BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 24(4), 418-425 (2016)

Antinociceptive Effects of Prim-O-Glucosylcimifugin in Inflammatory Nociception via Reducing Spinal COX-2

Liu-Qing Wu[1], Yu Li[1,*], Yuan-Yan Li[1], Shi-hao Xu[1], Zong-Yong Yang[1], Zheng Lin[1] and Jun Li[2]

[1]*Department of Anesthesiology, Wenzhou Central Hospital,*
[2]*Department of Anesthesiology, Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China PR 325000*

Abstract

We measured anti-nociceptive activity of prim-o-glucosylcimifugin (POG), a molecule from *Saposhnikovia divaricate* (Turcz) Schischk. Anti-nociceptive or anti-inflammatory effects of POG on a formalin-induced tonic nociceptive response and a complete Freund's adjuvant (CFA) inoculation-induced rat arthritis pain model were studied. Single subcutaneous injections of POG produced potent anti-nociception in both models that was comparable to indomethacin analgesia. Anti-nociceptive activity of POG was dose-dependent, maximally reducing pain 56.6% with an ED_{50} of 1.6 mg. Rats given POG over time did not develop tolerance. POG also time-dependently reduced serum TNFα, IL-1β and IL-6 in arthritic rats and both POG and indomethacin reduced spinal prostaglandin E2 (PGE_2). Like indomethacin which inhibits cyclooxygenase-2 (COX-2) activity, POG dose-dependently decreased spinal COX-2 content in arthritic rats. Additionally, POG, and its metabolite cimifugin, downregulated COX-2 expression *in vitro*. Thus, POG produced potent anti-nociception by downregulating spinal COX-2 expression.

Key Words: Prim-o-glucosylcimifugin, Nociception, Inflammation, Cytokine, Prostaglandin E2, Cyclooxygenase-2

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting about 1% of the global population (Hua *et al*., 2005). Osteoarthritis and rheumatoid arthritis are the most common forms and RA is characterized by excessive synovial hyperplasia, vasculogenesis and cartilage destruction (Agarwal *et al*., 2013). Pain, bone destruction and joint malformation are common manifestations and non-steroid anti-inflammatory drugs (NSAIDs) are routinely used to treat RA to alleviate and edema, although they have gastric and renal side effects (Beaugerie *et al*., 2001). Monoclonal antibodies that target pro-inflammatory cytokines can ameliorate RA suffering for patients (Klimiuk *et al*., 2006; Kageyama *et al*., 2012; Keiserman *et al*., 2014; Kim *et al*., 2015) but they are expensive and depending on administration route, endogenous antibodies and infection may occur. Thus, a potent and safe molecule for treating RA is still needed.

Saposhnikovia divaricate (Turcz) Schischk (Fangfeng) is a traditional Chinese medicine purportedly used to treat RA and spasms (Deng *et al*., 2005). Pharmacological studies suggest that Schischk extracts have anti-inflammatory, analgesic, anti-convulsion and anti-tumor effects (Wang *et al*., 1999; Deng *et al*., 2005; Jiang *et al*., 2006) but anti-nociception of prim-o-glucosylcimifugin (POG) is unclear. POG, a chromone extracted from Schischk root (Fig. 1) comprises 0.24% (g/g) of the entire root (Xue *et al*., 2000; Dai *et al*., 2008) and is the active ingredient standardized in the Chinese Pharmacopoeia (Dai *et al*., 2008; He *et al*., 2009). Therefore, we measured the anti-nociception effects of POG in a formalin or arthritis-induced nociception rat model and measured prostaglandin E2 (PGE_2) which mediates pro-inflammatory cytokine production and cyclooxygenase 2 (COX-2).

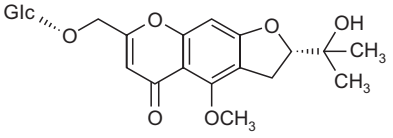

Fig. 1. Chemical structure of POG.

Open Access **http://dx.doi.org/10.4062/biomolther.2015.168**

Received Oct 15, 2015 **Revised** Jan 25, 2016 **Accepted** Mar 24, 2016
Published Online Jul 1, 2016

***Corresponding Author**
E-mail: daiyueyue888@163.com
Tel: +86-577-88816173, Fax: +86-577-88816255

MATERIALS AND METHODS

Drugs and reagents

POG was provided by Best Reagent (Chengdu, China) and was ≥98% pure. Cimifugin was from Shanghai PureOne Biotechnology (Shanghai, China), and was ≥98% pure. Indomethacin was from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Male Wistar rats (180-220 g, AGE) were obtained from Wenzhou Medical University Animal Facility. Rats were housed in a standard animal environment with a 12-h light/dark cycle and *ad lib* access to food and water. Prior to the experiments, rats were acclimated to a laboratory environment for 5 days and treatment groups were assigned randomly with researchers blinded to behavioral testing. Research protocols were approved by the Animal Care and Welfare Committee of Wenzhou Medical University and were performed according to the animal care guidelines of the National Institutes of Health.

Cell culture

Human SGC7901 GC cell lines were purchased from Cell Resource Center of the Shanghai Institutes for Biological Sciences, Chinese Academy of Science (Shanghai, China) and cultured in RPMI1640 medium (containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate) and maintained at 37°C with 5% CO_2.

Formalin-induced nociception in rat

As published (Esfahani e*t al*., 2012; Han e*t al*., 2012), a rat formalin model was established. First, 6 groups of rats (n=10/group were treated with saline (10 ml/kg), indomethacin (10 mg/kg) or POG (1, 3, 10, 30 mg/kg). After 30 min, 50 μl of 5% formalin was injected (sc) to left dorsal hind paws. Flinch times in each epoch from 0-90 min were recorded at 10-min intervals. For long-term nociception tests, two groups of rats (n=10/group) were given saline (10 ml/kg) or POG (10 mg/kg) consecutively for 7 days and a formalin challenge was performed 30 min minutes after the last drug administration.

Rotarod test

Motor performance after POG treatment was measured using a ZH-PL accelerating rotarod (Zhenghua Bio-instruments, Anhui, China). Rats were placed on a rotating drum with speed increasing from 0 to 30 rpm within 1 min followed by a consistent 30 rpm for 4 more min and they were forced to move forward to avoid falling (Li e*t al*., 2016). Fall latencies every 5 min were measured. After 3 days' training, rats were given test agents and the rotarod test was given and recorded at 0.5, 1, 2, and 4 h post-injection.

CFA-induced inflammatory nociceptive model

Rat arthritis models were established with Complete Freund's Adjuvant (10 mg/ml of *Mycobacterium butyricum*) (Costa e*t al*., 1981; Nagakura e*t al*., 2003). Each rat was treated in the left hind paw with 0.1 ml CFA (sc) (Life Technologies, US) for 14 days and then divided into 5 groups (n=10/group): normal (saline, 10 ml/kg), model (saline, 10 ml/kg), POG (10 mg/kg), POG (30 mg/kg) or indomethacin (10 mg/kg). Paws then became swollen, and after two weeks of inoculation, rats were considered to have sustained chronic pain and they were randomized to subsequent experiments.

Measurement of mechanical allodynia and thermal hyperalgesia

The contralateral or ipsilateral hind limb withdrawal threshold of mechanical allodynia was measured using a 2290 CE electrical von Frey monofilament test (IITC Life Science, CA, USA). Rats were placed on a metal grid, and hind paw withdrawal was evoked by stimulation. Monofilaments were applied to the foot pad with increasing force until the rat withdrew the hind limb and the lowest force producing a withdrawal response was considered the threshold. Measurements were performed in triplicate at 30-sec intervals and means were calculated.

The contralateral or ipsilateral hind paw withdrawal threshold of thermal hyperalgesia was measured using a heated glass plantar test apparatus (IITC Life Science). Rats were placed on a glass plate, and a hot illuminant was applied to the foot pad and withdrawal time was considered the threshold. Measurements were performed in triplicate at 30-sec intervals and means were calculated.

The maximal possible effect (MPE) was calculated as (postdrug threshold in ipsilateral hind limb-baseline threshold in ipsilateral hind limb) * 100/(baseline threshold in contralateral hind limb-baseline-threshold in ipsilateral hind limb).

Western blot

According to published methods (Schafers e*t al*., 2003), spinal lumbar enlargements of each rat (50 mg) were harvested and homogenized in 1 ml RIPA lysis buffer (Beyotime Institute of Biotechnology, Suzhou, China). Homogenate was centrifuged at 12,000×g at 4°C for 10 min. Supernatant was collected and protein concentration was measured using a BCA kit (Beyotime Institute of Biotechnology). Total sample protein (30 μg/10 μl) were separated by SDS-PAGE (10%) and then transferred to a PVDF membrane (Millipore, USA) using an electrophoretic method. PVDF membranes were then blocked in TBST with 5% non-fat milk powder at room temperature for 60 min and incubated with the goat monoclonal primary antibody raised against COX-2 (Abcam, Cambridge, England) at 1:500 and rabbit monoclonal antibody against β-actin (Abcam) at 1:1,000 at 4°C overnight. After washing with TBST, membranes were incubated with rabbit anti-goat HRP secondary antibody (Santa Cruz, Dallas, TX, USA) at 1:10,000 and goat anti-rabbit HRP (Santa Cruz) secondary antibody at 1:2,000 at 37°C for 60 min. Protein bands were visualized using an ECL chromogenic reaction detection system (BioRad, Hercules, CA, USA) and band density was quantified using Image- Pro Plus software (Media Cybernetics, Rockville, MD, USA).

Serum TNFα, IL-1β and IL-6

TNFα and IL-1β and IL-6 in serum were measured using ELISA. On day 14, 21 and 28 post-CFA-inoculation, blood was collected from the tail vein and stored at 37°C for 30 min. Then, blood was centrifuged at 3,000 rpm for 10 min then sera was removed and stored -20°C for later measurement. Serum TNFα, IL-1β and IL-6 were assessed according to kit instructions.

CSF PGE_2

Rats were anesthetized with pentobarbital (50 mg/kg) and fixed in a stereotaxic apparatus. The head was wiped with a cotton swab to facilitate exposure of the foramen magnum and a 25-gauge needle connected to a 1 ml syringe was inserted

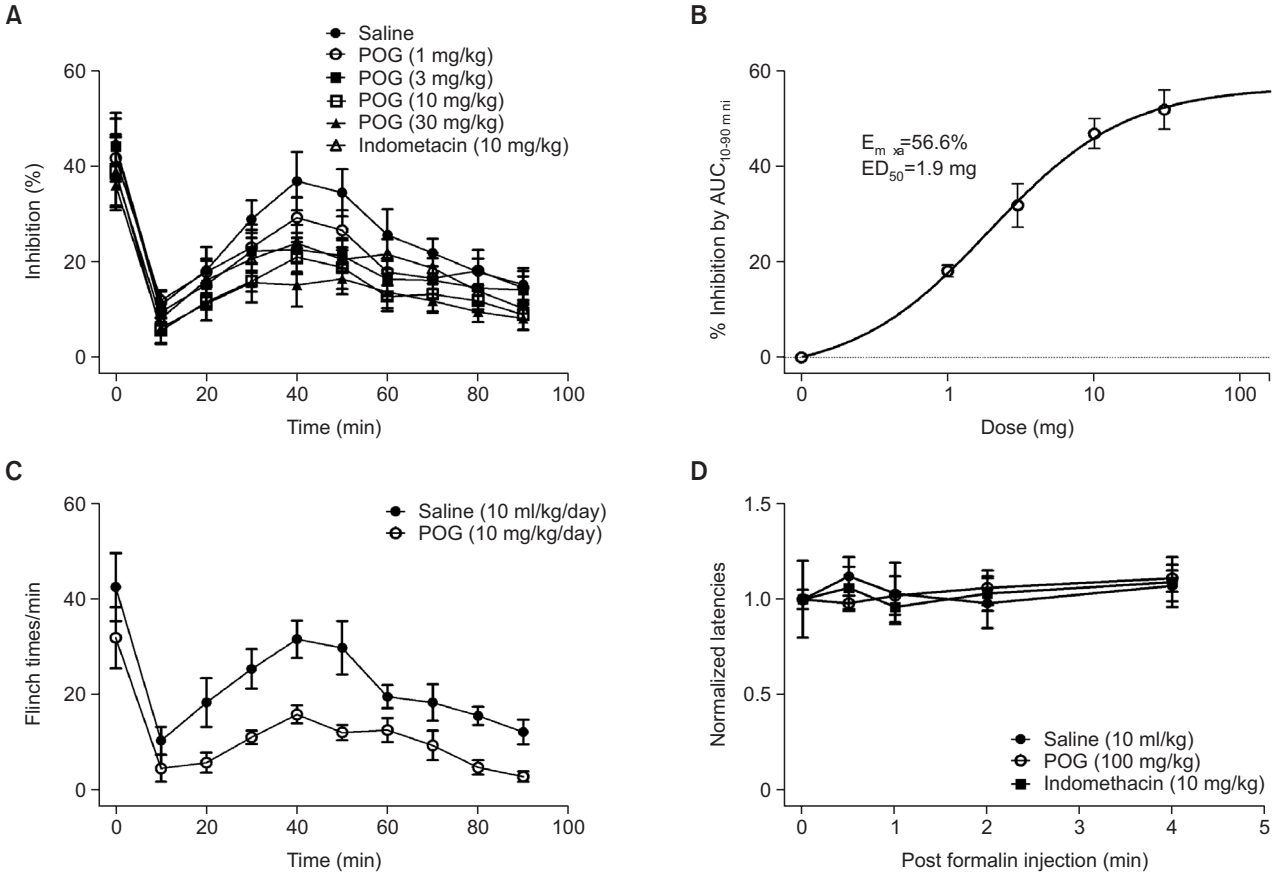

Fig. 2. Effects of POG on formalin-induced acute or tonic nociception. Flinch times in each 1-min epoch were quantified as nociceptive behavior. (A) Treatment with 1, 3, 10, or 30 mg/kg of POG (sc) once for formalin-induced nociception. (B) Dose-response analysis of anti-nociception of POG on formalin-induced tonic pain ($AUC_{10\text{-}90\ min}$). (C) Effects of POG after 7 days of administration. (D) Rotarod test of indomethacin (10 mg/kg) and POG (100 mg/kg). Falling latencies were normalized by latency at 0 min in each group. Data are presented as Means ± SEM, n=10 in each group.

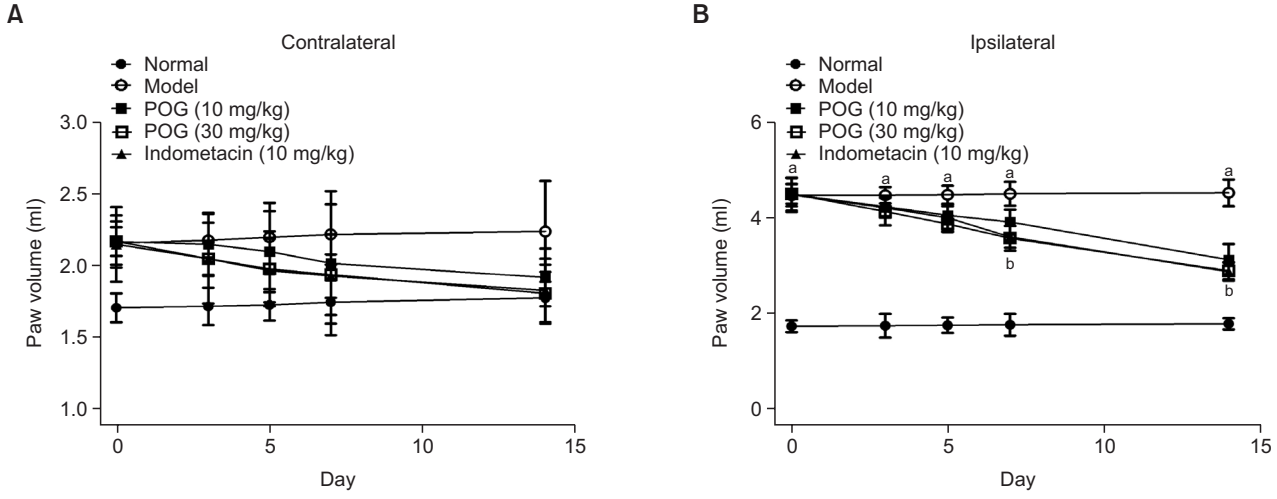

Fig. 3. Effects of POG on paw swelling in arthritic rats. Rats were assessed 14 days after CFA inoculation. Effect of POG on ipsilateral (A) and contralateral (B) paw volumes at 0, 3, 5, 7, or 14 days after dosing. Data are means ± SEM (n=10/group). [a,b]Denote statistical significance compared with normal or model groups, respectively (p<0.05, two-way repeated-measures ANOVA followed by SNK *post hoc* SNK test).

gently into the cisterna magna and CSF was removed via syringe aspiration (50 to 100 μl CSF). CSF samples were mixed with indomethacin (10 mM) to prevent prostaglandin production (Samad e*t al*., 2001). Samples were kept in the dark and on ice until centrifugation at 1,300×g for 15 min at 4°C, and stored at -20°C until measurement. PGE_2 was measured with ELISA according to the kit instructions (Cayman Chemical, Ann Arbor, MI, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism Project (Version 5.0, GraphPad Software Inc., La Jolla, CA, USA) (Ocvirk e*t al*., 2008), and data are means ± SEM. Statistical significance was assessed using a one-way or two-way ANOVA followed by *post hoc* Student Newman Keuls (SNK) test. Dose-response was confirmed by fitting nonlinear least-squares analysis. The maximal effect (E_{max}) and half-effective dose (ED_{50}) were determined by fitting nonlinear least-squares curves to Y=a+bx, where x=[D]n/(ED_{50}n+[D]n). ED_{50} and b (E_{max}) were determined by yielding a minimum residual sum of squares of deviations from theoretical curves (Gong e*t al*., 2014) and $p<0.05$ was considered statistically significant.

RESULTS

After establishing pain models, POG and indomethacin were used to treat animals and POG dose-dependently inhibited tonic formalin-induced nociception (Fig. 2A) and the acute phase as well (E_{max} and ED_{50} in the tonic phase were 56.6% and 1.6 mg, respectively; Fig. 2B). Long-term anti-nociception of POG was also measured in the formalin pain model and (Fig. 2C) POG inhibited pain and no tolerance was observed A rotarod test to test drug effects on motor coordination revealed that saline, indomethacin, and POG at 0.5, 1, 2 and 4 h after injection (Fig. 2D) did not alter motor coordination.

A chronic pain model was established and indomethacin and POG were compared by measuring paw volume, thermal hyperalgesia and mechanical allodynia of both paws pre- and post-drug administration. Serum cytokines were also measured. Swelling in ipsilateral hind paws was approximately 4 ml (Fig. 3A) and contralateral hind paws were swollen slightly (Fig. 3B). POG (10 mg/kg) suppressed swelling in a time- and dose-dependent manner (Fig. 3) and this was comparable to indomethacin (10 mg/kg) at each time point, and lower than that of high-dose POG (30 mg/kg). Thus, CFA-induced inflammation can be prevented by POG.

Compared with normal controls model rats had 2.4-, 1.6- or 1.7-fold more TNFα, IL-1β or IL-6, respectively (Fig. 4). POG (10 or 30 mg/kg) elevated TNFα, IL-1β or IL-6 in a dose-dependent manner (Fig. 4B, 4C) and this was comparable with or more potent than indomethacin (10 mg/kg). Thus, POG ameliorated inflammation in CFA-treated rats.

Thermal hyperalgesia data appear in Fig. 5A, and paw withdrawal latency in ipsilateral paw was approximately 8 sec, compared to contralateral (17.5 s) or normal control (15.8 s) limbs. POG dose-dependently elevated bilateral paw withdrawal latencies and the maximal effect (%) of indomethacin (10 mg/kg), POG (10 mg/kg) and POG (30 mg/kg) at 14 days post-administration were 69.4%, 70.2%, and 78.2%, respectively. Elevated paw withdrawal latencies were also observed in contralateral paws after treatment (Fig. 5B).

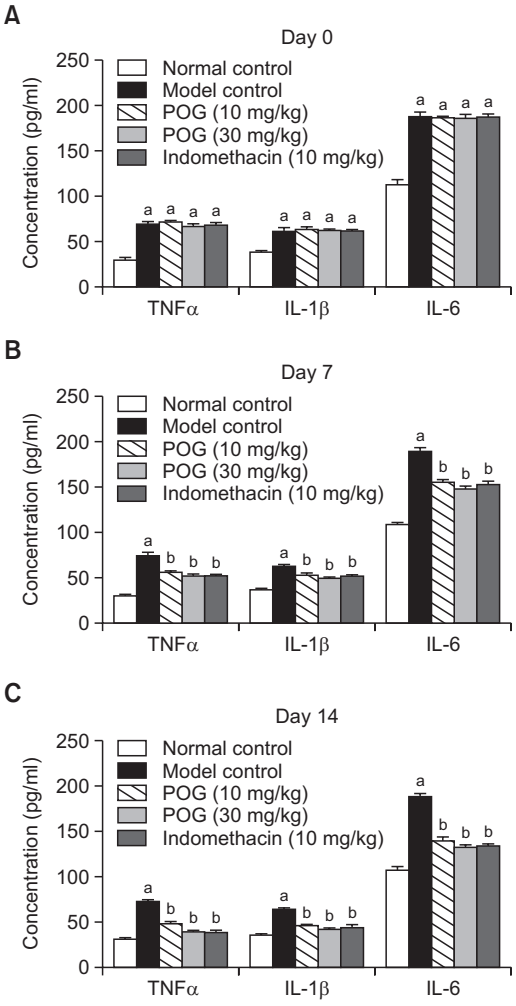

Fig. 4. Effects of POG on the TNFα, IL-1β and IL-6 concentrations in arthritic rats at 0 (A), 7 (B) or 14 (C) days after dosing. Serum pro-inflammation cytokines. Data are means ± SEM (n=10/group). [a,b]Denote statistical significance compared with normal or model groups, respectively ($p<0.05$, one-way ANOVA followed by a *post hoc* SNK test).

Mechanical allodynia data appear in Fig. 5C, 5D and the paw withdrawal threshold in the ipsilateral paw was approximately 7.3 g, which was lower than for contralateral (42.1 g) or normal control (44.9 g) paws. POG enhanced in a dose-dependent manner paw withdrawal thresholds. The maximal effect (%) of indomethacin (10 mg/kg), POG (10 mg/kg) and POG (30 mg/kg) at 14 days post-administration were 46.3%, 46.5%, and 65.1%, respectively. Elevated paw withdrawal thresholds were also observed for contralateral paws after treatment (Fig. 5B). Thus, POG had potent anti-nociceptive effects against arthritis-induced pain.

Spinal PGE_2 was consistent with CFA-induced nociception (Samad e*t al*., 2001; Zeilhofer, 2007) and was greater in model animals. POG dose-dependently decreased spinal PGE_2 ($p<0.05$ one-way ANOVA), as did indomethacin ($p<0.05$ one-way ANOVA) (Fig. 6A). Spinal COX-2 expression data show that (Fig. 6B) COX-2 protein expression in model rats was greater than in controls ($p<0.05$ one-way ANOVA). POG

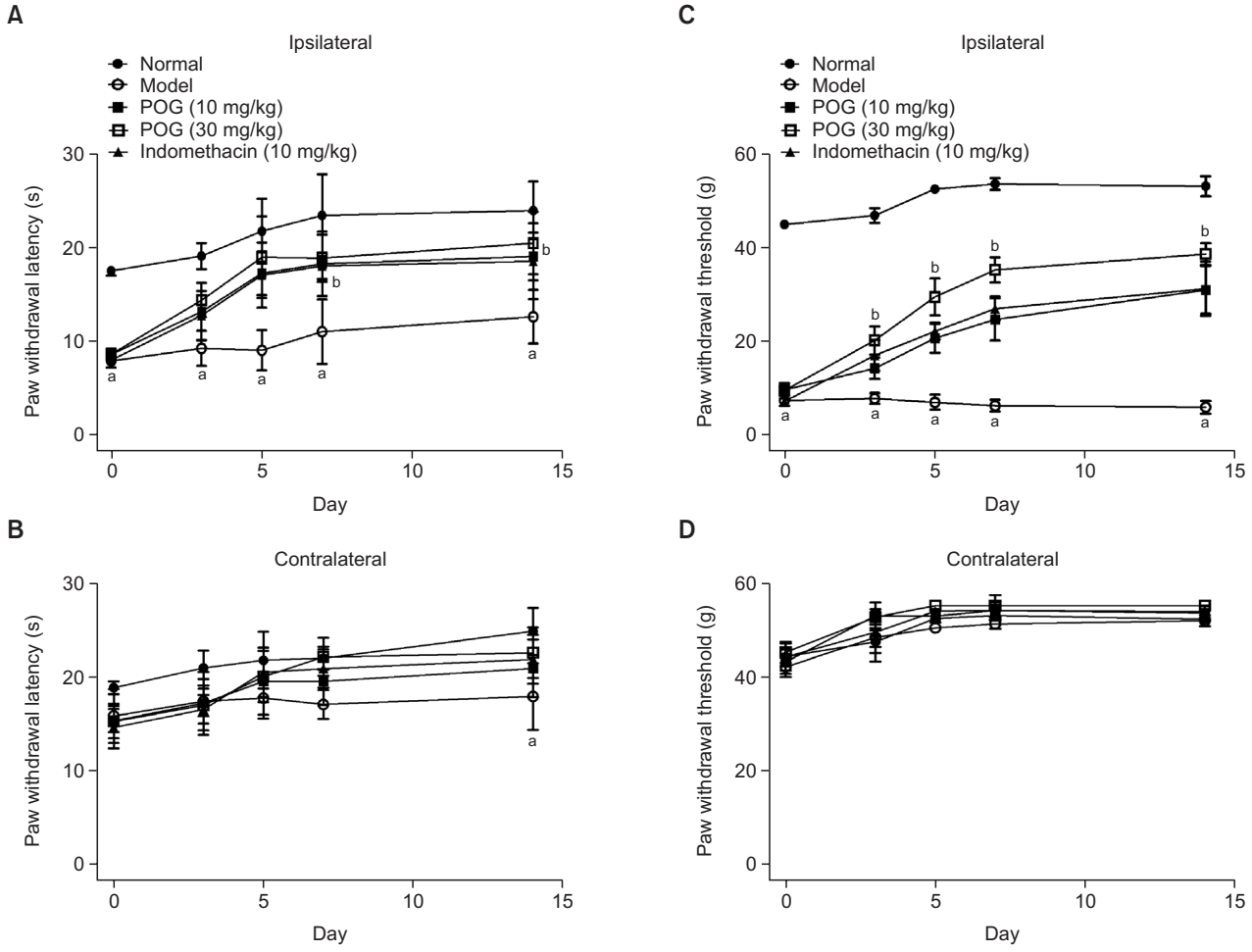

Fig. 5. Effects of POG on nociception in arthritic rats. Paw thermal hyperalgesia and mechanical allodynia were measured at 0, 3, 5, 7, or 14 days after dosing. Bilateral nociception response are represented as paw withdrawal latencies (A, B) and paw withdrawal thresholds (C, D). Data are means ± SEM (n=10/group). [a,b]Denote statistical significance compare with normal group or model group, respectively (p<0.05, two-way repeated-measures ANOVA followed by an SNK *post hoc* test).

decreased spinal COX-2 protein dose-dependently compared with indomethacin (p<0.05 one-way ANOVA). Thus, POG decreased spinal PGE_2 concentrations by decreasing COX-2 expression.

POG is metabolized to cimifugin *in vivo* and this has been measured in rat brains (Li e*t al*., 2014). The inhibitory effects of POG or cimifugin on COX-1/2 activity were not observed at 100 μM (data not shown), so COX-1 or COX-2 expression in a human GC cell line SGC7901 were assessed. Fig. 7 show that expression of COX-2 was downregulated by POG and cimifugin.

DISCUSSION

Schischk is said to be widely used in traditional Chinese medicine for thousands of years and may have pharmacological properties (Xue e*t al*., 2000) and its extract is said to have anti-bacterial, antipyretic, anti-inflammatory and analgesic properties (Xue e*t al*., 2000; Deng e*t al*., 2005). We assessed analgesic effects of POG which we hypothesized had specific anti-nociceptive effects on inflammatory pain as tested in rat tonic and chronic pain models. However, POG mildly inhibited formalin-induced nociception in the acute phase (13.6%). Interestingly, POG has an E_{max} of 56.6% and an ED_{50} of 1.6 mg, which is comparable with indomethacin. Seven-day treatment with POG also produces anti-nociception without tolerance.

The rodent formalin pain model is first described by Dubuisson and Dennis and has been used to study analgesic compounds with respect to acute and tonic responses to a noxious chemical stimulus (Dubuisson and Dennis, 1977). Injection of 5% formalin into a dorsal rodent paw provokes a bi-phasic response with an immediate or acute outcome due to nociceptors and a tonic inter-phase that arises from afferent input and central sensitization in the dorsal horn (Vissers e*t al*., 2003). These attributes are common to chronic pain syndromes such as chronic neuropathic pain (Granados-Soto e*t al*., 1997). Prostaglandin is key to the tonic phase and can be blocked by NSAIDs (Hunskaar and Hole, 1987).

The CFA-induced arthritic rat model is well-characterized in the literature and peripheral tissue injury followed by inoculation of CFA usually increases sensitivity to noxious heat as

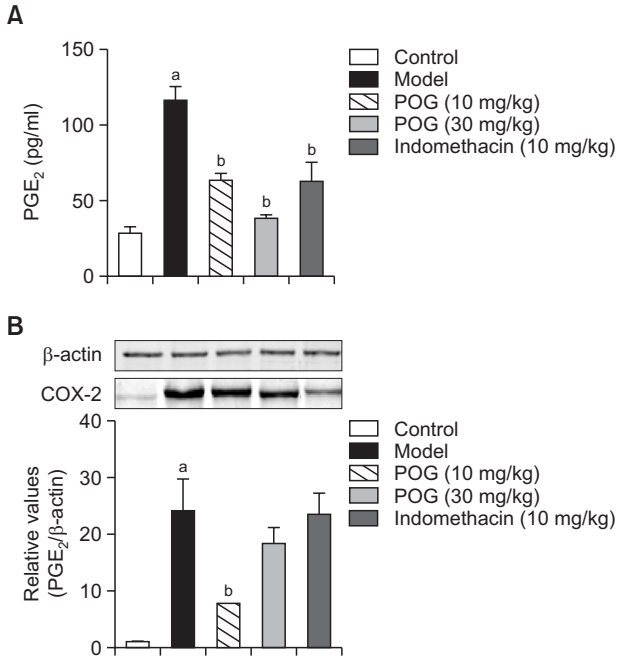

Fig. 6. Effects of POG on spinal PGE_2 (A) and COX-2 (B) in arthritic rats. PGE_2 was measured in rat CSF (ELISA) and spinal COX-2 expression was measured with Western blot. Data are means ± SEM (n=10/group). [a,b]Denote statistical significance compared with normal or model groups, respectively (p<0.05, one-way ANOVA followed by a *post hoc* SNK test.

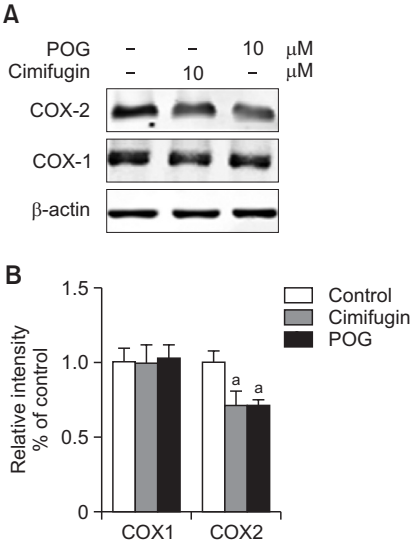

Fig. 7. Inhibitory effect of cimifugin or POG on expression of COXs in SGC-7901. (A) SGC-7901 cells were treated as depicted in Methods and Western blot was used to assess results with β-actin as a loading control. (B) Blots were denistometrically quantified after normalization with β-actin and all data are means ± SEM of three experiments. [a]Denotes statistical significance compared with controls (p<0.05, one-way ANOVA followed by a *post hoc* SNK test.

well as heightens sensitivity to mechanical tactile stimulation. Primary inoculation of CFA provokes an acute partial inflammatory response and subsequent chronic injury occurs after 10-20 days and persists for 28 days, which mimics a chronic inflammatory condition (van Eden e*t al*., 2001). CFA-induced arthritis is a well-characterized model for studying arthritic pain and has typical features manifested by paw swelling, thermal hyperalgesia and mechanical allodynia. Activation of T cells by adjuvant also stimulates macrophage and monocytes to produce pro-inflammatory cytokines such as TNFα, IL-1β and IL-6. Serum TNFα, IL-1β and IL-6 aggravate bone and joint injury in arthritic patients (Kong e*t al*., 1999) and blockage with monoclonal antibodies of pro-inflammatory cytokines, such as adalimumab for TNFα, tocilizumab for IL-6 can reduce swelling and pain in clinical osteoarthritis patients (Weinblatt e*t al*., 2003; Hammoudeh e*t al*., 2015). POG also has specific anti-inflammation and similar to indomethacin can reduce serum TNFα, IL-1β and IL-6 in arthritic rats, which may explain POGs analgesic effects.

PGE_2 is produced by COX-2 during inflammation (Ebersberger e*t al*., 1999) and this prostanoid production requires the conversion of arachidonic acid to PGH2 by COX. Two isoforms of COX have been identified, and COX-1 is widely expressed in many cell types, whereas COX-2 is mainly expressed in the kidney and parts of the central nervous system and induced at inflammation sites (Crofford, 1997; McAdam e*t al*., 1999). COX-2 is critical to the inflammatory state, and PGE_2 is an important marker of inflammation (Anderson e*t al*., 1996). Prostanoids contribute to the development of peripheral sensitization in nociceptor terminals, increasing excitability and reducing pain thresholds (Anderson e*t al*., 1996; McCoy e*t al*., 2002). Moreover, hyperalgesia was confirmed in the spinal cord (Samad e*t al*., 2001). Targeting COX-2, NSAIDs produce specific analgesia for inflammatory pain both in central and peripheral sites (Anderson e*t al*., 1996). Spinal elevation of COX-2 in arthritic pain rat models shows that downregulation of spinal COX-2 expression occurs after POG treatment but not after indomethacin (Samad e*t al*., 2001; Lee e*t al*., 2004). Although spinal COX-2 expression was downregulated in surgical pain rat models (Yamashita e*t al*., 2006), previous data indicate that NSAIDs do not alter spinal COX-2 expression in arthritic rats (Beiche e*t al*., 1998; Ebersberger e*t al*., 1999). POG and cimifugin inhibited little COX-1 or COX-2 activity even at 100 μM (data not shown) but data show that POG and its metabolite cimifugin downregulated COX-2 expression *in vitro*. Distinct from inhibiting COX-2 activity by NSAIDs, antinociception from POG was due to reduction of spinal COX-2. However, POG's effect on COX-2 activity is unclear and requires study. Thus, POG offers anti-nociceptive effects against inflammatory pain without tolerance by downregulating spinal COX-2 expression.

ACKNOWLEDGMENTS

We thank LetPub (http://www.letpub.com) for its linguistic assistance during the preparation of this manuscript. This project is supported by Zhejiang Science Foundation for Yu Li (No. Z132003).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Agarwal, S. and Das, S. K. (2013) Rheumatoid arthritis with increased bone mineral density. *Int. J. Rheum. Dis.* **16**, 762-763.

Anderson, G. D., Hauser, S. D., McGarity, K. L., Bremer, M. E., Isakson, P. C. and Gregory, S. A. (1996) Selective inhibition of cyclooxygenase (Cox)-2 reverses inflammation and expression of Cox-2 and interleukin 6 in rat adjuvant arthritis. *J. Clin. Invest*. **97**, 2672-2679.

Beaugerie, L., Berenbaum, F., Berrebi, D., Gendre, J. P., Prier, A., Kaplan, G. and Chatelet, F. P. (2001) Chronic use of non-steroidal anti-inflammatory drugs does not alter colonic mucosa of patients without diarrhoea. *Aliment. Pharmacol. Ther.* **15**, 1301-1306.

Beiche, F., Brune, K., Geisslinger, G. and Goppelt-Struebe, M. (1998) Expression of cyclooxygenase isoforms in the rat spinal cord and their regulation during adjuvant-induced arthritis. *Inflamm. Res.* **47**, 482-487.

Costa, M. D. C., De Sutter, P., Gybels, J. and Van Hees, J. (1981) Adjuvant-induced arthritis in rats: a possible animal model of chronic pain. *Pain* **10**, 173-185.

Crofford, L. J. (1997) Cox-1 and Cox-2 tissue expression: implications and predictions. *J. Rheumatol. Suppl.* **49**, 15-19.

Dai, J., Chen, X., Cheng, W., Liu, X., Fan, X., Shen, Z. and Bi, K. (2008) A sensitive liquid chromatography-mass spectrometry method for simultaneous determination of two active chromones from saposhnikovia root in rat plasma and urine. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **868**, 13-19.

Deng, C., Yang, X. and Zhang, X. (2005) Rapid determination of panaxynol in a traditional chinese medicine of saposhnikovia divaricata by pressurized hot water extraction followed by liquid-phase microextraction and gas chromatography-mass spectrometry. *Talanta* **68**, 6-11.

Dubuisson, D. and Dennis, S. G. (1977) The formalin test: a quantitative study of the analgesic effects of morphine, meperidine and brain stem stimulation in rats and cats. *Pain* **4**, 161-174.

Ebersberger, A., Grubb, B. D., Willingale, H. L., Gardiner, N. J., Nebe, J. and Schaible, H. G. (1999) The intraspinal release of prostaglandin E2 in a model of acute arthritis is accompanied by an upregulation of cyclo-oxygenase-2 in the spinal cord. *Neuroscience* **93**, 775-781.

Esfahani, H. M., Esfahani, Z. N., Dehaghi, N. K., Hosseini-Sharifabad, A., Tabrizian, K., Parsa, M. and Ostad, S. N. (2012) Anti-inflammatory and anti-nociceptive effects of the ethanolic extracts of alkanna frigida and alkanna orientalis. *J. Nat. Med.* **66**, 447-452.

Gong, N., Fan, H., Ma, A.-N., Xiao, Q. and Wang, Y.-X. (2014) Geniposide and its iridoid analogs exhibit antinociception by acting at the spinal Glp-1 receptors. *Neuropharmacology* **84**, 31-45.

Granados-Soto, V., Rufino, M. O., Gomes Lopes, L. D. and Ferreira, S. H. (1997) Evidence for the involvement of the nitric oxide-cgmp pathway in the antinociception of morphine in the formalin test. *Eur. J. Pharmacol.* **340**, 177-180.

Hammoudeh, M., Al Awadhi, A., Hasan, E. H., Akhlaghi, M., Ahmadzadeh, A. and Sadeghi Abdollahi, B. (2015) Safety, tolerability, and efficacy of tocilizumab in rheumatoid arthritis: an open-label phase 4 study in patients from the middle east. *Int. J. Rheumatol.* **2015**, 975028.

Han, Y. K., Lee, S. H., Jeong, H. J., Kim, M. S., Yoon, M. H. and Kim, W. M. (2012) Analgesic effects of intrathecal curcumin in the rat formalin test. *Korean J. Pain* **25**, 1-6.

He, Q. J., Gao, Y. H. and Wei, Z. X. (2009) Determination of primo-glucosylcimifugin in biyan tablets by Hplc. *Zhongyao Xinyao Yu Linchuang Yaoli* **3**, 25-28.

Hua, J., Suguro, S., Hirano, S., Sakamoto, K. and Nagaoka, I. (2005) Preventive actions of a high dose of glucosamine on adjuvant arthritis in rats. *Inflamm. Res.* **54**, 127-132.

Hunskaar, S. and Hole, K. (1987) The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain* **30**, 103-114.

Jiang, Y. Y., Liu, B. and Shi, R. B. (2006) Hplc determination of the contents of four components in the chromone fraction of saposhnikovia divaricata (turcz.) Schischk. *J. Beijing Univ. Trad. Chin. Med.* **2**, 15-17.

Kageyama, Y., Torikai, E., Tsujimura, K. and Kobayashi, M. (2012) Involvement of Il-33 in the pathogenesis of rheumatoid arthritis: the effect of etanercept on the serum levels of Il-33. *Mod. Rheumatol.* **22**, 89-93.

Keiserman, M., Codreanu, C., Handa, R., Xibille-Friedmann, D., Mysler, E., Briceno, F. and Akar, S. (2014) The effect of antidrug antibodies on the sustainable efficacy of biologic therapies in rheumatoid arthritis: practical consequences. *Expert Rev. Clin. Immunol.* **10**, 1049-1057.

Kim, G. W., Lee, N. R., Pi, R. H., Lim, Y. S., Lee, Y. M., Lee, J. M., Jeong, H. S. and Chung, S. H. (2015) Il-6 inhibitors for treatment of rheumatoid arthritis: past, present, and future. *Arch. Pharm. Res.* **38**, 575-584.

Klimiuk, P. A., Sierakowski, S., Domyslawska, I. and Chwiecko, J. (2006) Regulation of serum chemokines following infliximab therapy in patients with rheumatoid arthritis. *Clin. Exp. Rheumatol.* **24**, 529-533.

Kong, Y.-Y., Feige, U., Sarosi, I., Bolon, B., Tafuri, A., Morony, S., Capparelli, C., Li, J., Elliott, R., McCabe, S., Wong, T., Campagnuolo, G., Moran, E., Bogoch, E. R., Van, G., Nguyen, L. T., Ohashi, P. S., Lacey, D. L., Fish, E., Boyle, W. J. and Penninger, J. M. (1999) Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* **402**, 304-309.

Lee, K. M., Kang, B. S., Lee, H. L., Son, S. J., Hwang, S. H., Kim, D. S., Park, J. S. and Cho, H. J. (2004) Spinal NF-κB activation induces Cox-2 upregulation and contributes to inflammatory pain hypersensitivity. *Eur. J. Neurosci.* **19**, 3375-3381.

Li, T.-X., Hu, L., Zhang, M.-M., Sun, J., Qiu, Y., Rui, J.-Q. and Yang, X.-H. (2014) A Sensitive Uplc-Ms/Ms method for simultaneous determination of eleven bioactive components of Tong-Xie-Yao-Fang decoction in rat biological matrices. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **944**, 90-100.

Li, X., Hu, Y., Wu, Y., Huang, Y., Yu, S., Ding, Q., Zhangsun, D. and Luo, S. (2016) Anti-hypersensitive effect of intramuscular administration of Ao-Conotoxin Gexiva [1,2] and Gexiva [1,4] in rats of neuropathic pain. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **66**, 112-119.

McAdam, B. F., Catella-Lawson, F., Mardini, I. A., Kapoor, S., Lawson, J. A. and FitzGerald, G. A. (1999) Systemic biosynthesis of prostacyclin by cyclooxygenase (Cox)-2: the human pharmacology of a selective inhibitor of Cox-2. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 272-277.

McCoy, J. M., Wicks, J. R. and Audoly, L. P. (2002) The role of prostaglandin E2 receptors in the pathogenesis of rheumatoid arthritis. *J. Clin. Invest.* **110**, 651-658.

Nagakura, Y., Okada, M., Kohara, A., Kiso, T., Toya, T., Iwai, A., Wanibuchi, F. and Yamaguchi, T. (2003) Allodynia and hyperalgesia in adjuvant-induced arthritic rats: time course of progression and efficacy of analgesics. *J. Pharmacol. Exp. Ther.* **306**, 490-497.

Ocvirk, R., Pearson Murphy, B. E., Franklin, K. B. and Abbott, F. V. (2008) Antinociceptive profile of ring A-reduced progesterone metabolites in the formalin test. *Pain* **138**, 402-409.

Samad, T. A., Moore, K. A., Sapirstein, A., Billet, S., Allchorne, A., Poole, S., Bonventre, J. V. and Woolf, C. J. (2001) Interleukin-1β-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature* **410**, 471-475.

Schafers, M., Svensson, C. I., Sommer, C. and Sorkin, L. S. (2003) Tumor necrosis factor-α induces mechanical allodynia after spinal nerve ligation by activation of p38 MAPK in primary sensory neurons. *J. Neurosci.* **23**, 2517-2521.

van Eden, W., Wagenaar-Hilbers, J. and Wauben, M. H. (2001) Adjuvant arthritis in the rat. *Curr. Protoc. Immunol.* **Chapter 15**, Unit 15.4.

Vissers, K., Hoffmann, V., Geenen, F., Biermans, R. and Meert, T. (2003) Is the second phase of the formalin test useful to predict activity in chronic constriction injury models? A pharmacological

comparison in different species. *Pain Pract.* **3**, 298-309.
Wang, C.-C., Chen, L.-G. and Yang, L.-L. (1999) Inducible nitric oxide synthase inhibitor of the Chinese Herb I. Saposhnikovia Divaricata (Turcz.) schischk. *Cancer Lett.* **145**, 151-157.
Weinblatt, M. E., Keystone, E. C., Furst, D. E., Moreland, L. W., Weisman, M. H., Birbara, C. A., Teoh, L. A., Fischkoff, S. A. and Chartash, E. K. (2003) Adalimumab, a fully human anti-tumor necrosis factor alpha monoclonal antibody, for the treatment of rheumatoid arthritis in patients taking concomitant methotrexate: the armada trial. *Arthritis Rheum.* **48**, 35-45.
Xue, B. Y., Li, W., Li, L. and Xiao, Y. Q. (2000) A pharmacodynamic research on chromone glucosides of fangfeng. *Zhongguo Zhong Yao Za Zhi* **25**, 297-299.
Yamashita, K., Fukusaki, M., Ando, Y., Fujinaga, A., Tanabe, T., Terao, Y. and Sumikawa, K. (2006) Preoperative administration of intravenous flurbiprofen axetil reduces postoperative pain for spinal fusion surgery. *J. Anesth.* **20**, 92-95.
Zeilhofer, H. U. (2007) Prostanoids in nociception and pain. *Biochem. Pharmacol.* **73**, 165-174.