Anti-nociceptive effect of aspirin-pharmaceutical acupuncture in animal models

Hyun-Ja Jeong[1], Min-Gi Kim[2], Jeong-Hwa Kim[3], Hyung-Min Kim[2]*

[1]Biochip Research Center, Hoseo University 165, Sechul-ri, Baebang-myun, Asan, Chungnam, 336-795, Republic of Korea
[2]Department of Pharmacology, College of Oriental Medicine, Kyung Hee University, Seoul,130-701, Republic of Korea
[3]Department of Social Welfare, The Graduate School of Ewha Womans University,
11-1 Daehyun Dong, Seodaemun-gu, Seoul, 120-750, Republic of Korea
*Correspondence: hmkim@khu.ac.kr

Although the oral administration of aspirin has an anti-nociceptive effect, the clinical use of aspirin is limited by its side effects and often by the patient's conditions. Here, we investigate the anti-nociceptive effect of aspirin-pharmaceutical acupuncture (PA). Aspirin was subcutaneously injected into the ST36 acupoint (PA), and the effect of PA was compared with the effects of intraperitoneal (IP) and intramuscular (IM) injections. PA, IP, and IM significantly inhibited abdominal constrictions induced by acetic acid. PA (10 mg/kg) showed significant anti-nociceptive activity compared to IM. PA prolonged the latency period to 60 min in a tail-flick test (TFT) and showed attenuated sciatic nerve crash injury-induced hypersensitivity and the flinching responses in the late phase of the formalin test compared with the IP and IM. The anti-nociceptive effects by PA were reverted by naloxone administration and denervation. PA also decreased interleukin-6 and cyclooxygenase-2 expressions. The findings from this study will inform those who plan the aspirin therapies.

Key words: Pain – Aspirin – Pharmaceutical acupuncture – Tail-flick test – Formalin test – Interleukin-6 – Cyclooxygenase-2.

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage [1, 2]. Many researchers are finding and inventing effective chemical and methods to control pain. Acupuncture is best known in the United States as an alternative therapy for chronic pain [3]. Acupuncture is a procedure during which fine needles are inserted into an individual at discrete points for treatment. These discrete points are usually called acupoints, and matching acupoints to a specific organ is called a meridian [4]. In particular, the stomach meridian 36 zusanli acupoint (ST36) has been used in many studies for relieving pain and electronic acupuncture has been developed to improve the anti-nociceptic effects, finding applications in many fields [5]. It has been reported that the major systemic anti-nociceptive mechanisms of ST36 acupuncture are the release of opioid peptides in the central nervous system (CNS) in response to the long-lasting activation of ascending sensory tracks during intermittent stimulation. Simply, the insertion of the needle is insufficient to relieve pain. Therefore, rotation, electric stimulation, or heating are needed to relieve pain sufficiently [6-8]. In addition, it has been reported that acupuncture down-regulates inflammation and immune systems such as splenic natural killers (NKs) cell activity [9] and T-helper cell type 1 and 2 responses [10, 11]. There are few variations of acupuncture, including electronic acupuncture, heat-stimulated acupuncture, and pharmaceutical acupuncture (PA). PA is a traditional oriental therapeutic technique that combines acupuncture with an herbal treatment. This technique involves injecting an herbal extract into certain acupuncture points, according to oriental medical theory [12]. PA is effective for dealing with various diseases, such as allergies, arthritis, rheumatism, general pain, and lower back pain [13]. Specially, bee venom pharmaceutical acupuncture, called apipuncture, refers to the injection of a small amount of bee venom into ST36 as a treatment. It reportedly relieves pain, and it is mediated to the alpha-2 adrenergic receptor [14, 15]. Silva *et al*. recently reported that the tail flick latency was increased by an injection of 1 % formalin at the ST-36 acupoint [16].

Aspirin is commonly used throughout the world as an over-the-counter analgesic medication used to treat various painful conditions and to reduce fever through the regulation of cyclooxygenase (COX) [17,18]. Aspirin has antithrombotic activity by the inhibition of COX-1 in platelets and the production of nitric oxide from mononuclear cells [19, 20]. High doses are more effective, but the oral administration of aspirin can cause serious side effects including drowsiness and gastric irritation. As a result of these side effects, the clinical use of aspirin is limited. In this study, we investigated the effect of aspirin administered through PA on the ST-36 acupoint to manage pain more efficiently and safely in various pain animal models. Aspirin was also injected intraperinoneally (IP) or intramuscularly (IM) in various pain animal models. In this study, we show that PA has more anti-nociceptive activity than IP and IM injection.

I. MATERIALS AND METHODS

1. Animal

Experiments were performed on male ICR mice (31-35 g) and male SD rats (250-300 g). Each animal was housed three to five per cage at a controlled temperature (20 ± 2°C) and was maintained on a 12 h light and 12 h darkness schedule (lights on from 07:00 h to 19:00 h), with food and water made available *ad libitum*. The animals were euthanized after the end of the experiment, and blood samples or tissues were taken as needed for the experiments. Mouse care and experimental procedures were performed under approval from the Animal Care Committee of Kyung Hee University.

2. Writhing test

The mice were injected intraperitoneally with 0.25 mL of 1 % acetic acid. Mice were divided into six groups: a control group, an acetic acid group, an aspirin oral group, a PA group, an IP group, and an IM injection group (n = 6-7 in each group). Aspirin (3, 10, 30, and 100 mg/kg) was given and then the acetic acid was injected. The mice were placed in a large glass cylinder. The numbers of abdominal stretches were counted during 30 min after the acetic acid injection.

3. Tail-flick test (TFT)

Anti-nociception was determined using a tail-flick test. In brief, the

distal one third of the tail was immersed in a water bath maintained at 50 °C. Latency times until a tail-flick response were recorded before and at different time points after the aspirin injection. Rats were divided into aspirin-injection groups and the different vehicle groups. The cut-off time was 12 s to avoid tissue damage. Latency times were measured at 0, 15, 30, 60, 90, and 120 min. To confirm the results, additional test was carried out. The area under the curve (AUC) between 30 to 60 min was calculated following the equation: [(mean latency time of 30 min + mean latency time of 60 min)/2 × 1800 s].

4. Surgery for a sciatic nerve crushed injury (SCI) and axotomy

To induce a crush injury on the sciatic nerve in rats, a surgical procedure based on a previously described method was performed [21]. In brief, the right sciatic nerve was exposed through a splitting incision on the gluteal muscle under anesthesia with Zoletil 50, 2 % Rompun and distilled water at a ratio of 1:2:3. The sciatic nerve was carefully exposed and crushed for 30 s using a surgical clip between the sciatic notch and the point of trifurcation. Subsequently, the surgical wound was sutured and recovered. For total denervation of the right leg, the surgery was similar to that for the SCI but the sciatic nerve and saphenous nerve were sectioned and removed from the sectioned terminal at a length of 1 mm.

5. Touch test

After the SCI surgery, 3 weeks were allowed to pass until the sensory nerve recovered. The SCI ipsilateral side was stimulated with a Touch-Test (North Coast Medical, Inc. Morgan Hill, CA, United States) NC12775-18 (100 g), and the rats were selected based on similar numbers of foot withdrawals per 10 attempts. The rats were then divided into groups (n = 4) as TFT. The number of foot withdrawals for each group was measured at 0, 30, 60, and 90 min.

6. Formalin test

Tonic inflammatory pain was induced in the mice by a subcutaneous injection of a formalin solution (20 μL of 2 % in PBS/mouse) into the right hind paw. All of the aspirin treatment groups were pre-treated 30 min beforehand, and the naloxone treatment group was 45 min pre-treated at 2 mg/kg. After the formalin injection, the numbers of flinches and attempts biting or licking were observed for 60 min, and the results were tabulated for successive 5-min intervals. The number of attempts at licking and biting were counted every 7 s. when there was continuous licking and biting. The total number of flinches and instances of licking or biting during the early phase (0-10 min) and the late phase (10.01-60 min) were summed every 5 min in both cases [10, 22]. At the end of the experiment, the mice were euthanized and the researchers took blood samples and the formalin-injected hind paws.

7. Enzyme-linked immunosorbent assay (ELISA)

The cytokines of the serum and the homogenized hind paw total protein levels were measured by an ELISA. The ELISA was performed by coating 96-well plates with 1 μg/well of capture Ab. Before the subsequent steps in the assay, the coated plates were washed twice with 1 × PBS containing 0.05 % tween-20 (PBST). All reagents and coated wells used in this assay were incubated for 2 h at room temperature. The standard curve was generated from known concentrations of cytokine, as provided by the manufacturer. After exposure to the medium, the assay plates were exposed sequentially to each of the biotin-conjugated secondary antibodies, avidin peroxidase, and a 2-AZINO-bis (3-ethylbenzithiazoline-6-sulfonic acid) substrate solution containing 30 % H_2O_2. The plates were read at 405 nm. Appropriate specificity controls were included, and all samples were run in duplicate. Cytokine levels in the hind paws were divided according to the total protein amounts. Protein levels were determined using a nanodrop photospectrometer 2000 (Thermo Scientific).

8. Western blot analysis

Western blot analysis was used for hind paw extracts prepared by a detergent lysis procedure. Samples with a loading buffer were heated at 95 °C for 5 min and then briefly cooled on ice. Following centrifugation at 15,000 × g for 5 min, 50 μg aliquots were resolved by 10 % SDS-PAGE. The resolved proteins were electro-transferred overnight to nitrocellulose membranes in 25 mM Tris, pH 8.5, 200 mM glycine, and 20 % methanol at 25 V. Blots were blocked for at least 2 h with PBST containing 5 % nonfat dry milk and then incubated with COX-2 and actin antibodies (Santa Cruz, CA, United States) for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibodies, and proteins were visualized by enhanced chemiluminescence procedures (Amersham Biosciences, Piscataway, NJ, United States) according to the manufacturer's instructions.

9. Statistical analysis

Data were expressed as the mean ± standard error mean (S.E.M). For comparisons between groups, a one-way ANOVA and Duncan's post-hoc test were performed with $p < 0.05$ as an indication of statistical significance.

10. Drugs

Acetic acid (0.25 mL of 1 % acetic acid), aspirin (3, 10, 30, or 100 mg/kg), naloxone, formalin (20 μL of 2 % in PBS/mouse), and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Aspirin or vehicle was injected into the Zusanli (ST36, PA, United States) point located on the lateral side of the stifle joint adjacent to the anterior tubercle of the tibia. In the IM group, aspirin or vehicle was injected into the thigh. In the IP group, aspirin or vehicle was injected into the abdominal cavity. The axotomy group was injected once contralaterally or ipsilaterally. Naloxone (2 mg/kg) was intraperitoneally pretreated. All syringe needles used were 29-gauge ultrafine needles with 20 μL for mice and 40 μL for rats in each single dosage. The aspirin was dissolved in DMSO. The acetic acid and naloxone were dissolved in normal saline, and the formalin was diluted with phosphate-buffered saline (PBS).

II. RESULTS

1. PA was more effective than IP or IM

We investigated the effect of PA in an acetic acid-induced writhing model. The acetic acid injection significantly increased the amount of writhing compared to the control group. Vehicle groups had no effect on the amount of writhing. All aspirin-injection groups showed significant decreases in the amount of writhing in a dose-dependent manner (*Figure 1A*), but the 3 mg/kg aspirin-injection groups did not show a statistical significant decrease, and the 30 and 100 mg/kg aspirin-injection groups showed statistical significant decreases in the PA, IP, and IM groups compared to acetic acid group. For the 10 mg/kg aspirin injection, a significant decrease was noted in the PA and IP groups, whereas the IM group showed no effect compared to the acetic acid group. When 10 mg/kg of aspirin was orally administered, the aspirin did significantly not affect the amount of writhing (*Figure 1B*). Therefore, the PA was significantly more effective than the IM and oral administration groups. Aspirin 10 mg/kg was chosen for the subsequent pain test.

We investigated the effect of PA in SCI-induced hypersensitivity. SCI-induced mechanical hypersensitivity persisted for about 10 weeks after the operation, especially in the first 1 to 4 weeks [19]. The number of foot withdrawals was increased in the vehicle group which underwent SCI ipsilateral side stimulation. The PA, IP, and IM significantly decreased the number of withdrawals after 30 min of stimulation. The PA and IM groups showed a prolonged anti-nociceptive effect of aspirin up to 60 min after the injection. The PA was more effective than the IM (*Figure 2*).