Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

Analgesic action of Rubimaillin *in vitro* and *in vivo*

Wanli Yan[1#], Zhonglei Jiang[1#], Fengyun Fan[1*]

Department of Rehabilitation, Yantai Hospital of Traditional Chinese Medicine, Yantai, PR China

*Correspondence to: qhsm14@163.com

Received October 18, 2019; **Accepted** May 20, 2020; **Published** June 5, 2020

[#] These authors contributed equally to this work as co-first author.

Doi: http://dx.doi.org/10.14715/cmb/2020.66.3.27

Abstract: Pain, a common symptom in clinics, is a serious impediment to quality of life. The analgesic drugs presently in use have poor efficacy, and are associated with undesirable side effects. Rubimaillin (*Rub*) is a naphthoquinone compound extracted from Chinese herbal medicine, and it has various biological activities. In this study, the analgesic effect of *Rub*, and its mechanism of action were investigated using glacial acetic acid-induced mice writhing model and a mice model of neurogenic and inflammatory bipolar pain. Analgesic effects were measured in different experimental groups. *In vitro*, RAW 264.7 cells were used to investigate the release of nitric oxide (NO), iNOS and COX-2 protein in RAW 264.7 cells stimulated with lipopolysaccharide (LPS). The results revealed that *Rub* reduced the number of acetic acid-induced writhing in mice, inhibited formalin-induced biphasic pain response, and suppressed the production of NO in RAW 264.7 cells. The mechanisms involved in the analgesic and anti-inflammatory effects of rub may be related to the inhibition of cyclooxygenase-2 (COX-2), endogenous inflammatory mediators, and reduction in the content of pain-induced mediators.

Key words: Rubimaillin; Analgesic; Inflammation; PEG2.

Introduction

Pain is a prevalent medical problem that confronts human populations worldwide (1). However, very little is known on the mechanisms involved in pain. Existing analgesic drugs are have poor efficacies, and some are associated with unwanted side effects. There are no effective and specific pain treatment methods in clinical practice (2, 3). Bioactive substances in various organisms have anti-inflammation effects, and some even alleviate inflammatory pain. However, very little research has been done on the related molecular mechanisms involved in the analgesic properties of these substances. Natural medicines have been widely studied because of their availability, affordability and minimum side effects. The anti-inflammatory and analgesic effects of some natural drugs have been recognized (4). Thus, the search for analgesic and anti-inflammatory active ingredients from traditional Chinese medicine has become a subject of intensive research.

Madder is the dry root and rhizome of *Rubia cordifolia* L. which is distributed in most parts of China. It has high yields in Weinan, Shaanxi, Xingtai, Hebei, and Luoyang, Henan. For long, *madder* has been used as a natural dye, food coloring agent and medicinal plant (5). It has arrests bleeding and promotes blood circulation, and it has analgesic effects (6). In addition, *madder* has antibacterial, anti-cancer, immune-enhancing and hepatoprotective effects, and it exerts inhibitory effects on proliferation of human epidermal cells (7). In recent years, steroids and their glycosides, naphthoquinone derivatives, cyclohexyl peptides, and polysaccharides have been isolated from valerian. Moreover, steroids and their glycosides, naphthoquinone derivatives, cyclohexyl peptides, and polysaccharides have been isolated from *madder*. Rubimaillin (*Rub*, Fig.1) is a naphthoquinone compound extracted from Chinese herbal medicine; it has antibacterial, anti-oxidant and anti-inflammatory effects (8, 9). Rubimaillin has a long history of application as a national medicine, but so far, not much research has been done on its analgesic effect. Therefore, this study was carried out to evaluate the analgesic effect of *Rub in vitro* and *in vivo*, and to determine its mechanism of action, so as to provide a scientific basis for the development and utilization of valerian.

Materials and Methods

Drugs and reagents

Rubimaillin was provided by Guangzhou Laipu Dake Biotechnology Co. Ltd. Aspirin was purchased

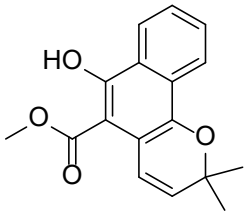

Figure 1. Chemical structure of Rubimaillin.

from Hubei Minkang Pharmaceutical Co. Ltd. Formaldehyde and glacial acetic acid were obtained from National Pharmaceutical Group Chemical Reagent Co. Ltd., while prostaglandin E2 (PEG2) and cAMP ELISA Kits were purchased from Tianjin Anorui Kang Biotechnology Co. Ltd. Antibodies for COX-2, iNOS and β-actin were provided by Santa Cruz, CA, USA.

Experimental animals

SPF Kunming mice (18-22g) were purchased from Hubei Experimental Animal Center [(animal production license number was SCXK (E) 2008-0005]. The mice were raised in the SPF animal room of the School of Pharmacy of Central South University for Nationalities, in strict compliance with the Hubei Provincial Laboratory Animal Quality Control Standards. The temperature in the animal room was 22 ± 2°C. The mice were allowed free access to feed and water, but were fasted for 12 h (with access to water) before the commencement of the experiment.

Induction of writhing in mice using glacial acetic acid

A total of 50 Kunming mice (25 male and 25 female) were randomly divided into 5 groups of 10 animals each: model group, positive control group, 3 Rub administration groups (50, 100 and 200 mg/kg), and aspirin positive control group. The positive control group was given aspirin at a dose of 200 mg/kg body weight, while the control group was given an equivalent volume of distilled water (5 mL/kg). After 30 min, each mouse was intraperitoneally injected with 1.0 % glacial acetic acid 10 mL/kg to induce pain. The number of writhes in each mouse within 20 min was recorded, and the degree of inhibition of writhing reaction was calculated as shown below:

$$Inhibition\ of\ writhing = \frac{(Mean\ writhes\ in\ control) - (Mean\ writhes\ in\ drug-treated\ mice) \times 100\%}{(Mean\ number\ of\ writhes\ in\ drug-treated\ mice)}$$

Determination of cAMP content in acetic acid-induced mice

After glacial acetic acid-induced analgesia in mice, blood samples were taken from each group. The blood was centrifuged at 3000 rpm for 10 min, and cAMP contents of the serum samples were determined using ELISA.

Formalin-induced pain test

One hour after treatment of each group, 5% formalin (10 μL/mouse) was injected into the right hind paw of the mice. Immediately, the mice were placed in a large beaker, and the total time taken for lameness in mice in the phase I neuropathic pain period (0-5 min) and phase II inflammatory pain period (15-30 min) were recorded using a stopwatch.

Assay of PEG2 in foot tissue of mice with formalin-induced inflammation

After the formalin test, the mice were sacrificed via cervical dislocation. The inflamed swollen foot was cut 0.5 cm above the ankle joint. After washing with physiological saline, 10% tissue homogenate was prepared, and PEG2 content was determined using ELISA.

Cell culture

Mouse mononuclear macrophage cell line RAW264.7 (ATCC; Manassas, VA, USA) was cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin. Cells in logarithmic growth phase were incubated at 37 ℃ in ad 5% CO_2 incubator for subsequent testing.

Assay of NO release levels in RAW264.7 cells with Griess reagent

RAW264.7 cells were inoculated into 12-well plates at a density of 2×10^5 cells per well. After adherence for 24 h, they were divided into blank control group, LPS treatment group, and drug + LPS treatment group. The cells were treated with the indicated concentrations of *Rub* in serum-free medium for 2 h prior to the addition of LPS (1 μg/ml). After 24 h of culture, the cell culture supernatant was collected and centrifuged at 3000 rpm for 20 min. Then, 100 μL of the supernatant was taken according to instructions on the NO detection kit, and the OD was read at 550 nm in an ultraviolet spectrophotometer. The NO content of each group of RAW264.7 cells was calculated using the OD readings. The test was repeated three times.

Western blot assay for protein expression levels of iNOS and COX-2

The RAW264.7 cells were placed in a 6-well plate and cultured for 24 h. Then, LPS (1 μg/ml) was added, and the cells were treated with different concentrations of Rub for 18 h. Thereafter, the cells were lysed with RIPA cell lysate reagent, and the total protein content of the lysate was determined using Coomassie Brilliant Blue method (10). Then, 50 μg of total protein samples from each group were loaded and subjected to 10% polyacrylamide gel electrophoresis. The separated proteins were electro-transferred to nitrocellulose membranes, and the membranes were blocked with blocking solution at room temperature for 2 h. The membranes were thereafter incubated overnight with rabbit anti-mouse COX-2 and iNOS monoclonal antibodies (diluted 1:1000 with TBS) at 4°C. After washing thrice with TBST (pH 7.6), the membrane was incubated with horseradish peroxidase-labeled anti-rabbit IgG secondary antibody (1:5000 dilution with TBS) for 2 h at room temperature. Then, the membrane was rinsed in TBST (pH 7.6). Enhanced chemiluminescence was used to illuminate the blots in the dark, and the relative expressions were analyzed using Image J software. Each experiment was repeated 3 times.

Statistical analysis

Graphpad Prism 7.0 statistical software was used for statistical analysis. Measurement data are expressed as mean ± SD, and *t*-test was used for two-group comparisons, while one-way analysis of variance (ANOVA) was used for comparison amongst groups. Statistical significance was assumed at $p<0.05$.

Results

Effect of Rub on acetic acid-induced writhing reaction, and serum cAMP in mice

The writhing data showed that, compared with the

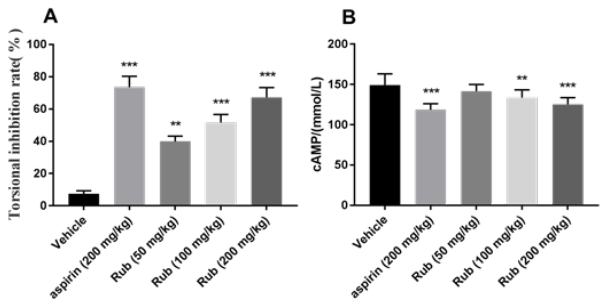

Figure 2. (A) Effect of *Rub* on acetic acid-induced writhing reaction. (B) Effect of *Rub* on serum cAMP content in mice subjected to acetic acid-induced writhing. $^{**}p < 0.01$, $^{***}, p < 0.001$ vs vehicle group.

model group, *Rub* at the three doses inhibited acetic acid-induced writhing reaction to different degrees ($p<0.01$; Fig. 2A). At the same time, serum cAMP content decreased significantly *(*$p<0.01$; Fig. 2B), indicating that *Rub* clearly exerts analgesic effects.

Effect of Rub on formalin-induced pain response and PEG2 content in mice foot tissue

Pain in the hind paws in the *Rub* group was significantly decreased ($p<0.01$; Fig. 3A). This indicates that *Rub* has a dose-dependent inhibitory effect on formalin-induced biphasic pain (neuropathic pain and inflammatory pain) response in mice. The analgesic effect of *Rub* on phase II inflammatory pain was more pronounced than that of phase I. Compared with the aspirin group in the positive control group, different doses of *Rub* significantly reduced PEG2 content ($p<0.01$; Fig. 3B). These results indicate that Rub exerts anti-inflammatory and

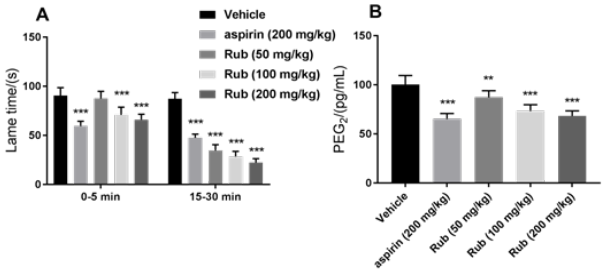

Figure 3. (A) Analgesic effect of *Rub* on phases 1 and 2 after formalin injection in mice. (B) Inhibitory effect of *Rub* on the release of PGE 2 in mice after formalin injection. $^{**}p < 0.01$; $^{***}p < 0.001$ vs vehicle group.

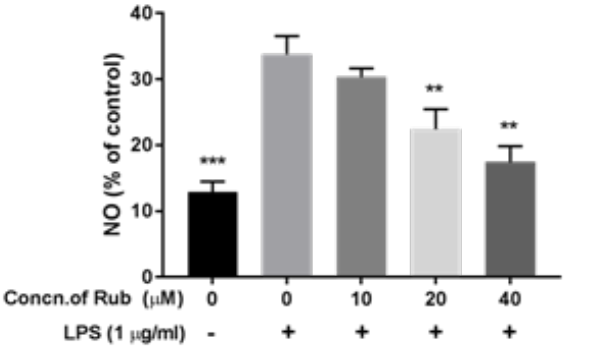

Figure 4. Effect of *Rub* on LPS-induced NO release in RAW264.7. $^{**}p < 0.01$; $^{***}p < 0.001$) vs LPS alone.

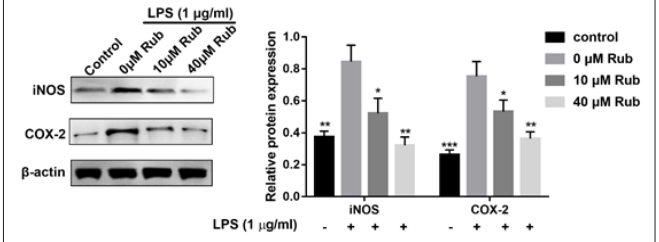

Figure 5. Effect of *Rub* on the LPS-induced iNOS and COX-2 protein levels in RAW264.7 cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs LPS alone.

analgesic effects.

Determination of release of NO in RAW264.7 cells using Griess reagent

Griess reagent method was used to measure the effect of *Rub* on the release of NO from RAW264.7 cells. The results showed that 1μg/mL LPS increased NO secretion level after 48 h of RAW264.7 cells, when compared with the blank control group *(*$p < 0.01$). Compared with the LPS treatment group, pretreatment with *Rub* significantly and dose-dependently inhibited the secretion of NO to varying degrees (Fig. 4).

Western blot assay of protein expression levels of iNOS and COX-2

Western blot was used to determine the effect of *Rub* on the protein expression levels of iNOS and COX-2 in RAW264.7 macrophages. The results are shown in Figure 5. Stimulation with LPS at a dose of 1 μg/ml significantly induced up-regulation of iNOS and COX-2 protein expressions in RAW264.7 macrophages. However, the LPS-induced upregulation of protein expressions of iNOS and COX-2 decreased with the increase in *Rub* concentration ($p < 0.05$).

Discussion

Inflammation and pain are symptoms that often occur simultaneously in patients in the clinics. Pain is a protective defense response in the body in response to existing or potential tissue damage. The aim of research on analgesic efficacy is to evaluate the effects of drugs on pain response of experimental animals by applying pain stimulation to the experimental animals, and evaluating the efficacy of the drugs. Common methods of pain involve physical (thermal, electrical and mechanical) or chemical (H^+, K^+ and bradykinin) stimulation (11). In this study, Rubin-induced pain and formalin-induced neuropathic and inflammatory bipolar pain in mice were used to investigate the analgesic effect of *Rub*.

The acetic acid writhing test in mice is a large-area, long-term acetic acid-induced inflammatory pain on the visceral and parietal peritoneum. It is a writhing-based reaction and a classic non-steroidal anti-inflammatory drug analgesic screening model. It is known that cAMP is the most important second messenger in the cell. It activates PKA after its formation, and enhances excitability and hypersensitivity of neurons through the cAMP-PKA pathway (12). The results obtained in his study showed that Rub inhibited the writhing reaction induced by acetic acid in a dose-dependent manner, and

that the cAMP content decreased in a dose-dependent manner. These results suggest that Rub exerts analgesic effect against inflammatory pain.

The formaldehyde-induced mouse pain test is a classic animal model of persistent somatic pain, and the pain properties are similar to those of clinical chronic pain (13). It is divided into first pain phase (0-5 min after formaldehyde injection) and the second pain phase (15-30 min after formaldehyde injection). The first pain phase is the pain caused by direct stimulation of the plantar nociceptors by formaldehyde, while the second is secondary inflammatory stimuli caused by the release of inflammatory mediators, including PEG2 (14). The analgesic effect of *Rub* on phase II inflammatory pain was more obvious than that of phase I, suggesting that *Rub* inhibits the secondary inflammatory stimuli caused by the release of inflammatory mediators. Further studies showed that different doses of *Rub* inhibited PEG2 in mouse foot tissue.

Bacterial lipopolysaccharide (LPS) is the most common proinflammatory factor. It stimulates macrophage synthesis and release of various inflammatory mediators (15). Nitric oxide (NO) is an inflammatory mediator involved in the pathogenesis of various inflammation-related diseases (16). Moderate amounts of NO help to reduce inflammatory reactions and kill pathogenic microorganisms. However, macrophages produce large amounts of NO under inflammatory or immune stimuli. These NOs react with oxygen radicals such as superoxide anions to increase their cytotoxic effects on tissue cells. The results of Griess assay showed that *Rub* inhibited the production of NO in LPS-induced mouse peritoneal macrophages, resulting in decreased levels of NO released in cells, thereby reducing cytotoxicity. It is known that iNOS catalyzes NO production in inflammatory reactions (17). The synthesis of prostaglandins is catalyzed by COX, a rate-limiting enzyme in the synthesis of PGE2 (18). There are two different COX isoenzymes in the human body, namely COX-1 and COX-2. It is generally believed that COX-1 is a normal physiological enzyme which regulates physiological prostaglandins (PG) in tissues and organs. In contrast, COX-2 is an inducible enzyme (pathological enzyme) that is involved in induction of inflammatory responses and inflammatory PG synthesis at the site of inflammation (19, 20). Western blot results showed that the secretion of iNOS and COX-2 decreased gradually with increase in Rub concentration.

In summary, different methods were used to study the analgesic effect of *Rub in vitro* and *in vivo*. It was found that *Rub* has significant analgesic and anti-inflammatory effects, and the analgesic mechanism is related to the content of endogenous inflammatory mediators and pain-induced mediators, including PEG2. Due to the fact that the molecular mechanism underlying inflammatory pain is very complicated, there is need for further research on the anti-inflammatory and analgesic effects of *Rub*, and the mechanisms involve.

Acknowledgements
None.

Conflict of Interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Fengyun Fan; Wanli Yan, Zhonglei Jiang, Fengyun Fan collected and analysed the data; Wanli Yan and Zhonglei Jiang wrote the text and all authors have read and approved the text prior to publication.

References

1. Guildford BJ, Daly-Eichenhardt A, Hill B, Sanderson K, McCracken LM. Analgesic reduction during an interdisciplinary pain management programme: treatment effects and processes of change. Br J Pain 2018;12: 72-86.
2. Wiese AD, Griffin MR, Schaffner W, Stein CM, Greevy RA, Mitchel EF, et al. Opioid analgesic use and risk for invasive pneumococcal diseases: a nested case–control study. Ann Intern Med 2018; 168: 396-404.
3. Cairat M, Fournier A, Murphy N, Biessy C, Scalbert A, Rinaldi S, et al. Nonsteroidal anti‐inflammatory drug use and breast cancer risk in a European prospective cohort study. Int J Cancer 2018; 143: 1688-1695.
4. Tan JY, Chen XQ, Kang BJ, Qin ZX, Chen JH, Hu RD, et al. Myricetin protects against lipopolysaccharide-induced disseminated intravascular coagulation by anti-inflammatory and anticoagulation effect. Asian Pac J Trop Med 2018; 11: 255.
5. Cai Y, Sun M, Xing J, Corke H. Antioxidant phenolic constituents in roots of Rheum officinale and Rubia cordifolia: structure–radical scavenging activity relationships. J Agric Food Chem 2004; 52: 7884-7890.
6. Idhayadhulla A, Xia L, Lee YR, Kim SH, Wee YJ, Lee CS. Synthesis of novel and diverse mollugin analogues and their antibacterial and antioxidant activities. Bioorg Chem 2014; 52: 77-82.
7. Tse WP, Che CT, Liu K, Lin ZX. Evaluation of the anti-proliferative properties of selected psoriasis-treating Chinese medicines on cultured HaCaT cells. J Ethnopharmacol 2006; 108: 133-141.
8. Matsuda D, Ohshiro T, Ohba M, Jiang W, Hong B, Si S, et al. The molecular target of rubimaillin in the inhibition of lipid droplet accumulation in macrophages. Biol Pharm Bull 2009; 32: 1317-1320.
9. Lu Y, Liu R, Sun C, Pan Y. An effective high‐speed countercurrent chromatographic method for preparative isolation and purification of mollugin directly from the ethanol extract of the Chinese medicinal plant Rubia cordifolia. J Sep Sci 2007; 30: 1313-1317.
10. Chen MJ, Liang T, Zhou KY. [Effect of proanthocyanidins on COX-2 enzyme activity and COX-2 mRNA /protein expression in LPS-induced RAW264.7 cells]. Yao Xue Xue Bao 2005; 40: 406.
11. Peng B, Liu XM, Wang LW. Survey on pharmacological methodology of sedative Chinese materia medica. Chin Tradit Herbal Drugs 2005; 36: 1257-1260.
12. Cao S, Li Y, Wang L, Cui J, Jia N, Li R, et al. Synergistic analgesic effect of propofol–alfentanil combination through detecting the inhibition of cAMP signal pathway. J Pharm Pharmacol 2016; 68: 1170-1176.
13. Xu Y, Ku B, Geng X, Yao H, Zhang Y, Han J, et al. Analgesic effects of tetrodotoxin combined with acetylsalicylic acid on acetic acid-induced abdominal constriction and formalin test in mice. J Chin Pharm Sci 2005; 14: 186.
14. Florentino IF, Silva DP, Galdino PM, Lino RC, Martins JL, Silva DM, et al. Antinociceptive and anti-inflammatory effects of Memora nodosa and allantoin in mice. J Ethnopharmacol 2016; 186: 298-304.

15. Prasad AS, Beck FW, Grabowski SM, Kaplan J, Mathog RH. Zinc deficiency: changes in cytokine production and T-cell subpopulations in patients with head and neck cancer and in noncancer subjects. Proc Assoc Am Physicians 1997; 109: 68-77.
16. Arnold RJ, Layton A, Massanar M. Cost impact of monitoring exhaled nitric oxide in asthma management. Allergy Asthma Proc 2018; 39: 338-344.
17. Jiang YX, Dai YY, Pan YF, Wu XM, Yang Y, Bian K, et al. Total flavonoids from Radix Glycyrrhiza exert anti-inflammatory and Antitumorigenic effects by inactivating iNOS signaling pathways. Evid Based Complement Alternat Med 2018; 2018: 22.
18. Loynes CA, Lee JA, Robertson AL, Steel MJ, Ellett F, Feng Y, et al. PGE2 production at sites of tissue injury promotes an anti-inflammatory neutrophil phenotype and determines the outcome of inflammation resolution in vivo. Sci Adv 2018; 4: 8320.
19. Aoki T, Narumiya SJA, Supplements A. Prostaglandins and chronic inflammation. Trends Pharmacol Sci 2012; 33: 304-311.
20. Streicher JM, Kamei K, Ishikawa T, Herschman H, Wang Y. Compensatory hypertrophy induced by ventricular cardiomyocyte-specific COX-2 expression in mice. J Mol Cell Cardiol 2010; 49: 88-94.