Anti-pruritic and anti-inflammatory effects of dihydromyricetin in a mouse model of allergic contact dermatitis

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Research Article

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Abstract

Background

Allergic contact dermatitis (ACD) is a highly prevalent inflammatory disease of the skin with limited treatment options. Its pathogenesis is believed to be driven by activation of inflammasome induced by allergens and irritants. Dihydromyricet(DHM) is a wild woody vine extract of Vitis viridis in the family Vitis. The main active ingredient is flavonoids, which exhibit a wide range of pharmacological effects such as anti-inflammation and anti-oxidation. In this study, we investigated the anti-inflammatory and antipruritic effects of DHM and its mechanism in ACD mouse models.

Methods

Sixty ICR male mice were randomly divided into control group, DHM-treated control group (250 mg·kg−1), ACD model group, and three DHM-treated ACD groups (50, 150, 250 mg·kg−1). To induce ACD, 1-fluoro-2, 4-dinitrofluorobenzene (DNFB) was applied to the neck surface of ICR mice, which were treated with DHM by gavage. Cervical skin changes and scratching behaviors were recorded. HE staining was used for pathological observation, immunohistochemistry and western blot were used to determine the expression level of spinal cord glial cells, and Real-time qPCR was used to determine the level of local and central cytokines.

Results

DHM treatment significantly reduced skin inflammation and scratching episodes. It repaired epidermal keratinization and inflammatory cell infiltration in ACD mice. DHM treatment inhibited the activation of microglia and astrocytes to a certain extent. In addition, it reduced toll-like receptor (TLRs) 4 protein expression levels. At the same time, it significantly reduced the mRNA expression levels of TNF-α, IL-1β and IL-6 in local area and in central area.

Conclusion

This study demonstrates that DHM exhibits anti-pruritus and anti-inflammatory effects in ACD mice by modulating inflammatory mediators. DHM may be a potential treatment for itching and skin inflammation in patients with ACD.

1. Introduction

Allergic contact dermatitis (ACD) is a common skin disease caused by immune responses to various substances that contact the skin. It has a high incidence in the work environment and is often characterized by erythema, blisters and pruritus[1, 2]. Allergic contact dermatitis is common, occurring in
up to one in five people. Although anyone can be affected at any age, people who have eczema from an early age may be more likely to develop ACD. They are most intense in places where the skin comes into direct contact, but may spread sometimes[3]. Intractable pruritus caused by ACD is an uncomfortable feeling that can trigger the urge to scratch, which occurs repeated and negative effects on concentration and sleep quality [4]. Meanwhile, the exact mechanism of pathogenesis and effective treatment of allergic contact dermatitis is still unknown[5].

Currently, the treatment of pruritus contact dermatitis mainly includes local and systemic corticosteroids, antibiotics and antihistamines[6]. However, long-term use of these drugs can lead to a series of unnecessary and serious side effects[7] and the treatment effect is not accurate. The lack of effective treatments places a heavy burden on patients with refractory pruritus. Therefore, the development of safe, effective and treatable pruritus compounds with minimal side effects, preferably a natural product, represents an unmet medical need to treat ACD[8].

For thousands of years, traditional Chinese medicines (TCM), such as oxymatrine (OMT), curcumin, cordyceps, etc., have been known to treat itch and pain symptoms with minimal side effects, providing valuable experience for the development of new compounds for the treatment of ACD refractory itch[9, 10]. Dihydromyricetin (Dihydromyricetin, DHM) is a 2, 3-dihydroflavonol compound mainly extracted from vitis ophidiae, which has many biological activities such as scavenging free radicals, anti-oxidation, anti-thrombosis, anti-tumor, anti-inflammation and so on. DHM has been used as an anti-inflammatory in China and other Asian countries for many years[11] and has been confirmed to play an anti-inflammatory role by inhibiting NF-κB signaling pathway in macrophages[12], suggesting its potential as a bioactive compound for the treatment of ACD. This study investigated the potential anti-inflammatory and anti-pruritus effects of DHM in ACD mice and its related mechanisms. Conclusions from this study will guide the development of new compounds for the treatment of refractory itch associated with ACD.

2. Methods

2.1 Animals

A total of 60 ICR male mice aged 6 weeks (20-25g) were used in this study, which were purchased from The Animal Experimental Center of Nantong University. All animals were placed on a 12-hour light/dark cycle. All animal feeding and experimental procedures were strictly in accordance with Nantong University "Guide to The Care and Use of Experimental Animals" and related ethical standards, in accordance with international standards. The mice were given a standard diet and water at will. The mice were divided into 6 groups (n = 10), Control group, DHM positive control group (250 mg· kg$^{-1}$), ACD model group, and 3 DHM positive ACD model groups (50 150 250 mg· kg$^{-1}$).

2.2. Reagents

DHM (Acme, China, lot #D62880), Acetone (Sigma Inc, America, lot# P3761), 2,4-Dinitrofluorobenzene (DNFB) (Alfa Aesar, China, lot #F-A11871), Olive oil (Acme, China, lot # O39310), Physiological saline
(Changzhou Lanling Pharmaceutical Co., Ltd, lot #H52020069), soflurane(Acmec, China, lot # I40690),
Trizol (Invitrogen Corp, Carlsbad, CA, USA, lot #103106), HiScript III RT SuperMix for qPCR (+ gDNA wiper)
(Vazyme, China, lot # R323-01), TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Japan, lot #
RR820A), Methanol (Acmec, China, lot # M14854), 4% Paraformaldehyde fixator (4% PFA) (Acmec, China,
lot # P39200), 180 kDa Prestained Protein Marker (Vazyme, China, lot # MP102-01), RIPA Lysis Buffe
(Millipore, Germany, lot # 20–188), BeyoECL Plus (Beyotime, China, lot #P0018S), anti-GFAP antibody
produced in mouse (Millipore, Germany, lot # MAB360), anti-IBA1 antibody produced in rabbit (Wako,
Japan, lot # 013-26471), anti-TLR4 antibody produced in rabbit (Millipore, Germany, lot # SAB5700684),
KitHematoxylin-Eosin /HE Staining Kit(Acmec, China, lot # AC11546), Bicinchoninic Acid (BCA) Protein
quantification kit(Beyotime, China, lot # P0012S), Mouse TNF alpha ELISA Kit (Abcam, Britain,ab208348),
Mouse IL-1β ELISA Kit (Abcam, Britain, ab197742), Mouse IL-6 ELISA Kit (Abcam, Britain, ab100713). All
other chemicals are analytical or high-performance liquid chromatography grade.

2.3. Establishment of ACD model, drug therapy and
evaluation of scratching behavior

As previously reported, mouse models of ACD induced by application of 2, 4-dinitrofluorobenzene (DNFB)
to the skin were stable and reliable[13, 14]. Briefly, abdominal hair was shaved on day 0 (2×2cm²) and
sensitized on day 1 with 50 µl 0.5% DNFB (dissolved in a mixture of acetone and olive oil, 4:1). On day 5,
the neck was shaved (2×2cm²), and on day 6, the shaving area was attacked with 30µl 0.25% DNFB. The
control group was given a mixture of acetone and olive oil (4:1). With the same method, the drug was
applied every other day, and the ACD model was successfully established for 4 times until day 12.
Meanwhile, the scratching behavior of the mice was measured 24 hours after each attack. On day 13,
DHM (dissolved in 0.5% sodium carboxymethyl cellulose (CMC-Na)) was administered by gavage after
behavior measurement. According to relevant literature[15], they were divided into three groups: 50 mg·
kg⁻¹, 150 mg· kg⁻¹ and 250 mg· kg⁻¹, the control group was given normal saline by gavage. The same
method was used for daily gavage, and scratching behavior was measured 24 hours after each
treatment. The related processes are schematically summarized in Fig. 2. For behavior measurements,
mice were housed in separate, clear plastic containers of 9 × 9 × 13 cm³. The video recorder was placed
above mice to record the behavior of multiple mice simultaneously. The experiment was carried out in a
soundproof room. All mice received adaptive training 6 days before the experiment and then adapted to
the laboratory 1 hour before the video recording. An uninformed investigator scored the number of
scratches that occurred during the 30-minute observation period. The number of times that mice
scratched their cheeks was counted. The scratching begins by lifting the back paw to the affected area
and ends by placing the back paw back on the floor or in the mouth. All experimenters were blind to
experimental group during data collection. Criterion of itch-like scratching behavior was used as reported
previously[16]. The optimal therapeutic dose of DHM and perfusion sampling time were determined by
behavior.

2.4. Evaluation of dermatitis
Assess the severity of dermatitis on the face, ears, and cephalic parts of the body using the table below. The total score (minimum 0, maximum 12) is expressed as the sum of each score of the four symptoms[17].

2.5 Histopathological examination

Neck skin was collected and histologically examined. The skin was fixed in 4% paraformaldehyde solution for 24 hours, then dehydration, embedding, slicing and dewaxing were carried out in sequence. Subsequently, paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and examined by Motic digital microscope (Olympus). Images were selected from appropriately representative areas.

2.6 Immunofluorescence Cell Staining

Isoflurane (5% in O₂) was percolated with 40 ml of normal saline, followed by 40 ml of cold 4% PFA solution. The spinal cord (C3-C4) was removed and fixed and overnight with the same 4% PFA at 4°C. The spinal cord was then transferred to 30% sucrose in PBS for at least 24 hours. Transverse sections of spinal cord (10 µm thick) were prepared on gelatine-coated slides with a frozen slicer. Sections were sealed with 5% goat serum and 0.3% Triton X-100 (Sigma) in TBS buffer for 1.5 h. The sections were incubated overnight with anti-GFAP antibody produced in mouse (1:1000) and anti-IBA1 antibody produced in rabbit (1:500) primary antibodies at 4°C, and then the sections were incubated. Corresponding secondary antibodies (Alexa Fluor 555 and 488, Invitrogen) were used at room temperature for 5 h. Sections were fixed with Fluoromount-G (SouthernBiotech) and fluorescence images were obtained using a confocal microscope (LSM510, Zeiss).

2.7 Western Blotting Analysis

It was cracked with a (RIPA) cracking buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), a mixture of protease and phosphatase inhibitors. Protein concentration was measured by using BCA protein detection kit. Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) (Millipore, Bedford, MA, USA). After sealing with 5% skim milk, the membrane was incubated overnight at 4°C with the desired primary antibody against IBA1, GFAP, TLR4 or GAPDH, and then incubated with the appropriate HRP conjugated secondary antibody. Detection by enhanced chemiluminescence. The sample loading quantity was standardized by the GAPDH quantity detected in parallel.

2.8 Real Time Quantitative PCR

The mRNA levels of IL-1β, IL-6 and TNF-α in cervical skin and spinal dorsal horn were measured by real-time fluorescence quantitative PCR (RT-QPCR). According to the manufacturer’s instructions, total RNA was extracted by using Trizol reagent. cDNA was synthesized from 1000 ng total RNA using HiScript III RT SuperMix for qPCR (+ gDNA Wiper) kit. The target gene and reference gene 18S of each cDNA sample were amplified in a 20 µL reaction volume using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus). All primers used are listed in Table 2. Real-time quantitative PCR was performed at 95°C for 30 seconds,
followed by 40 cycles at 95°C for 5 seconds, 55°C for 30 seconds, and 72°C for 60 seconds. Standardize mRNA levels for all genes.

Table 1
Dermatitis scoring table.

<table>
<thead>
<tr>
<th>Observation area</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Face, ears, and the skin of neck</td>
<td>No symptoms (score 0)</td>
</tr>
<tr>
<td></td>
<td>Mild (score 1)</td>
</tr>
<tr>
<td></td>
<td>Moderate (score 2)</td>
</tr>
<tr>
<td></td>
<td>Severe (score 3)</td>
</tr>
<tr>
<td>Erythema/hemorrhage of the back skin</td>
<td>No erythema hemorrhage</td>
</tr>
<tr>
<td></td>
<td>Local erythema no hemorrhage on back skin</td>
</tr>
<tr>
<td></td>
<td>Disseminated erythema, no hemorrhage</td>
</tr>
<tr>
<td></td>
<td>Erythema on the entire back skin or Hemorrhage caused by repeated scratching</td>
</tr>
<tr>
<td>Edema in the ear pinna</td>
<td>No increase in ear thickness</td>
</tr>
<tr>
<td></td>
<td>Slight increase in thickness in either the left or right ear pinna</td>
</tr>
<tr>
<td></td>
<td>Marked increase in thickness of both sides of ear pinna</td>
</tr>
<tr>
<td></td>
<td>Marked increase in thickness and stiffness of both sides of ear pinna</td>
</tr>
<tr>
<td>Excoriation/erosion in the ear pinna</td>
<td>No excoriation and tissue deficit</td>
</tr>
<tr>
<td></td>
<td>Local (not continuous) excoriation, no tissue deficit</td>
</tr>
<tr>
<td></td>
<td>Small scale continuous excoriation, no tissue deficit</td>
</tr>
<tr>
<td></td>
<td>Continuous excoriation and tissue deficit</td>
</tr>
<tr>
<td>Scaling/dryness of the rostral back skin</td>
<td>No scaling or dryness</td>
</tr>
<tr>
<td></td>
<td>Local scaling and slight exfoliation of skin</td>
</tr>
<tr>
<td></td>
<td>Disseminated scaling/markd exfoliation of skin</td>
</tr>
<tr>
<td></td>
<td>Scaling of the entire area and marked exfoliation of skin</td>
</tr>
</tbody>
</table>

Table 2
Primers used for RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense sequence</th>
<th>Anti-sense sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>GACAGGATTGACAGATTGATAG</td>
<td>CGTTATCGGAATTAACCAGAC</td>
</tr>
<tr>
<td>IL1-β</td>
<td>TGTCTTGCGGAGGACTAAG</td>
<td>TGGGCTGGACTGGTTTCTAATG</td>
</tr>
<tr>
<td>IL-6</td>
<td>TCCATCCAGTTGCTTTTCTTG</td>
<td>CCACGATTTCCAGAGAACATG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CCCCCAAGGGATGAGAAGTT</td>
<td>CATTGGTGGTTTGCTACGA</td>
</tr>
</tbody>
</table>

2.9 ELISA

ELISA Serum supernatant was separated after centrifugation and the levels of TNF-α, IL-1β and IL-6 were tested using ELISA test kits in accordance with the manufacturer’s instructions. The optical density value
was measured at an excitation wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.) with the blank well serving as the control. Results were expressed as pg/ml of supernatant.

2.10 Statistics

All data were presented as mean ± SEM. Data were analyzed via one-way ANOVA with Dunnett t3 test. $P < 0.05$ was considered as statistically significant.

3. Results

3.1 Successful establishment of ACD model

Consistent with the published report[18], repeat application of DNFB to the nape skin 4 times resulted in scratching behavior and damage to the treated area (Fig. 3). These indicate that ACD model has been successfully established.

3.2 Dihydromyricetin reduced scratching behavior in ACD mice

As demonstrated in Fig. 4, the ACD group experienced significantly more scratching bouts compared to the control group ($P < 0.001$). DHM treatment was given after successful modeling. The number of scratching in the treatment group was lower than that in the ACD group, significantly decreased in 250 mg·kg-1 group after the second treatment ($P < 0.001$). The other treatment dose groups were not statistically significant compared with the ACD group. Therefore, 250 mg·kg-1 was selected as the therapeutic dose of the ACD + DHM group in the follow-up experiment, and the samples were collected by perfusion after the second treatment.

3.3 DHM alleviated neck skin injury and inflammatory cell infiltration in ACD mice

As shown in Fig. 5A, compared with the model group, the neck skin erythema, chyme erosion, bleeding and auricle edema of ACD mice were alleviated after DHM treatment. At the same time, HE staining showed that the epidermal cells in the control group and the DHM group were arranged regularly, without large gaps and morphological abnormalities. Compared with these two groups, the ACD model group had increased scratching behavior, severe hyperkeratosis of neck skin, and increased lymphocytic infiltration at the junction between dermis and epidermis. After treatment with DHM (250 mg·kg-1), keratinization and inflammatory cell infiltration of ACD mice were relieved, but still higher than those of healthy control mice. The administration time may be too short, and the mice in the administration group are still in the recovery period. As shown in Figure B, the dermatitis score again confirmed that DHM effectively alleviated the progression of dermatitis.

3.4 Dihydromyricetin inhibited the activation of microglia and astrocytes in the spinal cord of ACD mice
We evaluated the activation of astrocytes and microglia by detecting the protein levels of GFAP and Iba1 by immunohistochemical staining and Western blot. The final results show that the results of the two methods are consistent.

3.5 Dihydromyricetin treatment can partially rescue the elevated expression of inflammatory cytokines in ACD mice

As shown in Fig. 7, compared with the blank control group, the mean expression levels of inflammatory cytokines in serum, neck skin and spinal dorsal horn of the ACD model group were significantly increased. Compared with the ACD model group, the average expression levels of inflammatory cytokines in serum, neck skin and spinal cord dorsal horn in DHM (250 mg·kg\(^{-1}\)) treatment group were decreased, but still higher than those in blank control group.

3.6 DHA can alleviate Toll-like receptor 4 (TLR4) activation in the spinal dorsal horn of ACD mice

As demonstrated in Fig. 8, western blot analysis shown that the expression of TLR4 protein in ACD mice spinal dorsal horn was significantly increased compared with the two control groups \((P < 0.001)\). However, after DHM treatment (250 mg·kg\(^{-1}\)), the expression of TLR4 protein in ACD mice spinal dorsal horn was inhibited \((P < 0.01)\), but still higher than that in the control group.

4. Discussion

Chronic pruritus, also known as refractory pruritus, is often associated with recurrent episodes, but treatment has limited effect and the mechanism behind the resistance remains elusive. Pruritus is an unpleasant sensation that causes a desire or reflex to scratch, often as a self-protective mechanism against harmful external factors. However, excessive and repeated scratching can lead to skin damage, which can aggravate the itch sensation and lead to further scratching (malignant itch-scratch cycle). Pruritus is a subjective sensation that cannot be accurately represented in animal models. However, scratching is a response to pruritus and can be studied by using a mouse skin model\[19\]. Therefore, we successfully used DNFB to establish an ACD model and used hindlimb scratching as an index of pruritus \[20\], which finally verified that DHM treatment alleviated pruritus in ACD mice.

The clinical manifestations of contact dermatitis are varied, but the epidermal barrier defect is the center. Therefore, restoration of epidermal barrier function is an important prevention and control objective\[21\]. Skin injury and inflammation lead to the recruitment of immune cells (e.g., various innate immune cells, T lymphocytes). Activated immune cells release proinflammatory mediators to sensitize itch receptors. This peripheral sensitization can lead not only to chronic pain but also to chronic pruritus\[22, 23\]. In this study, we found that administration of DHM could rescue the defects of skin injury and inflammatory cell infiltration in ACD mice by dermatitis score and H&E staining analysis.
Microglia-mediated chronic inflammation is involved in the pathological process of a variety of chronic neurodegenerative diseases. In the process of chronic inflammation, microglia are activated for a long time and then continuously release a series of inflammatory mediators, leading to oxidative stress response [24]. Studies have confirmed that intrathecal injection of minocycline, an inhibitor of activated microglia, can inhibit scratching behavior in DNFB-induced contact dermatitis mouse model[25], reflecting the role of microglia activation in ACD pruritus[26]. Meanwhile, studies have proved that activated microglia are powerful modulators of astrocyte and neuronal function[27], and the neurotoxic phenotype of astrocytes is induced by factors secreted by activated microglia[28]. Astrocytes, the most abundant glial cell type in the central nervous system (CNS), are responsible for a series of functions required to support the activity of cortical circuits and play a key role in neuronal function under physiological and pathological conditions[29]. Studies have demonstrated that astrocytes are induced to activate in spinal cord segments corresponding to skin lesions in refractory pruritus[30, 31]. Recent findings suggest that scratching can promote chronic pruritus by inducing astrogliosis, which is an important mechanism in the pruritus -scratching cycle. At the same time, studies have shown that activated glial cells, including microglia and astrocytes, produce a variety of cytokines and chemokines[32]. In this study, we demonstrated that DHM inhibited the activation of microglia and astrocytes in the spinal cord by immunofluorescence staining and Western blot analysis.

TLR4, a member of the TLR family, mediates innate and adaptive immunity by identifying exogenous ligands, pathogen-related molecular patterns after viral and bacterial infection, and detecting endogenous ligands and risk-related molecular patterns generated by tissue injury[33]. Previous studies have shown that TLR4 knockdown or intrathecal treatment with TLR4 antagonists reduces spontaneous scratching in a model of chronic pruritus associated with contact dermatitis. Researchers believe that spinal TLR4 signaling is important for spinal astrocyte activation and astrogliosis, which may underlie chronic pruritus. In this experiment, Western blot verified that DHM treatment effectively inhibited TLR4 expression in the spinal cord.

Studies have shown that chemokines, inflammatory cytokines, histamine, and other substrates from mast cells produce pruricity-related scratching behavior in ACD[34]. In addition, abundant production of pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α contributes skin inflammation as well as a control of itch sensation[35]. IL-6 and TNF-α are also known to mediate the initiation of AD[36]. IL-1β, one of the initial markers of Langerhans cells, is involved in the progression of many allergic diseases, and its concentration can reflect the severity of inflammation[37]. In addition, TNF-α, as a central mediator of inflammation, plays an important role in immune regulation. In this study, we demonstrated that DHM administration significantly downregulated peripheral and central inflammatory cytokine expression.

5. Conclusion

Previous studies have shown that the interaction of TLR4, spinal cord glial cells, inflammatory cytokines, and scratching behavior promotes the disease progression of ACD pruritus. In this study, we found that DHM inhibited the activation and expression of glial cells and TLR4 in the spinal cord of ACD mice, and
reduced the production of inflammatory cytokines, which effectively alleviated the pruritus symptoms of ACD mice, thus improving the skin injury. In conclusion, these results fully validated the antipruritic and anti-inflammatory effects of DHM in a mouse model of allergic contact dermatitis. Therefore, DHM may be a potential drug for the treatment of pruritus contact dermatitis and other chronic pruritus related diseases as well as skin inflammation.

Declarations

Ethical Approval

The experimental protocol was in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, NIH) and was approved by The Standard Operating Procedures for Laboratory Animal Center of NTU (Nantong, China). protocol number: S20211130-904(China).

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

Chunwei Yin designed and carried out the experiment. Chunwei Yin and Jiacheng Zhao analyzed the behavioral data, wrote the draft of manuscript and revised the manuscript. Xiang Zhu was in charge of the experiment design, supervision of the project, and final approval of the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Availability of data and materials

The data used to support the findings of this study are available from the corresponding authors upon request.

References


**Figures**
Figure 1

Structure of dihydromyricetin (DHM). This picture was downloaded from pubchem

Figure 2
The schematic experimental schedule.

Figure 3

Allergic contact dermatitis of skin lesions in inflammatory dermatitis are erythema, erosion, edema, and dryness. The main pathological manifestation of allergic contact dermatitis is inflammatory cell infiltration. The top picture shows skin damage, and the bottom picture shows skin pathological changes.
Figure 4

Scratching bouts of mice 30 min after administration of the compound (n = 10). Adjusted $P$ values were showed as: ns, no significance; $***$, $P < 0.001$; $###$, $P < 0.0001$. 
Figure 5

Skin injury of mice in each group (n = 5). (a) Skin injury after DHM treatment and HE staining of attacked neck skin (200X). Arrows indicate areas of inflammatory cell infiltration. (b) Dermatitis scores in each group after treatment. Adjusted P values were showed as: ns, no significance; ***, P < 0.001; ###, P < 0.0001.
Figure 6

Activation of glial cells in spinal cord of mice in each group (n = 5). (a, b) Immunoreactivity of GFAP (green) and Iba1 (red) in the superficial dorsal horn of the cervical segment (C3-C4) injected by the treated skin. Bar = 100μm. (c, d) Western blotting analysis of GFAP and Iba1 expression in spinal dorsal horn of C3-C4 mice. Quantitative analysis of protein band strength (as multiples of controls, normalized to GAPDH). Adjusted P values were showed as: ns, no significance; ***, $P < 0.001$; ###, $P < 0.0001$. 
Figure 7

The expression level of inflammatory cytokines in each tissue. (a) The expression level of inflammatory cytokines in serum was detected by ELISA. (b) The expression levels of inflammatory cytokines in skin were detected by RT-qPCR. (c) The expression levels of inflammatory cytokines in spinal dorsal horn of C3-C4 mice were detected by RT-qPCR (n = 6). Adjusted $P$ values were showed as: ns, no significance; ***, $P < 0.001$; ###, $P < 0.0001$. 
Figure 8

Western blotting analysis of TLR4 expression in spinal dorsal horn of C3-C4 mice (Picture above) (n = 4). Quantitative analysis of protein band strength (as multiples of controls, normalized to GAPDH) (Picture below). Adjusted $P$ values were showed as: ns, no significance; ***, $P < 0.001$; ###, $P < 0.0001$. 