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Original Reports

Injectable PLGA-Coated Ropivacaine Produces A Long-Lasting Analgesic Effect on Incisional Pain and Neuropathic Pain

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Abstract: **The management of persistent postsurgical pain and neuropathic pain remains a challenge in the clinic. Local anesthetics have been widely used as simple and effective treatment for these 2 disorders, but the duration of their analgesic effect is short. We here reported a new poly lactic-co-glycolic acid (PLGA)-coated ropivacaine that was continuously released in vitro for at least 6 days. Perisciatic nerve injection of the PLGA-coated ropivacaine attenuated paw incision-induced mechanical allodynia and heat hyperalgesia during the incisional pain period, and spared nerve injury-induced mechanical and cold allodynia for at least 7 days postinjection. This effect was dose-dependent. Perisciatic nerve injection of the PLGA-coated ropivacaine did not produce detectable inflammation, tissue irritation, or damage in the sciatic nerve and surrounding muscles at the injected site, dorsal root ganglion, spinal cord, or brain cortex, although the scores for grasping reflex were mildly and transiently reduced in the higher dosage-treated groups.**

Perspective: **Given that PLGA is an FDA-approved medical material, and that ropivacaine is used currently in clinical practice, the injectable PLGA-coated ropivacaine represents a new and highly promising avenue in the management of postsurgical pain and neuropathic pain.**

Key Words: *Microparticles, neuropathic pain, poly lactic-co-glycolic acid, postsurgical pain, ropivacaine, long-lasting analgesia.*

Despite great efforts in research on the control of persistent postsurgical pain during past decades, management of this disorder remains a challenge in a large number of patients.[59] Systemic administration of analgesic drugs (eg, opioids) may cause severe side effects, especially when given repeatedly.[8] Local anesthetics (LA; eg, ropivacaine [RVC]) have been used widely as simple and effective treatment for persistent

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pain with absent or reduced adverse effects.[39] However, their analgesic effect lasts only several hours after single injection.[41] Given that postsurgical pain may persist for several days, months, and in some cases, even more than 1 year,[60] prolonged, continuous infusions of LA through a catheter implanted near the target nerve tissue are often required, although long-term catheter use is limited due to adverse effects including tissue damage and infection.[16,48] A sustained release strategy has also been employed using polymeric microparticles 5 to 500 μm in diameter as implanted carriers. However, clinical application as a non- or minimally invasive approach to postsurgical pain management has not been achieved, as these microparticles cannot be injected directly.[21,30,31] Therefore, there is a compelling need for new LA formulations to produce a prolonged analgesic effect.[55]

Previous research has also focused on encapsulating LA within nanocarriers to prolong analgesia and decrease toxicity. Various nanosized drug delivery systems for LA have been developed, including liposomes, hydrogel and polymeric nanoparticles, nanostructured lipid carriers and etc.[13,18,27,38] However, these formulations have limitations related to efficacy, toxicity and tissue reactions.[42] For example, perisciatic nerve injection of Exparel (DepoFoam bupivacaine), a liposomal LA formulation, produced long-lasting nerve blockade, but also led to significant inflammation and myotoxicity.[32] Under some circumstances, nanoparticles themselves enhanced local myotoxicity and augmented inflammatory responses at the nerve.[20,33] In addition, due to nanoparticles' fast diffusion rate, nanoformulations often could not meet the need for prolonged duration of analgesic effect.

In the present study, we have developed an injectable LA formulation using a degradable polymer poly lactic-co-glycolic acid (PLGA) with average size of 1.7 μm in diameter as a drug delivery carrier. This carrier is small enough for minimally invasive injection, but large enough to stay at injection sites as a drug depot for prolonged release. RVC was used as a model of anesthetic drug because it is one of the safest LA in the market and widely used in the management of pain. We used RVC-loaded PLGA particles to realize sustained-release RVC (SRR) and to observe the effect of SRR on pain after hind paw incision or spared nerve injury (SNI), 2 commonly used rodent models of postsurgical pain and neuropathic pain, respectively.

Methods

Preparation and Characterization of RVC•HCl Microparticles

Materials

PLGA was purchased from Absorbable Polymers International. Poly vinyl alcohol (PVA, 87−89% hydrolyzed, MW 13,000−23,000), dichloromethane (DCM), and RVC hydrochloride (RVC•HCl) were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of RVC•HCl-Loaded PLGA Microparticles

RVC•HCl-loaded PLGA microparticles were prepared using a water-in-oil-in-water (w/o/w) double emulsion, solvent evaporation technique. Briefly, 100 mg of 50:50 PLGA was dissolved in 5 mL of DCM. Twenty mg/mL of RVC•HCl was prepared using .5% PVA. One milliliter of the RVC•HCl was mixed with PLGA/DCM solution using a probe sonicator (Q700 Sonicator, Qsonica, Llc) for 30 seconds at 30% amplitude to form the first emulsion. This emulsion was then rapidly added to 20 mL of.5% (w/v) PVA solution by stirring at 10,000 rpm for 30 seconds using a homogenizer (Bio-Gen PRO200, Pro Scientific) to form the second emulsion. The mixture was stirred overnight to allow the DCM solvent to evaporate. The particles were collected by washing 3 times with distilled water using a centrifugal filter device (Amicon Ultra 15 mL Centrifugal Filter, Millipore).

RVC•HCl Loading on PLGA Microparticles

To determine the encapsulation efficiency (EE) and drug loading capacity (DLC) of RVC•HCl, 10 mg samples of RVC•HCl microparticles were dissolved in.2 mL acetonitrile and.8 mL distilled water. This mixture was agitated using a vortex mixer and centrifuged at 13,000 rpm for 5 minutes to remove PLGA precipitates. The RVC•HCl content in the supernatant was analyzed by high pressure liquid chromatography (HPLC; Agilent 1200 series) with an Agilent Eclipse XDB-C18 column (150 mm $\times$ 4.6 mm, 5 μm) using a mobile phase consisting of acetonitrile/water (20:80) containing.1% trifluoroacetic acid (TFA) and UV detection at 260 nm. EE and DLC of RVC•HCl microparticles were calculated according to the following formula: EE (%) = (weight of RVC•HCl loaded into PLGA microparticles)/(weight of RVC•HCl in the system) $\times$ 100%. DLC (%) = (weight of entrapped drug/weight of all materials in the system) $\times$ 100%.

Ropivacaine Nanoparticle Preparation

The nanoparticles encapsulated with a payload of RVC were formulated via the double-emulsion solvent evaporation technique. In brief, copolymer PLGA-PEG was dissolved in DCM. RVC solution (.5 mL) was added drop-wise into 1 mL of PLGA-PEG solution and emulsified by probe sonification to form the first emulsion. The emulsified mixture was then added into 3 mL of aqueous solution containing 1% PVA, followed by probe sonification to form the double emulsion. The final emulsion solution was poured into 15 mL of water and stirred for 3 hours to allow the DCM solvent to evaporate and the particles to harden. The remaining organic solvent and free molecules were removed by washing the particle solution 3 times using an Amicon Ultra-4 centrifugal filter (MWCO 100 kDa; Millipore). The nanoparticle size and zeta potential were determined using a Zeta-PALS dynamic light-scattering (DLS) detector (15-mW

laser, incident beam of 676 nm; Brookhaven Instruments Corporation). The particle size was characterized as 200 nm using DLS.

Microparticles Size and Surface Morphology Analysis

Microparticles' sizes and zeta potentials were measured using DLS technique on Zetasizer Nano ZS (Malvern, Southborough, MA). Briefly, the particles were suspended in deionized water at a concentration of 1 mg/mL. The mean diameter of the hydrodynamic volume size was confirmed by cumulative analysis. The zeta potential was also measured based on the electrophoretic mobility of the microparticles in aqueous solution, and was confirmed with folded capillary cells. Microparticles' morphologies were assessed by scanning electron microscopy (SEM, LEO 1530 VP). Air-dried microparticles were placed on adhesive carbon tabs mounted on SEM specimen stubs. The specimen stubs were coated with ~5 nm of carbon before examination in the SEM.

In Vitro Drug Release Study

Drug release tests were carried out at constant body temperature (37°C). Briefly, RVC•HCl-loaded microparticles (10 mg) were added to 1 mL of 10 mM phosphate-buffered saline (PBS) and incubated at 37°C by shaking in a Thermomixer (Eppendorf, Germany) at 300 rpm. At predetermined time intervals, the sample was centrifuged at 2,000 rpm for 3 minutes. The supernatant was collected, and the medium was replaced with 1 mL of fresh PBS. The amount of RVC•HCl released into each medium was quantified by HPLC analysis. All experiments were performed in triplicate (n = 3 repeats).

Animal Experiments

Animal Preparation

Male Sprague Dawley rats weighing 200 to 250 g were obtained from Charles River Laboratories (Wilmington, MA). All rats were housed in an animal facility that was kept in a standard 12-hour light/dark cycle, with standard laboratory water and food pellets available ad libitum. Animal experiments were conducted with the approval of the Animal Care and Use Committee at Rutgers-New Jersey Medical School, and consistent with the ethical guidelines of the U.S. National Institutes of Health and the International Association for the Study of Pain. To minimize intra- and interindividual variability of behavioral outcome measures, animals were trained for 1 to 2 days before behavioral testing was performed. The experimenters were blinded to treatment condition during behavioral testing.

Perisciatic Nerve Injection

Perisciatic nerve injections were carried out with a 25-gauge needle attached to a 2 mL syringe under isoflurane-oxygen anesthesia as described.[50] The needle was inserted percutaneously from the posterior aspect of the upper leg and advanced anterolaterally toward the greater trochanter. Once bone was contacted, the drug was injected with total volume of 2 mL.

Incisional Pain Model

The incisional surgery was carried out with minor modification as described.[7] Rats were anesthetized with 2% isoflurane delivered via a nose cone. The plantar aspect of the left hind paw was prepared in a sterile manner with a 10% povidone-iodine solution. A 1-cm longitudinal incision was made with a number 11 blade through skin and fascia of the plantar aspect of the foot, starting .5 cm from the proximal edge of the heel and extending toward the toes. The muscle was elevated and separated bluntly and longitudinally. After hemostasis with gentle pressure, the skin was sutured with 5-0 nylon thread.

Spared Nerve Injury Model

The SNI surgery was carried out with minor modification as described.[14] Rats were anesthetized with 2% isoflurane delivered via a nose cone. A 1 cm skin incision was applied in the longitudinal direction proximal to the posterior knee. The muscle layers were separated to expose the sciatic nerve. The trifurcation of the sciatic nerve was identified, and the common peroneal and tibial branches exposed and ligated with 5-0 silk suture. Nerve segments distal to the ligature approximately 2 mm in length was transected from both branches. Special care was taken to avoid damaging the sural nerve. Sham surgery was carried out in the same manner as described above, except for nerve ligation and transection.

Behavioral Analysis

Mechanical allodynia was tested by measuring paw withdrawal thresholds in response to mechanical stimuli using the up–down testing paradigm as described.[5,10,24,26,63,64] Briefly, rats were placed in Plexiglas chambers on an elevated mesh screen. Calibrated von Frey filaments in log increments of force (.69, 1.20, 2.04, 3.63, 5.50, 8.51, 15.14, and 26.00 g) were applied to the center (for incision) or lateral (for SNI) aspect of the plantar surface of the rats' left and right hind paws. The 2.04-g stimulus was applied first. If a positive response occurred, the next smaller von Frey hair was used; if a negative response was observed, the next larger von Frey hair was used. The test was terminated when 1) a negative response was obtained with the 26.00-g hair or 2) 3 stimuli were applied after the first positive response. Paw withdrawal threshold was determined by converting the pattern of positive and negative responses to the von Frey filament stimulation to a 50% threshold value with a formula provided by Dixon.[9]

Heat hyperalgesia was tested by measuring paw withdrawal latencies to noxious heat with a Model 336 Analgesic Meter (IITC Inc/Life Science Instruments,

Woodland Hills, CA) as described previously.[40,64] Rats were placed in a Plexiglas chamber on a glass plate. Radiant heat was applied by aiming a beam of light through a hole in the light box through the glass plate to the center of the plantar surface of each hind paw. When the animal lifted its foot, the light beam was turned off. The length of time between the start of the light beam and the foot lift was defined as the paw withdrawal latency. Each trial was repeated 5 times at 5-min intervals for each side. A cut-off time of 20 seconds was used to avoid tissue damage to the hind paw.

Cold allodynia was examined by measuring paw withdrawal latencies to noxious cold with a cold plate, which was set at 0°C as described.[40,64] The length of time between the placement of the hind paw on the plate and the animal lifting its hind paw, with or without paw licking and flinching, was defined as the paw withdrawal latency. Each trial was repeated 3 times at 10-min intervals for the paw on the ipsilateral side. A cut-off time of 60 seconds was used to avoid paw tissue damage.

Locomotor Function Test

Tests of locomotor function, including placing, grasping, and righting reflexes, were performed before and after incision surgery or SNI according to the previously described protocol.[37,46,64] 1) *Placing reflex*: The rat was held with hind limbs slightly lower than the forelimbs, and the dorsal surfaces of the hind paws were brought into contact with the edge of a table. Whether the hind paws were placed on the table surface reflexively was recorded; 2) *Grasping reflex*: The rat was placed on a wire grid and whether the hind paws grasped the wire on contact was recorded; 3) *Righting reflex*: The rat's back was placed on a flat surface and whether it immediately assumed the normal upright position was recorded. Scores for placing, grasping, and righting reflexes were based on counts of each normal reflex exhibited in 5 trials. In addition, the animal's general behaviors, including spontaneous activity (eg, walking and running), were observed.

Morphological/Biochemical Experiments

Electron Microscopy

Rats (n =3 per group) from the vehicle-, PLGA-,.25% free RVC-, and.25% SRR-treated groups were anesthetized with isoflurane (for harvesting of the sciatic nerve) and perfused with 300 mL of 2.5% glutaraldehyde in .1 M sodium cacodylate buffer (pH 7.4). After perfusion, sciatic nerves were dissected and postfixed at 4°C for 3 hours. After dehydration by gradient ethanol elution, samples were cleared by propylene, then infiltrated with resin and embedded in molds. Three ultrathin sections (50 nm in thickness) from each rat were collected by grouping every tenth section and counterstained with uranyl acetate and lead citrate. The sections were examined with a Hitachi H-7500 transmission electron microscope (Hitachi, Ltd., Japan). Images from 3 randomized fields per section were taken. A total of 27 images per group were examined.

Immunohistochemistry

Immunohistochemistry was performed as described previously.[47] Three rats from each treated group as indicated above (4 groups; N = 3 rats/group) were anesthetized with isoflurane and perfused with 300 mL of 4% paraformaldehyde in .1 M PBS (pH 7.4). After perfusion, sciatic nerves, or adjacent muscles were dissected, postfixed at 4°C for 4 hours and cryoprotected in 30% sucrose overnight. The transverse or longitudinal sections were cut on a cryostat at a thickness of 20 μm and collected from each tissue by grouping every third sections. The sections were first blocked for 1 hour at 25°C in .01 M PBS containing 5% goat serum and .3% Triton X-100 and then incubated overnight at 4°C with mouse antimyelin basic protein (MBP; RRID: AB_10120129; catalog number: SMI-99P; lot number: 808401. 1:500; BioLegend, San Diego, CA)[17] or rabbit anti-CD68 (RRID: AB_10975465; catalog number: ab125212; lot number: 43780. 1:800; Abcam, Cambridge, MA).[56] The sections were finally incubated with goat antimouse or antirabbit antibody conjugated to Cy2 or Cy3 (1:500; Jackson ImmunoResearch, West Grove, PA) for 2 hours at room temperature. Immunofluorescence-labeled images were randomly taken using a Leica DMI4000 fluorescence microscope with a DFC365FX camera (Leica, Germany). At least 5 sections per rat (15 sections/group) were examined.

TdT-Mediated dUTP Nick End Labeling and Cresyl Violet Histochemical Staining

After rats (3 rats/group) from 4 treatment groups as described above were anesthetized with isoflurane, sciatic nerve, lumbar dorsal root ganglions (DRGs), lumbar spinal cord, and brain cortex were harvested and postfixed in 4% paraformaldehyde. The tissues were cryoprotected in 30% sucrose overnight. Two sets of the sections at thickness of 20 μm were collected from each tissue by grouping every third sections. TdT-Mediated dUTP Nick End Labeling (TUNEL) histochemical staining was performed on one set of sections using an in situ cell death detection kit (Roche Molecular Biochemical, IN). Briefly, the sections were incubated with proteinase K solution (20 μg/mL) for 20 minutes at room temperature, and then with a TUNEL reaction mixture composed of terminal deoxynucleotidyl transferase (TdT) at 37°C in a humidified chamber for 60 minutes. TdT enzyme-incorporated fluorescein was detected with converter-alkaline phosphatase (AP), consisting of sheep antifluorescein antibody conjugated with AP. The signal was detected using nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate as color substrate. Another set of sections was stained with cresyl violet. The sections were rinsed in distilled water and incubated for 30 minutes in a solution of.2% cresyl violet (cresyl violet acetate; Sigma, St. Louis, MO) in acetate buffer, then washed in distilled water, dehydrated through a graded series of ethanol, and cover-slipped. The images were randomly taken using a Nikon microscope (Nikon Eclipse 80i, Japan). At least 5 sections per rat (15 sections/group/test) were examined.

Western Blot Analysis

Protein extraction and Western blot analysis were carried out as described.[25] Briefly, the ipsilateral L4/5 spinal cords on day 12 post-SNI or sham surgery were harvested and homogenized in lysis buffer. After centrifugation at 4°C for 15 minutes at 1,000 g, the supernatant was collected. After protein concentration was measured, equal amounts of total protein from each sample were loaded.[22] The samples were heated at 99°C for 5 minutes and loaded onto a 4 to 15% stacking/7.5% separating SDS–polyacrylamide gel (Bio-Rad Laboratories). The proteins were then electrophoretically transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories). After being blocked with 3% nonfat milk in the Tris-buffered saline containing.1% Tween 20 for 2 hours, the membranes were then incubated at 4°C overnight with the rabbit antiphospho-ERK1/2 (RRID: AB_2315112; catalog number: 4370; lot number: 24. 1:1,000; Cell Signaling Technology), rabbit anti-ERK1/2 (RRID: AB_390779; catalog number: 4695; lot number: 25. 1:1,000, rabbit, Cell Signaling Technology),[23] mouse antiglial fibrillary acidic protein (GFAP; RRID: AB_561049; catalog number: 3670; lot number: 6. 1:1,000; Cell Signaling Technology) or goat anti-GAPDH (RRID: 641107; catalog number: sc-20357; lot number: A0714. 1:3,000; Santa Cruz Biotechnology).[54] The proteins were detected by horseradish peroxidase-conjugated antirabbit, antimouse, or antigoat secondary antibody (1:3,000, Bio-Rad Laboratories), and visualized by chemiluminescence reagent (enhanced chemiluminescence, Bio-Rad Laboratories). Images were generated using ChemiDoc XRS System with Image Lab software (Bio-Rad Laboratories). Band intensities were quantified with densitometry by System with Image Lab software (Bio-Rad Laboratories). Band intensities were normalized to the loading control GAPDH, which has been demonstrated to be stable even after peripheral nerve injury insult.[25,64]

Plasma Samples Analysis

Plasma concentrations of RPV were determined by HPLC (Agilent Technologies 1200 series, California) based on the method reported previously.[2] In brief, blood was withdrawn from a tail vein into heparinized tubes 1 day before.25% free RVC or.25% SRR injection and 10 minutes, 20 minutes, 40 minutes, 2 hours, 4 hours, 1 day, 3 days, 5 days, 7 days, and 14 days after.25% free RVC or.25% SRR injection. Plasma (50 μL) was separated by centrifugation and added to 150 μL acetonitrile to extract the RVC; this was followed by sonication and centrifugation. The supernatant of each sample was analyzed using HPLC equipped with a C18 column (5 μm, 4.6 $\times$ 150 mm) under 220 nm UV wavelength. The mobile phase consisted of water with .1% TFA (solvent A), acetonitrile with.1% TFA (solvent B), and mixed A/B (v/v) at ratio of 30:70, which were used at a flow rate of 1 mL/min. The column thermostat was set at 25°C, and injection volume was set to 10 μL. The concentration of RVC was calculated according to its concentration standard curve ($y = 30{,}014x + 1872.1$, $R^2 = .9927$). The experiments were repeated 3 times in each group (N = 3 repeats/group).

Statistical Analysis

All data were collected using random sampling, and given as means ± SEM. The data were analyzed with 2-

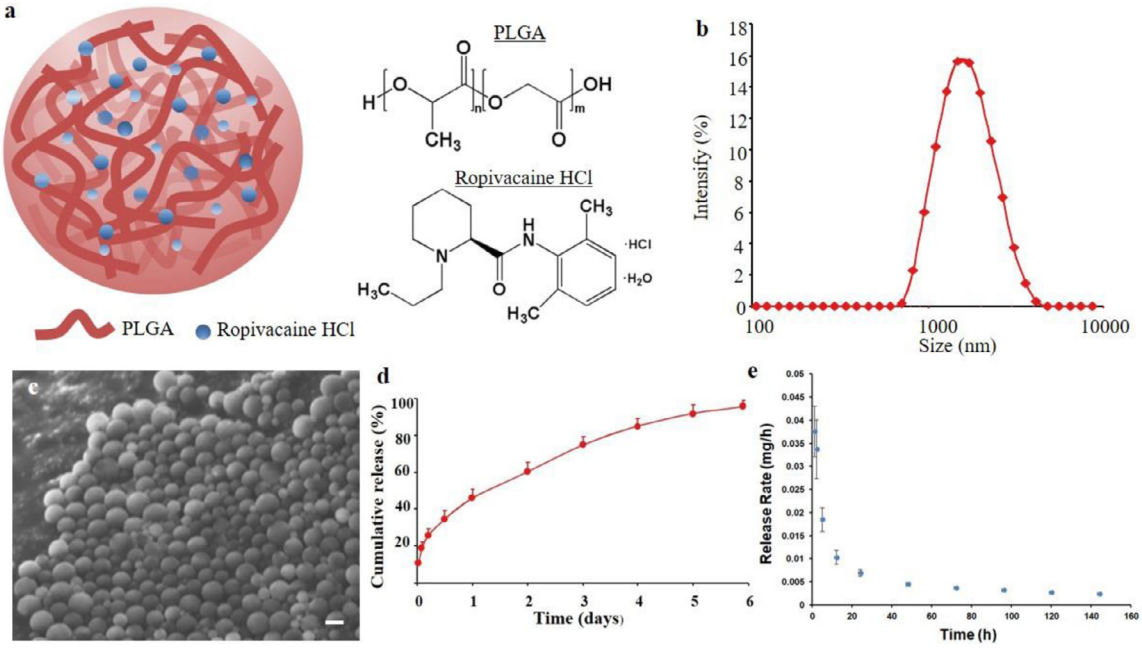

Figure 1. (**A**) Structure model of ropivacaine (RVC)•HCl microparticles. (**B**) Size distribution of PLGA microparticles containing RVC•HCl by dynamic light scattering. (**C**) Representative SEM image of the RVC•HCl microparticles. Scale bar: 2 μm. (**D**) In vitro cumulative release of RVC•HCl from PLGA microparticles. (**E**) In vitro release rate (mg/h) of RVC•HCl from PLGA microparticles. Data are expressed as the mean ± SEM of 3 biological repeats in D and E.

tailed, paired/unpaired Student's t-test and a 1-way or 2-way ANOVA. When ANOVA showed a significant difference, pairwise comparisons between means were tested by the post hoc Tukey method (SigmaStat, San Jose, CA). Locomotor function data were analyzed using Wilcoxon rank-sum test. Significance was set at $P < .05$.

Results

Preparation and Characterization of RVC•HCl Microparticles

In this study, we fabricated the RVC•HCl-encapsulated PLGA microparticles using water-in-oil-in-water double-emulsion solvent evaporation techniques (Fig 1A). The mean diameters of RVC•HCl microparticles were found as 1.7 ± .2 μm (Fig 1B). The RVC•HCl microparticles had a smooth morphology and spherical shape with some deviations and nominal aggregation (Fig 1C). In vitro release of RVC•HCl microparticles showed that the drug payload was released from the particle in a sustained release fashion (Fig 1D). About 25.6% of total RVC•HCl was rapidly released over the first 8 hours, followed by a sustained release after 12 hours. This lasting release of RVC•HCl from the microparticles extended over 6 days, reaching a maximum value of 95.7% thereafter. Consistently, the release rates were high over the first 8 hours, then gradually reduced after 12 hours, and subsequently maintained at persistently low levels for at least 6 days (Fig 1E).

Effect of Perisciatic Nerve Injection of SRR on Postsurgical Pain

We examined whether SRR had long-term analgesic effect when applies to the ipsilateral sciatic nerve after surgery. A unilateral hind paw plantar incision was performed in male rats immediately after perisciatic nerve injection of SRR (.25%,.125%, and 0.06%; dissolved in PBS), free RVC (dissolved in PBS), PLGA alone (dissolved in PBS) or vehicle (PBS). The RVC concentration in each formulation was characterized by HPLC per fixed volume of formulation. Consistent with previous reports,[53] paw incision led to persistent mechanical and heat pain hypersensitivities on the ipsilateral (but not contralateral) side, which occurred at 2 hours postincision, peaked around 4 hours to 1 day postincision, and lasted for 5 to 6 days postincision, in the vehicle-treated group (Fig. 2A and 2B). As expected, the injection of PLGA solution alone did not significantly affect incision-induced mechanical allodynia and heat hyperalgesia during the observation period (Fig. 2A and 2B). The injection of.25% free RVC produced an analgesic effect on incisional pain only within 12 hours postinjection (Fig. 2A and 2B). The injection of SRR at the same dose significantly blocked mechanical allodynia and heat hyperalgesia during the 5 to 6-day incisional pain period (Fig. 2A and 2B). None of.25% SRR, PLGA, free RVC and vehicle altered basal paw withdrawal responses to mechanical and heat stimuli on the contralateral side (Fig. 2C and 2D).

The analgesic effect of SRR was dose-dependent. The injection of.125% SRR produced a similar analgesic effect on incision-induced mechanical allodynia as the injection of.25% SRR, but SRR at the former dose led to an analgesic effect on incision-induced heat hyperalgesia only within 2 days postincision (Fig. 3A and 3B). The injection of.06% SRR did not produce any significant analgesic effects on incision-induced mechanical allodynia and heat hyperalgesia (Fig. 3A and 3B). As expected, neither SRR at doses used nor vehicle changed basal paw withdrawal responses to mechanical and heat stimuli on the contralateral side (Fig. 3C and 3D).

To further examine whether the long-lasting effect of SRR was related to the size of the microparticle, we manufactured different sizes of PLGA microparticles, which were then loaded with distinct concentrations of RVC-HCl. Microparticles larger than 2 μm in size could not be injected through 25-gauge syringe when the concentration of SRR exceeded.125%, a concentration required in clinical practice (Table 1). As expected, microparticles smaller than 2 μm in size could be injected through 25-gauge syringes, except when loaded with the highest concentration of 1.2% SRR (stock solution; Table 1). We also generated nanoparticle-coated RVC. Perisciatic nerve injection of .25% nanoparticle-coated RVC relieved incision-induced heat hyperalgesia, but not incision-induced mechanical allodynia (Fig. 4A and 4B). This analgesic effect occurred at 4 hours postincision and lasted only for 2 days postincision (Fig 3B). Neither nanoparticle-coated RVC, nor free RVC, nor vehicle altered basal paw withdrawal response on the contralateral side (Fig. 4C and 4D).

Effect of Perisciatic Nerve Injection of SRR on Neuropathic Pain

We also examined whether SRR had a long-term analgesic effect on SNI-induced neuropathic pain when injected on the ipsilateral perisciatic nerve. SRR at different concentrations (.25%,.125%, and.06%) or vehicle was injected on day 7 post-SNI. Consistent with a previous report,[44] mechanical allodynia and cold allodynia peaked on day 7 post-SNI and were maintained for at least 2 weeks after SNI on the ipsilateral (but not contralateral) side of the vehicle-treated SNI group (Fig. 5A −5C). Postinjection of SRR alleviated SNI-induced mechanical allodynia and cold allodynia in a dose-dependent manner (Fig. 5A and 5C). The analgesic effects lasted for up to 12 days postinjection on SNI-induced mechanical allodynia, and for up to 7 days postinjection on cold allodynia at the dose of .25% SRR (Fig. 5A and 5C). SRR at the dose of .125% led to analgesic effects on mechanical allodynia for up to 7 days postinjection and on cold allodynia for up to 3 days postinjection (Fig. 5A and 5C). There were no significant differences in SNI-induced decreases in paw withdrawal thresholds and latencies between the .06% SRR-treated SNI group and the vehicle-treated SNI group (Fig. 5A and 5C). As expected, injection of SRR at 3 doses did not change basal responses on the contralateral side

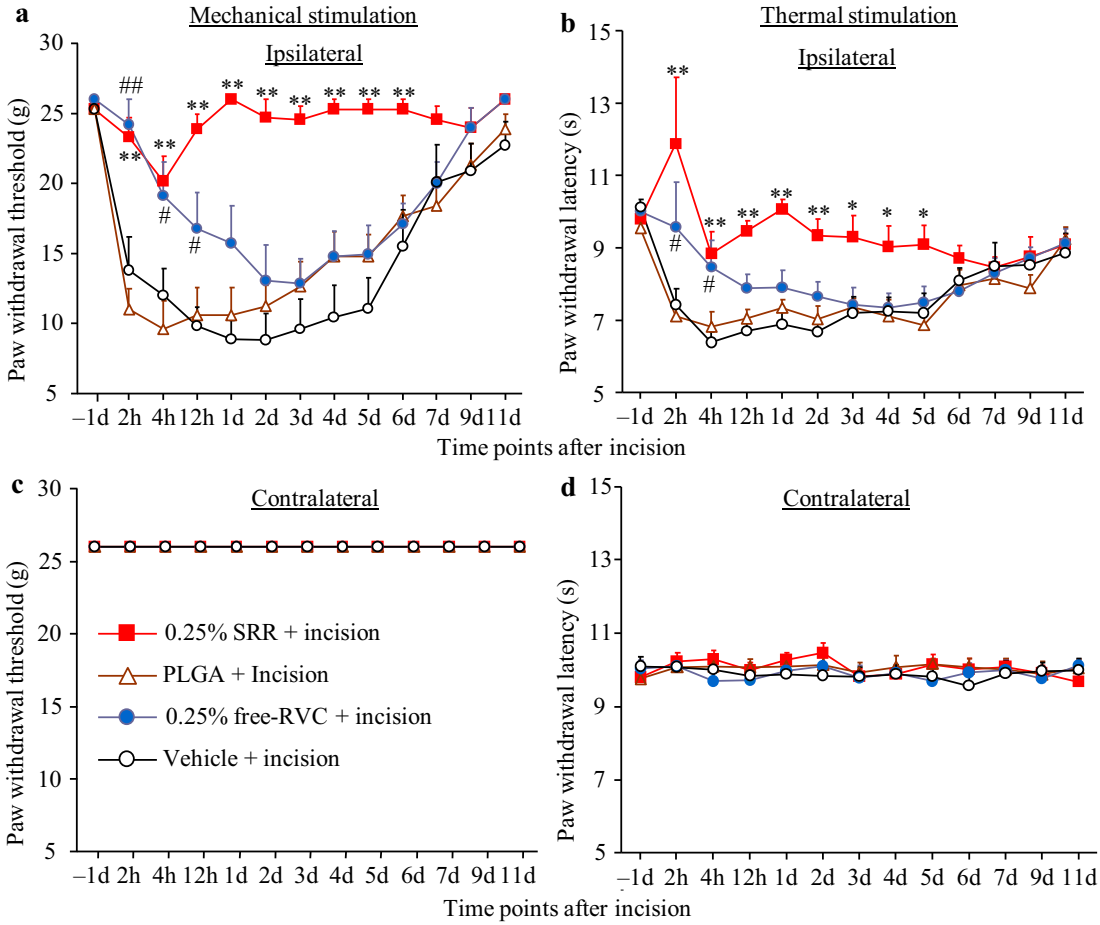

Figure 2. Effect of perisciatic nerve injection of .25% SRR, .25% free ropivacaine (RVC), PLGA, or vehicle on paw withdrawal responses to mechanical (**A** and **C**) and heat (**B** and **D**) stimuli on the ipsilateral (**A** and **B**) and contralateral (**C** and **D**) sides at the different time points as indicated after incision. Data are expressed as the mean ± SEM of 8 rats per group. Two-way ANOVA with repeated measures followed by post hoc Tukey test. **P* < .05 or ***P* < .01 comparison between the SRR-treated incisional group and the vehicle-treated incisional group at the corresponding time point. #*P* < .05 or ##*P* < .01 comparison between the free ropivacaine-treated incisional group and the vehicle-treated incisional group at the corresponding time point.

(Fig 5B). To further define that perisciatic nerve injection of SRR produced local effect, vehicle, .25% free RVC or .25% SRR were injected into perisciatic nerve on the contralateral side on day 7 after SNI or sham surgery (Fig. 5D–5H). As expected, contralateral injection of none of these solutions affected SNI-induced mechanical allodynia, heat hyperalgesia and cold allodynia on the ipsilateral side (Fig. 5D–5F). Contralateral injection of neither .25% SRR nor .25% free RVC altered basal paw withdrawal thresholds in response to mechanical stimulation on the contralateral side during the observation period (Fig 5G), as basal mechanical responses had reached the ceiling effect. However, contralateral injections of .25% SRR and .25% free RVC significantly increased basal paw withdrawal latencies in response to heat stimulation over 9 days and 4 hours, respectively, after perinerve injection on the contralateral side (Fig 5H). Finally, we monitored the plasma concentration of RVC before and after perisciatic nerve injection of .25% SRR or .25% free RVC. Consistent with the previous reports,[4,57] RVC was detected in blood plasma over 4 hours after perinerve injection, but was undetectable after 1 day (Fig 5I). There was no marked difference in the level of plasma RVC between .25% SRR- and .25% free RVC-treated groups (Fig 5I).

We further examined whether SRR affected SNI-induced dorsal horn central sensitization as indicated by increases in phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2) and GFAP in the dorsal horn. In line with previous studies,[25] the levels of p-ERK1/2 (but not total ERK 1/2) and GFAP were significantly increased in the ipsilateral L4/5 dorsal horn of the vehicle-treated SNI rats, but not in the vehicle-treated sham rats, on day 12 post-SNI or sham surgery (that is, 5 days after SRR or vehicle injection; Fig. 6A and 6B). These increases were dramatically blocked in the .25% SRR-treated SNI rats (Fig. 6A and 6B). Injection of .25% SRR did not affect basal expression of p-ERK1/2, ERK1/2, and GFAP in dorsal horn of sham rats (Fig. 6A and 6B).

Safety of SRR

We finally examined the safety of SRR. Locomotor function tests showed that only the scores for grasping reflex were reduced at 2 hours postincision in the .25% SRR-, .125% SRR-, and .25% free RVC-treated incisional

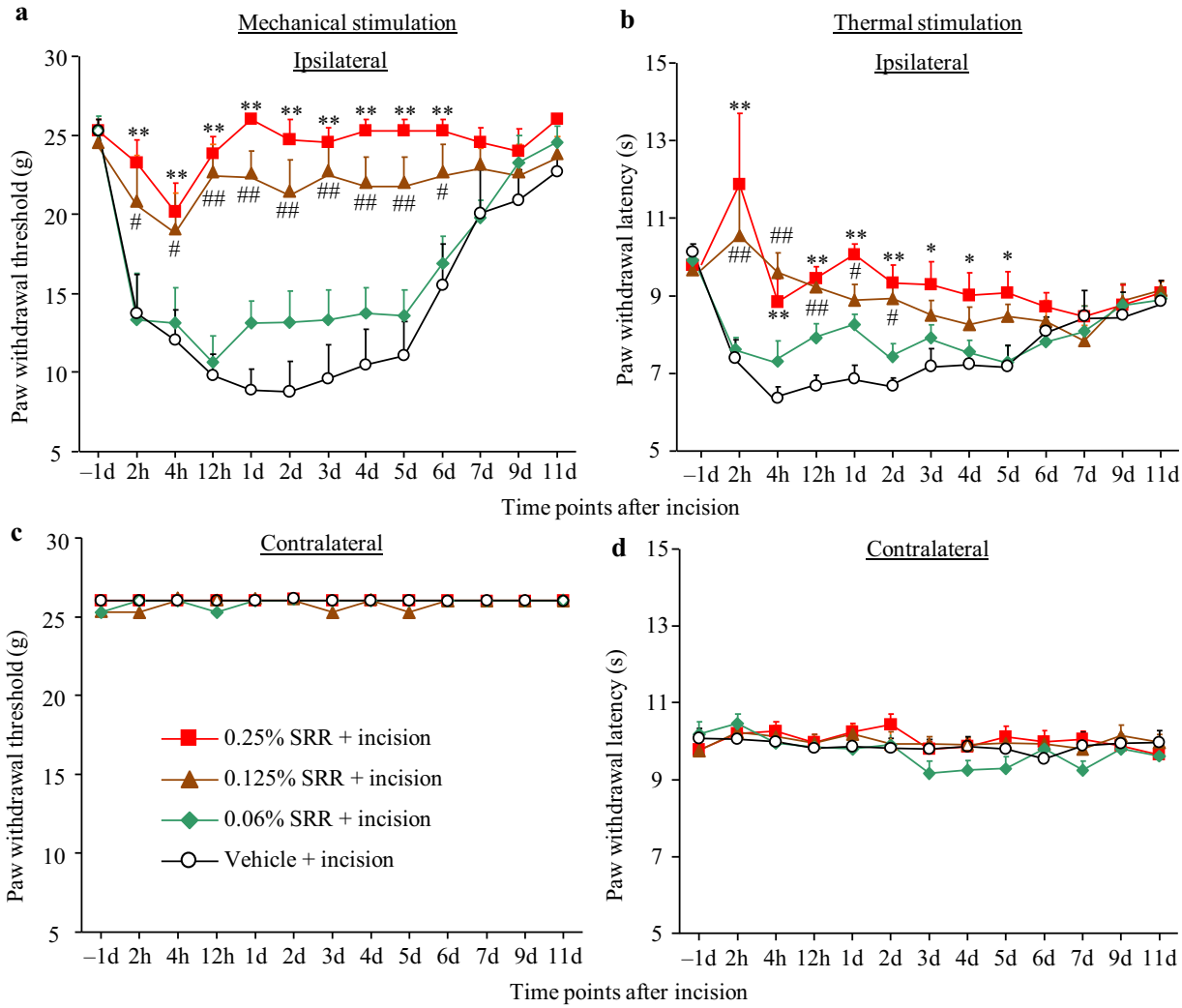

Figure 3. Dose-dependent effect of SRR on incisional pain. The injection of .25% SRR showed stronger analgesic effect than the injection of .125% SRR on both the duration and intensity of incision-induced mechanical allodynia (**A**) and heat hyperalgesia (**B**) on the ipsilateral side. The injection of .06% SRR did not produce significant analgesic effect on incision-induced mechanical allodynia (**A**) and heat hyperalgesia (**B**) on the ipsilateral side. None of the doses of SRR altered basal responses to mechanical (**C**) and thermal (**D**) stimuli on the contralateral side. Data are expressed as the mean ± SEM of 8 rats per group. Two-way ANOVA with repeated measures followed by post hoc Tukey test. *$P < .05$ or **$P < .01$ comparison between the .25% SRR-treated incisional group and the vehicle-treated incisional group at the corresponding time point. #$P < .05$ or ##$P < .01$ comparison between the .125% SRR-treated incisional group and the vehicle-treated incisional group at the corresponding time point.

Table 1. Microparticle Sizes and Drug Concentrations for 25-Gauge Syringe

Drug Concentrations / *Microparticle Size*	*12 ± 2.4 mg/μL (Stock Solution)*	*5 mg/mL*	*2.5 mg/mL*	*1.25 mg/mL*	*.6 mg/mL*
<2 μm	Not injectable	Injectable	Injectable	Injectable	Injectable
>2 μm	Not injectable	Not injectable	Not injectable	Not injectable	Injectable

groups, and 4 hours postincision in the .25% SRR-treated group (Table 2). However, these reductions were limited to the ipsilateral hind paw, and had no statistical significance compared to the vehicle-treated incisional group. The grasping reflex at the remaining time points after incision, and placing and righting reflexes at any time points after incision were normal in all 4 treatment groups (Table 2). In addition, SRR- or vehicle-injected SNI rats displayed normal placing, grasping and righting reflexes on day 8 (1 day postinjection) and 22 (15 days postinjection) after SNI surgery (Table 3). Additionally, except for the first 2 hours postincision in the.25% SRR-,.125% SRR-, and.25% free RVC-treated incisional groups, and 4 hour postincision in the.25% SRR-treated group, we did not observe any marked difference in general behaviors, including the gait and spontaneous activity among the treated groups. None of the treated rats exhibited the injected residuals at

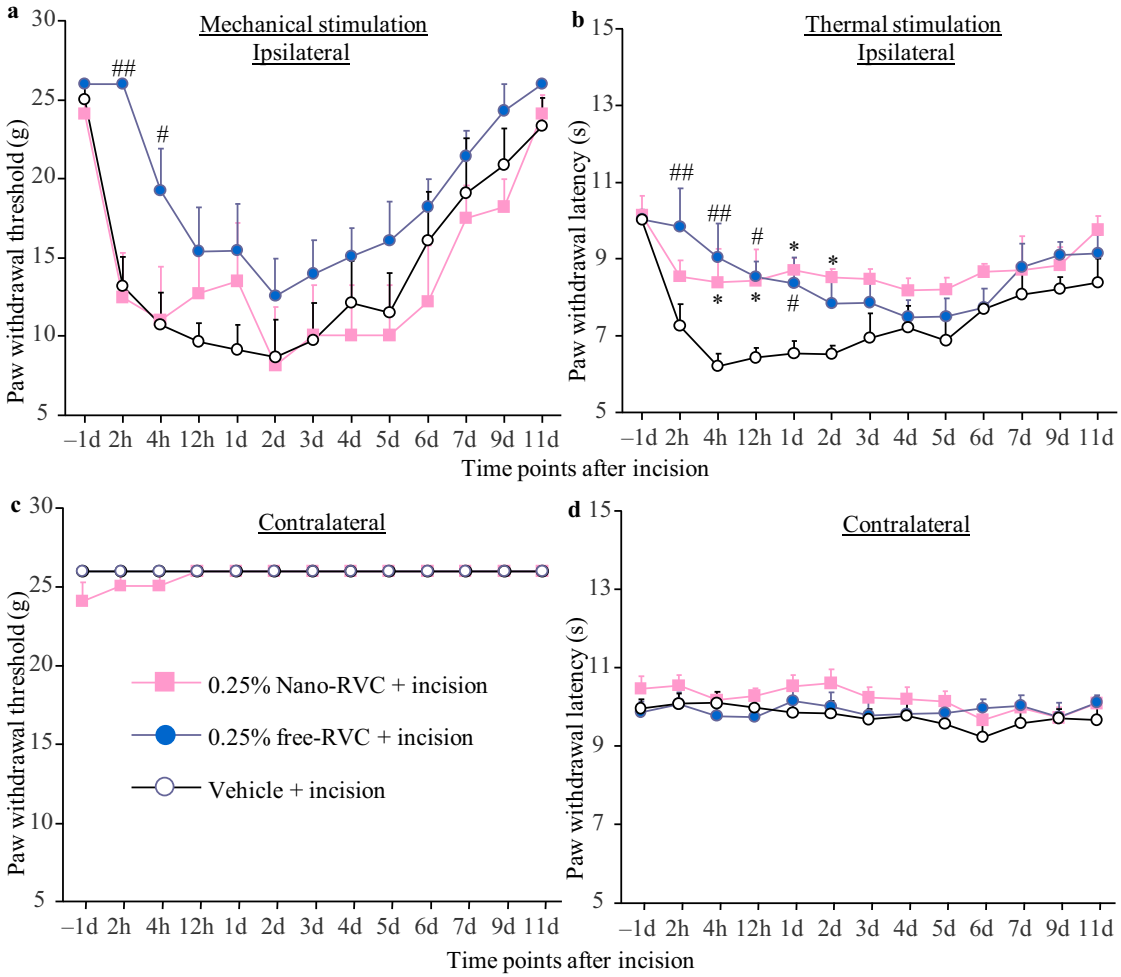

Figure 4. Effect of perisciatic nerve injection of .25% nanoparticle (nano)-coated ropivacaine (RVC), .25% free ropivacaine, or vehicle on paw withdrawal responses to mechanical (**A** and **C**) and heat (**B** and **D**) stimuli on the ipsilateral (**A** and **B**) and contralateral (**C** and **D**) sides at different time points as indicated after incision. Data are expressed as the mean ± SEM of 6 rats per group. Two-way ANOVA with repeated measures followed by post hoc Tukey test. *$P < .05$ comparison between the .25% nanoparticle-coated ropivacaine-treated incisional group and the vehicle-treated incisional group at the corresponding time point. #$P < .05$ or ##$P < .01$ comparison between the .25% free ropivacaine-treated incisional group and the vehicle-treated incisional group at the corresponding time point.

the injection site. Gross examination of surgical sites showed normal wound healing. No prolonged healing or muscle atrophy were found. The hematoxylin and eosin staining showed dense myelinated nerve fibers and fully developed axons in all 4 treatment groups on day 11 after perinerve injection (Fig. 7A–7D). The uniformity and thickness of myelin sheaths were similar among these 4 treatment groups. No swollen and/or shriveled axons were detected in any treated groups (Fig. 7A–7D). To further examine whether .25% SRR or .25% RVC injection damaged myelinated nerve fibers of sciatic nerves, we observed the expression pattern of MBP, a major constituent of the myelin sheath of oligodendrocytes and Schwann cells,[52] in the sciatic nerve at the injected site on day 11 after perinerve injection. The densities and distribution patterns of MBP-like immunoreactivity were similar among these 4 treatment groups (Fig. 7E–7H). Under electron microscopy, the fibers were observed to be myelinated, and the myelin sheaths were dense, uniform and arranged as concentric rings in all 4 treatment groups on day 11 after perinerve injection (Fig. 7I–7L). Specific features characteristic of the degeneration of myelin sheaths, including thinner lamellae, loose lamellae, split myelin lamellae with discontinuities, or vacuole-like inclusions in the cytoplasm of Schwann cells were not observed in these 4 groups (Fig. 7I–7L). Although a very slight separation of myelin sheath layers appears in a few sections in each group, there was no significant difference among 4 groups. To exclude the possibility that long-lasting release of local SRR caused neurotoxicity in peripheral and central nervous systems, we examined neuronal damages in the lumbar DRG, lumbar spinal cord dorsal horn and brain cortex using TUNEL histochemistry. The thymus was used as a positive control since, under normal conditions, it expresses many apoptosis-positive cells.[15] As shown in Fig 8A, many thymus cells were positive for TUNEL. In contrast, no TUNEL-positive cells were observed in the ipsilateral fourth lumbar DRG, fourth lumbar spinal dorsal horn or brain cortex on day 15 after perisciatic nerve injection of .25% SRR (Fig. 8B–8D). To determine whether long-lasting release of local SRR

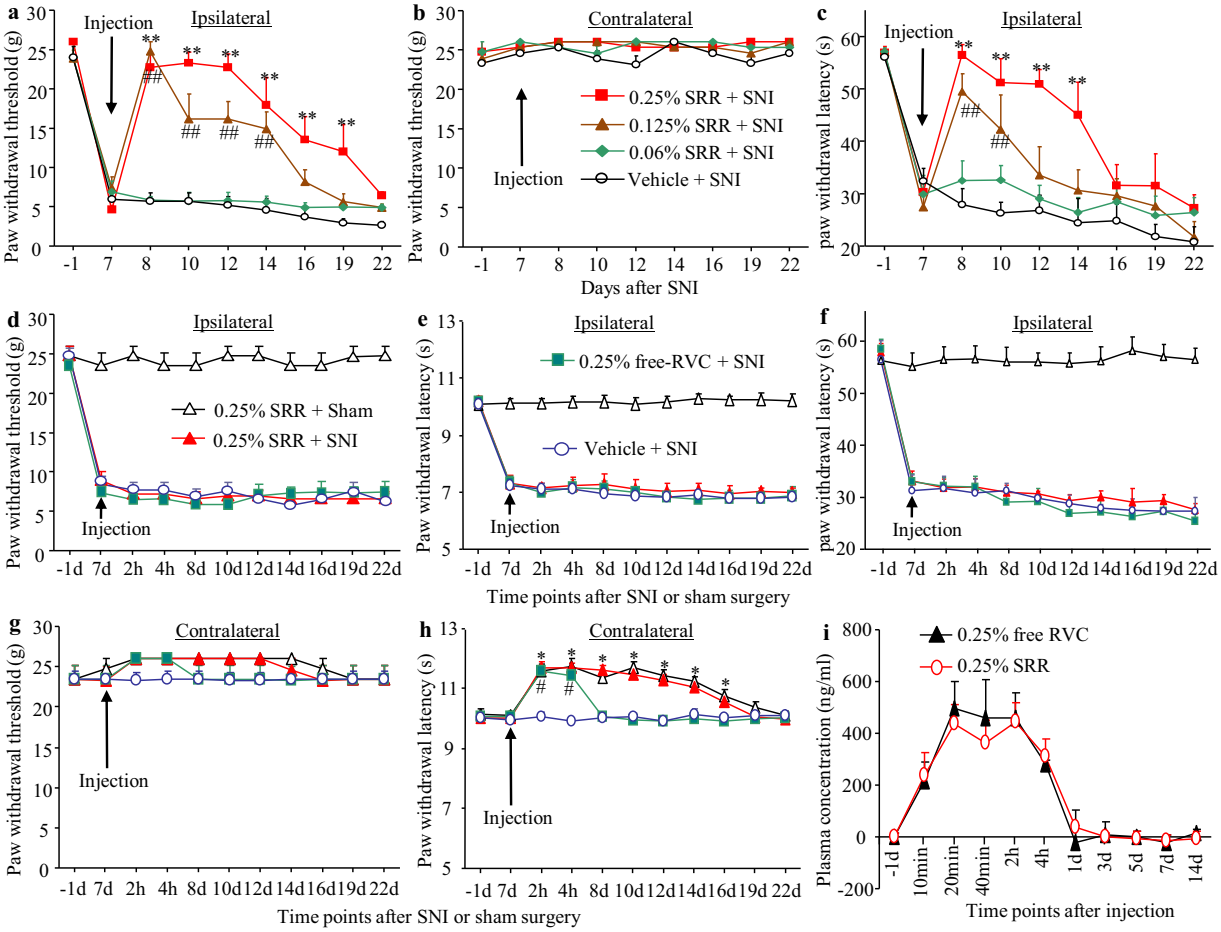

Figure 5. Behavioral responses and mean plasma concentrations of ropivacaine (RVC) after perisciatic nerve injection of SRR in rats with spare nerve injury (SNI) or sham surgery. (**A**–**C**) Effect of ipsilateral perisciatic nerve injection of.06% SRR,.125% SRR,.25% SRR, or vehicle on paw withdrawal responses to mechanical (**A** and **B**) and cold (**C**) stimuli on the ipsilateral (**A** and **C**) and contralateral (**B**) sides at different days as indicated after SNI. Data are expressed as the mean ± SEM of 8 rats per group. Two-way ANOVA with repeated measures followed by post hoc Tukey test. $^{**}P < .01$ comparison between the.25% SRR-treated SNI group and the vehicle-treated SNI group at the corresponding time point. $^{\#\#}P < .01$ comparison between the .125% SRR-treated SNI group and the vehicle-treated SNI group at the corresponding time point. (**D**–**H**) Effect of contralateral perisciatic nerve injection of .25% SRR, .25% free RVC or vehicle on paw withdrawal responses to mechanical (**D** and **G**), heat (**E** and **H**), and cold (**F**) stimuli on the ipsilateral (**D** and **E**) and contralateral (**G** and **H**) sides at different days as indicated after SNI or sham surgery. Data are expressed as the mean ± SEM of 8 rats per group. Two-way ANOVA with repeated measures followed by post hoc Tukey test. $^{*}P < .05$ comparison between the.25% SRR-treated SNI group and the vehicle-treated SNI group at the corresponding time point. $^{\#}P < .05$ comparison between the .25% free RVC-treated SNI group and the vehicle-treated SNI group at the corresponding time point. (**I**) Mean plasma concentrations of RVC at different time points as indicated after perisciatic nerve injection of .25% SRR or .25% free RVC. Data are expressed as the mean ± SEM of 3 biological repeats per group.

produced inflammation of tissue at the injected site, we examined the expression of CD68, a marker of microphages and monocytes,[49] in adjacent muscles at the injected site. Since complete Freund's adjuvant (CFA) produced persistent inflammation,[37] we used the CFA-injected muscles as a positive control. As expected, the muscles injected with 20 μL of 50% CFA displayed abundant expression of CD68 (Fig 8E). However, there was no expression of CD68 in the adjacent muscles on day 15 after perisciatic nerve injection of either .25% SRR or .25% free RVC (Fig 8F and 8G).

Discussion

The present study demonstrated that injectable PLGA-coated RVC produced prolonged analgesic effects on postsurgical pain and neuropathic pain after single-dose local administration without detectable inflammation, tissue irritation, or pathological nerve damage in the targeted nerve and adjacent muscles. Given that PLGA is an FDA-approved biomedical material, and that RVC is used currently in clinical practice, the injectable PLGA-coated RVC is a new and highly promising prolonged LA in the management of persistent pain.

PLGA has been one of the most attractive biodegradable polymers used to fabricate devices for controlled delivery of small drug molecules, therapeutic proteins, nucleic acid-based therapeutics and other macromolecules.[12,45] PLGA is biocompatible and can degrade in the body via hydrolysis of its ester linkages in the presence of water. Importantly, the by-products of the hydrolysis process are its original monomers, lactic acid and glycolic acid, which can be further

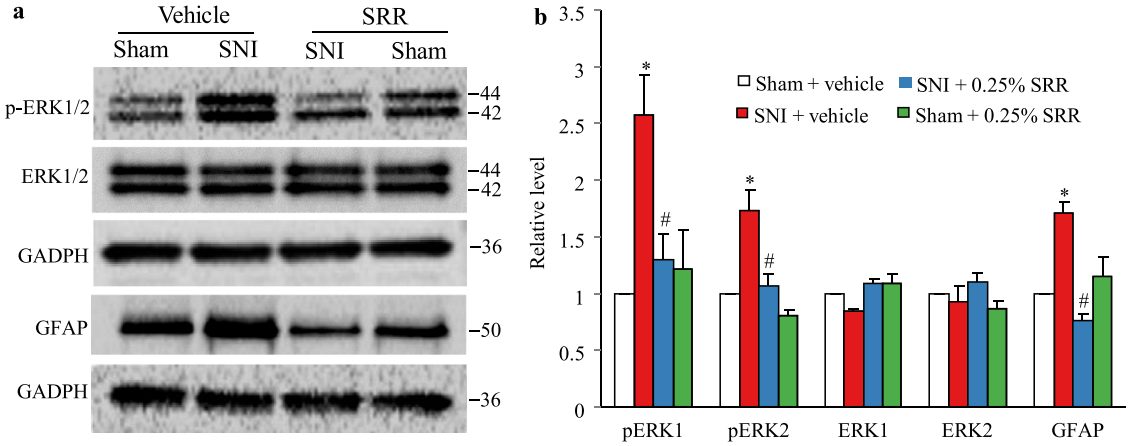

Figure 6. Effect of perisciatic nerve injection of .25% SRR or vehicle on spare nerve injury (SNI)-induced increases in the levels of phosphorylation of extracellular signal-regulated kinase 1/2 (p-ERK1/2) and glial fibrillary acidic protein (GFAP) in the ipsilateral L4/5 spinal cord on day 12 post-SNI or sham surgery (that is, 5 days after SRR or vehicle injection). Representative Western blots (**A**) and a summary of densitometric analysis (**B**) are shown. Data are expressed as the mean ± SEM of 3 biological repeats (6 rats) per group. One-way ANOVA with repeated measures followed by post hoc Tukey test. *$P < .05$ comparison between the vehicle-treated SNI group and the corresponding vehicle-treated sham group. #$P < .05$ comparison between the .25% SRR-treated SNI group and the corresponding vehicle-treated SNI group.

Table 2. Mean (±SD) Changes in Locomotor Function at the Different Time Points After SRR, Free RVC, or Vehicle Injection

	2 h			4 h			12 h			11 d		
Treatments	Placing	Grasping	Righting	Placing	Grasping	Righting	Placing	Grasping	Righting	Placing	Grasping	Righting
.25% SRR + incision	5 (0)	3.75 (.82)	5 (0)	5 (0)	4.75 (.25)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)
.25% SRR + incision	5 (0)	3.75 (.82)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)
.06% SRR + incision	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)
PLGA + incision	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)
.25% RVC + incision	5 (0)	4.38 (.63)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)
Vehicle + incision	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)

N = 5/group; 5 trials; mean (±SD).

Table 3. Mean (±SD) Changes in Locomotor Function at the Different Time Points After SRR or Vehicle Injection

	7 d (Before SRR or Vehicle Injection)			8 d			22 d		
Treatments	Placing	Grasping	Righting	Placing	Grasping	Righting	Placing	Grasping	Righting
.25% SRR + SNI	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)
.125% SRR + SNI	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)
.06% SRR + SNI	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)
Vehicle + SNI	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)	5 (0)

N = 5/group; 5 trials; mean (±SD).

eliminated by the normal metabolic pathways under normal physiological conditions. As such, PLGA has been widely used as an FDA-approved polymer for drug delivery and medical device fabrication.[29] In this study, we fabricated the RVC•HCl-encapsulated PLGA microparticles with average size of 1.7 μm in diameter and found that the in vitro lasting release of RVC•HCl from these microparticles extended over 6 days. We also tried different sizes of microparticle and found out that microparticles larger than 2 μm in size could not be injected through 25-gauge syringes when the concentration of SRR exceeded the .125% that is required in clinical practice. Interestingly, the previous studies generated PLGA-coated bupivacaine microspheres in the range of 45 to 105 μm in size (containing 5, 10, 20, or 40 mg bupivacaine-free base; .4 mL) for injection around the sciatic nerve with a 21-gauge needle, or for subcutaneous injection with a

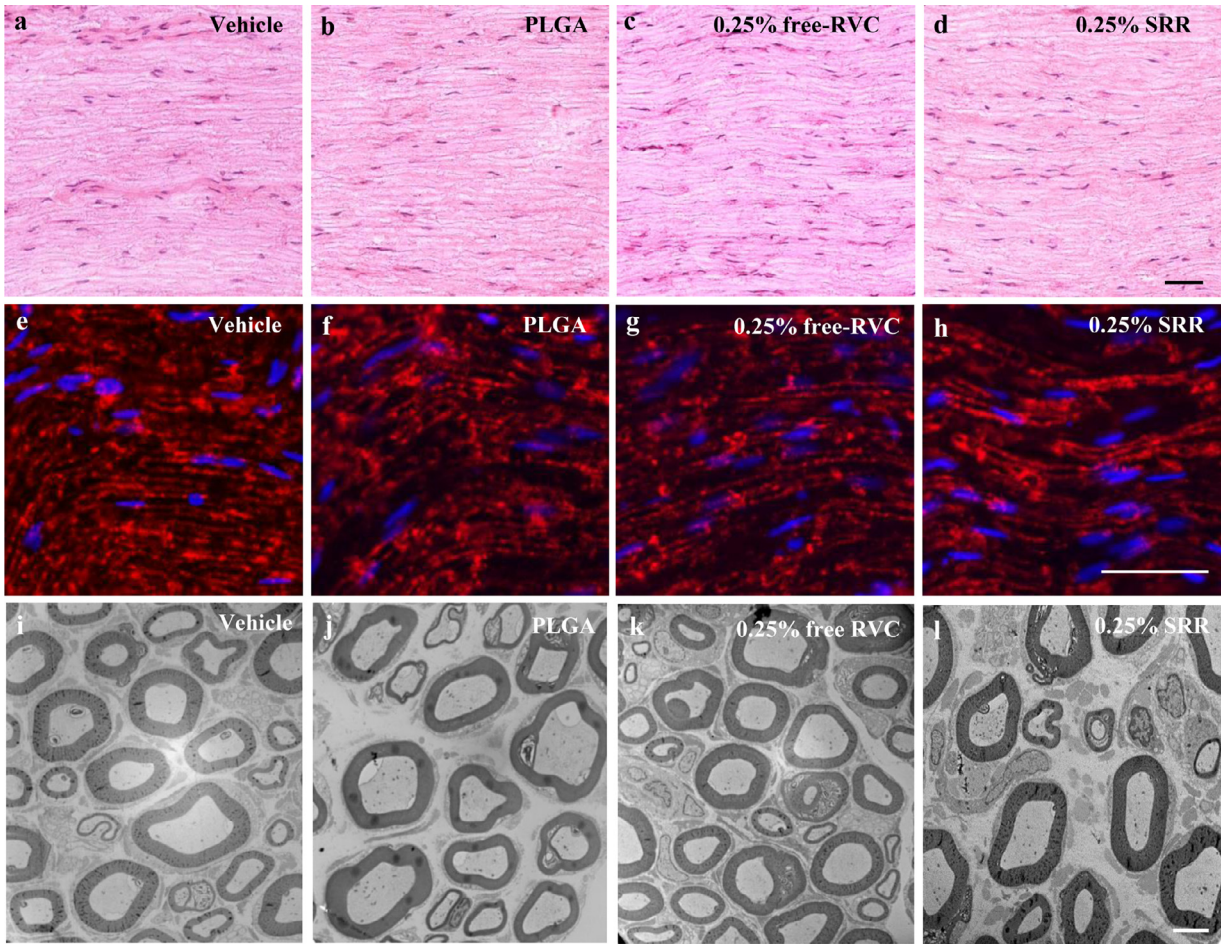

Figure 7. (**A**–**D**) Representative hematoxylin and eosin stained images of sciatic nerves at the injected site on day 11 after the ipsilateral perisciatic nerve injection of vehicle (**A**), PLGA (**B**), .25 free RVC (**C**), or.25% SRR (**D**). n = 3 rats (15 sections)/group. Scale bar: 10 μm. (**E**–**H**) Representative images of MBP-like immunoreactivities in sciatic nerves at the injected site on day 11 after the ipsilateral perisciatic nerve injection of vehicle (**E**), PLGA (**F**), .25 free RVC (**G**), or .25% SRR (**H**). n = 3 rats (15 sections)/group. Scale bar: 50 μm. (**I**–**L**) Representative ultrastructure images of sciatic nerves at the injected site on day 11 after the ipsilateral perisciatic nerve injection of vehicle (**I**), PLGA (**J**), .25 free RVC (**K**), or .25% SRR (**L**). n = 3 rats (9 ultrasections/27 images)/group. Scale bar: 6 μm.

25-gauge needle.[35,36] It is unclear how microspheres larger than 45 μm in size containing the higher concentration of bupivacaine (eg, 10%) were injected subcutaneously with a 25-gauge needle.

Current LA hardly covers the period of persistent postoperative pain, which usually lasts for several days, and neuropathic pain by 1 injection. Repeated LA administration or catheter implantation followed by continuous LA infusion may lead to adverse effects such as infection or tissue damage, and can also present an inconvenience for patients.[39] In addition, although the PLGA-coated bupivacaine microspheres injected around the sciatic nerve or subcutaneously as mentioned above significantly reduced postoperative pain for up to 4 days,[35,36] the toxicity of bupivacaine limited its clinical application. To this end, we examined whether SRR had a long-term analgesic effect on postoperative pain and neuropathic pain when applied to the ipsilateral sciatic nerve. As expected, a single injection of .25% SRR led to a dose-dependent analgesic effect on the development of incisional pain, and on the maintenance of SNI-induced neuropathic pain for at least 6 days. Peripheral nerve injury causes central sensitization indicated by the increases in the levels of p-ERK1/2 and GFAP in the spinal dorsal horn.[19,58] Our results indicate that perisciatic nerve injection of SRR also attenuated SNI-induced increases in the amounts of p-ERK1/2 and GFAP in the dorsal horn. These findings are consistent with a previous observation that perisciatic nerve injection of the sodium channel blocker saxitoxin plus the glucocorticoid agonist dexamethasone decreased the SNI-induced activation of astrocytes in the lumbar dorsal horn of the spinal cord.[44] Our findings indicate that administration of local SRR has a long-lasting antinociceptive effect on neuropathic pain. Although previous studies have reported that LA attenuated the development of neuropathic pain when applied to the injured nerve at the time of nerve injury,[61,62] our study may have more clinical application in patients seeking medical care after nerve injury-induced pain.

Due to the potential for neurotoxicity with administration of LA's, especially at high dosage and when long-lasting effect is desired,[43] and given that exposure to drug delivery materials may also result in tissue

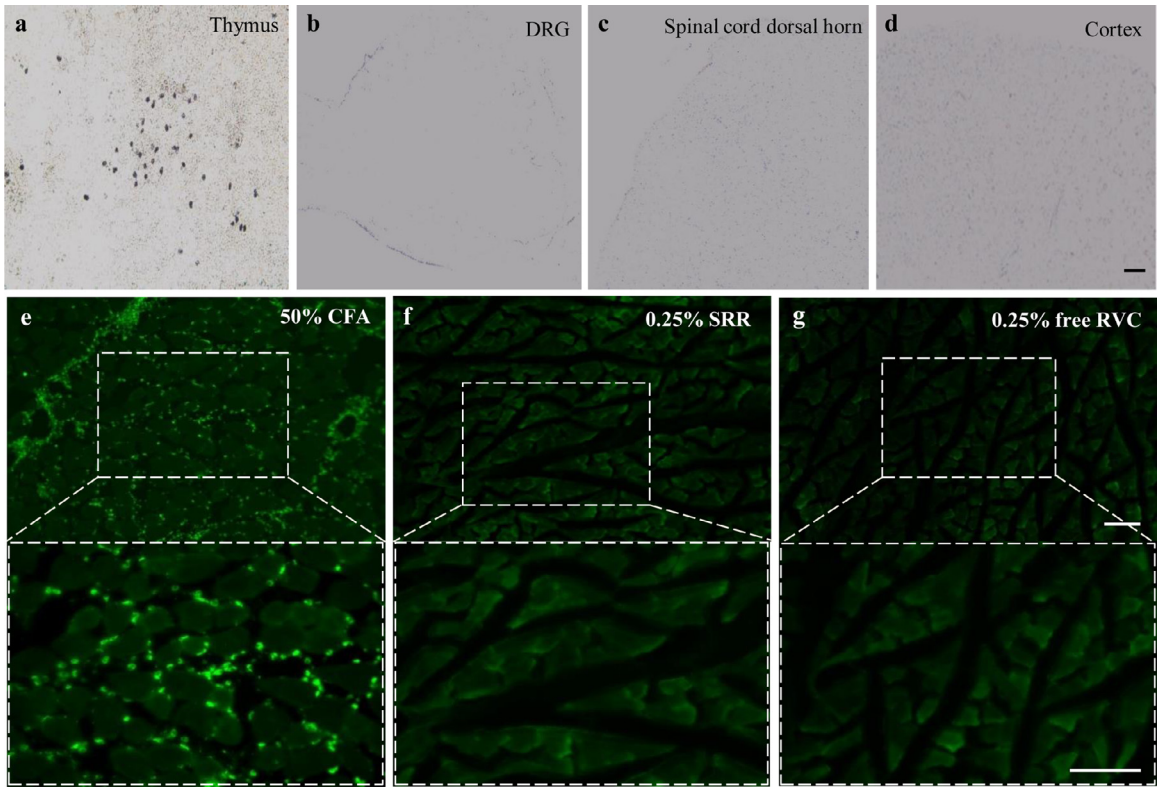

Figure 8. (**A**–**D**) Representative photographs showing TUNEL-positive cells in the thymus (**A**), the fourth lumbar dorsal root ganglion (**B**), the fourth lumbar spinal cord dorsal horn (**C**), and brain cortex (**D**) on the ipsilateral side on day 15 after the perisciatic nerve injection of .25% SRR. n = 3 rats (15 sections)/group. Scale bar: 100 μm. (**E**–**G**) Representative images of CD68-like immunoreactivities in the muscles on day 15 after 50% complete Freund's adjuvant (CFA) injection (**E**) and at the injected site after perisciatic nerve injection of .25% SRR (**F**) or .25% free RVC (**G**). n = 3 rats (15 sections)/group. Scale bar: 10 μm.

reaction and toxicity,[33] we examined the safety of SRR by observing the locomotor function, the morphology of sciatic nerve and surrounding muscle at the injected site, and cell damage in peripheral and central nervous systems. In our study, only transient abnormal grasping reflex was observed, which may be attributed to the effect of RVC at higher dosage on motor nerve function.[59] Normal nerve fibers, fully developed axons stained via hematoxylin and eosin, and similar expression of MBP and ultrastructure of nerve fibers in sciatic nerves were seen among all groups. There was no detectable cell damage in the DRG, dorsal horn and brain cortex after local injection of SRR. In addition, gross inflammation was absent in sciatic nerve and adjacent muscles at the injection site. Although prolonged nerve block may alter the process of wound healing and result in muscle atrophy in patients,[6,51] neither muscle atrophy nor the variation in the process of wound healing were observed among all treatment groups. Consistent with previous reports,[4, 57] RVC in blood plasma was transiently detected after local injection of SRR. We found that this detectable level of systemic RVC was too low to affect SNI-induced pain hypersensitivity and basal behavioral responses. Whether this detectable level of systemic RVC affects heart function remains to be further examined. Taken together, our findings suggest that the PLGA-coated RVC is biologically safe and well tolerated by the body.

Several controlled-release LAs have been developed in recent years.[41] Exparel is the only one that has been applied in clinical practice.[3,34] Exparel provides prolonged postsurgical analgesia for up to 3 days with a single-dose local administration at the surgical site in patients.[3,34] However, its 3-day analgesic effect still did not completely cover the total period of postsurgical pain. Rats receiving injections with.6 mL of Exparel (1.31% bupivacaine HCl) at the sciatic nerve exhibited maximal sensory blockage for up to 240 minutes, which was only slightly longer than 1.31% free-bupivacaine HCl (210 minutes).[32] Compared to Exparel, SRR used in the present study produced much longer-lasting analgesic effect for up to at least 6 days with a single-dose administered via local perisciatic nerve injection. Although our findings were not exactly comparable to the previous study,[32] we may conclude that SRR has the potential for a longer analgesic duration than Exparel. The analgesic dosages of SRR used in the present study did not exceed clinical dose and concentration of RVC. No evidence of active inflammation, tissue irritation, and pathological nerve changes was detected in the sciatic nerve and adjacent muscles at the injected site, indicating good tolerability and safety of the in-situ application of this drug. In addition, the PLGA-coated RVC can be drained into powder, making it easier to transport and providing a long preservation time (up to years), whereas Exparel has the limitation of short

preservation time (up to 30 days) according to its prescribing information. In addition, FDA-approved PLGA along with its by-products are biodegradable under proper conditions, and can be well tolerated by the body.

In conclusion, although present findings are based on male rat models, our research demonstrated that injectable PLGA-coated RVC can have the exact characteristics desired by physicians for application into their clinical practice: easy to use, achieving long-lasting analgesia, and providing a high margin of safety with minimization of complications and side-effects. Given that females have shown greater prevalence of chronic pain conditions compared with males,[1,11,28] the effect of the injectable PLGA-coated RVC on incisional/neuropathic pain in female rats will be further examined. In addition, gross and histological safety in large numbers of rodents and nonrodent species remains to be further confirmed before clinical trials can be undertaken. Even so, our findings provide a new and highly promising avenue in the management of postsurgical pain and neuropathic pain.

Authors' Contributions

Y.X.T. and X.X. conceived the project and supervised all experiments. X.T., H.Z., S.D., F.L., Z.L., Y.F., X.X., and Y.X.T. designed the experiments. X.T., H.Z., S.D., F.L., F. J., and Z.L. conducted the experiments. X.T., H.Z., S.D., Z.L., K.T., X.X., and Y.X.T. analyzed the data. X.X., X.T., H.Z., X.Q.Z., K.T., Y.H.T., S.H., and Y.X.T. interpreted the data. X.T., H.Z., X.X., and Y.X.T. wrote the manuscript. All authors read and discussed the manuscript.

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