Dihydromyricetin attenuates neuropathic pain via enhancing the transition from M1 to M2 phenotype polarization by potentially elevating ALDH2 activity in vitro and vivo

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Background: Treatment for neuropathic pain as a refractory disease remains unsatisfactory and represents a significant clinical challenge. A highly effective drug is thus urgently needed for neuropathic pain treatment. Dihydromyricetin (DMY) is a flavonoid with a wide range of biological activities. The purpose of this research is to explore the effects of DMY on neuropathic pain and the underlying mechanism of its effect.

Methods: The effect of DMY was investigated in BV-2 cells and lipopolysaccharide (LPS)-induced BV-2 cells. A neuropathic pain model was established via spared nerve injury (SNI) surgery in mice, and the protein expression level was detected via Western blot assay. The percent of M1 and M2 phenotype polarization cells were detected via flow cytometry assay. Immunohistochemical staining assay was also performed to measure the marker levels of the M1 and M2 phenotype polarization cells and aldehyde dehydrogenase 2 (ALDH2) level, and mechanical pain sensitivity was evaluated via measurement of the mechanical withdrawal threshold.

Results: We found that DMY promoted the transition from M1 to M2 polarization and upregulated the ALDH2 level in vitro and vivo. ALDA-1, an ALDH2 agonist, promoted the switching from M1 to M2 polarization in vitro and vivo. DMY alleviated pain hypersensitivity induced by SNI via enhancing M2 phenotype polarization by elevating ALDH2 activity in mice. After DMY- or ALDA-1-microglia were injected into SNI-induced pain hypersensitive mice, the mechanical withdrawal threshold was increased significantly when compared with the SNI group.

Conclusions: Our data demonstrated that DMY alleviated neuropathic pain via enhancing the polarization transition from the M1 to M2 phenotype by potentially elevating ALDH2 activity in vitro and vivo. DMY- or ALDA-1-microglia may have alleviative effects on neuropathic pain. The findings herein provide a promising avenue for neuropathic pain treatment, suggesting a new target, ALDH2, in the treatment of neuropathic pain.

Keywords: Neuropathic pain; dihydromyricetin (DMY); aldehyde dehydrogenase 2 (ALDH2); polarization; ALDA-1

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**Introduction**

Neuropathic pain is a chronic pain disease which arises from impairment or disease in the somatosensory nervous system (1-3). Neuropathic pain is characterized by hyperalgesia and allodynia and severely worsens patients’ quality of life, placing a heavy burden on society (4,5). The prevalence of neuropathic pain is about 5–10% of the general population (6,7). Neuropathic pain can result from complications in diseases like diabetes, cancer, and stroke (8-10). Its incidence is likely on the rise, which is attributable to the concurrent increase in diabetes. Thus, the mechanisms of neuropathic pain and its related therapeutic approaches urgently need to be elucidated.

The current perspectives of neuropathic pain pathogenesis include central sensitization, inflammatory mediator stimulation, metabolic damage, overactivation of microglia cells, and other factors (11-13). In this regard, convincing evidence has demonstrated that the activation of microglia cells plays a crucial role in neuropathic pain (14,15). Microglia function as the major immune cells in the nervous system. They are activated within 24 h of nerve injury (16,17) via polarization, specifically, M1 phenotype and M2 phenotype polarization. M1 polarization is a pro-inflammatory phenotype that contributes to neuropathic pain (18,19) while, the M2 phenotype, as the alternative path of polarization, exerts an anti-inflammatory effect, thus opposing M1 phenotype polarization (20). The relevant research indicates that switching from M1 phenotype polarization to M2 phenotype polarization is a promising avenue in neuropathic pain treatment (21,22). The process behind the regulation of the M1/M2 polarization process remains unclear, and gaining insight into its mechanism is crucial to developing new drugs for neuropathic pain therapy.

Aldehyde dehydrogenase 2 (ALDH2), the second enzyme of the major oxidative pathway of alcohol metabolism, is highly expressed in the brain (23). ALDH2 is reported to relieve inflammation via regulating autophagy (24) and has been shown to reduce dox-induced toxicity via suppressing oxidative stress and apoptosis (25). In microglial cells, ALDH2 functions downstream of TGR5, reducing oxidative stress and resisting neuronal apoptosis after activation (26) and can exert protective effects in chronic pain (27). However, the relation between ALDH2 and M1/M2 polarization in neuropathic pain remains unknown.

Dihydromyricetin (DMY) is an active extract of many plants including *Ampelopsis grossedentata* and *Hovenia dulcis*. Of these, *H. dulcis* has protective effects against hangover and liver injuries induced by alcohol through elevating ALDH2 activity, which may be a newly discovered strategy of neuroprotection (28,29). We speculated that DMY, as the main active compound of *H. dulcis*, may have effects on ALDH2 activity and thus has a role in neuropathic pain. DMY is also an extract with a variety biological activity, including anti-inflammatory and anti-oxidative effects. DMY was reported to have protective effects against neuroinflammation and microglial activation APP/PS1 transgenic mice (30), and other recent research has indicated that DMY has alleviative effects on diabetic neuropathic pain (31,32). Despite these findings, the effect and mechanism of DMY in neuropathic pain is not yet fully understood. Thus, this study aimed to explore the effects and potential mechanism of DMY on ALDH2 activity in neuropathic pain.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/atm-20-5838).

**Methods**

**Cell culture and treatment**

The BV-2 cells (cat. no. CL-0493, Procell Life science & Technology Co., Ltd., China) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL), and penicillin (100 U/mL). Cells were preserved in serum-free cell freezing medium (Teyebio, Shanghai, China). The BV-2 cells were seeded in 6-well plates at a density of 2×10⁵, at 37 °C in an incubator containing 5% CO₂ and 95% air. The cells were assigned into the following groups: control, 10 μM DMY, 100 μM DMY, 1 mM DMY, lipopolysaccharide (LPS), LPS + 10 μM DMY, LPS + 100 μM DMY, and LPS + 1 mM DMY. In the DMY group, the cells were treated with different concentrations of DMY (10 μM, 100 μM, and 1 mM). For the LPS group, the cells were treated with 100 ng/mg LPS for 24 h. For the LPS + DMY group, the cells were pretreated with LPS and then treated with different concentrations of DMY (10 μM, 100 μM, and 1 mM) for 24 h. For the ALDA-1 group, the cells were incubated with ALDA-1 (20 μM). For the ALDA-1 + LPS group, after LPS treatment, the cells were incubated with ALDA-1 (20 μM). After that, images of the cells were taken under a microscope.
**Western blotting**

The total proteins in BV-2 cells were extracted with lysis buffer, and then the lysates were centrifuged (15,000 x g, 10 min). Bicinchoninic acid assay kit (Teyebio, Shanghai, China) was used to detect the concentration of the proteins. Then, 30 μg of proteins were electrophoresed on a 15% SDS-polyacrylamide gels and then blotted on a polyvinylidene fluoride (PVDF) membrane. After blockage with 5% skim milk, the membranes reacted with the primary antibodies against nitric oxide synthase (iNOS) (#ab15323, Abcam), arginase 1 (Arg1) (#ab96183, Abcam), cluster of differentiation 206 (CD206) (#ab64693, Abcam), CD86 (#ab242142), and ALDH2 (#ab108306, Abcam). GAPDH (#ab181602, Abcam) was used as the internal reference. Then, IgG-HRP secondary antibody (#ab7090, Abcam) was incubated with the membranes. An enhanced chemiluminescence system (Bio-Rad Laboratories, USA) was used for visualization of the bands.

**Flow cytometry**

The percentage of M2/ M1 phenotype cells was determined via evaluating the level of their corresponding markers: CD86 and CD206, respectively. The percentage of CD86 and CD206 was determined via flow cytometry assay. After treatment in the study groups, the BV-2 cells were collected and suspended in phosphate-buffered saline (PBS). Subsequently, the BV-2 cells were stained with the fluorescence-labeled CD206 and CD86 antibody for half an hour in the dark at 4 °C. For the control group, isotypes of corresponding primary antibodies were incubated with the cells. FACS Calibur (Beckman Coulter, USA) was applied for analyzing the percentage of CD206 and CD86.

**Animals**

Male CD1 mice (8 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. and kept under the standard conditions (25±2 °C, humidity: 55%±5%, 12:12 h light/dark). The mice had free access to the standard laboratory diet and water. The mice were housed in the experiment environment for 48 h to adapt to the environment before testing began. Animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at China-Japan Union Hospital of Jilin University (#JU3874).

**Establishment of the neuropathic pain model and treatment**

The neuropathic pain model was established via spared nerve injury (SNI) surgery. The mice were randomly assigned to the study groups (10 mice per group). The anesthesia was carried out via using (sodium pentobarbital 60 mg/kg i.p.) in mice. After shaving and sterilization, an incision was made on the left hind limb. The sciatic nerve was exposed after the biceps femoris muscle was separated. Then, the common peroneal and tibial nerves at the terminal branches of sciatic nerve were ligated using suture silk 5.0, following the manufacturer instruction. The terminal of the nerve trunk (2–4 mm) was removed. For the sham group, after exposure of sciatic nerve and its branches as described above, no ligation or other operation was performed, apart from direct suture. All procedures were conducted in sterile conditions. After establishment of the model, the mice in the ALDA-1 Ii group were treated with ALDA-1 (10 mg/kg) via intraperitoneal injection, while mice in the ALDA-1 IL group were treated with ALDA-1 (10 μg/10 μL) via local injection as previously described (33). Mice in the SNI + DMY Ii group were administered with DMY (10 mg/kg) once a day via intraperitoneal injection, while mice in the SNI + DMY Li group, were given DMY (10 μg/10 μL) via local injection once a day for 6 days. After modeling, mice in the SNI + DMY microglial group were treated with 10^7 microglial cells which were pretreated with DMY. Mice in the SNI + ALDA microglial group were treated with 10^7 microglial cells which were pretreated with ALDA-1. For the microglial groups, 10^7 microglial cells were injected into the myelin sheath.

**Measurement of the mechanical withdrawal threshold**

The measurement of the mechanical withdrawal threshold was performed by using a series of von Frey filaments (2, 4, 6, 8, 10, 15 g). All the mice were placed in a cage for 30 min before tests to adapt to the experimental environment. Then, a series Frey filament stimulation (0.6, 1, 2, 4, 6, 8, 10, 15 g) with internal of 15 s treatment was performed on the left hind paw of the mice for 3–4 seconds. It was considered as a positive reaction when the mice withdrew their paw rapidly within 4 s. On the contrary, a negative reaction was characterized by no paw withdrawal. A threshold tracking algorithm method was used for calculation of the paw withdrawal threshold (PWT) (34).
**Immunohistochemistry assay**

After 10% fixation, tissue samples of the different study groups were embedded with paraffin. Then, the samples in the different study groups were made into 5 μm sections and subjected to dewaxing and dehydration. Then, the sections were incubated with the primary antibodies against INOS (Cat#15323, Abcam), Arg1 (Cat# ab272887, Abcam), CD206 (Cat#ab64693, Abcam), CD11b (Cat#ab133357, Abcam), and ALDH2 (Cat#ab194587, Abcam) at 4 °C, overnight. Next, the sections were reacted with the secondary antibodies at room temperature for 1 h. Subsequently, hematoxylin and eosin (HE) counterstaining was performed. An RFCA (Olympus, Japan) microscope was used for observation of the slides.

**Statistical analysis**

The results were analyzed via SPSS 11.0 and GraphPad version 6.0 software. All the data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with Tukey’s multiple test was used for comparison between groups. A P value <0.05 indicated significant difference. We present the following article in accordance with the ARRIVE reporting checklist.

**Results**

**DMY promoted the transition from M1 to M2 polarization and upregulated ALDH2 level in vitro**

As M1 phenotype polarization contributes to neuropathic pain and M2 phenotype polarization works in counter fashion, we explored the effects of DMY on M1/M2 polarization. INOS and CD86, which are the common M1 polarization markers, and Arg1 and CD206, which are M2 polarization markers, were subsequently measured. The polarized cells were gradually increased by DMY in a concentration-dependent manner when compared with controls (Figure 1A). Compared with controls, INOS and CD26, were all decreased by DMY, while Arg1 and CD206 were all increased by DMY (Figure 1B,C,D,E,F) in a concentration-dependent manner, suggesting that DMY promotes a polarization switch from M1 to M2. As neuropathic pain is mainly caused by M1 polarization, the M1 phenotype polarization cells served well as a cell model of neuropathic pain. The M1 phenotype polarization cells were induced by LPS. Compared with controls, there was a significant increase in the polarized cells induced by LPS. With the increase of DMY concentration, the polarized cells first decreased and then increased in LPS-induced cells (Figure 1A). Simultaneously, DMY reduced the levels of INOS and CD86 while elevating the levels of Arg1 and CD206 in LPS-induced cells, indicating that DMY promotes the transition from M1 to M2 polarization. With the increase of DMY concentration, there was an initial decrease and a subsequent increase in the number of polarized cells induced by LPS (Figure 1A), which reflected the transition from M1 to M2 under DMY treatment.

As ALDH2 is thought to be a protective enzyme in chronic pain, the effect of DMY on ALDH2 was also examined. The ALDH2 level was elevated significantly by DMY in a concentration-dependent manner in BV-2 cells with or without LPS treatment (Figure 1G,H). The results from flow cytometry showed that the M1 phenotype polarization, as indicated by the percent of CD86, was decreased, while M2 phenotype polarization, as indicated by CD206, was increased (Figure 2A,B), further confirming that DMY promotes a transition from M1 to M2 polarization.

**DMY promoted polarization from M1 to M2 via upregulating ALDH2 level in vitro**

As outlined previously, DMY can elevate ALDH2 level and promote cell polarization from M1 to M2. However, the relation between M1/M2 polarization and ALDH2 remains unknown. The effects of ALDH2 agonist, ALDA-1, was further investigated in BV-2 cells with or without LPS treatment. Compared with controls, the polarized cells were increased in the ALDA-1 group and LPS group (Figure 3A). As seen in the results from Western blotting (Figure 3B,C,D,E,F), the M1 polarization markers, iNOS and CD86, in the ALDA-1 group were decreased, while the M2 polarization markers, Arg1 and CD206, were increased when compared with controls (Figure 3B,C,D,E,F); this indicate that the polarized cells in the ALDA-1 group were mainly M2 polarization cells. Consistent with the results above, the polarized cells in LPS group were mainly M1 polarization cells, which was further supported by the increased levels of cell polarization markers CD86. In the ALDA-1 + LPS group, the M1 polarization markers were decreased and M2 polarization markers were increased when compared with LPS group, suggesting that ALDA-1, being an ALDH2 agonist, can promote the cell polarization switch from M1 to M2. In addition, we found that DYM could elevate the ALDH2 level, with cell polarization from M1 to M2 being simultaneously enhanced by the ALDH2
agonist, ALDA-1. We can therefore conclude that DYM promoted a cell polarization switch from M1 to M2 via elevating the ALDH2 level in vitro.

**DMY attenuated pain hypersensitivity induced by SNI by enhancing the transition from M1 to M2 phenotype polarization through elevation of ALDH2 activity in mice**

As shown by the immunohistochemistry results, the positive staining of INOS was increased while Arg1, CD206, and CD11b were decreased in the neuropathic pain model induced by SNI when compared with sham group (Figure 4A,B; 10 mice per group), indicating that M1 polarization was the major polarization phenotype in the neuropathic pain model induced by SNI (SNI group). After ALDA-1 or DMY treatment, the INOS staining-positive level via intraperitoneal injection was decreased more obviously than that via local injection when compared
ALDH2 level could be elevated by DMY. All these results had the same effects on M1/M2 polarization. Moreover, the ALDH2 level was decreased in the SNI group when compared with the sham group, meaning DMY attenuates neuropathic pain via elevating ALDH2 activity. We further explored the therapeutic effects of DMY- and ALDA-1-microglia on neuropathic pain via elevating ALDH2 activity.

The effect of DMY on mechanical hyperalgesia was also measured. The PWT in the SNI group was significantly lower than that in the sham group (Figure 5A), confirming that the neuropathic pain model was established successfully. The PWT was more obviously increased by ALDA-1 or DMY treatment via intraperitoneal injection than via local injection when compared with SNI group, confirming that DMY could alleviate neuropathic pain and that this effect is inferior to that of ALDA-1. In addition, DMY could elevate the ALDH2 level, meaning DMY attenuates neuropathic pain via elevating ALDH2 activity. We further explored the therapeutic effects of DMY- and ALDA-1-microglia on neuropathic pain. As shown by the results (Figure 5B), the

with the SNI group, revealing that M1 polarization was inhibited by ALDA-1 or DMY in the neuropathic pain model induced by SNI. Arg1, CD206, and CD11b were decreased in the SNI group when compared with the control group, and appeared elevated by ALDA-1 or DMY via intraperitoneal injection more obviously than via local injection when compared with the SNI group, demonstrating that both ALDA-1 and DMY could promote M2 polarization. Moreover, The ALDH2 positive-staining level was decreased in the SNI group when compared with control, and was more obviously elevated by ALDA-1 or DMY via intraperitoneal injection than via local injection when compared with the SNI group. DMY and ALDA-1 had the same effects on M1/M2 polarization. Moreover, the ALDH2 level could be elevated by DMY. All these results indicate that DMY functions on M1/M2 polarization via elevating ALDH2 activity.
PWT in the SNI group was significantly lower than that in the sham group, confirming that the neuropathic pain model was consistent. After microglia treatment, there was almost no change in PWT when compared with the SNI group, indicating that microglia has no therapeutic effect on neuropathic pain. Moreover, the DMY- or ALDA-1-microglia treatment elevated the PWT significantly, strongly supporting the notion that DMY- or ALDA-1-microglia can alleviate neuropathic pain, and thus offering a potential new strategy for neuropathic pain treatment.

**Discussion**

Neuropathic pain is caused by a lesion or disease of the somatosensory system, including peripheral fibres (Aβ, Aδ and C fibres) and central neurons (35). The mechanism of neuropathic pain including imbalances between excitatory and inhibitory somatosensory signaling (36), alterations in ion channels (37) and second-order nociceptive neuron alterations (38). Neuropathic pain remains a problematic refractory disease. Despite the progress that has been made in the therapy of neuropathic pain, the efficacy of treatment remains unsatisfactory (39). Studies have confirmed that microglial polarization plays a pivotal role in development of neuropathic pain (20,40-42), with the typical activated microglia being M1 phenotype polarization which contributes to progression of neuropathic pain via its pro-inflammatory role. On the other hand, M2 phenotype polarization, as an alternative path of polarization, has an anti-inflammatory effect. Regulation of polarization states towards the M2 phenotype has been considered a promising avenue for neuropathic pain treatment. Substantial evidence has shown that DHM can cross the blood-brain barrier, modulate inflammation, and exert neuroprotective effects (43). It has been reported that DHM can inhibit microglia-mediated neuroinflammation and play a neuroprotective role in Alzheimer’s disease by suppressing inflammation (44). However, the role of DMY in modulating neuropathic pain has not been studied. In the present study, we found that DMY induced a switch from M1 to M2 phenotype polarization in microglia cells, which attenuates neuropathic pain.
Figure 4 The effects of DMY/ALDA-1 on the levels of M1 and M2 polarization markers and ALDH2 level. The levels of M1 and M2 polarization markers and ALDH2 (A,B) detected by immunochemical staining assay. The scale bar is 100 μm. DMY, dihydromyricetin.
As neuropathic pain is mainly caused by M1 phenotype polarization, we constructed M1 phenotype polarization cells using LPS, as it is the common stimulator of M1 phenotype polarization (45,46). We further investigated the effects of DMY on M1/M2 phenotype polarization in the presence of LPS treatment, and found that the polarization of cells was increased with an increase in the DMY concentration in BV-2 cells. Flow cytometer analysis also demonstrated that DMY treatment could promote the M2 microglial phenotype by shifting microglial cells from the M1 to the M2 phenotype in a concentration-dependent manner, which was consistent with the previous study (30).

A growing body of evidence has indicated that oxidative stress and inflammation in many diseases (24,53-56); however, the role of ALDH2 in neuropathic pain remains elusive. Thus, this was the first study to examine the role of ALDH2 in neuropathic pain. We found that the ALDH2 level could be elevated by DMY in a concentration dependent-manner in BV-2 cells with or without LPS treatment, and, as ALDH2 was reported to work as a protective agent in chronic pain (27), ALDH2 may thus be a promising therapeutic target in neuropathic pain. Because DMY promoted M2 polarization, elevated the ALDH2 level, and thus had a protective effect in neuropathic pain, we speculated that there may be a certain relationship between microglia polarization and ALDH2. As expected, our data suggest that ALDA-1, the agonist of ALDH2, elevated M2 phenotype polarization cells and decreased M1 phenotype polarization cells. Furthermore, we found that DMY promoted the transition from M1 to M2 phenotype polarization via the elevation ALDH2.

In order to confirm the validity of our conclusions, the effects of DMY were examined in vivo. The immunochemical staining results showed that DMY could also elevate ALDH2 level in vivo. The M1 phenotype polarization cells were decreased by DMY and ALDA-1, as reflected by the positive staining level of INOS. The M2 phenotype polarization cells were increased by DMY and ALDA-1, as indicated by the markers’ positive staining level. All the observed effects of DMY in vivo were consistent with those observed in vitro. In addition, the mechanical withdrawal threshold was decreased significantly by DMY and ALDA-1, strongly supporting the supposition that DMY attenuates neuropathic pain via promoting M2 polarization by elevating ALDH2 activity. As M2 polarization has therapeutic effects, we further investigated the effects of microglial cells treated by DMY or ALDA-1. The mechanical withdrawal threshold was increased more significantly by the microglial cells treated by DMY/ALDA-1, demonstrating that using DMY- or ALDA-1-induced microglial cells may be a promising strategy for treating neuropathic pain.

**Conclusions**

In this study, DYM was found to attenuate neuropathic pain by switching from M1 to M2 phenotype polarization via potentially elevating ALDH2 activity. The findings provide a new strategy for neuropathic pain treatment, laying the foundations for future clinical implications.
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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at China-Japan Union Hospital of Jilin University (#JU3874).

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