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Prolonged analgesic effect of PLGA-encapsulated bee venom on formalin-induced pain in rats

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ABSTRACT

To enhance the medicinal activity of bee venom (BV) acupuncture, bee venom was loaded into biodegradable poly(D,L-lactide-co-glycolide) nanoparticles (BV-PLGA-NPs) by a water-in-oil-in-water-emulsion/solvent-evaporation technique. Rat formalin tests were performed after subcutaneous injection of BV-PLGA-NPs to the Zusanli acupuncture point (ST36) at 0.5, 1, 2, 6, 12, 24, and 48 h before plantar injection of 2% formalin. BV-PLGA-NPs treatment showed comparable analgesic activity to typical BV acupuncture during the late phase, compared with saline-treated controls, and the analgesic effect lasted for 12 h. PLGA-encapsulation was also effective in alleviating the edema induced by allergens in bee venom. These results indicate that PLGA-encapsulation provided a more prolonged effect of BV acupuncture treatment, while maintaining a comparable therapeutic effect.

1. Introduction

For optimum drug action, the most efficient method of administration is to deliver the drug to the desired site of action in the body, removing or minimizing side effects at non-target sites. There have been many reports addressing nano-engineered drug carrier systems, such as liposomes, micelles, and polymer micro/nanoparticles (Gupta et al., 2000), with advantages of high carrier capacity, efficient incorporation, targeted delivery, and controlled (sustained) release. Nanoparticle-based drug delivery systems using biodegradable polymers are strong candidates that can provide sustained, controlled, and targeted drug delivery to improve the therapeutic effect and reduce the side effects of a formulated drug (Soppimath et al., 2001). It also helps to increase the stabilities of chemical or protein drugs and possesses useful controlled release properties.

Nanoparticles are colloidal systems that range in size typically from 10 to 1000 nm in diameter and are formulated from a biodegradable polymer (Langer, 1997). Poly(D,L-lactide-co-glycolide) (PLGA) is one of the most extensively studied biopolymers and is particularly suitable for manufacturing a biocompatible drug delivery system. Although a number of different polymers have been investigated for formulating biodegradable nanoparticles, PLGA is the only one approved by the US Food and Drug Administration (FDA) as a biocompatible and biodegradable polymer (Jain, 2000). The biocompatibility, bioabsorbability, changeable biodegradability, and good mechanical properties of PLGA nanoparticles have been recognized in previous studies (Emerich et al., 1999).

In Korean traditional medicine, BV acupuncture is considered a useful therapeutic method for treating chronic diseases accompanied by severe pain and inflammation (Kwon et al., 2001b). In the BV acupuncture treatment, BV injection is conducted to stimulate an acupuncture point as well as to exert pharmacological effects by the biologically active compounds in BV (Kwon et al., 2001a). Although BV acupuncture therapy is relevant to treating various chronic pain diseases in Korean traditional medicine, the treatment sometimes results in erythema and eventually edema and pain, because of allergenic components in BV. It has been reported that melittin injection induces paw edema in mice (Hartman et al., 1991). Thus, the use of BV acupuncture therapy in treating disease has been somewhat limited.

The present study was performed to investigate the prolonged medicinal effects of BV acupuncture with a combination of BV and poly(D,L-lactide-co-glycolide) nanoparticles (BV-PLGA-NPs) in

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relieving formalin-induced pain in rats. BV-PLGA-NPs were injected to the Zusanli acupuncture point. The controlled release system of BV-PLGA-NPs was also evaluated by assessment of BV-induced edema. An emulsion/solvent-evaporation technique was applied to formulate PLGA nanoparticles and polyvinyl alcohol (PVA) was used as an emulsifier in this study (Chiba et al., 1997). BV-loaded PLGA nanoparticles were prepared and examined for analgesic efficacy in formalin-induced pain rats in comparison with plain BV acupuncture.

2. Materials and methods

2.1. Chemicals

Lyophilized powder of bee venom from *Apis mellifera* (V-3125, Sigma–Aldrich Co.), polyvinyl alcohol (PVA, MW 26,300∼30,000 kDa), poly(D,L-lactide-co-glycolide) (PLGA:PVA = 50:50; MW 40 kDa), formalin (35% formaldehyde), indomethacin and other chemical reagents were all purchased from Sigma–Aldrich Co. (St. Louis, MO, USA).

2.2. Animals

Male Sprague–Dawley rats, weighing 200–250 g, were purchased from Samtaco Animal Co. (Osan, Kyungki-do, Korea), housed in colony cages with free access to food and water, and maintained in temperature and light-controlled rooms (23 ± 0.5 °C, 12/12 h light/dark cycle with lights on at 07:00) for at least one week prior to the study. All methods used in the present study were approved by the Animal Care and Use Committee of Kyung Hee University. All procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals," published by the Korean National Institute of Health.

2.3. Preparation of bee venom-nanoparticles

BV-loaded PLGA/PVA nanoparticles were prepared, using a w/o/w emulsion/solvent-evaporation technique (Chiba et al., 1997). Briefly, aliquots of 200 μl of BV (2 mg/ml) or saline solution were emulsified with 2 ml of chloroform solution containing 40 mg of PLGA to produce a primary emulsion using a microtip probe sonicator (Sonicator XL; Misonix, Farmingdale, NY, USA) for 30 s at 50 W in an ice-bath. This emulsion was further emulsified in aqueous PVA solution (12 ml, concentration of PVA was 3%) to form a multiple w/o/w emulsion. Emulsification was performed again for 5 min at 50 W in an ice-bath. The emulsion was stirred overnight on a magnetic stirrer to allow the chloroform to evaporate. Nanoparticles were recovered by ultracentrifugation (35,000 rpm) for 20 min at 4 °C and washed twice with distilled water to remove un-entrapped PVA.

Particle sizes and size distribution were determined by the light scattering method (DLS-8000; Otsuka Electronics Co., Osaka, Japan). For particle size analysis, 0.2 ml of nanoparticle suspension was dispersed in 3 ml of distilled water and optically analyzed at a scattering angle of 90° and the temperature of 25 °C. The average particle size, expressed as a mean diameter, and polydispersity index were determined. The zeta potential of the nanoparticles was measured using an electrophoretic light-scattering spectrophotometer (ELS-8000; Otsuka Electronics Co., Osaka, Japan). All the measurements above were repeated three times. The amount of BV encapsulated in the nanoparticles was determined by analyzing the protein content in the washing step of nanoparticle formulation using a BCA protein assay kit (Pierce Co., Rockford, IL, USA). The appearance and shape of the nanoparticle surfaces were analyzed using a scanning electron microscope, JSM-6400 (JEOL Co., Tokyo, Japan). The release rate of BV was determined by suspending bee venom-loaded nanoparticles in 1 ml of phosphate-buffered saline (PBS) followed by incubation at 4 and 37 °C under mild agitation. After predetermined time intervals, samples were harvested and centrifuged. The supernatant was collected for analysis of BV and the precipitated nanoparticles were resuspended in 1 ml of fresh PBS, and then returned to the incubator for continuous release measurement.

The percentage of encapsulation efficiency was 70.6 ± 2.3% by the calculation as follows: encapsulation efficiency (%) = (actual BV loading/theoretical BV loading) × 100.

2.4. Rat formalin test

Anti-nociception was assessed using the rat formalin test, according to Dubuisson and Dennis (1977). To perform the test, 50 μl of 2% formalin was injected subcutaneously into the plantar surface of the right hind paw with a 30-gauge needle. The prolonged analgesic effects were examined at time points of 0.5, 1, 2, 6, 12, 24, and 48 h after the injections of BV, BV-PLGA-NPs (0.08 mg/kg in a volume of 50 μl of saline), PLGA, and indomethacin (10 mg/kg) to the Zusanli (ST36) acupuncture point to be ipsilateral to formalin injected hind paw. The acupuncture point was located at the proximal one-fifth point on the line from the depression lateral to the patella ligament to the anterior side of ankle (Yin et al., 2008). Nociceptive behaviors were quantified by counting the number of times the animal licked, bit, or shaked the formalin-injected paw at 5-min intervals during the period from 10 to 60 min after injection. Two phases of spontaneous nociceptive behavior were observed: an initial acute phase (early phase, during the first 10 min after formalin injection) was followed by a relative short quiescent period and then by a prolonged tonic response (late phase, beginning about 10 min after formalin injection).

2.5. BV-stimulated paw edema

The BV-stimulated paw edema rat model was used for quantitative measurement of the local inflammatory response in comparison with plain BV acupuncture. In this method, BV-PLGA-NPs powder equivalent to 0.4 mg BV was weighed and dissolved in 1 ml of saline solution, and 50 μl of BV-PLGA-NPs solution was injected into the subplantar muscle of the right paw to induce acute inflammation. Equivalent amounts of BV without PLGA and PLGA without BV were also used to induce edema as negative controls. The increase in volume of the injected hind paw was used as an index of the severity of inflammation. A plethysmometer (Ugo Basile, Comerio, Italy) was used to measure the volume of the hind paw prior to and up to 3 h after BV treatment. The increase in paw volume (IPV) was calculated as: %IPV = test volume − baseline volume/baseline volume.

2.6. Statistical analysis

All data in this study are presented as the means ± S.E.M. Data were analyzed by independent *t*-test or analysis of variance (ANOVA), followed by *post hoc* comparisons using Fisher's least significant difference test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Characterization of BV-loaded nanoparticles

The characterization and morphology of BV-loaded nanoparticles are indicated in Fig. 1. The BV nanoparticles were spherical in shape with smooth surfaces and without any aggregation or adhesion, as determined by scanning electron microscopy (Fig. 1(A)).

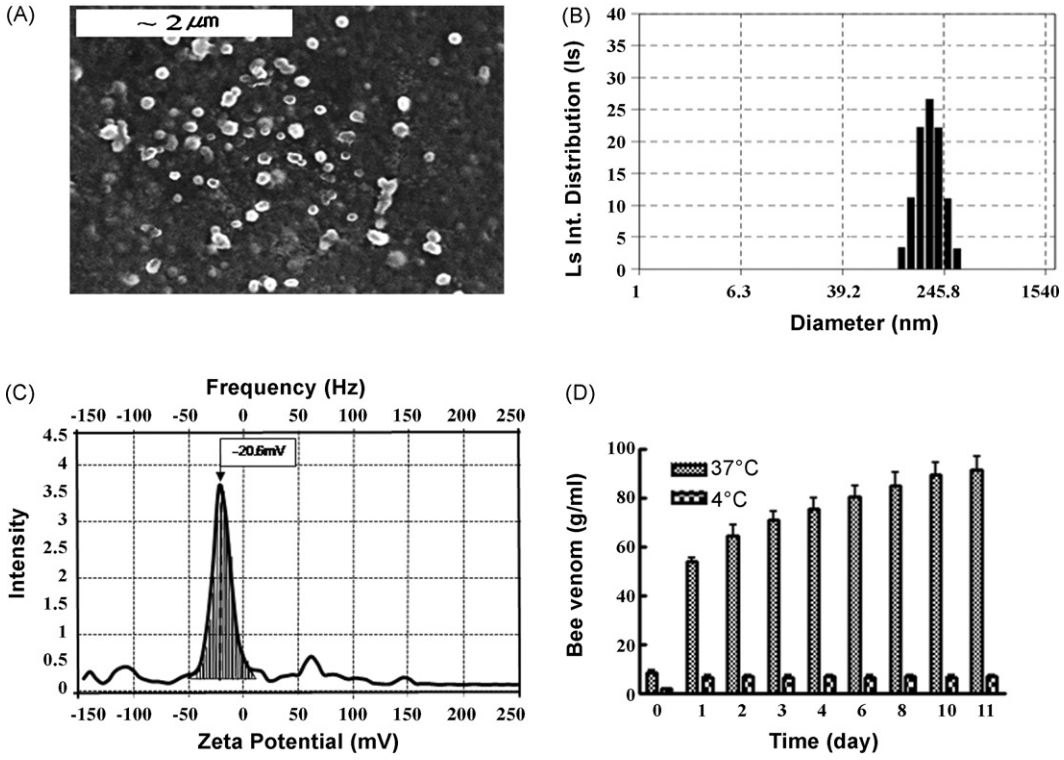

Fig. 1. Characterization of BV-loaded nanoparticles (BV-PLGA-NPs). Scanning electron microscopy (SEM) micrograph (29,000×; the bar indicates 2 μm) (A), particle size distribution profile (B), zeta potential measurement (C), and cumulative release of BV from the BV-loaded nanoparticles (D).

BV-loaded nanoparticles showed a mean diameter of 179.8 ± 2.1 nm (Fig. 1(B)) and polydispersity index of 0.126 ± 0.032, respectively. A lower polydispersity index means a narrower size distribution. Zeta potential was −20.6 ± 0.2 mV (Fig. 1(C)). The drug encapsulation efficiency into the nanoparticles was determined to be 70.6 ± 2.3%. The zeta potential is the overall charge a particle acquires in a specific medium and the magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. If all the particles have a large negative or positive zeta potential (more positive than +30 mV or more negative than −30 mV), they will repel each other and there is dispersion stability. If the particles have low zeta potential values then there is no force to prevent the particles

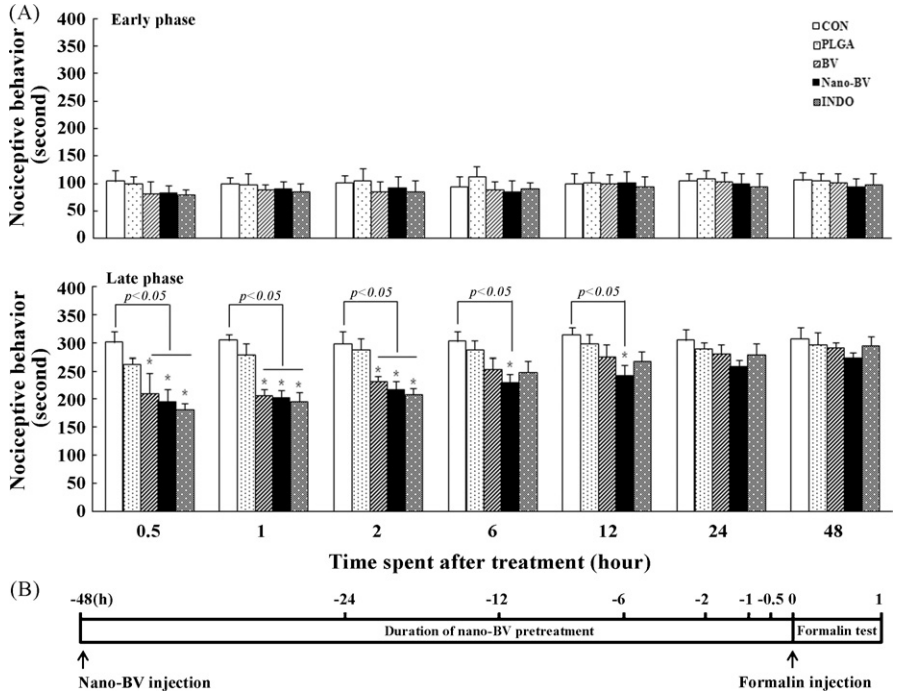

Fig. 2. (A) Prolonged analgesic effect of nanoparticle-encapsulated BV (Nano-BV, $n = 8$) acupuncture to the Zusanli acupuncture point (ST36) on formalin-induced nociceptive behavior as compared with saline (CON, $n = 8$)-, PLGA vehicle (PLGA, $n = 8$)-, plain BV (BV, $n = 8$)- and indomethacin (INDO, $n = 8$)-treated groups. $^*p < 0.05$, as compared with the CON group. (B) Schematic diagram of BV-PLGA-NPs treatment protocols at time points 0.5, 1, 2, 6, 12, 24, and 48 h before 2% formalin injection.

coming together and flocculating, and there is dispersion instability. The magnitude of the measured zeta potential is thus an indication of the repulsive force that is present and can be used to predict the long-term stability of the product (Lin et al., 2006; Veronesi et al., 2002). Fig. 1(D) shows the time courses of *in vitro* cumulative release of bee venom from nanoparticles at 4 and 37 °C. During the initial burst phase (day 0–1), BV was released rapidly from the nanoparticles and accumulated to a concentration of 53.9 μg/ml on the day after encapsulation at 37 °C. However, the release rate of BV markedly decreased after this time point and the cumulative amount of BV eventually reached about 91.5 μg/ml at day 11 post-encapsulation. Thus, the *in vitro* release profile of BV from PLGA nanoparticles was a typical biphasic release phenomenon; there was an initial burst release (day 0–1) and a subsequent slower release (day 1–11). On the other hand, after incubation at 4 °C, BV release from the nanoparticles was not significant over the time period examined.

3.2. Prolonged analgesic effect of nanoparticle-encapsulated BV

The time courses of nociceptive behavior in the rat formalin test after BV-PLGA-NPs injection at various time intervals are shown in Fig. 2. This figure shows the prolonged duration of the analgesic effect of BV-PLGA-NPs treatment, as compared to plain BV acupuncture treatment. The BV injected to the Zusanli (ST36) acupuncture point significantly suppressed the formalin-induced pain behavior in the late phase, at time points of 0.5, 1, and 2 h, as compared with saline-treated controls ($p < 0.05$). In the case of BV nanoparticles (BV-PLGA-NPs), the pain-relieving activity was comparable to that of plain BV treatment at 0.5, 1, and 2 h, and the effect lasted for up to 12 h ($p < 0.05$; Fig. 2(A)). Even at 24 and 48 h, BV-PLGA-NPs treatment was more likely than plain BV acupuncture to exhibit an analgesic effect, although the difference was not statistically significant. There were no significant changes of nociceptive behaviors in the early phase among groups (Fig. 2(A)).

3.3. Paw edema by nano-BV injection

Fig. 3 indicates that subplantar injection of BV induced an increase of 30% in paw volume at 30 min post-injection. This increased paw volume was lasted for up to 2 h and slightly decreased in 3 h. To exam the preventive effect of PLGA-encapsulation from inducing allergic and inflammatory response to BV injection, the BV-PLGA-NP powder containing the equivalent amount of BV was dissolved in PBS and injected to the subplantar muscle of the right paw. The BV-PLGA-NPs-induced edema formation was negligible in all phases of the experiment as compared with plain BV ($p < 0.05$). In case of BV-PLGA-NPs, however, a decreasing trend in paw volume in 3 h post-injection did not observed. No significant increase in paw volume was observed with PLGA injection as a vehicle control, which implied that a biodegradable polymer, PLGA did not affect the edema induction in itself.

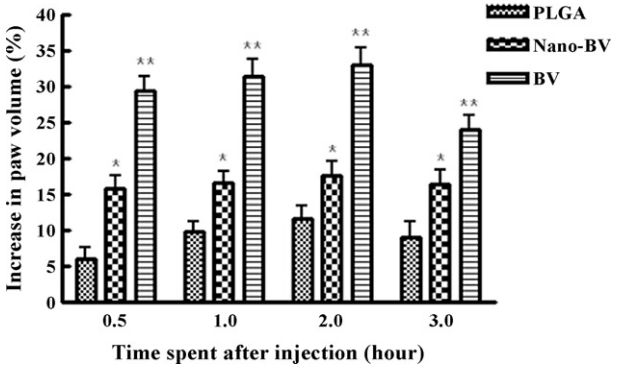

Fig. 3. Increments in paw volume after subcutaneous injection of Nano-BV ($n = 8$) into the hind paw, as compared with PLGA ($n = 8$) or plain BV ($n = 8$) injection. $^{*}p < 0.05$ and $^{**}p < 0.001$ as compared with the PLGA group.

4. Discussion

BV acupuncture has been known to exert a remarkable effect in relieving pain and inflammation in various acute or chronic diseases (Chen et al., 2001; Hartman et al., 1991; Kim et al., 2005; Kwon et al., 2001a,b; Son et al., 2007). On the other hand, the BV injection also induces a systemic or local allergic response, accompanying fever, tonic pain and edema, itching, etc. (Lariviere and Melzack, 1996). It was reported that the injection of melittin, a representative component in BV induced dose-dependent edema in mouse paws (Chen et al., 2003). In recent years, the drug delivery system using the nanoparticle encapsulation technique seems to be a promising strategy for reducing the side effects of BV injection as well as for enhancing the targeting or delivering efficiency of the drug.

In the present study, the *in vitro* release profile of BV from PLGA nanoparticles showed a typical biphasic pattern and there was an initial burst release of BV from BV-PLGA-NPs for the first 24 h post-encapsulation (Fig. 1(D)). The rapid initial release of BV from nanoparticles was probably the result of drug adsorbed on the surface of the nanoparticles (Magenheim et al., 1993). In the second stage of slower release, the BV from the nanoparticles was gradually and steadily released. A burst of release in the initial phase can be useful in improving the penetration efficiency of BV, and sustained and controlled release in the second phase also becomes important for minimizing the irritating effects of some ingredients in BV, which may occur in BV acupuncture treatment at higher concentrations. These releasing properties of BV-PLGA-NPs are necessarily required to provide a prolonged duration of effective BV therapy and to confine the nanoparticles to the limited area around the point of injection in the skin in BV acupuncture.

In order to verify the sustained and controlled release of BV from BV nanoparticles in vivo, the analgesic effect of BV-PLGA-NP injection to the specific acupuncture point was compared with either BV or the vehicle (PLGA) injection in the rat formalin test. In the late phase, the injection of BV nanoparticles to the acupuncture point ST36 exerted a significant analgesic effect for 12 h post-injection whereas the effect of plain BV acupuncture lasted for only 2 h. This result is in considerable agreement with the *in vitro* release profile of BV from BV-PLGA-NPs at 37 °C. Based on these results, it was concluded that the analgesic effect of BV acupuncture could be extended for more than 12 h by nanoparticle encapsulation, while maintaining activity comparable to that of BV acupuncture.

In the rat formalin test, pain behaviors during the early phase are thought to be due to direct chemical stimulation of nociceptors without any tissue damage, while those associated with the late phase are attributed to inflammatory pain induced by inflammatory mediators, including histamine and prostaglandin (Tjoelsen et al., 1992; Wheeler-Aceto and Cowan, 1991). BV can be a powerful regulator of anti-nociception and a potential therapeutic agent against a number of diseases. On the other hand, it has been reported that animals show most types of nociception and hypersensitivity following subcutaneous treatment of BV (Lariviere and Melzack, 1996). The BV test, a well-established experimental animal model mimicking honeybee sting-induced natural tissue injury, is produced by subcutaneous injection of a given dose of honeybee venom into one hind paw (Chen et al., 2001).

In the present study, BV-induced edema was controlled by PLGA-encapsulation. The main ingredients of BV responsible for pain include histamine, melittin, mast-cell degranulating (MCD) peptide, phospholipase A, and apamin (Lariviere and Melzack, 1996).

Melittin causes the release of histamine and serotonin from mast cells, erythrocytes, and thrombocytes. MCD peptide causes the release of histamine from destroyed mast cells. Phospholipase A potentiates melittin's effect, and apamin has neurotoxic effects on spinal cord. The BV components responsible for the inflammation and edema are also known to be histamine, serotonin, apamin, and hyaluronidase. Several experimental reports have elucidated the clinical importance of evoking an allergic (immune) response caused by these allergen components of BV (Lariviere et al., 2005; Calixto et al., 2003). The PLGA-encapsulation technology used here may be a promising strategy to minimize the irritating side effects of BV by preventing instant exposure to the allergen components of BV, such as histamine, melittin, phospholipase A, and apamin (Lariviere and Melzack, 1996), and by controlling the release rate of BV from BV nanoparticle.

Although the precise mechanism of analgesic effect of BV acupuncture to the acupuncture points has remained unclear, several mechanisms involving neuronal receptors have been suggested. Among them, the representative theory is that BV-induced anti-nociception is produced by the activation of the α_2-adrenergic and seronongeric components of the descending pain inhibitory system in the rat formalin pain model (Kim et al., 2005). It was also reported that the anti-nociceptive effect of BV pretreatment on the formalin-induced pain behavior is associated with the reduction of c-Fos expression in the rat spinal cords (Son et al., 2007).

In the present study, the injection of BV or BV-PLGA-NP provided diametrically opposing results: analgesic action and spontaneous nociception in two different animal models, respectively. The subcutaneous injection of BV to ST36 acupuncture point on the knee produced a therapeutic effect on the formalin-induced nociception without eliciting any sign of pain due to the BV injection. However, in the paw edema test, the intramuscular injection of BV to glabrous skin of plantar surface induced the significant inflammatory edema at the injection site. In contrast to the general induction of spontaneous and persistent inflammatory pain through the activation of nociceptor by chemical or physical stimuli to skin, the needle insertion to the specific acupuncture points on the skin, named acupuncture in the traditional Oriental medicine, does not induce any significant signs of pain behavior as well as biochemical and histological changes (Kwon et al., 2001a,b). Although the mechanism by which the single stimulation of BV elicits the different pain responses to the different skins has not been elucidated, it can be suggested that the nociceptors are differentially distributed between the hairy skin around ST36 acupoint and the glabrous skin of plantar surface. And in some pain-controlling acupoints, it might be explainable that the analgesic mechanism by acupuncture stimulation might be separated with the pain transmission neural pathway by acupuncture needle pricking.

In this study, BV or nano-BV was injected to the acupuncture point (ST36) on the hind legs of rats, which attempted to produce synergistic effects of acupuncture therapy as well as pharmacological action of BV components. The efficacy and the duration of medicinal effect of BV acupuncture to the acupuncture points were quite different from those of simple injection of BV intramuscularly or subcutaneously (data not shown). Since PLGA had little effects on the medicinal activities of BV acupuncture, it seems that PLGA-encapsulation of BV enhanced its analgesic by controlling the releasing rate of BV components from the BV nanoparticles, which resulted in extending the period of acupuncture point stimulation and the pharmacological action of the various BV components.

The results of this study demonstrated experimentally that acupuncture therapy using PLGA-encapsulated BV exhibits markedly prolonged suppression of nociceptive behavior in rats with formalin-induced pain, and minimizes the irritating side effects of BV by retarding the rate of BV release from the nanoparticles. Overall, PLGA-encapsulation in BV acupuncture therapy is a promising technique to extend the medicinal effect and to reduce BV-induced irritation and edema.

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