

Original Article

Houttuynia cordata Thunb reverses oxaliplatin-induced neuropathic pain in rat by regulating Th17/Treg balance

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Abstract: Oxaliplatin is a widely used anti-advanced colorectal cancer drug, while it could induce neuropathy. *Houttuynia cordata* Thunb (HCT) has a wide range of biological activities, such as anti-inflammation, anti-cancer, and immune regulation. In the present study, we investigated the effect of HCT on oxaliplatin-induced neuropathy in rat models. HCT (1000 mg/kg/day) significantly decreased the number of withdrawal responses and the withdrawal latency in oxaliplatin-treated rats. HCT could down-regulated the serum levels of Interleukin-6 (IL-6) and macrophage inflammatory protein1- α (MIP-1 α) in oxaliplatin-treated rats. Th17/Treg balance was reversed by HCT in oxaliplatin-treated rats by regulating PI3K/Akt/mTOR signaling pathway. The present results suggest that HCT is useful as a therapeutic drug for oxaliplatin-induced neuropathic pain.

Keywords: Oxaliplatin, *Houttuynia cordata* Thunb, neuropathic pain, Th17/Treg, PI3K/Akt/mTOR

Introduction

Oxaliplatin is a third-generation diaminocyclohexane (DACH) platinum drug, which is widely used in the treatment of colorectal [1], ovarian [2], and pancreatic cancer [3]. Oxaliplatin causes neurotoxicity predominantly within the peripheral nervous system [4]. Chronic neuropathy develops after long-term treatment with oxaliplatin, leading to loss of sensory and motor function [5]. Despite awareness of oxaliplatin-associated neuropathies and their severity, the underlying mechanisms are not well understood. Increasing evidence indicates a pivotal role of the immune system in neuropathic pain [6, 7]. Previous studies showed that neuropathic pain could be reduced by blocking pro-inflammatory or enhancing anti-inflammatory immune cells and cytokines [8-10].

Houttuynia cordata Thunb (HCT), a perennial herbaceous plant, grows in the wild in moist and shady locations in Asian countries, including China [11]. HCT has a wide range of biological activities, such as anti-inflammation [12], anti-cancer [13], and immune regulation [14]. To our knowledge, there have been no comprehensive studies of the protective effects of HCT

in oxaliplatin-induced neuropathic pain in experimental animals. The present study was conducted to evaluate whether HCT can be employed to potentially treat neuropathic pain.

Materials and methods

Preparation of HCT ethanol extract

HCT was purchased from Tong Ren Tang Group Co., Ltd (Shenyang, China). A 100 g ground powder was extracted twice with 80% v/v ethanol using a Sonicator XL-2020 Ultrasonic Homogenizer (Woburn, MA) for 30 min at room temperature. The resulting extract was filtered through a 0.22 μ m filter and concentrated to 100 ml under reducing pressure. The ethanol extract was evaporated at 40°C and freeze-dried for 72 h. The powder from the extract was dissolved in DMSO and stored in aliquots at 80°C until further analysis.

Experimental animals

All procedures were in accordance with the guiding principles established by the Animal Care Committee and the institutional ethical guidelines of China Medical University (Shenyang, China). Healthy male Sprague-

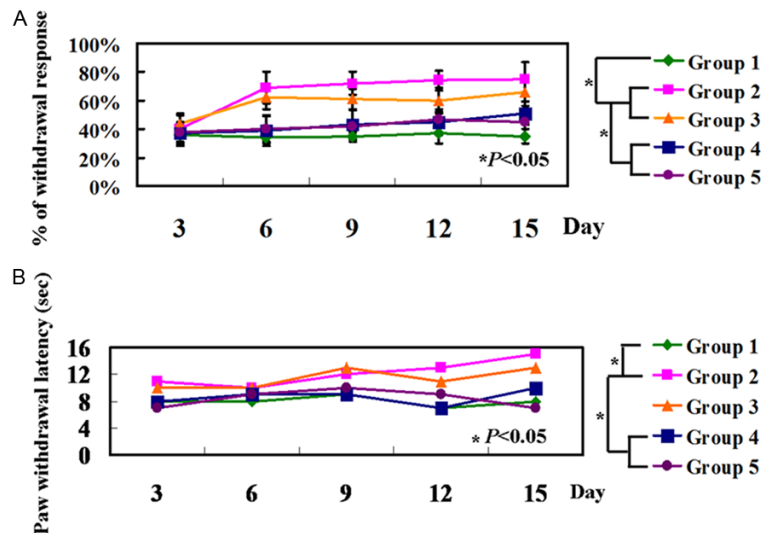


Figure 1. The effect of HCT on the withdrawal threshold (A) and paw withdrawal threshold latency (B) in oxaliplatin treated rats.

Dawley rats (8-10 weeks, 180-230 g, Better-Biotechnology Co., Ltd., Nanjing, China) were housed in an air-conditioned room at a temperature of 22-25°C, with unlimited access to tap-water and standard rat chow (BetterBiotechnology Co., Ltd.).

Oxaliplatin administration and HCT treatment

Oxaliplatin (Sigma, St. Louis, MO) was dissolved in a 5% glucose solution at a concentration of 2 mg/ml and was intraperitoneally (i.p.) injected in SD rats at 6 mg/kg [14]. The vehicle control group received the same volume of a 5% glucose solution through the same injection route. SD rats were divided into five equal groups randomly including 10 rats each as follows:

Group 1: SD rats received intraperitoneal injection of 5% glucose for 15 days; Group 2: SD rats received intraperitoneal injection oxaliplatin for 15 days; Group 3: SD rats received intraperitoneal injection oxaliplatin + oral administration HCT (400 mg/kg/day) for 15 days; Group 4: SD rats received intraperitoneal injection oxaliplatin + oral administration HCT (600 mg/kg/day) for 15 days; Group 5: SD rats received intraperitoneal injection oxaliplatin + oral administration HCT (1000 mg/kg/day) for 15 days.

Paw-pressure test and thermal hyperalgesia test

As the methods of Di Cesare et al. [15], a constantly increasing pressure was applied to a

small area of the dorsal surface of the hind paw using a blunt conical probe by a mechanical device. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. Rats scoring below 40 g or over 75 g during the test before drug administration were rejected (25%).

Thermal hyperalgesia was determined by measuring paw withdrawal latency in a thermal stimulation system (XR1102; Shanghai Xin Ruan Information Technology Co., Ltd., Shanghai, China) consisting of a clear plastic chamber (10 × 20 × 24 cm) on a clear smooth glass floor, at 30°C. Rats were placed individually in the chamber for 15 min, in order to acclimatize to the chamber conditions. A heat stimulus (150 mcal/sec/cm²) was delivered using a 0.5 cm diameter radiant heat source positioned under the plantar surface of the paw. Once a rat withdrew its paw from the heat stimulus, a photocell detected the interruption of a light beam, which automatically switched off the infrared generator and stopped the timer, providing the value for paw withdrawal latency. This method exhibits a 0.1 sec precision level for the measurement of paw withdrawal latency. If a rat failed to withdraw its paw the heat stimulus was automatically discontinued after 25 sec.

Enzyme-linked immunosorbent assay (ELISA)

Serum TNF-α level was measured by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Assaypro, St. Charles, MO), according to the instructions of the manufacturer and plates were read at 450 nm wavelength by a spectrophotometer (Bio-Rad Laboratories, Inc., Beijing, China). Levels of serum Interleukin-4 (IL-4), IL-6, and macrophage inflammatory protein1-α (MIP-1α) were measured with ELISA kits using monoclonal antibodies specific to rat IL-4, IL-6 and MIP-1α (R&D Systems, Minneapolis, MN).

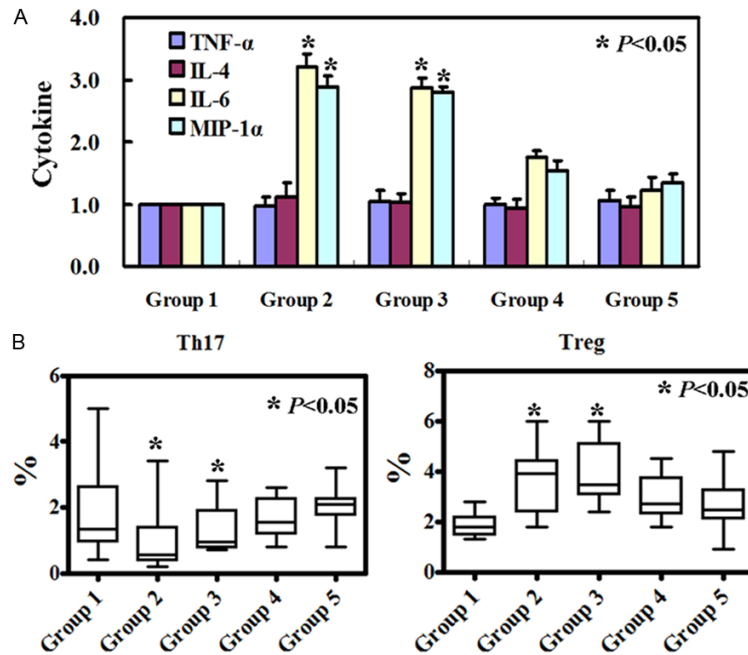


Figure 2. HCT reversed the balance of pro-/anti-inflammatory cytokines (A) and Th17/Treg (B) in serum of oxaliplatin treated rats.

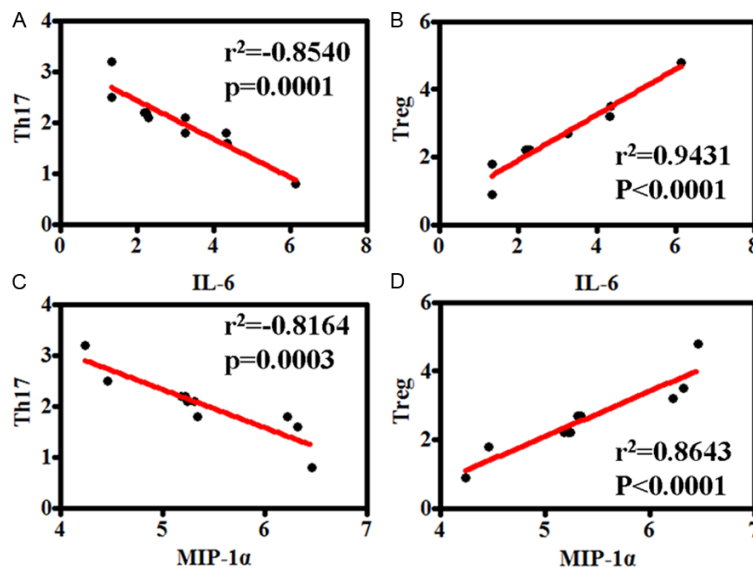


Figure 3. A positive correlation between IL-6 and MIP-1 α with Treg cells and a negative correlation between IL-6 and MIP-1 α with Th17 cells were confirmed by Spearman's rank test.

Isolation and culture of natural Th17 and Tregs

The Th17 cells were purified using an IL-17 Secretion Assay-Cell Enrichment and Detection kit (Miltenyi Biotec Technology & Trading Co., Ltd., Shanghai, China) according to the

manufacturer's instructions. PE-anti-CD25 and APC-anti-Foxp3 (eBioscience, San Diego, CA) were used for isolating CD4⁺CD25⁺Foxp3⁺ Tregs in rat. The purity of Th17 and Tregs was monitored via fluorescence-activated cell sorting (FACS, BD Biosciences, Baltimore, MD). Freshly isolated Th17 and Tregs were grown in RPMI-1640 medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin) and maintained in a humidified cell incubator with 5% CO₂ at 37°C.

Colony formation assay

Cells were seeded in 24-well plates at 300 cells/well. The plates were incubated in a humidified incubator at 37°C. After 24 h, cells were treated with various concentrations of HCT (e.g., 0, 50, 100, 200, 500 μ g/ μ l for each). Colonies were stained with 0.05% crystal violet containing 50% methanol, and counted. Photographic images were taken using a digital camera (Canon sx520, Canon, Beijing, China).

Western blot

Cell lysates were centrifuged at 14 000 \times g for 10 min, and cleared lysates were collected and separated by 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked in 5% milk-Tris-buffered saline

with Tween-20 and incubated with the following primary antibodies: phospho-PI3K (4228, Cell signaling technology, Danvers, MA), PI3K (4249, Cell signaling), phospho-Akt1 (2965, Cell signaling), Akt1 (9272, Cell signaling), phospho-mTOR (2971, Cell signaling), mTOR (4517, Cell

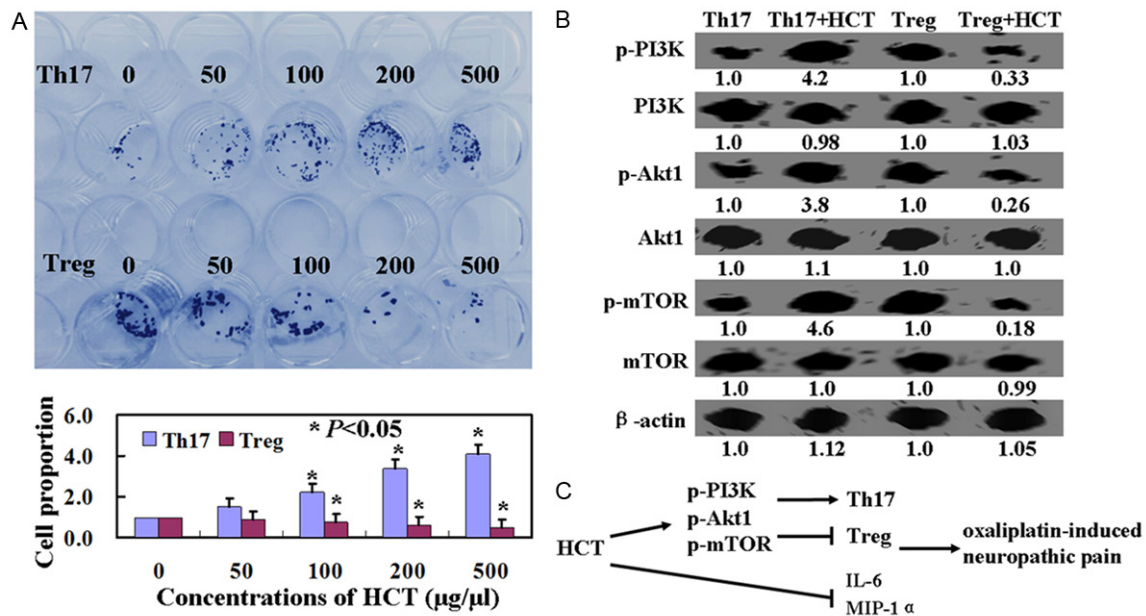


Figure 4. The effects and mechanisms of HCT on Th17 and Treg cells. A. The proliferation rate of Th17 and Treg cells was determined by colony formation assay. B. Western blot analysis of the PI3K/Akt/mTOR signaling pathway. C. Schematic model of the mechanism proposed for HCT in Th17 and Treg cells.

signaling), and β -actin (sc-47778, Santa Cruz, CA).

Statistical analyses

All results were expressed as mean \pm SEM and were analyzed by SPSS for Windows, version 17.0 (SPSS Inc, Chicago, IL). Differences were evaluated using an unpaired Student's *t* test between two groups. Spearman's rank test was used to analyze the association between cytokines and Treg or Th17 cell. For all comparisons, a value of *P* less than 0.05 was considered statistically significant.

Results

Effect of HCT on oxaliplatin-induced neuropathic pain

To assess peripheral neuropathy induced by oxaliplatin in rat models, we conducted behavioral tests for paw pressure and thermal hyperalgesia. The animals treated with oxaliplatin for 15 d (Group 2) showed a significant increase in the number of withdrawal responses and the withdrawal latency compared with that of the vehicle group (Group 1) (Figure 1, *p*<0.05). HCT (1000 mg/kg/day, Group 5) significantly decreased the number of withdrawal responses and the withdrawal latency in oxaliplatin-treated rats (Figure 1, *p*<0.05). These results

indicated that HCT reversed the development of oxaliplatin-induced neuropathic pain in rats.

Effects of HCT treatment on pro-/anti-inflammatory cytokines and Th17/Treg cells

In this study, we found that oxaliplatin-treatment coincided with an alteration in the serum levels of pro-/anti-inflammatory cytokines in rat models. The serum levels of TNF- α and IL-4 showed no changes, whereas IL-6 and MIP-1 α were significantly increased in oxaliplatin-treated group as compared with vehicle-treated group (Figure 2A, *p*<0.05). HCT could down-regulated the levels of IL-6 and MIP-1 α in oxaliplatin-treated group (Figure 2A, *p*<0.05). The serum levels of Tregs were significantly higher in oxaliplatin-treated group than that in vehicle-treated group, while Th17 were lower (Figure 2B, *p*<0.05). HCT treatment could reverse the levels of Th17 and Treg cells (Figure 2B, *p*<0.05). Furthermore, we found that a positive correlation between IL-6 and MIP-1 α with Treg cells and a negative correlation between IL-6 and MIP-1 α with Th17 cells (Figure 3, *p*<0.05).

The effects and mechanisms of HCT on Th17 and Treg cells in vitro

Cell viability was monitored using colony formation assay, and Figure 4A showed the prolifera-

tion rate of Th17 cells was induced. However, Treg cells was inhibited by HCT (**Figure 4A**, $p < 0.05$). Western blot assays were performed to identify the mechanisms of HCT on Th17 and Treg cells. HCT treated Th17 cells showed a higher expression level of p-mTOR, p-AKT1, and p-P13K, while total levels of mTOR, AKT1, and P13K showed no changes (**Figure 4B**). However, the opposite roles of HCT in Treg cells were observed (**Figure 4B**). These results suggest that HCT could influence PI3K/Akt/mTOR signaling pathway in both Th17 and Treg cells.

Discussion

Oxaliplatin is widely used for cancer treatment and many patients suffer from the development of peripheral neuropathy [2]. Thermal and mechanical hypersensitivity is a hallmark of oxaliplatin-induced neuropathy [16]. In our study, we constructed the rat model with neuropathic pain by using oxaliplatin and observed significant increased levels of IL-6 and MIP-1 α in a rat model. Other studies also showed that IL-6 and MIP-1 α blocker could reduce pain hypersensitivity in animal models of neuropathy [8, 17]. Treg is linked to increased pain sensitivity, while Th17 is mainly involved in the endogenous recovery in neuropathy [18]. Furthermore, we also found a clear anti-inflammatory T-cell shift in neuropathic pain.

Few previous reports showed the effective treatment and prevention of oxaliplatin-induced neuropathic pain. As noted in the Introduction, numerous antiinflammatory functions of HCT have been reported [11]. Our data in this study revealed that HCT completely reversed both thermal and mechanical hyperalgesia induced by oxaliplatin. Th17 frequency was increased and Treg frequency was decreased in rat with neuropathic pain after HCT treatment. Furthermore, we confirmed that HCT could influence PI3K/Akt/mTOR signaling pathway in both Th17 and Treg cells. Interestingly, we found that PI3K/Akt/mTOR signaling was activated in Th17 cells and was inhibited in Treg cells (**Figure 4C**). Previous studies have found that the differentiation of Th17 cells is controlled by PI3K/Akt/mTOR signaling pathway [19, 20]. In contrast, the inhibition of PI3K and/or mTORC1 increases Treg cell differentiation [20]. However, in our study, we didn't find why HCT plays an opposite role in Th17 cells and Treg cells.

In conclusion, the study presented here demonstrates, for the first time, that HCT reverses oxaliplatin-induced neuropathic pain in rats. The present results suggest that HCT is useful as a therapeutic drug for oxaliplatin-induced neuropathic pain.

Disclosure of conflict of interest

None.

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