Zhu C *et al*., also showed that the mechanism of DHA's effect on NP is not only limited to this pathway, and more studies are needed to explore it in depth. The study by Ling L *et al*., reported that sinomenine ameliorates microglial activation and NP following chronic constriction injury via the ALK5/Smad3 signaling pathway,[12] suggesting a certain potential relationship between ALK5/Smad3 and NP. For DHA, the ALK5/Smad3 signaling pathway is also considered a crucial mechanism of action.[13] Therefore, we hypothesized that DHA's effect on NP might also be related to this pathway.

Therefore, in the present study, we will establish a mouse model of NP, analyze the effects of DHA on inflammatory response and stress injury in NP, and observe whether its pathway is related to the ALK5/SMAD3 signaling pathway, so as to provide new references and guidelines for the treatment of NP in the future.

## MATERIALS AND METHODS

### Animal data

Thirty Specific Pathogen-Free (SPF)-grade C57BL/6N mice were procured from Wuhan Servicebio Technology Co., Ltd., with the animal use license number SYXK (E) 2023-0101. These animals, at 15 weeks of age and weighing between 25-28 g, were evenly divided between genders. They were housed in separate cages, with five mice per cage, in an environment maintained at a temperature of (21.0 week) and a humidity of (50.0h 5 mice per cage, in an environment maintained at a temper. All the experimental procedures involving animals were conducted in accordance with ARRIVE guidelines and approved by the Animal Ethics Committee of Yichang Central People's Hospital (NO. kl0056).

### Induction of NP in mice by NS1

Fifteen mice were randomly divided into three groups (5 mice per group) and intraperitoneally injected with 5 mL of Japanese Encephalitis Virus (JEV) NS1 suspension (purchased from Beijing Bioleaf Biotechnology Co., Ltd.,) at a concentration of $3\times10^6$ pfu/mL.[14] On the 3rd, 6th, and 9th days after the intervention, one group of mice were randomly selected at each time point to detect the mechanical pain threshold of the L5 reflex zone of the left foot using the Von Frey method, and the 50% paw-withdrawal threshold was calculated. A 50% paw-withdrawal threshold of less than 4 g was regarded as a successful establishment of the NP model.[15] Upon completion of the examination, the mice were humanely euthanized (carbon dioxide inhalation is used. Mice are placed in a clear airtight container and carbon dioxide is injected at a rate of 20%-30% of the container volume/minute. Observe mice until respiration stops and death is confirmed). Brain tissues were then harvested, fixed in 4% paraformaldehyde for 24 hr, and subsequently processed into tissue sections. Immunofluorescence staining was then performed as follows: After washing with Phosphate-Buffered Saline (PBS), the sections were blocked with 5% bovine serum albumin in Tris-Buffered Saline (BSAT) for 30 min. Rabbit anti-JEV envelope protein (JEV-E) antibody (1:200, abcam, USA) was added and incubated overnight at 4°C. Following washing with PBS containing Tween-20 (PBST), the sections were incubated with a fluorescent secondary antibody (1:200, abcam, USA) at room temperature for 1 hr. After that, 4',6-Diamidino-2-Phenylindole (DAPI) (1:1000, abcam, USA) was added for staining. After mounting the slides, fluorescence images were captured using a laser-scanning confocal microscope, and the images were analyzed with Image J software (National Institute of Mental Health, USA) to determine the appropriate intervention duration of the NS1 suspension.

### Grouping and modeling

After determining the intervention duration of the NS suspension, the remaining 15 mice were re-divided into a control group, a model group, and a DHA group, with 5 mice in each group. The model group and the DHA group both established the NP model induced by NS1 according to the method described above. The control group was treated with an equal volume of normal saline. After the modeling was completed, the DHA group was gavaged with 0.8 mL of a DHA solution with a concentration of 0.8 mg/mL (provided by Shaanxi Yihaochuan Biotechnology Co., Ltd.,) once a day for 2 weeks.[16] The control group and the model group were given an equal volume of normal saline by gavage.

### Behavioral tests

After the modeling was completed, the Tail-Suspension Test (TST),[17] Forced Swimming Test (FST),[18] Open Field Test (OFT),[19] and Morris Water Maze Test[20] were carried out on the mice. The struggling duration of the mice in the TST and FST, the staying time in the OFT, and the escape latency in the Morris Water Maze Test were recorded in detail. Among them, TST and FST were used to assess depressive behaviors in mice by measuring the immobility time of the animals in the suspended tail state/in

water, reflecting their desperate or helpless behaviors. OFT was used to assess the anxiety level of the animals by measuring the amount of activity in the open field. MWM was used to assess the learning and memory abilities of the animals by measuring the time they took to find the hidden platform (latency period).

### Detection of inflammatory response and stress injury

Upon completion of the behavioral tests, the mice were humanely euthanized. Subsequently, the brain tissues of the mice were carefully collected and processed into tissue homogenates. The homogenates were then subjected to centrifugation in a high-speed centrifuge under low-temperature conditions. Afterward, the supernatant was carefully collected, and an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Wuhan CUSABIO Bioengineering Co., Ltd.,) was employed to detect Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α), Superoxide Dismutase (SOD), Catalase (CAT), and Malondialdehyde (MDA).

### Neuroinflammation detection

The brain tissues were fixed using 10% formalin, followed by paraffin embedding and sectioning. Paraffin sections with a thickness of 5 μm were selected for Hematoxylin-Eosin (HE) staining. Additionally, transmembrane protein 119 (TMEM119) and chemokine (C-C motif) ligand 3 (CCL3) in the brain tissues were detected in accordance with the aforementioned fluorescence staining method. The antibody concentrations were set as follows: TMEM119 at a dilution of 1:200 (abcam, USA) and CCL3 at a dilution of 1:200 (abcam, USA).

### ALK5/SMAD3 pathway detection

The BCA protein assay kit (Thermo Fisher, USA) was employed to accurately quantify the proteins in the brain tissue homogenates. Following protein separation via electrophoresis, the separated proteins were meticulously transferred onto a PVDF membrane (Millipore, USA) for 1 hr of incubation with 5% fetal bovine serum at room temperature. After that, primary antibodies against NS1 (1:1000, abcam, USA), ALK5 (1:1000, abcam, USA), SMAD3 (1:1000, abcam, USA), pSMAD3 (1:1000, abcam, USA), and GAPDH (1:1000, abcam, USA) were added, and the incubation process was continued overnight at 4°C. The next day, a horseradish peroxidase-conjugated secondary antibody (diluted to 1:2000, abcam, USA) was introduced, and the incubation was carried out at room temperature for 1 hr. After thorough washing, the developing solution was carefully dropped onto the membrane. The gel images were analyzed for optical density using Image J software.

### Statistical Analysis

Statistical analysis was performed using SPSS 24.0 software (IBM, USA). The Shapiro-Wilk test was employed to confirm the data distribution. For data with a normal distribution (presented as mean±standard deviation,χ±s), one-way analysis of variance and LSD *post-hoc* test groups were used for comparison. For data with a non-normal distribution [presented as median (interquartile range)], the Kruskal-Wallis H test was applied for comparison. A *p*-value less than 0.05 was considered to indicate a statistically significant difference.

## RESULTS

### Induction of NP in mice by NS1

When compared to the pre-intervention period, the 50% mechanical paw-withdrawal threshold of mice decreased significantly after the intervention ($p<0.05$). Specifically, there was no notable difference in the 50% mechanical paw-withdrawal threshold between the 6-day and 9-day marks ($p>0.05$), yet both values were lower than that on the 3rd day ($p<0.05$). Fluorescence staining results indicated that the fluorescence intensity of JEV-E did not differ significantly between 6 days and 9 days ($p>0.05$), but was higher than that on the 3rd day ($p<0.05$). Considering these findings, we chose a 6-day intervention with NS1 suspension as the subsequent intervention duration (Figure 1).

### DHA can improve the behavior of NP mice

First, we observed that the 50% mechanical paw-withdrawal of the mice in the DHA group, although still lower than that of the control group, had been significantly higher than that of the model group ($p<0.05$), suggesting that DHA ameliorated pain in

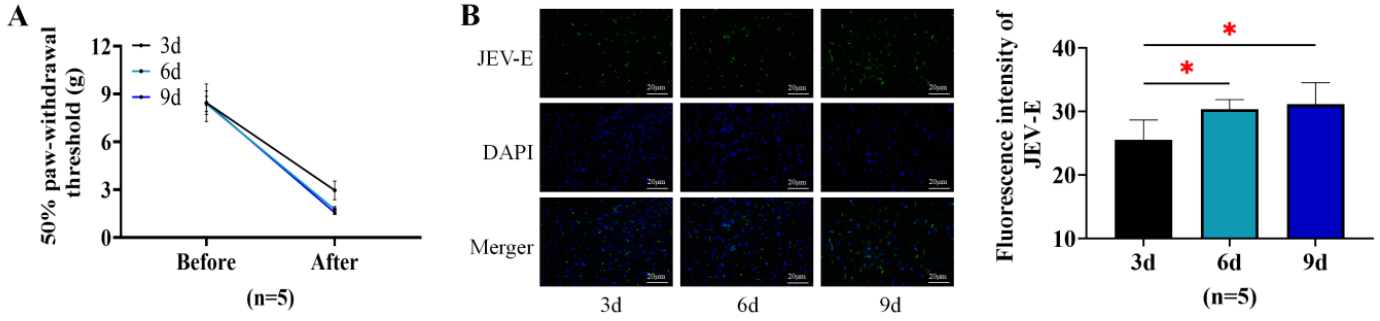


**Figure 1:** NS1-induced NP mouse model. (A) 50% mechanical paw-withdrawal of mice before and after NS1 intervention. (B) Effect of NS1 on JEV-1 in mouse brain tissue. *$p<0.05$.

the mice. In the behavior tests, both the model group and the DHA group demonstrated longer struggling times in the TST and the FST, along with an extended escape latency compared to the control group, while the staying time in the OFT was shortened ($p<0.05$). When compared with the model group, the DHA group showed a reduction in the struggling times in the TST and FST, a shorter escape latency, and extended staying time in the OFT ($p<0.05$). That is, DHA use improved the abnormal behavior of NP mice (Figure 2).

## DHA ameliorates inflammatory response and stress injury in NP mice

In the assessment of inflammatory cytokines, the levels of IL-1β, IL-6, and TNF-α were elevated in both the model group and the DHA group compared to the control group ($p<0.05$), while IL-1β, IL-6, and TNF-α in the DHA group are lower versus the model group ($p<0.05$), suggesting that DHA inhibits inflammatory responses in NP mice. Regarding stress injury, the activities of SOD and CAT in the DHA group were lower than those in the control group but higher than those in the model group. Conversely, the level of MDA was higher than that in the control group yet lower than that in the model group ($p<0.05$). These results suggest that DHA also plays a role in suppressing stress responses in NP mice (Figure 3).

## DHA ameliorates pathological damage in NP mice

When compared with the control group, the brain tissues of mice in the model group exhibited extensive activation of microglial cells, necrosis of neuronal cells, and pronounced inflammatory infiltration. In contrast, the DHA group demonstrated significant alleviation in pathological lesions, with a reduced degree of inflammatory infiltration. In fluorescence staining analysis, although the fluorescence intensities of TMEM119 and CCL3 in the DHA group were still higher than those in the control group, they were markedly lower than those in the model group ($p<0.05$). This finding implies that DHA alleviates microglia infiltration in NP mice (Figure 4).

## DHA activated the ALK5/SMAD3 signaling pathway in NP mice

Finally, Western blot analysis demonstrated that the NS1 protein expression in the DHA group was elevated relative to the control group, yet decreased compared to the model group ($p<0.05$). In addition, ALK5 and p-SMAD3/SMAD3 protein levels were markedly reduced in the model group compared to the control group, suggesting the inhibition of the ALK5/SMAD3 pathway in NP. On the other hand, the expression of p-SMAD3/SMAD3 protein in the DHA group was significantly higher than that in the control and model groups ($p<0.05$), indicating that DHA activates the ALK5/SMAD3 pathway expression (Figure 5).

# DISCUSSION

In the present study, we found that the abnormal behaviors of NP mice were improved under the intervention of DHA, and at the same time, the inflammatory response and stress injury of NP mice were reduced, and the pathological damage condition was repaired, confirming that DHA is a potential therapeutic drug for NP.

In this study, we developed a murine model of NP using JEV NS1. Although this is not a mainstream approach for NP model establishment, previous research has verified that the pathological features of Japanese encephalitis, including necrosis, morphological alterations, vascular anomalies, edema, the formation of glial nodules, and calcification,[21] are similar to those of NP. Moreover, JEV, a hallmark virus of Japanese encephalitis, is a positive-sense single-stranded RNA virus characterized by its capacity to cause neuro-invasive disorders and induce

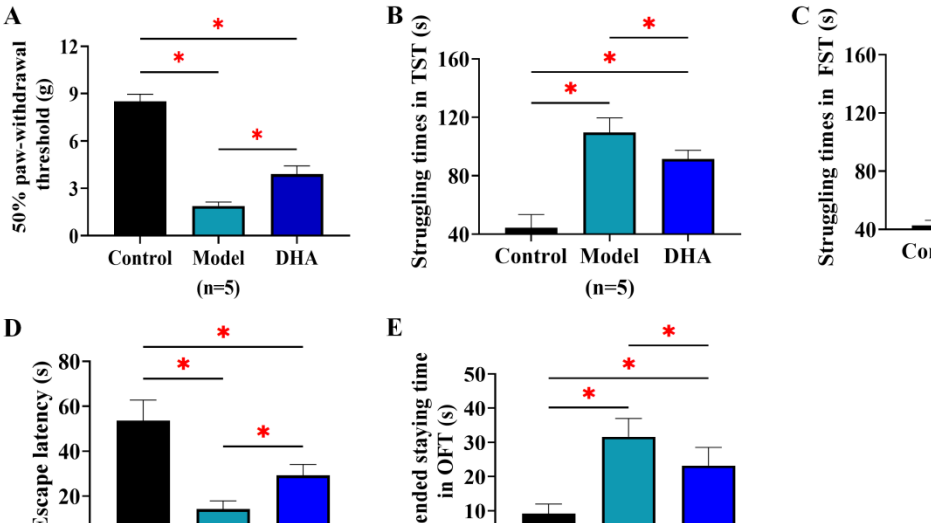


**Figure 2:** Effect of DHA on the behavior of NP mice. Results of 50% mechanical paw-withdrawal. (B) Results of TST. (C) Results of FST. (D) Results of Morris Water Maze Test. (E) Results of OFT. Mice in the DHA group showed improved pain and better behavioral test results than the model group. *$p<0.05$.

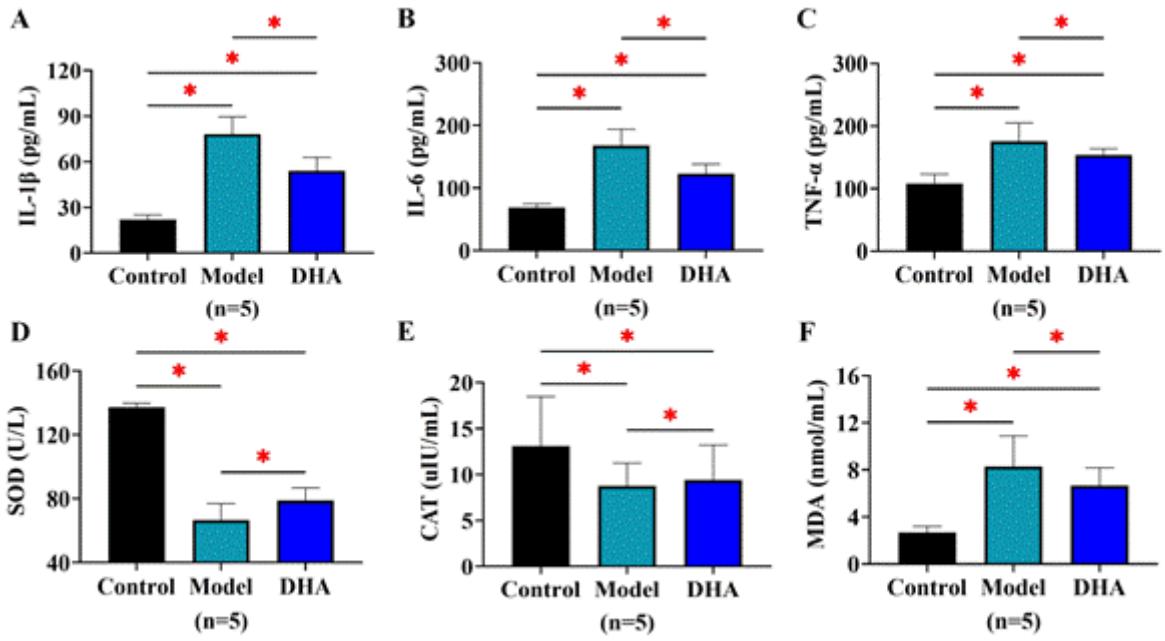


**Figure 3:** Impact of DHA on inflammatory response and stress injury in NP mice. (A) Effect of DHA on IL-1β. (B) Effect of DHA on IL-6. (C) Effect of DHA on TNF-α. (D) Effect of DHA on SOD. (E) Effect of DHA on CAT. (F) Effect of DHA on MAD. IL-1β, IL-6, TNF-α, and MDA were lower and SOD and CAT were higher in the DHA group than in the model group. *$p$<0.05.

synaptic dysfunctions.[22] NS1, a functional protein of JEV, has been demonstrated to be intricately involved in multiple viral processes, including replication, assembly, and the modulation of innate immune responses.[23] Compared with the traditional spinal nerve ligation technique for model establishment, the NP model induced by JEV NS1 does not require surgical procedures, thus ensuring higher experimental safety. Moreover, this non-surgical modeling approach is not only more convenient but also eliminates the need to set up a sham-operation group as a control, significantly reducing experimental costs and modeling requirements and enhancing its amenability to widespread popularization across different research settings. In the present investigation, we observed a significant reduction in the 50% mechanical paw-withdrawal threshold of NP mice induced by NS1, with all values falling below 4 g, validating the efficacy of this method in establishing the NP model. Through fluorescence staining analysis, we noted that on the sixth day of intervention, the rate of increase in the JEV-E fluorescence intensity in the mouse brain tissue decelerated, suggesting that the activation of the virus by NS1 had reached a critical threshold at this time point. Given the potential for exacerbating neurological damage with prolonged NS1 intervention, we determined that the optimal duration for subsequent NS1 intervention in this study was six days.

First of all, in the behavioral examination, we observed that in the DHA group, the struggling time in the TST and the FST, along with the escape latency, were notably shortened compared to the model group, while the OFT staying time was extended, suggesting that DHA exerts a significant ameliorative effect on depressive and anxious behaviors, as well as cognitive impairments, in mice with NP. Tang C *et al*., have also substantiated that DHA can protect mice from depressive behaviors induced by chronic unpredictable mild stress through the modulation of gut microbiota,[24] which is consistent with our findings. DHA has drawn extensive attention in the treatment of various diseases, attributable to its remarkable anti-inflammatory and anti-oxidative stress capabilities. We hypothesized that the excellent therapeutic effect of DHA on NP might also be associated with its anti-inflammatory and anti-oxidative stress properties. To validate this hypothesis, we conducted comprehensive assessments of inflammatory mediators and stress-induced damage. Our findings revealed that the model group manifested pronounced inflammatory responses and stress-induced damage, which are congruent with the established pathological manifestations of NP.[25] In contrast, a marked reversal of these parameters was evident in the DHA group, providing preliminary corroboration for our conjecture. In the research conducted by Gao X *et al*., artemisinin was identified as an outstanding candidate for the treatment of autoimmune disorders. Its derivative, DHA, has been shown to impede platelet aggregation induced by platelet-activating factor, thereby reducing blood coagulation. Moreover, it enhances the phagocytic function of macrophages, consequently augmenting the body's immune competence. It can be thus seen that its potent anti-inflammatory effect indeed constitutes the cornerstone for its clinical applications.[26] In a therapeutic study on prostatitis by Zhou Y *et al.*, they also confirmed the excellent anti-inflammatory effect of DHA,[27] which is consistent with our results. For NP, neuroinflammation represents one of its primary pathological

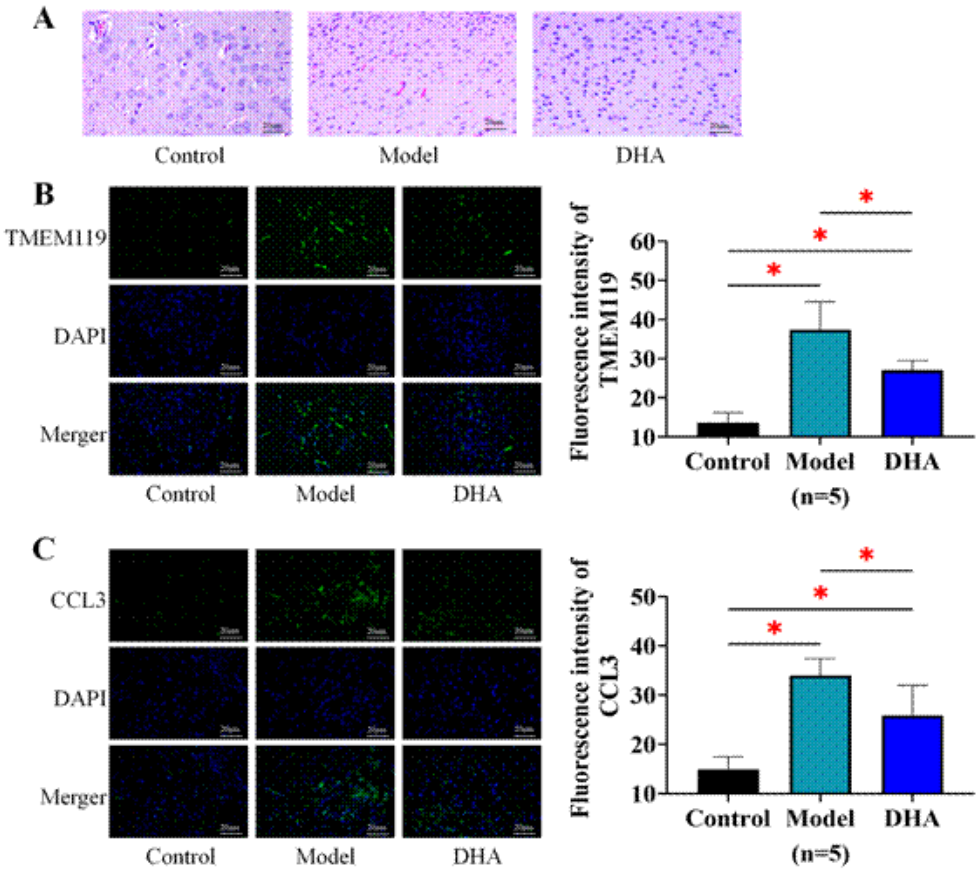


**Figure 4:** Influence of DHA on pathological injury in NP mice. (A) HE staining of mouse brain tissue. (B) Effect of DHA on TMEM119. (C) Effect of DHA on CCL3. Pathologic damage was ameliorated in the DHA group with reduced expression of TMEM119 and CCL3. *$p$<0.05.

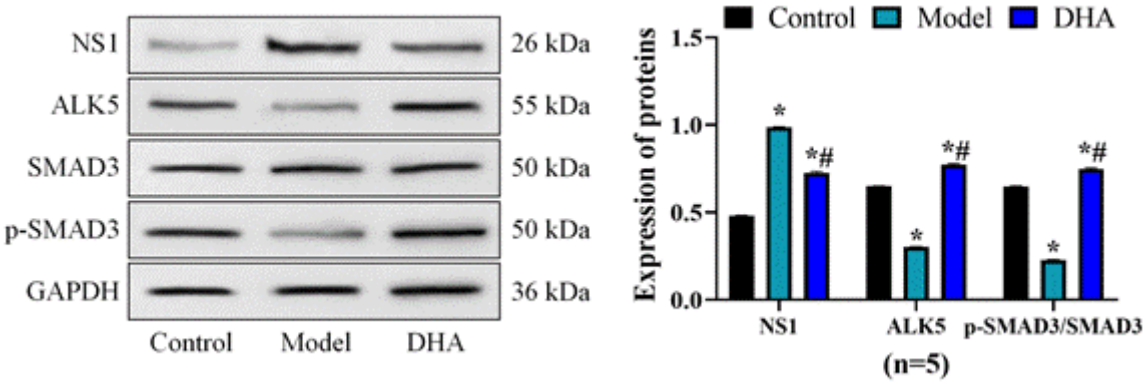


**Figure 5:** Effect of DHA on the ALK5/SMAD3 signal pathway. NS1 expression was reduced and the ALK5/SMAD3 pathway was activated in the DHA group. *vs* control group, *$p$<0.05, *vs* model group, #$p$<0.05.

mechanisms. Following nerve injury, there is an infiltration of immune cells and activation of glial cells.[28] This leads to an increase in inflammatory mediators such as pro-inflammatory cytokines and chemokines in both the peripheral nervous system and the central nervous system, thereby triggering neuroinflammation.[29] Consequently, intervening in microglial activation is regarded as a potential target for treating NP. Through fluorescence staining, we observed a significant reduction in the levels of TMEM119 and CCL3 in the DHA group compared with the model group. This indicates that the administration of DHA can inhibit microglia infiltration and activation, ameliorate neuroinflammation, and alleviate pain. The study by Gao Y *et al.*, similarly verified the inhibitory effect of DHA on LPS-induced neuroinflammation,[30] corroborating our view.

Finally, the study by Ling L *et al.*, reported that sinomenine ameliorates microglial activation and NP following chronic constriction injury via the ALK5/Smad3 signaling pathway,[12] suggesting a certain potential relationship between ALK5/

Smad3 and NP. For DHA, the ALK5/Smad3 signaling pathway is also considered a crucial mechanism of action.[13] Therefore, we hypothesized that DHA's effect on NP might also be related to this pathway. Our experimental analysis demonstrated a significant suppression of the ALK5/Smad3 pathway in the model group, consistent with the findings reported by Ling L *et al.*,[12] suggesting that the occurrence of NP leads to the downregulation of the ALK5/Smad3 pathway. In contrast, DHA intervention was found to upregulate the expression of ALK5 and enhance the phosphorylation of Smad3 in the DHA group, thereby activating the ALK5/Smad3 pathway. These results further elucidate the molecular mechanism underlying DHA's beneficial effects on NP.

In this study, we used the NS1-induced NP model of encephalitis B virus, which has the advantages of easy operation and no need for surgery, but whether its pathological mechanism completely mimics that of human NP (e.g., diabetic neuropathy or chemotherapy-induced NP) still needs to be further verified. In addition, only 5 mice were included in each group, and the small sample size may lead to insufficient statistical power and increase the risk of chance in the results. Finally, the physiological differences between the mouse model and human NP may lead to limited extrapolation of the results, and we need to start a clinical trial to verify its safety and efficacy as soon as possible.

## CONCLUSION

DHA exhibits remarkable efficacy in alleviating NP induced by the JEV NS1. The underlying mechanism appears to involve the activation of the ALK5/Smad3 pathway, which in turn suppresses the neuroinflammatory response in mice. Given these findings, DHA emerges as a promising novel therapeutic candidate for future management of NP.

## ABBREVIATIONS

**DHA:** Dihydroartemisinin; **NP:** Neuropathic pain; **JEV:** Japanese encephalitis virus; **HE:** hematoxylin-eosin; **TMEM119:** Transmembrane protein 119; **CCL3:** chemokine (C-C motif) ligand; **TST:** Tail-suspension test; **FST:** Forced-swimming test; **OFT:** open-field test; **HnRNPA1:** Heterogeneous nuclear ribonucleoprotein A1; **SPF:** Specific pathogen-free; **BSAT:** Tris-buffered saline; **PBST:** PBS containing Tween-20; **JEV-E:** Anti-JEV envelope protein; **DAPI:** 4',6-Diamidino-2-phenylindole; **TST:** Tail-Suspension Test; **ELISA:** Enzyme-Linked Immunosorbent Assay; **IL-1β:** Interleukin-1β; IL-6: interleukin-6; **TNF-α:** Tumor necrosis factor-α; **SOD:** Superoxide dismutase; **CAT:** Catalase; **MDA:** Malondialdehyde.

## FUNDING STATEMENT

The authors did not receive specific funding.

## CONFLICT OF INTEREST

The authors declare that they have no competing interest.

## ETHICAL APPROVAL

All the experimental procedures involving animals were conducted in accordance with ARRIVE guidelines and approved by the Animal Ethics Committee of Yichang Central People's Hospital (NO.kl0056).

## AUTHOR CONTRIBUTIONS

Jie Xia conceived and designed the study, Hui Wang wrote and revised the manuscript, collected and analyzed data, All authors read and approved the final submitted manuscript.

## SUMMARY

Neuropathic Pain (NP) is a disease that severely affects the health of patients and is characterized by a high recurrence rate. In this study, we induced a mouse model of NP using Japanese Encephalitis Virus NS1 and treated it with Dihydroartemisinin (DHA) for intervention, and found that the inflammatory response, stress injury, and pathological injury were significantly improved in NP mice, which shows that DHA has potential for treating NP. In addition, we found that the mechanism of DHA's effect on NP was related to the activation of the ALK5/SMAD3 pathway. These findings lay the foundation for the future clinical application of DHA.

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