

# Preclinical Evaluation of Ropivacaine in 2 Liposomal Modified Systems

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**BACKGROUND:** Our research group has recently developed liposomes with ionic gradient and in a combined manner as donor and acceptor vesicles containing ropivacaine (RVC; at 2% or 0.75%). Looking for applications of such novel formulations for postoperative pain control, we evaluated the duration of anesthesia, pharmacokinetics, and tissue reaction evoked by these new RVC formulations.

**METHODS:** The formulations used in this study were large multivesicular vesicle (LMVV) containing sodium acetate buffer at pH 5.5 or in a combined manner with LMVV as donor and large unilamellar vesicles (LUVs) as acceptor vesicles with an external pH of 7.4. Wistar rats were divided into 6 groups (n = 6) and received sciatic nerve block (0.4 mL) with 6 formulations of RVC (LMVV<sub>RVC0.75%</sub>, LMVV/LUV<sub>RVC0.75%</sub>, LMVV<sub>RVC2%</sub>, LMVV/LUV<sub>RVC2%</sub>, RVC 0.75%, and RVC 2%). To verify the anesthetic effect, the animals were submitted to the pain pressure test and the motor block was also monitored. Histopathology of the tissues surrounding the sciatic nerve region was also assessed 2 and 7 days after treatment. Rats (n = 6) were submitted to a hind paw incision, and mechanical hypersensitivity was measured via the withdrawal response using von Frey filaments after injection of the 6 formulations. Finally, New Zealand white rabbits (n = 6) received sciatic nerve block (3 mL) with 1 of the 6 formulations of RVC. Blood samples were collected predoze (0 minutes) and at 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, 480, and 540 minutes after injection. RVC plasma levels were determined using a triple-stage quadrupole mass spectrometer.

**RESULTS:** Duration and intensity of the sensory block were longer with all liposomal formulations, when compared to the plain RVC solution ( $P < .05$ ). Histopathological evaluation showed greater toxicity for the positive control (lidocaine 10%), when compared to all formulations ( $P < .05$ ). After the hind paw incision, all animals presented postincisional hypersensitivity and liposomal formulations showed longer analgesia ( $P < .05$ ). LMVV<sub>RVC0.75%</sub> presented higher time to reach maximum concentration and mean residence time than the remaining formulations with RVC 0.75% ( $P < .05$ ), so LMVV was able to reduce systemic exposure of RVC due to slow release from this liposomal system.

**CONCLUSIONS:** All new liposomal formulations containing 0.75% RVC were able to change the pharmacokinetics and enhance anesthesia duration due to slow release of RVC from liposomes without inducing significant toxic effects to local tissues. (Anesth Analg XXX;XXX:00–00)

## KEY POINTS

- **Question:** Can liposomes change the pharmacokinetics and anesthesia of ropivacaine (RVC) in vivo?
- **Findings:** These novel RVC liposomal formulations altered pharmacokinetics and enhanced the duration of anesthesia.
- **Meaning:** Liposomal RVC can be an interesting tool for pain control during trans- and postoperative periods.

The unsatisfactory treatment of postoperative acute pain has been accepted as an important issue in health care.<sup>1</sup> Researchers have estimated that only 1

in 4 surgical patients in the United States received adequate pain relief.<sup>2</sup> Despite the large diversity of treatments and side effect profiles associated with such treatments, mismanagement of postoperative pain is frequently associated with several deleterious consequences, including excessive analgesic use.<sup>3,4</sup>

Opioids are still the basis for the treatment of postoperative pain, despite their potential side effects and shortcomings. Another possibility is a multimodal technique based on >1 class of analgesic drugs to improve analgesia and reduce opioid-induced side effects. Despite the advantages of multimodal analgesia, further evidence is needed to prove that using various analgesics is better than single-drug regimens.<sup>1</sup>

One promising strategy would be the use of local anesthetics administered via multiple routes, such as neuraxial regional block as well as peripheral regional anesthesia.<sup>5,6</sup>

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Single-injection, peripheral nerve block with local anesthetics provided better pain control and fewer side effects when compared to opioids<sup>4</sup> enhancing early postoperative physical rehabilitation particularly after orthopedic surgery.<sup>1,2,6</sup> Nevertheless, due to their low molecular weight, local anesthetics are short lasting and larger doses are often associated with local and systemic toxicity.<sup>7,8</sup>

Drug delivery systems can prolong local anesthetic action or allow equivalent analgesia with lower doses as a result of slow drug release at the site of injection.<sup>5,7</sup> An extended-release bupivacaine formulation, Exparel (Pacira Pharmaceuticals, Inc, San Diego, CA), is commercially available in United States, and various authors described that the use of a liposomal-based delivery system was more effective to decrease postoperative pain when compared to free bupivacaine.<sup>9-11</sup> Moreover, this new formulation also decreased the need for opioids.<sup>9</sup> Despite the aforementioned advantages, the analgesic efficacy and safety profile of such ionic-gradient liposomal bupivacaine has not been thoroughly studied after perineural, intrathecal, and epidural administration.<sup>4</sup>

Recently, our research group has developed liposomes prepared with ionic gradient and in a combined manner as donor and acceptor liposomes for encapsulation of ropivacaine (RVC).<sup>12,13</sup> Large multivesicular vesicle (LMVV) liposomes showed approximately 80% of RVC encapsulation efficiency and *in vitro* release of RVC for 25 hours.<sup>12</sup> The second type of liposomes is a combined donor (LMVV) and acceptor (large unilamellar vesicle [LUV]) vesicles. Donor liposomes were LMVVs, containing ammonium sulfate and RVC in their inner aqueous core. Acceptor vesicles were LUVs, prepared with an internal (acetate buffer) pH 5.0 gradient. *In vitro* assays showed sustained release for RVC 2% in this system for up to 72 hours producing analgesia for approximately 9 hours in mice.<sup>13</sup>

Considering that postoperative pain control is still a challenge, and RVC is a safe option for the management of acute pain,<sup>8,13</sup> our study was designed to evaluate a new strategy for postoperative pain control in animal models. We evaluated, *in vivo*, whether such ionic-gradient liposomal formulations can provide the sustained release and prolonged analgesia required for effective pain control. Preclinical pharmacokinetics and pharmacodynamics (effectiveness) evaluation of RVC encapsulated in ionic-gradient liposomal systems was performed in animal models. Also, to verify the safety of these formulations, *in vivo* and *in vitro* toxicity was investigated using histological evaluation of the injection site and Schwann cultured cells, respectively.

## METHODS

### Preparation of Liposomal RVC Formulations

Dry lipid films composed of egg phosphatidylcholine/cholesterol/ $\alpha$ -tocopherol (4:3:0.07 molar ratio) were obtained after solvent evaporation under a flow of nitrogen and vacuum for  $\geq 2$  hours. Film hydration was performed with 50 mM sodium acetate buffer at pH 5.5 producing large multilamellar vesicles. These vesicles were first extruded 12 times through a 100-nm polycarbonate membrane, at room temperature; then, the extruded vesicles were submitted to 10 freeze-thaw agitation cycles (using liquid nitrogen and a thermostatic bath at 37°C) to form LMVVs, with a 15-mM lipid concentration.<sup>12,14</sup> Active incorporation of the anesthetic was

achieved by incubating the liposomes overnight with 0.75 or 2% RVC, under gentle agitation, at room temperature.<sup>12</sup>

Two other types of ionic-gradient liposomes produced from hydrogenated soy phosphatidylcholine and cholesterol (2:1 mol %) were prepared. Dry lipid films were prepared under nitrogen flow, followed by vacuum for 2 hours. LMVVs were produced by hydration of the lipid film with 250 mM ammonium sulfate, while LUVs were prepared with acetate buffer at pH 5.5 inside. LMVV (donor) and LUV (acceptor) liposomes were combined. The final formulations had 15 mM total lipids and 0.75% (LMVV/LUV<sub>RVC0.75%</sub>) or 2% RVC (LMVV/LUV<sub>RVC2%</sub>).<sup>13</sup>

To create the ionic gradients, the 3 types of liposomes were ultracentrifuged twice (at 120,000g for 2 hours at 4°C) and the pellet was suspended in 50 mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) buffer pH 7.4 (external pH).

Therefore, we used in this study 2 kinds of ionic-gradient liposomes with 2 RVC concentrations (0.75% and 2%). The first kind was composed by LMVVs and the second type by the combination of LMVV (donor) and LUV (acceptor) liposomes. Thus, the liposomal formulations used in this study were LMVV<sub>RVC0.75%</sub>; LMVV<sub>RVC2%</sub>; LMVV/LUV<sub>RVC0.75%</sub>; and LMVV/LUV<sub>RVC2%</sub>. Control groups were also included as follows: RVC 0.75% and RVC 2% (aqueous solutions) and LMVV and LMVV/LUV liposomes (without drug).

RVC (attested purity of 98.5%) was donated by Cristália Produtos Químicos Farmacêuticos Ltda (Itapira, Brazil). Egg phosphatidylcholine and hydrogenated soy phosphatidylcholine were purchased from Avanti Lipids Inc (Alabaster, AL). Cholesterol, HEPES, perchloric acid, sodium acetate, ammonium sulfate,  $\alpha$ -tocopherol, and uranyl acetate were purchased from Sigma-Aldrich Co (St Louis, MO).

### Cell Culture and Viability Assay: Sciatic Nerve Schwann Cells

The neuronal Schwann cell line was obtained from sciatic nerve samples from Wistar rats UNIB at 6 weeks of age. The explant of nerves was removed under aseptic conditions, and the epineurium and adjacent neuronal tissues were dissected according to the methods previously described.<sup>15</sup> The neuronal cell phenotype was confirmed via immunofluorescence using the antibodies antivimentin (1:300; Dako, Glostrup, DN) and anti-S100 (1:300; Dako, Glostrup, DN) which were positive, and by negativity for the epithelial cell marker AE1/AE3 (1:75; Dako, Glostrup, DN).

Cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO) supplemented with 1% antimycotic-antibiotic solution, containing 10% donor calf serum (Gibco, Buffalo, NY), plated onto 60-mm diameter plastic culture dishes (15  $\times$  10<sup>4</sup> per well), and incubated under standard cell culture conditions (37°C, 100% humidity, 95% air, and 5% carbon dioxide) according to the protocol for culture of this cell lineage.<sup>15</sup> After cellular semiconfluence was obtained, the purified Schwann neuronal cell lines were incubated for 2 and 4 hours, with the 2 vehicles (LMVV and LMVV/LUV) at 5 mM or with plain and the 2 types of liposomal RVC at 6 different concentrations RVC concentrations (0.8, 3.2, 6.4, 8.0, 12.0, and 16.0 mM). Similarly, untreated cells were used as control group. After treatment, the growth medium was replaced by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl

tetrazolium bromide (MTT, Sigma) solution at 10% in culture medium (5 mg/mL) at 37°C for 4 hours. The MTT solution was then discarded by aspiration, and 200  $\mu$ L of dimethyl sulfoxide (Sigma) was added to each well followed by gentle agitation for 5 minutes on a plate shaker to dissolve the formazan crystal. Subsequently, 150  $\mu$ L from each sample solution was transferred onto a fresh 96-well plate. The formazan absorbance was assessed at 570 nm on a spectrophotometer (Epoch; BioTek, Winooski, VT). Mitochondrial dehydrogenase activity was determined by measuring the MTT converted to formazan ( $n = 6$  replicates in 3 experiments for each formulation, plain liposome or RVC in the 2 different periods of treatment—2 and 4 hours), and data were expressed as optical density (at 570 nm) after the different treatments.

### Animals Used in the In Vivo Studies

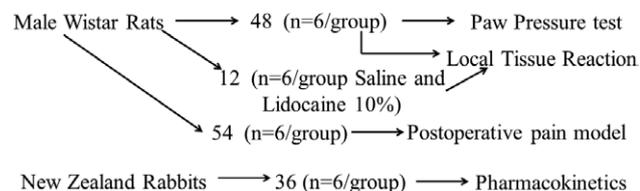
The animals used in this study were male Wistar rats (Unib:WH) (250–350 g) and New Zealand White rabbits (2.5–3.0 kg). The experimental protocols with rats were approved by the Institutional Committee for Ethics in Animal Research of University of Campinas (protocol No. n.4033-1). The experimental protocol in rabbits was approved by the Institutional Committee for Ethics in Animal Research of São Francisco University (protocol No. n. 002.04.2016). Animals were housed 5 per cage (rats) or 1 per cage (rabbits) and received water and food ad libitum with a 12:12-hour light–dark cycle at  $23 \pm 2^\circ\text{C}$ . Before experimentation, the animals were handled for 7 days for acclimatization with their accommodation site, equipment, and the researchers. The animals were divided into groups according to the flowchart represented in Figure 1.

### Sciatic Nerve Block in Rats: Paw Withdrawal Threshold to Pressure

Forty-eight Wistar rats (300–350 g) were used to perform the paw pressure test using an analgesiometer (Ugo Basile, Varese, Italy) to determine sensory block. This device applies increasing pressure (in grams) to the paw, and the paw withdrawal threshold to pressure (PWTP) was used to measure the withdrawal reflex.

The animals were divided into 6 groups ( $n = 6$ ) and received 0.4 mL at the sciatic nerve<sup>16,17</sup> of 1 of the 6 formulations as described: LMVV<sub>RVC0.75%</sub>, LMVV<sub>RVC2%</sub>, LMVV/LUV<sub>RVC 0.75%</sub>, LMVV/LUV<sub>RVC2%</sub>, RVC 0.75%, and RVC 2%. We also used the 2 types of liposomes LMVV and LMVV/LUV ( $n = 6$ ) as control groups, that is, without RVC.

The injected volume was equivalent to a 5.6 mg/kg RVC dosage.<sup>18</sup> The pain threshold was determined before the injections of the formulations or control preparations. Once the injections were administered, measurements were performed at 15-minute intervals until there was no difference between the test and control groups.<sup>19</sup>



**Figure 1.** Flow chart of animal distribution across the tests.

Motor block was assessed based on the loss of motor control in the injected limb according to the following scores: 0 (normal movement), 1 (unable to flex the limb completely), and 2 (unable to use the limb).<sup>20</sup> The efficacy of motor block was evaluated every minute, from 1 to 5 minutes, and every 10 minutes thereafter until full recovery. Total effect was evaluated as time for motor function recovery (area under the effect curve versus time). Latency (time between injection and loss of motor function) and time to reach the maximum score were also assessed.<sup>19</sup>

### Local Toxicity and Histological Evaluation

The same 48 animals that were submitted to the PWTP test were euthanized under anesthesia (urethane 1 g/kg and  $\alpha$ -chloralose 50 mg/kg) after 2 or 7 days of the injections ( $n = 3$ ). Further 12 animals received Lidocaine 10% or saline solution as control groups and were also euthanized after 2 and 7 days after injections ( $n = 3$ ). The soft tissue surrounding the sciatic nerve was removed, and the samples were prepared to obtain 5 cross-sections (5  $\mu$ m thick, 40  $\mu$ m deep) stained with hematoxylin and eosin.<sup>21</sup>

The cross-sections were submitted to qualitative analysis by a blinded subject using a specific scoring system to evaluate the intensity of the leucocytic infiltration and/or any area of necrosis. The cross-sections were photographed using a photomicroscope, and the analyzed region was the site of the injection, which included the soft tissue surrounding the sciatic nerve. The score of local tissue inflammation was defined based on the following descriptions: (1) <25% of the total area presented no infiltrate, injury, or necrosis (mild inflammation); (2) 25%–50% of the evaluated area presented inflammatory infiltrate, injury, or necrosis (moderate inflammation); (3) >50% of the area was injured or presented necrosis areas (severe inflammation).

### Postoperative Pain Model in Rats

Fifty-four male Wistar rats (300–350 g) divided into 9 groups were placed in clear plastic cages with an elevated wire mesh floor and the basal withdrawal responses to the Von Frey aesthesiometer (Ugo Basile) were recorded. The withdrawal response consists in the elevation of the paw caused by probing the plantar region with increasing pressure measured in grams. Anesthesia was then induced with sevoflurane,<sup>16</sup> and animals ( $n = 6$ ) received the same formulations or controls (including 1 group that received saline) at the sciatic nerve (0.4 mL) as described earlier.

A paw incision was performed through skin, fascia, and muscle at the most lateral plantar aspect of the hind paw.<sup>22</sup> The wound was closed with a 5-0 nylon suture. The animals stayed in their cages for 1 hour for recovery. Mechanical hypersensitivity was assessed every 10 minutes, for the first hour, and then every 30 minutes until the basal values were achieved. The absence of withdrawal response with a stimulus equal or more intense than the basal levels was considered successful anesthesia and was used to determine the duration of anesthesia.<sup>16,23</sup> To estimate the total analgesic effect<sup>23</sup> of each individual formulation, a graph of the tolerated stimulus versus time was generated and the area under the curve (AUC) was calculated by trapezoidal approximation, beginning at time zero up to the last time at which the tolerated stimulus was above the basal threshold.

### In Vivo Pharmacokinetics Evaluation

Thirty-six New Zealand white rabbits divided into 6 groups ( $n = 6$ ) received a sciatic nerve block (3 mL) with 1 of the 6 RVC formulations as follows: LMVV<sub>RVC0.75%</sub>; LMVV<sub>RVC2%</sub>; LMVV/LUV<sub>RVC 0.75%</sub>; LMVV/LUV<sub>RVC2%</sub>; RVC 0.75%; and RVC 2% in aqueous solutions. An intravascular catheter (Safety-Lok 25 G; BD, Juiz de Fora, MG, Brazil) in the ear vein was used to collect blood samples with approximately 1 mL volume in vacuum tubes with 2,2',2'',2'''-(Ethane-1,2-diylidinitrilo) tetracetic acid (EDTA) (Vacuette; Greiner Bio-one, Monroe, NC). The samples were collected at intervals defined to provide 13 samples between the baseline (0 minute) and 540 minutes. This time interval was designed to collect blood for  $\geq 4$  times the  $t_{1/2}$  (half-life time) of RVC (approximately 2 hours).<sup>24</sup> Immediately after each blood collection, plasma was separated and stored at  $-70^{\circ}\text{C}$  until analysis.<sup>25</sup>

RVC plasma levels were determined using an LC-20AD (Shimadzu Scientific Instruments; Nishinokyo Kubabara-cho, Nakagyo-ku, Kyoto, Japan); coupled to a triple quadrupole mass spectrometer (Micromass Quattro LC; McKinley Scientific, Sparta, NJ) equipped with an electrospray source. To validate the method, quality control samples with 3 RVC concentrations (1200, 600, and 6 ng/mL) were prepared by mixing drug-free plasma with appropriate volumes of working solutions. Precision and accuracy were calculated based on intra- and interbatch variability at 3 quality controls concentration levels (1200, 600, and 6 ng/mL), using 5 replicates. Accuracy was based on the difference between the mean calculated concentration and the nominal concentration, whereas precision was determined as the relative standard deviation.<sup>12</sup> Three calibration curves were plotted in the range of 2.0–1500.0 ng/mL, with precision and accuracy calculated from the variability within the curves. The limit of quantification was defined as the lowest concentration at which precision and accuracy were within 20% of the true value.

Analytical method used Polaris C18 (Agilent, Santa Clara, CA;  $50 \times 2$  mm id,  $5 \mu\text{m}$  particle size) for all separations. The mobile phase was 80% acetonitrile and 20% water with 0.1 mL of formic acid ( $\text{pH} = 3.5$ ). The total run time was 2.5 minutes, and retention time for RVC was 0.75 minutes. Mass spectrometer was run in the positive mode (ES+) and set for multiple reaction monitoring. The full-scan single-mass spectrum and the daughter ion-mass spectrum for RVC and mepivacaine (Sigma Chem Co [Sigma-Aldrich Co, St Louis, MO]; internal standard) were ( $m/z$ )  $275.30 > 125.90$  and  $247.13 > 98.04$ , respectively.

Sample preparation was performed after frozen plasma samples ( $50.0 \mu\text{L}$ ) were thawed at room temperature, followed by the addition of internal standard work solution ( $1 \mu\text{g/mL}$ ). One thousand microliters of dichloromethane (1:1; V/V) and  $50 \mu\text{L}$  of sodium hydroxide (NaOH) (1 M) were added, and then the sample was vortexed for 5 minutes and centrifuged at 1200g, for 5 minutes at  $-4^{\circ}\text{C}$ . The organic liquid ( $0.8 \mu\text{L}$ ) layers were transferred to microtubes; the samples were dried under nitrogen flow; samples were reconstituted in  $200 \mu\text{L}$  mobile phase, vortexed for 1 minute; and  $150 \mu\text{L}$  was transferred to equipment system vials for further injection ( $5.0 \mu\text{L}$ ). The data were integrated using the MassLynx 4.1 (Waters Corporation, Milford, MA) software.

### Statistical Analysis

For all statistical calculations, GraphPad Prism 6.0 (GraphPad Software, Inc, La Jolla, CA) was used. Cell culture assays data were analyzed using 2-way analysis of variance (ANOVA) with Tukey post hoc test. The results obtained from the histological toxicity evaluation in each time interval (2 and 7 days after the injections) were compared using the Kruskal–Wallis test considering each group. The concentration–time data were analyzed using the noncompartmental approach. The pharmacokinetic parameters were calculated using WinNonlin 5.3 version software (Certara, Princeton, NJ) using a noncompartmental analysis approach. The pharmacokinetics parameters, plasma RVC concentrations, and PWTP were analyzed using 1-way ANOVA and the Tukey test (post hoc) considering each RVC concentration separately ( $\alpha = .05$ ). The results from the postoperative pain model were submitted to 2-way ANOVA, followed by Bonferroni post hoc test. The sample size calculation was based on the findings from our pilot study. It was based on the equation for a finite population<sup>26</sup> and indicated a sample size of 6 animals per group, providing 80% power with  $\alpha = .05$ .

## RESULTS

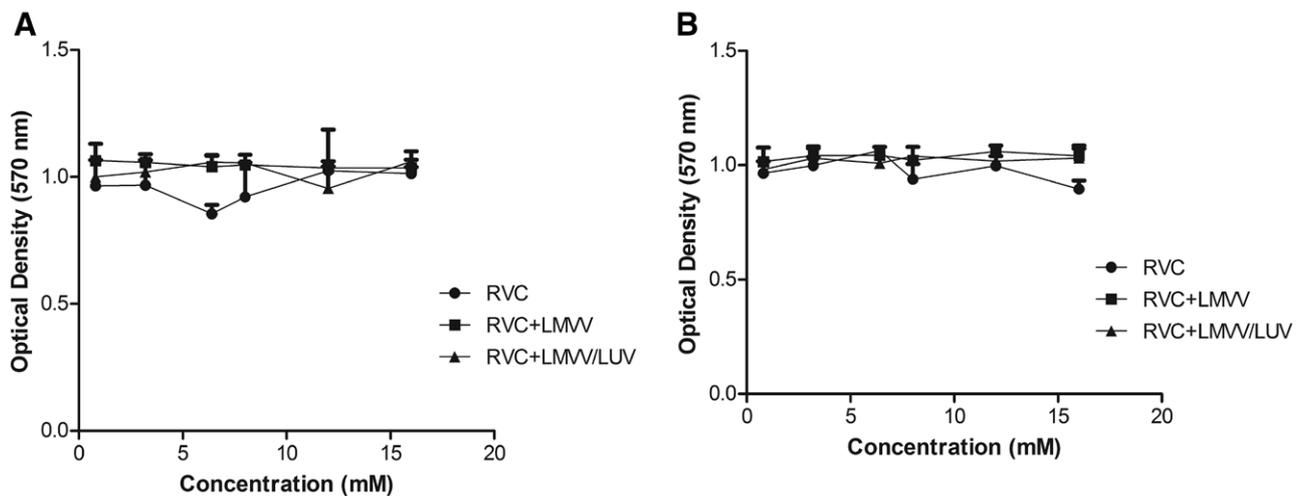
### New Liposomal Formulations Are Safe for Tissues and Neuronal Cells

There was no difference in neuronal cell viability between the liposomal formulations and free RVC at any concentration or treatment times (2–4 hours), nor was there any difference between the different liposome types ( $P > .05$ ). The optical density measured at 570 nm was similar for all tested formulations and concentrations (Figure 2A, B). RVC-free liposomes were used as controls and presented no difference in optical density measurements after the MTT assay (data not shown).

Figures 3 and 4 show cross-sections of the sciatic nerve and their surrounding soft tissues after 2 and 7 days of the injections of the tested formulations, respectively. Considering the liposomal formulations, with 0.75% and 2% RVC, no significant difference was detected between them, regarding local tissue reaction. Generally, 10% lidocaine (used as positive control) induced greater median inflammatory reaction scores than the liposomal formulations after 2 and 7 days of the administration ( $P < .05$ ). After 2 days, all 0.75% groups (free and encapsulated RVC) showed lower scores than 10% lidocaine ( $P < .01$ ), whereas 2% RVC induced greater inflammation than saline ( $P < .01$ ). The liposomal formulations, with or without RVC, had similar scores to saline injections after 7 days.

### Enhanced Analgesia Was Obtained With the Liposomal Formulations

Injection of 2 types of control liposomes (without RVC) did not promote any effect on sensory and motor functions after sciatic nerve block. The 2 formulations of free RVC induced shorter duration of sensory block (approximately 100 minutes shorter) when compared to the liposomal formulations of equivalent drug concentration ( $P < .001$ ). Considering the 2 concentrations of RVC separately, no significant difference was observed between the duration of anesthesia obtained with the 2 liposomal systems ( $P > .05$ ). In both cases, the



**Figure 2.** Cell culture assay. Optical density at 570 nm (mean  $\pm$  SD) after MTT assay on neuronal Schwann cells treated with plain RVC and encapsulated RVC with the 2 types of liposomes (RVC + LMVV and RVC+ LMVV/LUV), after 2 (A) and 4 (B) h. ( $P > .05$ , 1-way analysis of variance with Tukey post hoc test). LMVV indicates large multivesicular vesicle; LUV, large unilamellar vesicle; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; RVC, ropivacaine; SD, standard deviation.

mean duration was around 250 minutes (Figure 5A, B). In general, no differences between the liposomal and the free RVC formulations were observed, considering motor block ( $P > .05$ ).

The paw incision model was effective to produce hyperalgesia because the basal responses to the aesthesiometer in the control groups (saline, LMVV and LMVV/LUV, before and after the paw incision) showed a remarkable difference in pain tolerance. There was a significant difference ( $P < .0001$ ) comparing time zero (before paw incision) with all the subsequent times, for all the groups. The liposomal formulations of RVC in both concentrations presented longer duration compared with free RVC at the same concentration ( $P < .001$ ).  $LMVV_{RVC0.75\%}$  and  $LMVV/LUV_{RVC0.75\%}$  anesthesia persisted for approximately 370 minutes, while pure RVC at 0.75% lasted 120 minutes.  $LMVV_{RVC2\%}$  and  $LMVV/LUV_{RVC2\%}$  persisted for approximately 360 and 330 minutes, respectively, while plain RVC 2% lasted 130 minutes. Also, comparing the AUCs, there was a significant difference between free and liposomal RVC formulations ( $P < .001$ ). The overall results showed that liposomal formulations presented larger AUC (greater total analgesic effect) and longest analgesic duration.

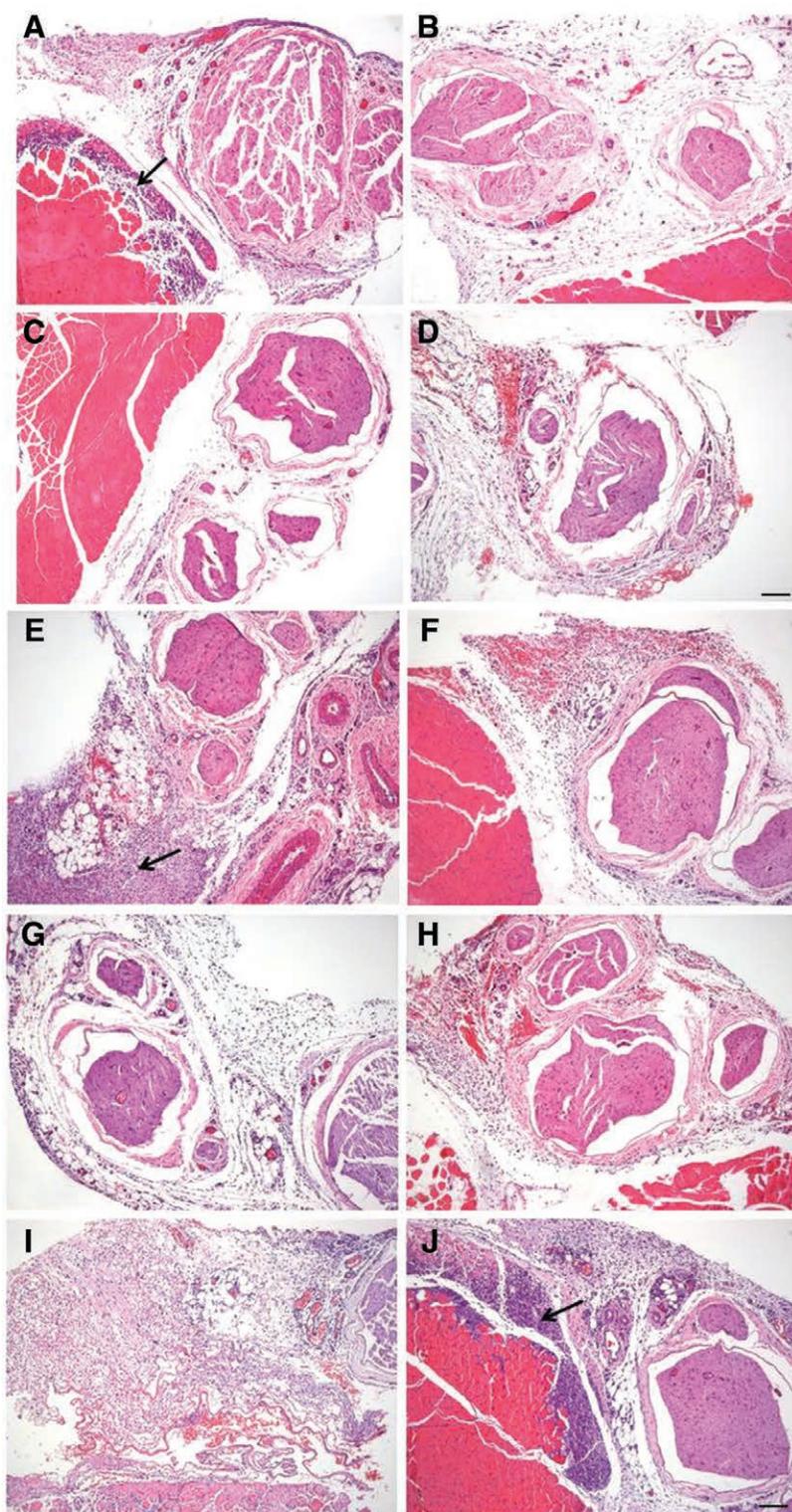
### Liposome Encapsulation Alters the Pharmacokinetics of RVC

The analysis of RVC presented neither interfering compounds nor ion suppression. The assays were linear, and the coefficients ( $r^2$ )  $>0.99$  for all the calibration curves range from 2.0 to 1500.0 ng/mL. The relative standard deviation in the calibration curves ranged from 0.13% to 2.84%. Intra- and interbatch accuracy of quality control plasma samples ranged from 91.60% to 108.90%, whereas precision was  $<0.45\%$  and  $3.79\%$  for the intrabatch and interbatch variation, respectively. The limit of quantification and detection for RVC was 2.00 ng/mL with a relative error of  $<3.36\%$ . Thus, the analytical methodology was reliable and reproducible within its analytical range for RVC.

After 15 minutes of sciatic nerve block in rabbits, RVC was detected in the systemic circulation of all animals. Regarding the 0.75% anesthetic formulations, plain RVC presented significantly higher plasma concentrations than  $LMVV_{RVC0.75\%}$  and  $LMVV/LUV_{RVC0.75\%}$  after 60–120 minutes ( $P < .05$ ). After 300 minutes, plain RVC at 0.75% still induced higher concentrations than the formulations with combined liposomes ( $P < .05$ ). No statistically significant difference was observed between plasma concentrations for the 2% RVC formulations in any time periods ( $P > .05$ ). Table shows the pharmacokinetics parameters obtained after injection of the 6 formulations. The 2% liposomal formulations did not alter any pharmacokinetic parameter when compared to plain RVC at the same concentration ( $P > .05$ ). Regarding the 0.75% concentration, LMVV reduced systemic exposure to RVC. This formulation showed the longest time to reach maximum plasma concentration, mean residence time, and lower maximum plasma concentration than the remaining formulations ( $P < .05$ ).

### DISCUSSION

The present study introduces novel liposomal formulations with RVC that are effective and safe. In general, we were able to demonstrate that all liposomal formulations evoked neither neurotoxicity in vitro nor tissue inflammation after injection near the sciatic nerve area. In vivo pharmacokinetic studies showed a delayed release profile of 0.75% RVC from the site of injection with liposomal formulations. Liposome encapsulation of RVC (2% and 0.75%) with ionic gradients enhanced analgesia when compared to the conventional drug. The presence of an ionic gradient in our liposome systems provided an acidic pH inside the vesicles acting as a “cage” for the anesthetic, enhancing the efficiency of encapsulation, as the protonated RVC species with high aqueous solubility prevails at acidic pH.<sup>12,13</sup> Furthermore, in a more clinically applicable pain scenario, that is, inflammation and hyperalgesia, the liposomal formulations promoted greater and longer analgesia than RVC in aqueous solution. Another interesting point is that not only did our

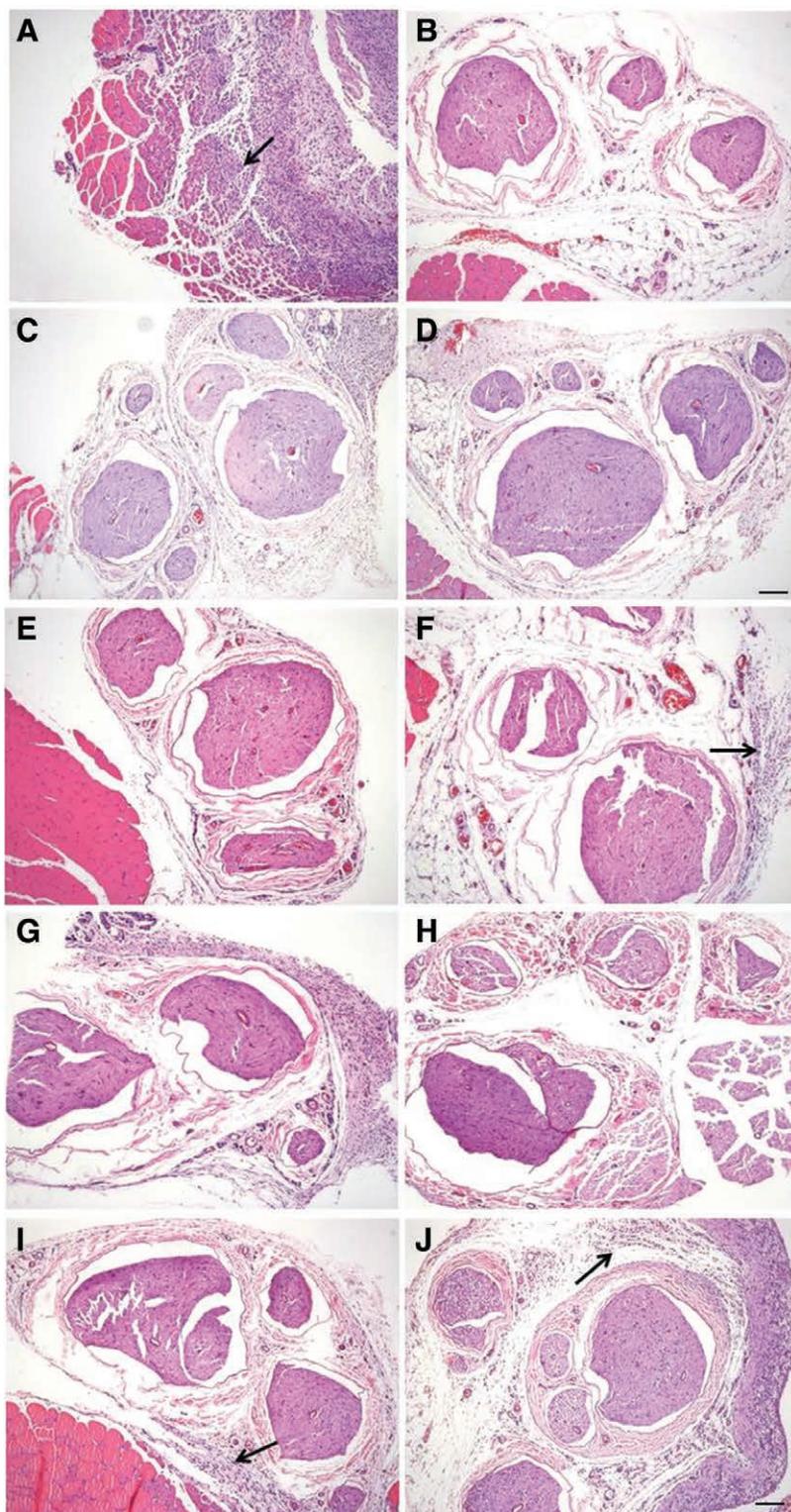


**Figure 3.** Local toxicity after 2 days. Histological analysis of the sciatic nerve region in rats after 2 d of administration of the control groups and treatment groups [median (minimum–maximum limits)] (A) Lidocaine 10% [3(3-3)] (B) Saline [1 (1-1)]; (C) LMVV [1 (1-1)]; (D) LMVV/LUV [1 (1-1)]; (E) LMVV<sub>RVC0.75%</sub> [3 (2-3)]; (F) LMVV/LUV<sub>RVC0.75%</sub> [2 (2-3)]; (G) RVC 0.75% [2 (1-2)]; (H) LMVV<sub>RVC2%</sub> [2 (2-3)]; (I) LMVV/LUV<sub>RVC2%</sub> [2 (2-3)]; and (J) RVC 2% [3 (3-3)]. Observe the local tissue inflammation (arrow). Scale bar: 100  $\mu$ m. LMVV indicates large multivesicular vesicle; LUV, large unilamellar vesicle; RVC, ropivacaine.

formulations evoke higher antinociception in 2 models, but such effect also started at the same time with conventional RVC. Thus, our formulation was able to prolong the analgesic effect, while still keeping the rapid initial drug release, which is important to maintain a viable latency period for future clinical use.

To access the safety of these new formulations, we used 2 models that cover the main target for local anesthetics

(neuronal cells) and the surrounding tissues of the injection site. Local anesthetics can promote chemical nerve injury evoked by the solution itself or its additives, with the degree of neurotoxicity being concentration or dose dependent.<sup>27</sup> Because we evaluated formulations with new additives, such as ammonium sulfate, our first experimental model was a neuronal cell culture. Similar to the results previously described<sup>13</sup> for 3T3 fibroblasts, the encapsulation of RVC

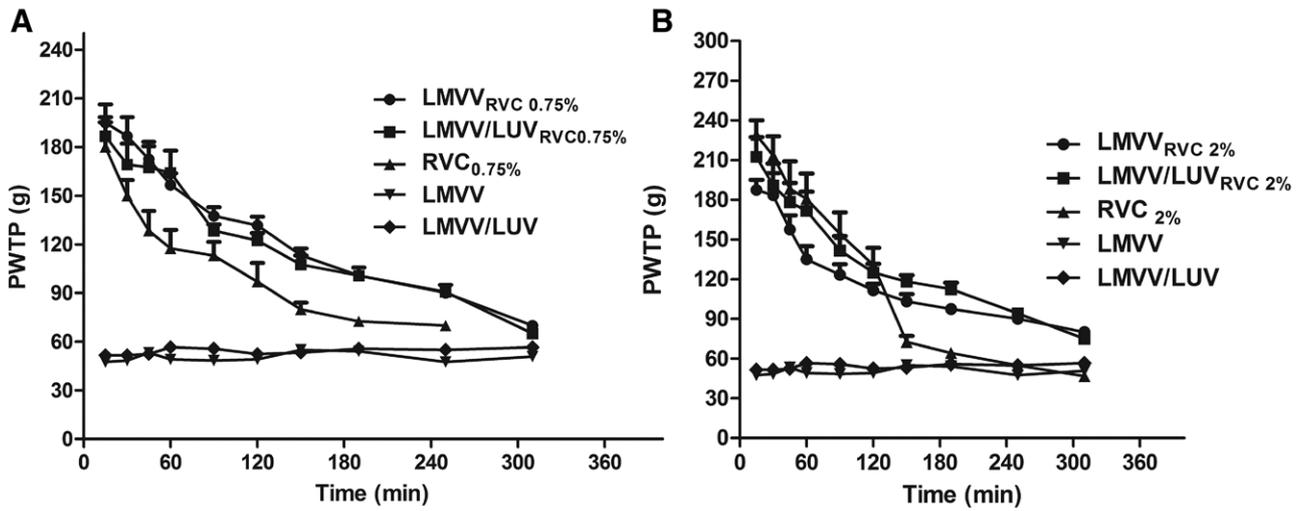


**Figure 4.** Local toxicity after 7 days. Histological analysis of the sciatic nerve region in rats after 7 d of administration of the control groups and treatment groups (A) Lidocaine 10% [3 (3–2)]; (B) Saline [1 (1–1)]; (C) LMVV [1 (1–1)]; (D) LMVV/LUV [1 (1–1)]; (E) LMVV<sub>RVC0.75%</sub> [1 (1–1)]; (F) LMVV/LUV<sub>RVC0.75%</sub>; (G) RVC 0.75% [1 (1–2)]; (H) LMVV<sub>RVC2%</sub> [1 (1–1)]; (I) LMVV/LUV<sub>RVC2%</sub> [2 (1–3)]; and (J) RVC 2% [2 (2–2)]. Note the local tissue inflammation (arrow). Scale bar: 100 μm. LMVV indicates large multivesicular vesicle; LUV, large unilamellar vesicle; RVC, ropivacaine.

in liposomes did not show any effect in the cultured cells after 2 and 4 hours of treatment. Also, liposomal formulations generally presented similar tissue reaction when compared to free RVC and saline. In fact, liposomes are made of biocompatible and biodegradable phospholipids that present low toxicity and such feature has been previously described by our group.<sup>15,28,29</sup> The methods reported herein

were convenient yet neuraxial administration was not used to ascertain safety of the aforementioned formulations.

For efficacy and analgesic profile evaluation, 2 different models were used after sciatic nerve block. The first model was performed with a sciatic nerve block injection, with subsequent evaluation of both sensor and motor block. Using the first model, we observed that the liposomal formulations



**Figure 5.** Paw withdrawal threshold to pressure. Time (min) vs PWTP (g) of sensory function evaluated by sciatic nerve block with RVC 0.75% (A) and 2% (B).  $LMVV_{RVC\ 0.75\%} > RVC\ 0.75\%$  from 90 to 250 min;  $LMVV/LUV_{RVC\ 0.75\%} > RVC\ 0.75\%$  from 60 to 250 min;  $LMVV/LUV_{RVC\ 2\%}$  and  $LMVV/LUV_{RVC\ 2\%} > RVC\ 2\%$  from 120 up to 250 min ( $P < .01$ ) (ANOVA/Tukey–Kramer). ANOVA indicates analysis of variance; LMVV, large multivesicular vesicle; LUV, large unilamellar vesicle; PWTP, paw withdrawal threshold to pressure; RVC, ropivacaine.

Table. $t_{1/2}$ , $C_{max}$ , $AUC_{0-480}$ , $AUC_{0-\infty}$ , $T_{max}$ , $V_d$ , $MRT$ After Sciatic Nerve Block of $LMVV_{RVC\ 0.75\%}$ , $LMVV_{RVC\ 2\%}$ , $LMVV/LUV_{RVC\ 0.75\%}$ , $LMVV/LUV_{RVC\ 2\%}$ , RVC 0.75%, and RVC 2% in Rabbits						
Pharmacokinetic Parameters	$LMVV_{RVC\ 0.75\%}$	$LMVV_{RVC\ 2\%}$	$LMVV/LUV_{RVC\ 0.75\%}$	$LMVV/LUV_{RVC\ 2\%}$	RVC 0.75%	RVC 2%
$C_{max}$ (ng/mL)	396 (313–531) <sup>a,b</sup>	2282 (1358–3463)	947.9 (565–1272)	2184 (1751–2470)	715 (540–880)	1632 (1291–2167)
$T_{max}$ (min)	135 (90–180) <sup>a,b,c,d</sup>	15 (15–30)	30 (30–60)	30 (15–60)	45 (30–90)	15 (15–30)
$AUC_{0-540}$ (ng·min/mL)	114,090 (103,132–143,957)	357,192 (255,621–599,106)	117,712 (105,234–122,996) <sup>d,e</sup>	450,122 (388,782–474,375)	156,081 (200,803)	268,943 (252,259–405,091)
$AUC_{0-}$ (ng·min/mL)	212,227 (145,513–257,478)	1,004,000 (582,017–1,744,000)	136,540 (103,447–618,469)	485,234 (156,625–852,430)	212,871 (173,545–489,036)	292,094 (285,524–699,331)
$t_{1/2}$ β (min)	264 (163–475)	1083 (634–1489)	110 (95–249) <sup>d,e</sup>	400 (40.35–639.1)	382 (315–507)	178 (114–404)
$MRT$ (min)	449 (360–593) <sup>a,b</sup>	1617 (860–2407)	207 (135–321) <sup>d,e</sup>	612 (228–800)	403 (357–487)	227 (202–382)
$V_d$ (L)	55 (29–15)	149 (39–337)	44 (24–77)	44 (73–385)	51 (44–102)	80 (48–126)

Data expressed as median and interquartile range (25th–75th).  
 Abbreviations: ANOVA, analysis of variance; AUC, area under the curve;  $C_{max}$ , maximum plasma concentration; LMVV, large multivesicular vesicle; LUV, large unilamellar vesicle; MRT, mean residence time; RVC, ropivacaine;  $t_{1/2}$ , half-life time;  $T_{max}$ , time to reach maximum plasma concentration;  $V_d$ , volume of distribution.  
 ANOVA/Tukey–Kramer: <sup>a</sup> $LMVV_{RVC\ 0.75\%}$  vs  $LMVV/LUV_{RVC\ 0.75\%}$ .  
 Statistical analysis: <sup>b</sup> $P < .01$ .  
 ANOVA/Tukey–Kramer: <sup>c</sup> $LMVV_{RVC\ 0.75\%}$  vs  $RVC\ 0.75\%$ .  
 Statistical analysis: <sup>d</sup> $P < .05$ .  
 ANOVA/Tukey–Kramer: <sup>e</sup> $LMVV/LUV_{RVC\ 0.75\%}$  vs  $RVC\ 0.75\%$ .

were able to increase the anesthetic duration of RVC at 0.75% and 2%. Furthermore, the motor block obtained was similar between free and liposomal RVC. These results corroborate previous findings that RVC induces sensory rather than motor block,<sup>30,31</sup> and this property was not altered by the use of liposomes.

The second in vivo model was the paw incision associated with sciatic nerve block.<sup>22</sup> This model was similar to that reported by Ickowicz et al<sup>20</sup> (2014) and Ohri et al<sup>16</sup> (2012) to evaluate bupivacaine formulations for acute postoperative pain control. All RVC liposomal formulations were able to reduce hyperalgesia caused by the paw incision in a greater

and longer manner when compared to the free drug. Using ionic-gradient liposomes, Grant et al<sup>23</sup> reported the analgesic effectiveness of a 2% bupivacaine formulation in liposomes compared to the standard 0.5% bupivacaine. Considering the 4-fold increase in concentration, the duration reported was 8 times greater for the encapsulated drug. In the present research, comparing RVC separately in both concentrations, a 3-fold increase in anesthesia duration was observed, even with the same concentration of RVC. An issue should, however, be highlighted in that regardless of the higher concentration, 2% RVC did not increase the duration of anesthesia when compared to the 0.75% formulations.

Almost all currently available analgesics were tested in small rodents before use in humans. The pain response might, however, vary considerably between species and between individual animals.<sup>32</sup> Furthermore, dose rates based on weight in rodents are not always comparable to other mammals because of their small body size and fast metabolic rate.<sup>33</sup> Despite such variations and limitations, during analgesic drug development, the pain models used in our study are very useful and able to provide invaluable data regarding analgesia.

Preclinical pharmacokinetics evaluation is an important (and mandatory) aspect of new formulations development because the *in vitro* results may not be replicable in *in vivo* studies. Rabbits are good models for pharmacokinetic studies, especially because they feature higher blood volume and easy ways to collect it when compared to rats.<sup>25</sup> The results obtained in our study with the 0.75% formulations are in accordance with the *in vitro* findings reported previously.<sup>12,13</sup> The encapsulation of RVC in the 2 ionic-gradient liposomal systems modulated the kinetic release of the drug. *In vitro* evaluation determined approximately 80% encapsulation of RVC 0.75% in LMVV liposomes and a delayed release profile (around 25 hours) when compared to free RVC. The use of this formulation, in fact, promoted lower plasma concentration than free RVC, as an indication that RVC was slowly released from the liposomes at the site of injection. Another interesting point was the lower plasma concentration observed for LMVV/LUV<sub>RVC0.75%</sub> when compared to RVC 0.75% after 300 minutes of injection, indicating that this liposomal system promoted tissue retention and slow release of RVC from the site of injection. Despite the significant increase in encapsulation efficiency as well as an increase in upload capacity from donor and acceptor liposomes *in vitro*,<sup>13</sup> the 2% formulation did not show significant differences in plasma concentrations *in vivo*.

Although Ginosar et al<sup>34</sup> reported the same peak concentration as the standard RVC 0.5% with a proliposomal oily RVC formulation with an 8-fold increase in concentration, no differences between pharmacokinetics parameters were observed considering the 2% formulations. In addition, the maximum concentration values were near the toxicity threshold for RVC of 2200 ng/mL.<sup>35</sup> Our findings for all 2% RVC formulations did not indicate that higher anesthetic concentrations would be safe or more effective. In addition, considering local tissue reaction, the 2% formulation induced a more intense inflammation than saline after 2 days.

The lack of superiority of the 2% liposomal formulations compared to the 0.75% liposomal formulations regarding the duration of anesthesia could be explained by the 2 types of liposome systems. The first one is an LMVV which showed approximately 80% of RVC encapsulation efficiency.<sup>12</sup> The second liposome type (LMVV as donor and LUV as acceptor) showed approximately 72% and 42% of encapsulation efficiency for RVC with LUV and LMVV, respectively.<sup>13</sup> Therefore in each 2% liposomal formulations, we have a larger amount of nonencapsulated RVC when compared to the same 0.75% formulations. These data show that such large amount of nonencapsulated RVC with the 2% concentration in both liposomal systems might be rapidly absorbed at the injection site and not contribute

to prolonging local anesthesia. The pharmacokinetic assay also presented similar pattern because the 2% formulations did not alter the absorption rate of RVC. Thus, it seems that the fraction of nonencapsulated local anesthetic of the 2% liposome systems is quickly transferred and redistributed from the site of injection.

The sustained release of 0.75% RVC from LMVVs prepared with ionic-gradient was able to positively alter the pharmacokinetic profile of this local anesthetic, as demonstrated by lower plasma concentrations and reduced systemic exposure. This slow release probably evoked greater analgesic effect in both animal models ( $\approx 3$  times), as it may produce a longer drug presence in the target site. Another important aspect is that the 0.75% formulations were well tolerated at the injection site and in neuronal cell cultures. Recently, a new formulation of RVC in nanoparticles of polyethylene glycol-copoly(lactic acid)<sup>36</sup> produced analgesic effect for over 3 days after single administration, but it also induced foreign body reactions to the surrounding areas of the sciatic nerve after 7 days of application. Considering the data presented herein, LMVV<sub>RVC0.75%</sub> showed the best overall pharmacological profile, with reduced systemic exposure, greater total analgesic effect, and minor tissue reaction. In spite of the novelty and encouraging results, our study evaluated a novel tool for postoperative pain management in animal models, foreseeing a potential clinical application. Therefore, clinical studies should be welcomed in the near future to validate whether such liposomal formulations containing 0.75% RVC could provide adequate postoperative pain management in humans. ■■

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