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Inhibition of the PKC-TRPV1-SP axis by propolis ameliorates chronic inflammatory pain in mice

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ABSTRACT

Objective(s): Chronic inflammatory pain significantly affects patient quality of life; however, existing treatment methods have limitations. Propolis is rich in various bioactive ingredients, such as flavonoids and phenolic acids. This study aimed to explore the effects and action mechanisms of propolis against chronic inflammatory pain.

Materials and Methods: A mouse model of chronic inflammatory pain was established using Complete Freund's adjuvant, with the mice divided into normal control, model, and propolis-treated groups. Body weight, pain score, foot volume, thermal pain threshold, mechanical pain threshold, and pathological changes in the toe tissue were recorded before injection (day 0) and on days 1, 7, 14, 21, and 28 after injection.After the experiment, changes in inflammatory factors (TNF-α, IL-4, IL-6, and IL-10) were detected, and the expression and distribution of proteins associated with the PKC-TRPV1-SP signalling axis in the toe, spinal cord, and hippocampus were detected by Western blot and immunofluorescence.

Results: Propolis treatment significantly inhibited foot swelling, alleviated tissue inflammatory lesions, relieved pain, and increased heat and mechanical pain thresholds in mice. In addition, propolis inhibited the secretion of pro-inflammatory cytokines TNF-α, IL-4, and IL-6, promoted the secretion of the anti-inflammatory cytokine IL-10, and inhibited the expression of proteins related to the PKC-TRPV1-SP signalling axis.

Conclusion: This study demonstrated that propolis exerts anti-inflammatory and analgesic effects by regulating the PKC-TRPV1-SP signalling axis. The findings offer new perspectives for the clinical treatment of chronic inflammatory pain and provide a reference for the development of new medications.

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Introduction

Chronic inflammatory pain is a prevalent and complex pain condition that has a negative impact on quality of life (1, 2). It not only causes persistent pain and discomfort in daily activities but also severely disrupts patients' sleep quality, leading to psychological problems such as depression, anxiety, and depression, thereby comprehensively diminishing the quality of life of patients (3, 4).

Current clinical treatments for chronic inflammatory pain mainly rely on drug intervention (5, 6). However, these conventional treatment methods have many limitations that cannot be ignored (7). For example, the long-term use of commonly prescribed non-steroidal anti-inflammatory drugs may cause serious side effects, such as gastrointestinal discomfort and liver and kidney function damage (8-10). Moreover, although opioid drugs can provide powerful pain relief, they carry a huge risk of addiction (11, 12). Once the patient has become addicted, these drugs not only adversely affect physical and mental health but also cause a series of social problems (13, 14).

Propolis contains a variety of bioactive components, such as flavonoids and phenolic acids (15, 16). Previous studies have demonstrated that these components endow propolis with remarkable pharmacological effects, including anti-inflammatory, antioxidant, and antibacterial effects (17-19). In research on multiple inflammation-related diseases, such as arthritis and enteritis, propolis showed significant ameliorative effects, indicating its potential therapeutic value (20, 21). However, despite the progress made in research on propolis in the field of inflammation, studies on its effects on chronic inflammatory pain are relatively scarce (22, 23).

Therefore, the objective of this study was to clarify the alleviating effect of propolis on chronic inflammatory pain

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and reveal its potential molecular mechanisms. Moreover, this study offers new perspectives for the clinical treatment of chronic inflammation and provides a solid theoretical basis for developing new anti-chronic inflammatory pain medications.

Materials and Methods

Preparation of a mouse model of chronic inflammatory pain

Thirty male C57BL/6 mice (specific pathogen-free grade, weighing 20±2 g) were purchased from the Nanjing Qinglongshan Animal Farm. Before the experiment, the mice were provided *ad libitum* access to food and water and acclimatised for one week. Subsequently, the mice were randomly divided into three groups (n=10 each): (1) normal saline (normal control, NC): 20 µl of normal saline was subcutaneously injected into the left hind paw; (2) chronic inflammatory pain model (Mod): 20 µl of Complete Freund's adjuvant was subcutaneously injected into the left hind paw; and (3) propolis administration (Pro): 75 mg/kg total flavonoids of propolis administered continuously by gavage for 28 days to Mod group mice. This study was approved by the Animal Ethics Committee of Wannan Medical College (LLSC-2021-024) and performed in strict accordance with the guidelines of the Animal Care and Use Committee of Wannan Medical College.

Reagents

Water-soluble propolis (lot number: 20210325-B) was obtained from Guangzhou Jiehe Bee Industry Co., Ltd. (China). The primary antibodies used for immunofluorescence were anti-PKC (ab184746; Abcam), Anti-TRPV1 (ab203103; Abcam), and anti-SP (PA5-106934; Invitrogen). The secondary antibodies used for western blotting were BL001A and BL003A (Biosharp). The secondary antibodies used for immunofluorescence were 594-conjugated AffiniPure Donkey Anti-Mouse (715-585-150) and 488-conjugated AffiniPure Donkey Anti-Rabbit (711-545-152)(Jackson).

Assessment of mouse characteristics under chronic pain

During the experiment, changes in the left hind limb were observed, including redness and swelling in the injected paw as well as exudate and infection at the injection site. The impact on food intake was also monitored. Gait and posture were observed along with licking, biting behavior, and limb paralysis. The mice were weighed before injection (day 0) and on days 1, 7, 14, 21, and 28 after injection, and the pain scores were recorded. The pain scoring criteria (24, 25) were as follows. 0 score: The hind paw on the side injected with complete Freund's adjuvant and contralateral hind paw touched the ground, and the mouse could walk freely. 1 score: Both hind paws touched the ground, but there was a slight limp on the injected side during walking. 2 score: Both hind paws touched the ground, but the body's centre of gravity shifted to the healthy side and an obvious limp was observed on the injected side during walking. 3 score: The hind paw on the injected side slightly touched the ground but could not bear weight and was lifted during walking. 4 score: The hind paw on the injected side was lifted and did not touch any surface. 5 score: The mouse licked, bit, or shook the hind paw on the injected side.

Measurement of thermal pain threshold

Mice were placed in an experimental cage for 10 min for acclimatisation. The focal point of the thermal nociceptor stimulator was the sole of the foot. The stimulator was turned on, and the time from the start of irradiation to the paw-withdrawal reflex (latency) was recorded. If no response occurred within 20 sec, irradiation was stopped immediately to avoid burning the foot sole. This measurement was conducted three times, with 10–15 min intervals between each measurement. The average of the three values was considered the latency of the paw withdrawal response to thermal stimulation (26, 27).

Measurement of mechanical pain threshold

Mice were placed in an experimental cage for 10 min for acclimatisation. The sole of the injected foot was stimulated using an appropriate filament, and the stimulus intensity that triggered the paw withdrawal reflex was recorded. If no response occurred at 20 g, stimulation was ceased immediately to prevent foot sole stabbing. This measurement was repeated three times, with 10-15 min intervals between each. The average of these three values was used as the threshold for mechanical pain (26, 27).

Sample collection

On the 28th day after injection, the animals were sacrificed after fasting for 24 hr with *ad libitum* access to water. Blood was collected from the orbital sinuses of the mice. Blood samples were left to stand at room temperature for 2 hr and then centrifuged at 3000 rpm for 10 min. The supernatant was aliquoted and stored at -80 ˚C. The inflamed toe tissue was excised with a scalpel and skinned, and 0.1 g of the tissue was quickly stored at -80 ˚C. The L4-L6 segments of the spinal cord and 0.1 g of hippocampal tissue were rapidly frozen in liquid nitrogen and stored at -80 ˚C. In each group, two mice were perfused with 4% paraformaldehyde. The injected foot tissue, L4–L6 segments of the spinal cord, and hippocampus were collected for cryosectioning, haematoxylin-eosin staining (HE staining), and immunofluorescence experiments.

HE staining

Foot tissue fixed with 4% paraformaldehyde was dehydrated in 15% and 30% sucrose solutions for one day each and then embedded in OCT compound. The embedded tissue was sectioned into slices with a thickness of 20 µm using a Leica cryostat microtome. The slices were fixed in PBST for 10 min, stained with haematoxylin for 6 min, and washed with water. Subsequently, the cells were differentiated with hydrochloric acid for 10 sec, stained with eosin for 20 sec, and washed again with water. Subsequently, the slices were dehydrated using an alcohol gradient (70, 75, 80, 85, 90, and 100%) for 1 min each, cleared with xylene, and mounted (28, 29). Finally, structural changes in the foot tissue were observed using a Leica fluorescence microscope.

Western blot

One hundred micrograms of tissue were added to 1 ml of lysis buffer, minced, homogenised, and left to stand for 30 min. The mixture was then centrifuged at 12,000 rpm for 30 min at 4 ˚C, and the supernatant was extracted. The supernatant was mixed with 5× loading buffer at a 4:1 ratio, incubated at 95 ˚C for 10 min, cooled, and stored. A 5 µl sample was subjected to SDS-PAGE electrophoresis, and the proteins were transferred to a PVDF membrane. The membrane was blocked with skim milk, incubated with primary antibody at 4 ˚C overnight, washed three times with

TBST, incubated with secondary antibody for 1 hr (30, 31), developed with ECL solution for imaging, and analysed for protein band grey values using ImageJ.

Immunofluorescence

Sections stored at -20 ˚C were first air-dried at room temperature and then baked in a 37 ˚C oven for 2 hr. Next, peroxisomes were blocked with 3% H_2O_2 for 10 min, followed by three 5-min washes with PBST on a shaker. Antigen retrieval was carried out by immersing the sections in citrate buffer in a 95 ˚C water bath for 8 min. Next, the sections were allowed to equilibrate to room temperature and underwent another three 5 min PBST washes. Subsequently, they were blocked with horse serum for 30 min and incubated with the primary antibody at 4 ˚C overnight. The sections were then brought back to room temperature and baked at 37 ˚C for 30 min, followed by three 5-min PBS washes. Incubation with the secondary antibody was performed in a dark room for 2 hr, and DAPI was added for 30 min (29, 30). Fluorescent expression of the PKC/TRPV1 and TRPV1/SP protein pairs was observed under a laser confocal microscope. Co-localisation of these two protein pairs in the foot tissue, spinal cord, and hippocampus was analysed using the Image Pro Plus 6.0 software.

Data analysis

Quantitative data were expressed as the mean ± standard deviation. Statistical analyses were performed using SPSS version 18.0. To evaluate the differences among multiple groups, one-way analysis of variance (ANOVA) and repeated-measures ANOVA were applied. For pairwise comparisons within these multiple groups, the least significant difference t-test (LSD t-test) was adopted. Statistical significance was set at a two-tailed *P*-value<0.05.

Results

Administration of propolis alleviated foot swelling

As depicted in Figure 1, foot volume did not discernibly differ among the three groups on day 0. Starting from day

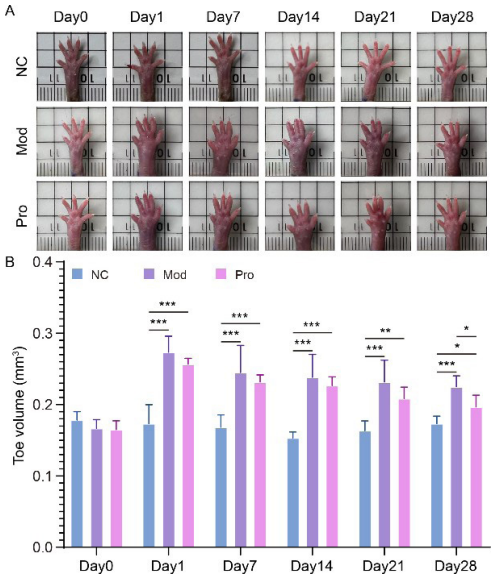

Figure 1. Effects of propolis on toe swelling in mice
A. Appearance of the foot. B. Quantitative data of foot volume. NC represents the normal control group, Mod represents the model group, and Pro represents the propolis treatment group. * *P*<0.05, ** *P*<0.01, *** *P*<0.001

1 and at subsequent time points, the foot volume of the Model (Mod) group significantly increased, signifying the successful induction of foot swelling by the modelling procedure. In contrast, when compared with the Mod group, the propolis (Pro) group exhibited a reduction in foot volume to varying degrees on days 1-28. Notably, on day 28, foot volume in the Pro group decreased significantly relative to the NC group (*P*<0.05). This clearly demonstrated that propolis effectively mitigated foot swelling and had a substantial and positive effect on the management and reduction of foot swelling.

Propolis can alleviate inflammatory pathological changes and improve weight loss

Pathological observations of the foot tissues (Figure 2A) indicated that the tissue structure of the NC group remained

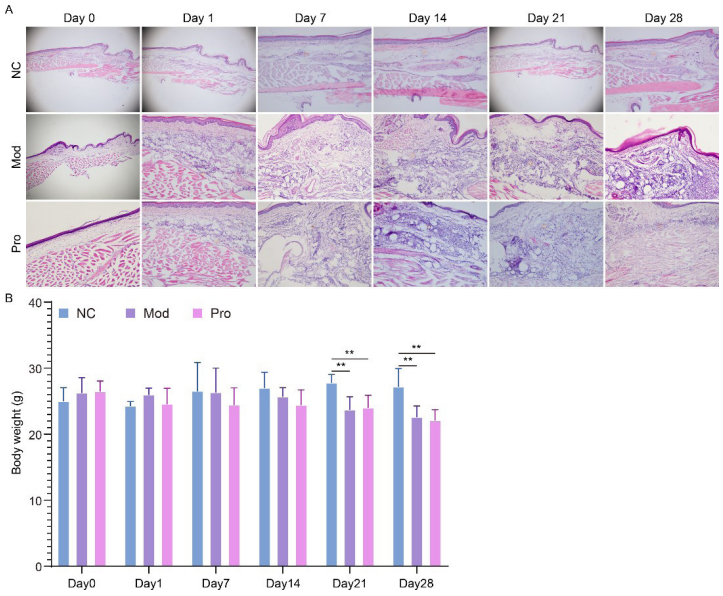

Figure 2. Effects of propolis on histopathological changes (HE staining) and body weight in mice
A. Histopathological sections of foot tissues at designated time points (day 0-day 28). B. Body weight data chart. NC represents the normal control group, Mod represents the model group, and Pro represents the propolis treatment group. * *P*<0.05, ** *P*<0.01, *** *P*<0.001

stable from days 1 to 28. The cells maintained a normal morphology and were free from notable inflammation-associated alterations such as edema, congestion, or cellular degeneration. Conversely, beginning on day 1, the Mod group exhibited a progressive disarray in cellular organisation. The epithelial layer was thickened, presumably because of cell proliferation induced by inflammation. Simultaneously, interstitial edema developed, accompanied by inflammatory cell infiltration, with the cells either scattered or aggregated within the tissue. From day 7 to day 28, the inflammatory symptoms were exacerbated. Tissue congestion became pronounced, blood vessels were dilated, the extent of inflammatory cell infiltration broadened, and signs of tissue necrosis emerged in some regions. These symptoms are characteristic of classic inflammatory injury pathology. When juxtaposed with the Mod group, the Pro group displayed some degree of disrupted cellular alignment and mild edema starting from day 1; however, these manifestations were significantly less severe. Inflammatory cell infiltration was relatively scarce, and the degree of tissue congestion and vascular dilation was muted. From day 7 to day 28, the Pro group demonstrated a distinct tendency towards tissue injury repair. Inflammatory cell infiltration waned, tissue edema subsided, and the architecture of both the epithelial layer and underlying tissues reverted toward normalcy, underscoring the propitious role of propolis treatment in mending inflammation-damaged tissues.

Regarding the weight changes illustrated in Figure 2B, on day 0, the average body weights of the NC, Mod, and Pro groups were comparable with the average weight of the NC group serving as the baseline. Over the course of the experiment from day 1 to 28, the NC group showed a consistent upward weight trend, following a typical growth pattern under standard feeding conditions, with a steadily increasing growth curve that reflected normal physiological development. In contrast, although the Mod group began with an average weight similar to that of the NC group, inflammation-induced injury led to a gradual decline in weight gain rate starting on day 1. The growth curve of the Mod group flattened significantly compared with that of the NC group, indicating a marked slowdown in growth, likely due to the negative impact of inflammation on metabolism. The Pro group, which also started with an equivalent average weight as the NC group on day 0, demonstrated a more stable weight-change pattern than the Mod group despite the presence of inflammation.

Propolis alleviates pain

The findings depicted in Figure 3A illustrate that on day 1, the pain behavior scores of both the Mod and Pro groups exhibited a notable increase ($P<0.001$), signifying successful induction of pain-associated behaviors through the modelling process. At subsequent timeframes, the scores of the Pro group on days 7 ($P<0.001$), 14 ($P<0.01$), 21 ($P<0.05$), and 28 ($P<0.05$) were progressively lower than those of the Mod group. This strongly suggests that propolis can mitigate pain-related behaviors. Moreover, the data presented in Figures 3B and 3C reveal that from day 1, the heat and mechanical pain thresholds of the Mod group declined substantially. Although the Pro group also experienced a decrease in these thresholds, the values remained higher than those in the Mod group on days 7, 14, 21, and 28 ($P<0.01$, $P<0.001$, respectively).

Propolis regulates cytokine secretion

The results in Figure 4 show that starting from day 1, the levels of TNF-α, IL-4, and IL-6 in the Mod group increased significantly. Although these indicators also increased in the Pro group, the magnitude of increase was smaller than that observed in the Mod group. In the subsequent time periods, the levels of these indicators in the Pro group were significantly lower than those in the Mod group on day 7

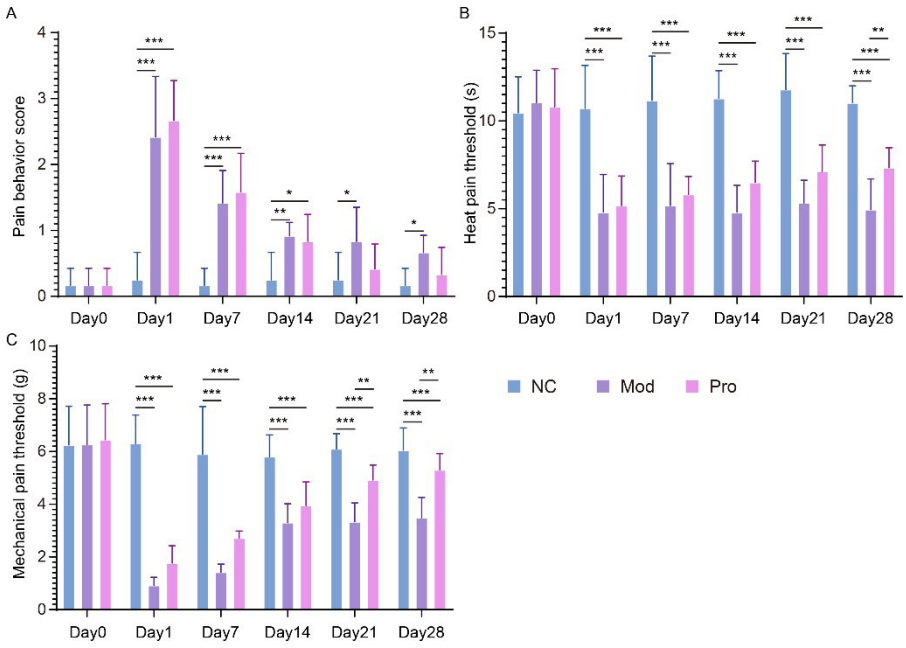

Figure 3. Effects of propolis on pain-related behaviors, heat pain thresholds, and mechanical pain thresholds in mice
A. Evaluation of pain behavior scores. B. Measurement of heat pain thresholds. C. Measurement of mechanical pain thresholds. NC represents the normal control group, Mod represents the model group, and Pro represents the propolis treatment group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

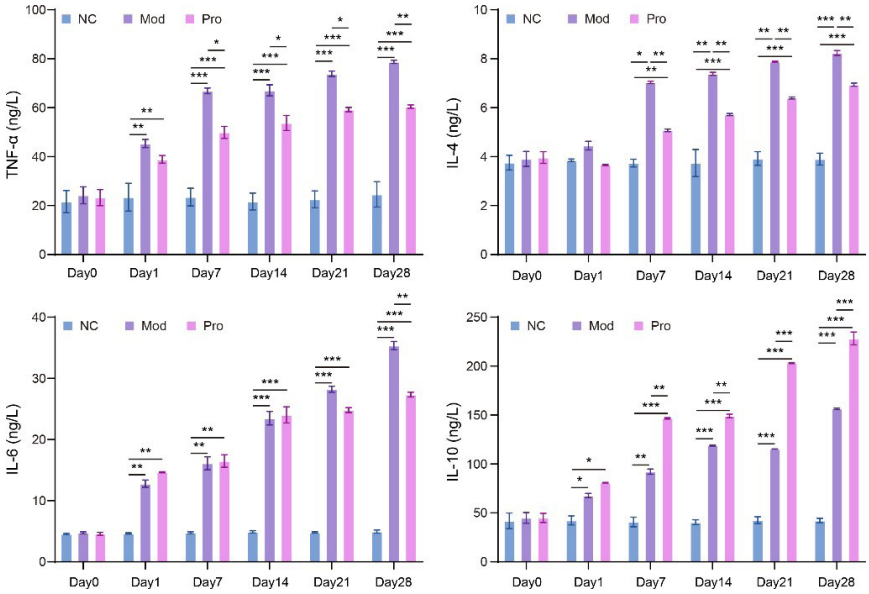

Figure 4. Effects of propolis on the secretion of cytokines TNF-α, IL-4, IL-6, and IL-10 in mice
NC represents the normal control group, Mod represents the model group, and Pro represents the propolis treatment group. * P<0.05, ** P<0.01, *** P<0.001.

(P<0.01), day 14 (P<0.001), day 21 (P<0.001), and day 28 (P<0.001), indicating that propolis can effectively inhibit the secretion of pro-inflammatory cytokines TNF-α, IL-4, and IL-6. Regarding IL-10, starting on day 1, the levels in both the Mod and Pro groups increased, with a more pronounced increase in the Pro group. On days 7 (P<0.01), 14 (P<0.001), 21 (P<0.001), and 28 (P<0.001), the IL-10 levels in the Pro group were significantly higher than those in the Mod group, suggesting that propolis can promote the secretion of the inhibitory inflammatory cytokine IL-10.

Anti-inflammatory mechanism of propolis via the PKC-TRPV1-SP signalling axis

To further explore the anti-inflammatory mechanism of propolis, we measured the expression levels of the three proteins: protein kinase C (PKC), transient receptor potential vanilloid 1 (TRPV1), and substance P (SP) in the toes, spinal cord, and hippocampus. As shown in Figure 5, the expression levels of TRPV1, PKC, and SP in the Mod group were significantly higher than those in the NC group (P<0.01). The expression of these three proteins was lower in the Pro group than in the Mod group, indicating that propolis inhibited the overexpression of relevant proteins in the toe, spinal cord, and hippocampus.

Expression and distribution of proteins associated with the PKC-TRPV1-SP signalling axis in the toe, spinal cord, and hippocampus

Finally, we detected the expression and distribution of the PKC, TRPV1, and SP proteins in the toes, spinal cord,

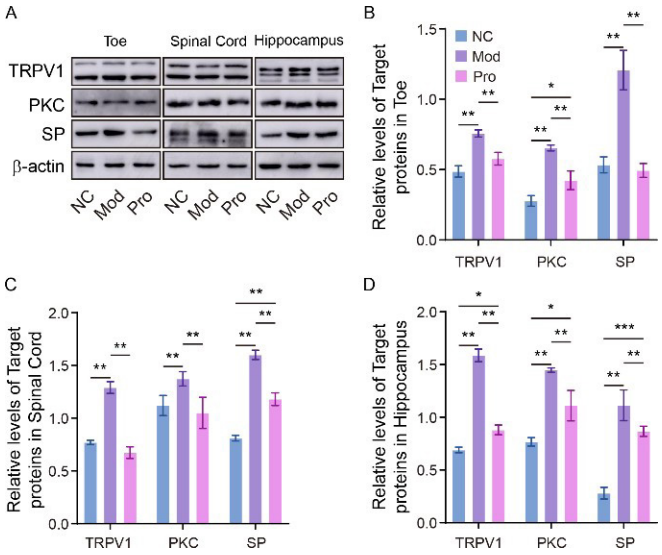

Figure 5. Effects of propolis on the expression of PKC, TRPV1, and SP in the toe, spinal cord, and hippocampus
A. Western blot results for the expression of PKC, TRPV1, and SP in the toe, spinal cord, and hippocampus. B-C. Quantification of the relative expression of PKC, TRPV1, and SP proteins in the toe (B), spinal cord (C), and hippocampus (D) as shown in A. NC represents the normal control group, Mod represents the model group, and Pro represents the propolis treatment group. * P<0.05, ** P<0.01, *** P<0.001

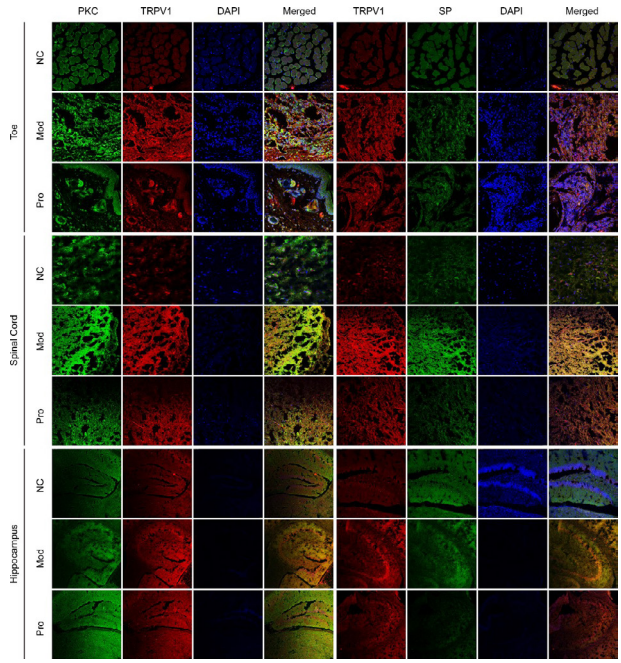

Figure 6. Expression and distribution of proteins associated with the mice PKC-TRPV1-SP signaling axis
NC represents the normal control group, Mod represents the model group, and Pro represents the propolis treatment group

and hippocampus by immunofluorescence staining. These results in Figure 6 were consistent with our expectations. In the NC group, the expression and distribution of PKC, TRPV1, and SP were relatively uniform in the toes, spinal cord, and hippocampus. In the Mod group, the expression levels of these three proteins significantly increased, and their distribution showed a certain degree of disorder. In the Pro group, the expression of the three proteins was significantly weaker than that in the Mod group, and their distribution became relatively regular, approaching the level observed in the NC group. Overall, the proteins associated with the PKC-TRPV1-SP signalling axis were overexpressed, and their distribution was disordered in the toes, spinal cord, and hippocampus of the Mod group.

Discussion

In this study, a mouse model of chronic inflammatory pain was established to observe the effect of propolis on chronic inflammatory pain and conduct an in-depth exploration of its underlying mechanisms. The experimental results revealed that propolis remarkably inhibited foot swelling and mitigated tissue inflammatory lesions in the mouse model of chronic inflammatory pain. This outcome is consistent with previous literature and our prior research on the anti-inflammatory characteristics of propolis.

Suran *et al.* pointed out that numerous bioactive components of propolis, such as flavonoids, phenolic acids, and their esters, exhibit potent anti-inflammatory effects. In the present study, propolis likely improved the pathological condition of the inflamed tissues by suppressing the infiltration of inflammatory cells and tissue edema (32). This is consistent with the findings of Li *et al.*, who showed that propolis alleviates the inflammatory response by inhibiting the release of inflammatory mediators (33).

Regarding the changes in body weight, the chronic inflammatory pain model led to a significant decrease in the body weight of the mice. However, the degree of body weight loss in the Pro group was significantly lower than that in the Mod group, indicating that propolis contributes to maintaining the overall health of the animals. This may be related to its ability to relieve pain and reduce the inflammatory response, thereby minimising its negative impact on metabolism. Relevant research has shown that when metabolism is disordered and body weight decreases in an inflammatory state, propolis can regulate the inflammatory response and thus play a positive role in maintaining body weight (23).

The results of the pain-related behaviors and pain thresholds further corroborate the analgesic effects of propolis. In the Mod group, the pain behavior scores of the mice increased significantly while the heat and mechanical pain thresholds decreased remarkably. This indicated the successful establishment of the model and the extreme sensitivity of the mice to pain stimuli. In contrast, in the Pro group, the pain behavior scores of the mice gradually decreased and the pain thresholds gradually rebounded. These findings suggest that propolis effectively alleviates chronic inflammatory pain and enhances pain tolerance in mice. Studies on the pain-relieving effects of other natural products have shown that their mechanisms of action are similar to those of propolis, with both achieving analgesic effects by regulating pain perception in the nervous system (34).

The results of inflammatory cytokine level detection demonstrated that propolis inhibits the production of pro-inflammatory cytokines such as TNF-α, IL-4, and IL-6. It also promoted an increase in the anti-inflammatory cytokine IL-10, which is related to immune regulation. This indicated that propolis has a favourable regulatory effect on the inflammatory response and contributes to maintaining the balance of inflammatory reactions, which is consistent with research on the immune- and anti-inflammatory mechanisms of propolis reported by Xie *et al.* (35). These findings further validate the effectiveness of propolis in regulating inflammation.

At the molecular level, this study found that the chronic inflammatory pain model activated the expression of proteins associated with the PKC-TRPV1-SP signalling axis while propolis significantly inhibited the expression of these proteins in the toes, spinal cord, and hippocampus. PKC, TRPV1, and SP play crucial roles in the transmission and processing of pain signals (36, 37). Qian *et al.* indicated that propolis mitigates ulcerative colitis injury by inhibiting the PKC-TRPV1-CGRP/SP signalling axis (38). In the present study, propolis demonstrated a remarkable inhibitory effect on the PKC-TRPV1-SP signalling axis during the onset and progression of chronic pain, thus providing robust evidence for its analgesic mechanism.

However, this study has certain limitations that should be noted. First, although the inhibitory effect of propolis on the PKC-TRPV1-SP signalling axis has been determined, whether there are other regulatory factors upstream and downstream of this signalling axis and whether propolis exerts its effects through other signalling pathways remain unclear. Yam *et al.* pointed out that multiple signalling pathways may be intertwined in complex pain signal transduction networks; thus, the mechanism of action of propolis may be more complex. Second, this study was

only conducted in a mouse model, and the effectiveness and safety of extrapolating the results to humans need to be further verified (39, 40). Numerous animal experiments have shown differences from human clinical trials (41, 42). Therefore, future research should further investigate the specific molecular targets and signalling pathways of propolis and conduct clinical trials to provide a more solid theoretical and practical basis for the clinical application of propolis for the treatment of chronic inflammatory pain.

Conclusion

Based on the findings of our behavioral experiments (pain score, thermal pain threshold, and mechanical pain threshold), we conclude that propolis can effectively alleviate chronic inflammatory pain induced by CFA. The mechanism is related to regulating the balance of cytokines (inhibiting the secretion of pro-inflammatory factors and promoting the secretion of anti-inflammatory factors) and inhibiting the PKC-TRPV1-SP signalling pathway. This study offers new perspectives for the clinical treatment of chronic inflammatory pain and provides a reference for the development of new medications.

Acknowledgment

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Authors' Contributions

Y J and Y S designed and performed the experiments and collected and analyzed data; C H, L M, D J, and S L performed the experiments and discussed the results and strategy; Y X analyzed and interpreted the results; W J supervised, directed, and managed the study and drafted the manuscript. All authors approved the final version to be published.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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