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Pharmacological and local toxicity studies of a liposomal formulation for the novel local anaesthetic ropivacaine

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Abstract

This study reports an investigation of the pharmacological activity, cytotoxicity and local effects of a liposomal formulation of the novel local anaesthetic ropivacaine (RVC) compared with its plain solution. RVC was encapsulated into large unilamellar vesicles (LUVs) composed of egg phosphatidylcholine, cholesterol and α-tocopherol (4:3:0.07, mole %). Particle size, partition coefficient determination and in-vitro release studies were used to characterize the encapsulation process. Cytotoxicity was evaluated by the tetrazolium reduction test using sciatic nerve Schwann cells in culture. Local anaesthetic activity was assessed by mouse sciatic and rat infraorbital nerve blockades. Histological analysis was performed to verify the myotoxic effects evoked by RVC formulations. Plain (RVC_{PLAIN}) and liposomal RVC (RVC_{LUV}) samples were tested at 0.125%, 0.25% and 0.5% concentrations. Vesicle size distribution showed liposomal populations of 370 and 130 nm (85 and 15%, respectively), without changes after RVC encapsulation. The partition coefficient value was 132 ± 26 and in-vitro release assays revealed a decrease in RVC release rate (1.5 fold, $P < 0.001$) from liposomes. RVC_{LUV} presented reduced cytotoxicity ($P < 0.001$) when compared with RVC_{PLAIN}. Treatment with RVC_{LUV} increased the duration ($P < 0.001$) and intensity of the analgesic effects either on sciatic nerve blockade (1.4–1.6 fold) and infraorbital nerve blockade tests (1.5 fold), in relation to RVC_{PLAIN}. Regarding histological analysis, no morphological tissue changes were detected in the area of injection and sparse inflammatory cells were observed in only one of the animals treated with RVC_{PLAIN} or RVC_{luv} at 0.5%. Despite the differences between these preclinical studies and clinical conditions, we suggest RVC_{LUV} as a potential new formulation, since RVC is a new and safe local anaesthetic agent.

Introduction

Ropivacaine (RVC) is an amino-amide, enantiomerically pure (S-isomer), novel local anaesthetic largely used in surgical procedures. RVC presents physico-chemical and therapeutic properties similar to those of bupivacaine, but with lower toxicity to the cardiovascular and the central nervous systems. In addition, a slight decrease in the lipid solubility of RVC confers to it a greater selectivity or differential block for sensory over motor function in isolated nerve preparations or epidural administration, in relation to bupivacaine (Rosenberg & Heinonen 1983; Bader et al 1989; Brockway et al 1991). These features point to RVC as an important option for regional anaesthesia and management of postoperative pain (McClure 1996; Simpson et al 2005).

Commercially available local anaesthetic formulations are used in a variety of doses and routes of administration. Despite the advances, the relatively short duration of analgesia (due to the transfer and redistribution from the site of injection) (Grant & Bansinath 2001; Grant 2002) and the severe side effects (evoked by large doses or inadvertent intravascular injections) restrict their clinical use (McClure 1996; McLure & Rubin 2005). Thus, the use of drug delivery systems, such as liposomes, would be highly desirable for the clinical use of local anaesthetics, offering the possibility to control the release of these drugs, to prolong the duration of action, especially for the newer and safer agents such as RVC.

Liposomes consist of one or more concentrically organized assemblies of phospholipid bilayers where the fatty acid tails are in the core of the bilayer while the hydrophilic heads are oriented to the aqueous phase. Because of their amphiphilic nature, local anaesthetics interact with these model membrane systems, sitting mainly in the bilayer (lipid) region, and they also retain a fraction of molecules in the aqueous phase (de Paula & Schreier 1995, 1996). In fact, works in the literature show that the sustained release of local anaesthetics in liposomes has advantages such as biocompatibility, low toxicity and biodistribution controlled by their size (Grant & Bansinath 2001). Studies with bupivacaine, for instance, report prolonged effect (Boogaerts et al 1993, 1994, 1995; Malinovsky et al 1999; Yu et al 2002; Grant et al 2003, 2004), changes in biodistribution (Boogaerts et al 1995), decreased plasma concentrations and low systemic toxicity (Boogaerts et al 1993; Malinovsky et al 1999) after encapsulation in large multilamellar liposomes (MLV), when compared to plain bupivacaine solution.

Our research group observed that the duration and intensity of sensory blockade induced by mepivacaine (de Araujo et al 2004), prilocaine and lidocaine (Cereda et al 2004, 2006) were enhanced by encapsulation in large unilamellar liposomes (LUV) even though these effects were not observed with bupivacaine. In another recent study, a liposomal-encapsulated RVC topical gel effectively reduced pain during needle insertion and increased the duration of soft-tissue anaesthesia in dentistry (Franz-Montan et al 2007); however, the efficacy of a liposomal system for RVC was not studied considering an infiltrative route and its possible local toxic effect. Thus, the purpose of this preclinical study was to investigate the pharmacological activity of a parenteral liposomal formulation for RVC, using the sciatic and infraorbital nerve blockade models, as well as to assess the cytotoxic and the myotoxic local effects in comparison with its plain solution.

Materials and Methods

Drugs

RVC hydrochloride and sodium thiopental were donated by Cristália Prod. Quím. Farm. Ltda (Itapira, SP, Brazil). Egg phosphatidylcholine (EPC), cholesterol (Ch), α-tocopherol (α-T), bovine serum albumin (BSA) and HEPES buffer were purchased from Sigma Chemical Company (St Louis, MO, USA). 3,4,5-Dimethylthiazol-2-yl-2,3-diphenyltetrazolium bromide (MTT) was obtained from Calbiochem Corp. (La Jolla, CA, USA) and antibody anti-S-100 polyclonal from DAKO (Glostrup, Denmark). Dulbecco's Modified Eagle's Medium (DMEM), collagenase and trypsin were obtained from Nutricell (Campinas, SP, Brazil).

Animals

Male adults Swiss mice, Wistar rats (30–35 g and 250–350 g, respectively) and newborn Sprague–Dawley rats were obtained from CEMIB-UNICAMP (Centro de Bioterismo, State University of Campinas – UNICAMP, Campinas, São Paulo). Protocols were approved by the UNICAMP Institutional Animal Care and Use Committee, which follows the recommendations of the Guide for the Care and Use of Laboratory Animals. Rats or mice, divided in groups of 6 or 7 animals each, were randomly selected for the pharmacological assays and treated by infiltration (0.1 mL) with $LUV_{ROPIVACAINE\text{-}FREE}$ (5 mM) or with RVC_{PLAIN} or RVC_{LUV} at 0.125%, 0.25% or 0.5% concentrations.

Liposomal ropivacaine

EPC–Ch–α-T (4:3:0.07, mole %) films were obtained by evaporating stock chloroform solutions under a stream of wet nitrogen followed by vacuum, for 2 h. Films were suspended in HEPES buffer (20 mM, 154 mM NaCl, pH 7.4) and MLVs were obtained after vortexing at ambient temperature (5 min, 25°C). LUVs were prepared by repeated extrusion (15 cycles) of the MLVs within 0.4-μm membrane filters (25°C), in a Lipex Biomembranes Inc. (Vancouver, Canada) extruder. The total phospholipid concentration, determined by inorganic phosphate quantification, was 5 mM (de Paula & Schreier 1995). RVC was added directly to the liposomes after extrusion at the same final concentrations of RVC_{PLAIN}: 0.125% (4.02 mM), 0.25% (8.04 mM) and 0.5% (16.08 mM).

The mean diameter and size distribution of the LUV, stored at 4°C were analysed by laser light-scattering (Malvern Mastersizer-Malvern Instruments, France) before and after RVC encapsulation. The polydispersity index was also evaluated as a measurement of the homogeneity of the dispersion (ranging from 0 to 1, representing a homogeneous or a heterogeneous distribution, respectively) (Barth & Flippen 1995).

The partition coefficient (P) was determined by ultracentrifugation (120 000 g for 2 h at 10°C) of samples containing 2 mM RVC and 4 mM liposomal suspensions. Four repetitions of duplicate tests were used for each P determination. The amount of RVC incorporated into the vesicles was optically determined at 260 nm (de Araujo et al 2008), by subtracting the supernatant concentration from the total RVC concentration, measured previous to phase mixing. P values were calculated using equation 1 (de Paula & Schreier 1995, 1996).

$$P = (n_m/V_m)/(n_w/V_w) \quad (1)$$

where n is the number of moles of RVC, V denotes volume (L), m refers to the membrane phase and w to the aqueous phase.

In-vitro release experiments were conducted at 37°C in a two-compartment dialysis system where the donor compartment (1 mL capacity, containing RVC_{PLAIN} or RVC_{LUV} sample) was separated from the acceptor compartment (100 mL, containing 20 mM HEPES buffer pH 7.4) by a cellulose membrane (Spectrapore, MWCO 12 000–14 000 Da). Samples were withdrawn from the acceptor compartment at regular intervals and drug concentration was determined by UV absorption (260 nm).

Cell culture and cytotoxicity assay: sciatic nerve Schwann cells

Schwann cells were isolated from the sciatic nerve of newborn Sprague–Dawley rats and purified as previously

described with minor modifications (Brockes et al 1979; Assouline et al 1983). Nerve segments were aseptically removed and dissected out from the epineurium and surrounding tissue, incubated in 0.05% collagenase for 30 min at 37°C and then in 0.15% trypsin for 20 min. The cell mixture was recovered by centrifugation in BSA 3% (300 *g*, 10 min) and resuspended in DMEM with 10% fetal calf serum supplement with glucose, insulin-like nerve growth factor (NGF), pituitary extract, forskolin and antibiotic (penicillin and streptomycin). Cells were seeded (15×10^4 cells/well) into a plastic cell culture dish with 48 wells (Corning-Costar Co., Cambridge, MA, USA) and cultured for 4 days (37°C, 5% CO_2). Cultures were fixed in 4% paraformaldehyde in phosphate buffer for 10 min (pH 7.4, 37°C). To avoid non-specific staining, the specimens were incubated for 45 min with 1% BSA in phosphate buffer containing 0.25% Triton X-100. The purity of the culture was evaluated by the antibody anti-S-100. Finally, the cells were rinsed in PBS (37°C), incubated with Cy3 antibody anti-rabbit conjugate for 45 min and observed under a Nikon eclipse TS100 microscope equipped for fluorescence analysis.

Purified Schwann cells were incubated for 2 h with the vehicle ($LUV_{ROPIVACAINE-FREE}$, 5 mM) or with RVC (RVC_{PLAIN} or RVC_{LUV}) at three different concentrations 0.125% (4.02 mM), 0.25% (8.04 mM) and 0.5% (16.08 mM). Cell viability was assessed by the tetrazolium reduction (MTT test). MTT (1 mg mL^{-1}) was incubated for 2 h with the treated Schwann cells (37°C). The number of viable cells was determined by measuring the amount of MTT converted to formazan by mitochondrial dehydrogenases. The produced formazan crystals were dissolved in 1 M HCl–isopropyl alcohol (1:24 v/v) and were shaken for 20 min. After that, the dye-containing solution was removed and the sample absorbance was determined at 570 nm (Denizot & Lang 1986; Park et al 2005).

Infraorbital nerve blockade

Rat infraorbital nerve blockade was performed as previously described (Fink et al 1975) and was used with minor adaptations. The rats were anaesthetized with 25 mg kg^{-1} sodium thiopental (intraperitoneal route) and a unilateral injection of the vehicle ($LUV_{ROPIVACAINE-FREE}$) or RVC formulations was performed into the infraorbital notch. The intact left side served as control. The analgesic effect was assessed by observation of aversive response to rat upper lip pinching according to the scores: 0 (aversive response) or 1 (no aversive response). The rats were tested every 5 min up to the time when the first aversive sign in the injected side was detected. Score values were expressed as percent of rats with analgesia. The efficacy of infraorbital nerve block was analysed by the time for sensory function recovery and the total local anaesthetic effect. Local anaesthetic effect was estimated by the area under the time curve (AUC) expressed as score/hour (Cereda et al 2006) and calculated using Origin 6.0 Software (Microcal TM Software Inc., Northampton, MA, USA).

Sciatic nerve blockade

Before the experiment, the ability of each mouse to walk normally with four limbs on both the top and inverted side of a wire mesh screen (1 mm diameter wire, 5 mm mesh) was evaluated. Mice showing this behaviour were selected for the experiment. Vehicle, plain solution or liposomal formulations were injected by inserting a needle into the popliteal space on the posterior surface of the knee, in the area of the sciatic nerve. Motor blockade intensity was assessed by the loss of motor control in the injected limb according to the scores: 0 (normal movement), 1 (unable to flex the limb completely) and 2 (total paralysis). The efficacy of motor blockade was evaluated every minute, from 1 to 5 min, and thereafter every 10 min up to at least 1 h following the injection. Latency (time between injection and the loss of motor function), time to reach the maximum score (Tmax), time for motor function recovery and the total local anaesthetic effect (area under the effect curve vs time, expressed as score/h) were evaluated (Leszczynska & Kau 1992; Gantenbein et al 1997).

Sensory blockade evaluation was performed by the paw pressure test (Randall & Selitto 1957) using an analgesymeter (Ugo Basile, Varese, Italy), which exerts a force (in grams) on the paw. The withdrawal reflex was considered representative of the pain threshold or paw withdrawal threshold to pressure (PWTP). The baseline of the PWTP test was measured before vehicle or drug injection, to determine the pain threshold of the mouse. Baseline values of 30–50 g were selected as the pain threshold and mice that presented lower or higher values than that baseline were excluded. The established antinociception cut-off value was 150 g, considered to be representative of the anaesthetic state (de Araujo et al 2004, 2008). After drug or vehicle administration, measurements were carried out at intervals of 15 min during the first hour, 30 min in the second and third hour and finally 60 min up to 5 h after treatment.

Histological assays

Mice were sacrificed by cervical dislocation 3 days after injection of $LUV_{ROPIVACAINE-FREE}$ (5 mM), RVC_{PLAIN} or RVC_{LUV} at 0.125, 0.25 and 0.5%. To evaluate the surroundings of the site of injection, gastrocnemius and soleus muscles were dissected out, fixed in 10% paraformaldehyde (pH 7.6) for 24 h and transferred to 70% ethanol solution. Samples were embedded in paraffin and transverse sections (6 μm) were obtained from the muscle bellies adjacent to the popliteal space and stained with haematoxylin and eosin (H&E). The presence of inflammatory cells, degenerating and regenerating myofibres was analysed using light microscopy. Low-power video-images (10 × objective) of the entire cross-section were taken with a highly sensitive video camera (Sony CCD) linked to a light microsce and enhanced with an image processor system (CoolSnap, Media Cybernetics, USA).

Statistical analysis

Size distribution of liposomes and in-vitro release tests were analysed by two-tailed unpaired *t*-test. Sciatic (motor function) and infraorbital nerve blockade data (latency, Tmax, time for recovery and AUC) were analysed by the Kruskall–Wallis test and expressed as medians (minimum and maximum limits). Sciatic nerve blockade (sensory function) and cytotoxic assay

data were analysed by one-way analysis of variance with Tukey–Kramer as a post-hoc test (Zar 1996). Statistical significance was defined as $P < 0.05$. Data were analysed using Origin 6.0 Software (Microcal TM Software Inc., USA) and Graph Pad Instat (Graph Pad Software Inc., USA) programs.

Results

Liposomal ropivacaine

Size distribution analysis by laser light-scattering assays showed two different populations of liposomes. The main population, representing 85%, had an average size of 371 ± 7.9 nm while a smaller fraction (15%) included liposomes with diameters of 128 ± 6.3 nm; the size did not change after RVC encapsulation (356 ± 8.3 nm and 138 ± 7.8 nm, respectively). All the measurements presented a polydispersity index of 0.12–0.17, reflecting the homogeneous distribution of the liposomal population obtained. The partition coefficient of RVC into the liposomes was 132 ± 26, corresponding to an encapsulation efficiency of 23.8 ± 3.5% (mean ± s.d.). Encapsulation of RVC significantly reduced (1.3 fold, $P < 0.001$) its rate of release from one dialysis compartment to another, as compared with RVC_{PLAIN}. One hour after dialysis, 58.1 ± 0.88% of RVC was released from the liposomes against 76.1 ± 6.2 % from the plain solution. At the time noted for total release (100%) of RVC_{PLAIN}, which was observed at 180 min of dialysis, only 68.2 ± 0.72% of RVC was released from the liposomal system (Figure 1).

Cell culture and cytotoxicity assay: sciatic nerve Schwann cells

The treatment with the vehicle ($LUV_{ROPIVACAINE-FREE}$) did not reduced the cell viability. On the other hand, RVC_{PLAIN} reduced the cell viability by 70% (0.5% RVC), while RVC_{LUV}, similar to $LUV_{ROPIVACAINE-FREE}$, had no effect on cell viability up to 2 h after treatment (100% cell viability, at 4.02 mM), compared to RVC_{PLAIN} ($P < 0.001$) (Figure 2).

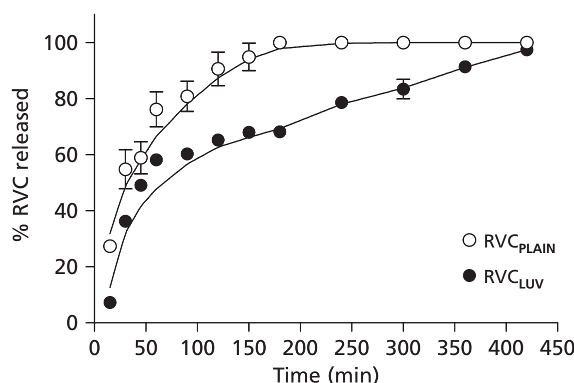

Figure 1 In-vitro release experiments for plain (RVC_{PLAIN}) and liposomal (RVC_{LUV}) ropivacaine in HEPES buffer, pH 7.4 at 37°C. Data are expressed as mean ± s.d., n = 4 experiments.

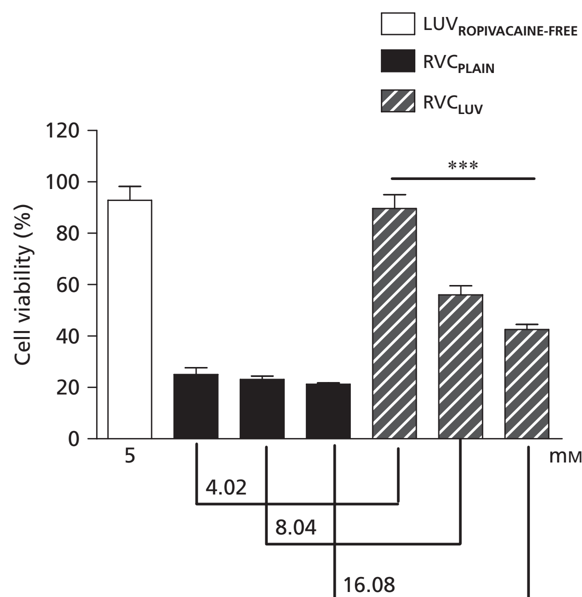

Figure 2 Cytotoxic effects of $LUV_{ROPIVACAINE-FREE}$ (ropivacaine-free large unilamellar liposomes, 5 mM) and RVC_{PLAIN} (plain ropivacaine) or RVC_{LUV} (liposomal ropivacaine) at 0.125, 0.25 and 0.5%, on a primary sciatic nerve Swann cells culture incubated for 2 h at 37°C and 5% CO_2 as evaluated by MTT reduction test. Data are expressed as % cell viability (mean ± s.d., n = 3 experiments). ***$P < 0.001$, RVC_{LUV} vs RVC (one-way analysis of variance with Tukey–Kramer post-hoc test).

Infraorbital nerve blockade

Table 1 summarizes the infraorbital test results for RVC_{PLAIN} and RVC_{LUV} regarding the local anaesthetic total effect (expressed as AUC) and time for recovery.

No signs of sensory blockade were observed on the intact left side of rats in any of the groups, punctuated with score 0 (i.e. aversive response to pinch) (data not shown). $LUV_{ROPIVACAINE-FREE}$, used as controls, presented no analgesic effect, whereas RVC_{LUV} induced an improvement on intensity of total local anaesthetic effect (27.5, 42.5 and 107.5 score/h for 0.125, 0.25 and 0.5% RVC_{LUV}, respectively) associated with prolonged times for recovery (duration of sensory blockade), since analgesia was observed until 37, 50 and 122 min after treatment with 0.125, 0.25 and 0.5%, as compared with RVC_{PLAIN}. Statistical analysis showed that RVC_{LUV} prolonged the time for recovery and enhanced the total effect of RVC at 0.125% ($P < 0.01$), 0.25% ($P < 0.05$) and 0.5% ($P < 0.01$).

Sciatic nerve blockade

The injection of $LUV_{ROPIVACAINE-FREE}$ into the mouse sciatic nerve did not cause any effect on motor blockade. However, even if the overall motor function was not significantly different between RVC_{PLAIN} and RVC_{LUV} formulations, dose-dependent effects were observed on latency, motor blockade duration and total effect of the local anaesthetic for each experimental group.

In this manner, statistical differences were observed after comparisons within the same groups (intra-group) for the

Table 1 Total local anaesthetic effect (AUC) and time for recovery for plain (RVC_{PLAIN}) and liposomal ropivacaine (RVC_{LUV}) in rat infraorbital nerve blockade test

Group	Concentration (%)	Time for recovery (min)	AUC (score/h)
RVC_{PLAIN}	0.125	22.0 (22.0–27.0)	17.5 (17.5–22.5)
	0.25	38.0 (32.0–40.0)	32.5 (27.5–32.5)
	0.5	81.0 (80.0–96.0)	72.5 (67.5–82.5)
RVC_{LUV}	0.125	32.0 (28.0–37.0)**	27.5 (22.5–332.5)**
	0.25	50.0 (39.0–50.0)*	42.5 (32.5–42.5)*
	0.5	115.0 (97.0–122.0)**	107.5 (92.5–117.5)**

Data expressed as median (minimum–maximum limits) (n = 6 or 7/group). *$P < 0.05$, **$P < 0.01$, RVC_{LUV} vs RVC_{PLAIN} (Kruskall–Wallis test).

three different concentrations used. Then, comparisons among RVC concentrations showed statistical differences in relation to time for recovery and AUC for RVC_{PLAIN} (0.5% vs 0.125% and 0.25% vs 0.125% with $P < 0.01$) and RVC_{LUV} (0.5% vs 0.25% with $P < 0.05$; 0.5% vs 0.125%, $P < 0.01$) (Table 2).

On the other hand, comparisons between RVC_{PLAIN} and RVC_{LUV} (inter-group) did not reveal statistical differences.

The sensory blockade data showed that the RVC formulations were statistically different from the $LUV_{ROPIVACAINE-FREE}$ group ($P < 0.001$). On the other hand, RVC_{LUV} had an increased duration and intensity of anti-nociceptive effect, when compared with the plain solution. Assessing individual time values, 0.125% RVC_{LUV} (Figure 3A) was different from RVC_{PLAIN} treatment from 45 up to 150 min ($P < 0.001$), increasing the intensity of analgesia (1.6 fold). The analgesia was observed until 180 min after injection of 0.125% RVC_{LUV}, when compared with RVC_{PLAIN}. The treatment of the mice with 0.25% RVC_{LUV} (Figure 3B) showed similar results to those at 0.125% concentration. Statistical differences in anti-nociceptive effects between 0.25% RVC_{LUV} and 0.25% RVC_{PLAIN} were observed from 45 up to 180 min ($P < 0.001$). At that time interval, the intensity of analgesia using RVC_{LUV} was 1.6 times higher than that with RVC_{PLAIN} and was observed up to 240 min after injection of 0.25% RVC_{LUV}. The group treated with 0.5% RVC_{LUV} (Figure 3C) presented different responses from 30 up to 240 min after infiltration, when compared with 0.5% RVC_{PLAIN} ($P < 0.001$). The intensity of analgesia reached with 0.5% RVC_{LUV} was 1.4- to 1.6-times higher than that with RVC_{PLAIN} and the duration was seen until 300 min.

Histological assays

Histological analysis of the mouse gastrocnemius and soleus muscles after the treatment with $LUV_{ROPIVACAINE-FREE}$, RVC_{PLAIN} or RVC_{LUV} at 0.125%, 0.25% or 0.5% revealed that these muscles appeared to be normal, with fibres round or roughly polygonal with rounded angles. No morphological tissue changes were detected in control mice, since the muscle fibres underlying the area of injection remained visibly unaffected and normal in all morphological aspects. Muscle fibres with peripheral nuclei location similar to control muscles were found and regenerated muscle fibres, characterized by central nuclei, were detected only on the needle track for all experimental groups. Sparse inflammatory cells were observed in only one of the mice treated with $LUV_{ROPIVACAINE-FREE}$ (5 mM) and RVC_{PLAIN} or RVC_{LUV} at the higher concentration (0.5%) (Figure 4).

Discussion

Despite being also a long-acting local anaesthetic, RVC is a relatively new drug with a similar clinical profile to bupivacaine but is associated with lower motor blockade and cardiotoxicity (Markham & Faulds 1996). The development of a liposomal system would be of interest to solve problems relating to the fast clearance of those molecules from the site of injection (Grant & Bansinath 2001; Grant 2002). In the development of a drug delivery system for local anaesthetics, aspects must be considered that determine the concentration and effect of the formulation on the nervous tissue: firstly, the

Table 2 Latency, T_{max}, time for recovery and total effect (AUC) from the motor blockade evaluation for plain (RVC_{PLAIN}) and liposomal ropivacaine (RVC_{LUV}) in sciatic nerve blockade test in mice

Group	Concentration (%)	Latency (s)	Tmax (min)	Time for recovery (min)	AUC (score/h)
RVC_{PLAIN}	0.125	50.0 (25.0–55.0)	1 (1–2)	30.0 (20.0–40.0)	25.0 (15.5–45.0)
	0.25	40.0 (30.0–50.0)	1 (1–3)	40.0 (30.0–50.0)	35.0 (15.0–50.0)
	0.5	30.0 (25.0–55.0)	1 (1–2)	55.0 (45.0–65.0)^{a**,b**}	66.0 (50.0–80.0)^{a***,b**}
RVC_{LUV}	0.125	45.0 (24.0–60.0)	1 (1–3)	30.0 (20.0–40.0)	25.0 (14.0–46.0)
	0.25	37.0 (20.0–60.0)	1 (1–4)	50.0 (40.0–80.0)^{d**}	44.0 (33.5–108.0)^{d*}
	0.5	34.0 (26.0–48.0)	1 (1–3)	60.0 (40.0–70.0)^{c,e**}	57.0 (34.0–108.0)^{c**}

Data are expressed as median (minimum–maximum limits) (n = 6 or 7/group). [a]RVC 0.5% and RVC 0.125%; [b]RVC 0.5% and RVC 0.25%; [c]RVC_{LUV} 0.5% and RVC_{LUV} 0.25%; [d]RVC_{LUV} 0.25% and RVC_{LUV} 0.125%; [e]RVC_{LUV} 0.5% and RVC_{LUV} 0.125%; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Kruskall–Wallis test).

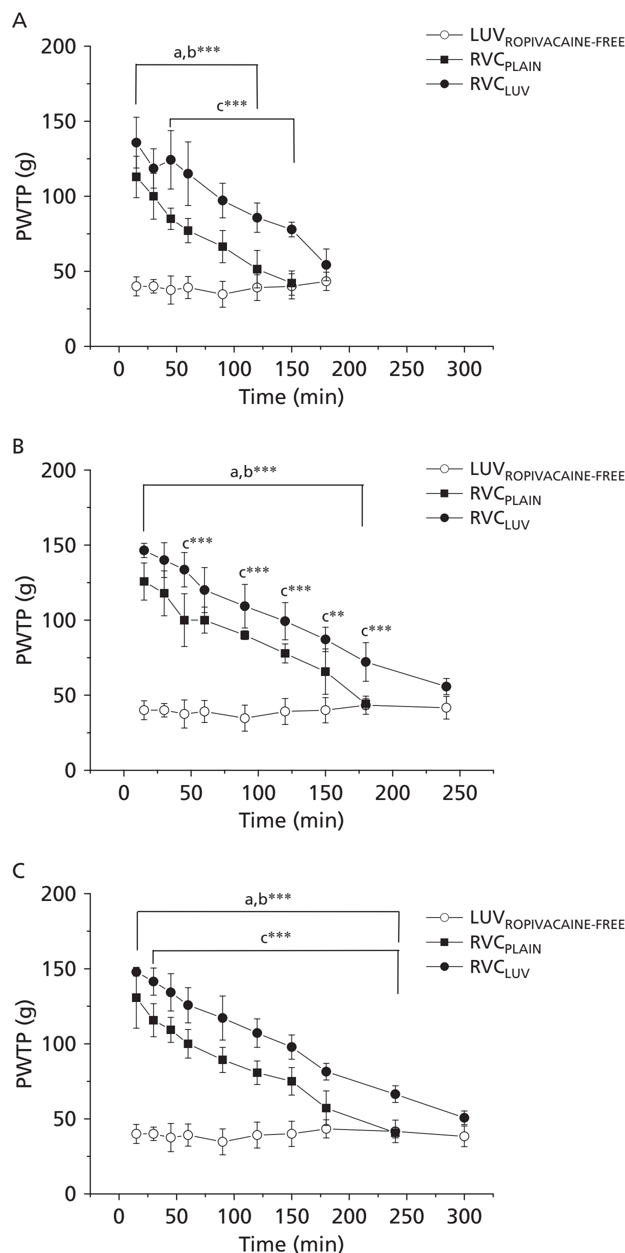

Figure 3 Time course (min) × PWTP (g) of sensory function evaluated by sciatic nerve blockade test in mice (n = 6 or 7/group). 5 mM ropivacaine-free large unilamellar liposomes ($LUV_{ROPIVACAINE-FREE}$), plain ropivacaine (RVC_{PLAIN}) and liposomal ropivacaine (RVC_{LUV}) at 0.125% (A), 0.25% (B) or 0.5% (C). Data are expressed as mean ± s.d. Statistical differences are shown between: [a]RVC and LUV; [b]RVC_{LUV} and LUV; [c]RVC_{LUV} and RVC_{PLAIN}. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (one-way analysis of variance with Tukey–Kramer post-hoc test).

drug must be sufficiently encapsulated to maintain the therapeutic concentration; secondly, the diffusion of the drug from the injected solution into the extracellular fluid and its uptake by nerve fibres must be slow; thirdly, clearance of the drug and the carrier should be sustained to allow a prolonged effect. Regarding local anaesthetics, features such as local concentration, diffusion and uptake by the nervous tissue determine the latency, spread and intensity of the blockade; however, the clearance rate influences the duration of action (Grant & Bansinath 2001).

Liposomes have been effectively used as slow-release systems for local anaesthetics. Encapsulation of these drugs has led to reduced systemic toxicity (Boogaerts et al 1993, 1995; Malinovsky et al 1997) and to longer duration of action (Booagerts et al 1994; Malinovsky et al 1999; Grant et al 2003, 2004; de Araujo et al 2004; Cereda et al 2004, 2006).

As shown here, the RVC_{LUV} system was not able to modify the motor blockade duration (since comparisons between RVC_{PLAIN} and RVC_{LUV} did not reveal statistical differences), but improved the intensity ($P < 0.001$) and duration of the analgesic effect ($P < 0.001$ at the final point of analgesic effect induced by RVC_{PLAIN}) in a mouse sciatic nerve blockade model at the three concentrations used.

Considering this, we here provided an initial assessment of the drug distribution and the pharmacological effects of a liposomal system for the novel local anaesthetic RVC, since the results showed a decrease in drug release rate and a slow delivery of RVC in-situ caused by its encapsulation into the liposomes. In fact, this slower release rate of RVC_{LUV} was confirmed by the increase in intensity and duration of sensory nerve blockade observed in rats and mice. The gradual drug release changes the rate of distribution of local anaesthetic molecules among membrane/water compartments, with possible therapeutic advantages such as prolonged effects, low plasmatic levels and reduced systemic toxicity (Grant & Bansinath 2001). Thus, we postulate that by changing the drug–membrane equilibrium, it is possible to reduce the latency without prolonging the duration, nor enhancing the intensity of motor blockade, but remaining at the site of action at sufficient concentration to improve the duration of analgesia. To explain this and the other factors involved in this purpose, pharmacokinetic and bioavailability studies are underway with this formulation.

Additionally, the rat infraorbital nerve blockade test provides information about intensity and duration of sensory blockade induced by local anaesthetic agents used as plain solutions (Fink et al 1975) or as pharmaceutical associations (vasoconstrictors, dextrans) (Hassan et al 1985 a, b), being an important experimental model, especially in dentistry for orofacial surgeries.

We have reported that the encapsulation in LUV intensified the analgesic effects of prilocaine (Cereda et al 2004, 2006), mepivacaine and lidocaine (Cereda et al 2006), showing that mepivacaine was affected to the greatest extent of analgesic effect, and that liposomes provided effective carriers for intermediate-duration local anaesthetics. In this study, our results also showed that RVC_{LUV} induced an improvement in intensity of total local anaesthetic effect associated with prolonged times for recovery at 0.125% ($P < 0.01$), 0.25% ($P < 0.05$) and 0.5% ($P < 0.01$), pointing out this liposomal system as a great advantage for the possible future use of RVC in dentistry.

Another important consideration is that comparisons among the partition coefficient values previously determined for other local anaesthetic molecules, such as bupivacaine (136 ± 32), lidocaine (114 ± 16), mepivacaine (93 ± 7) and prilocaine (57 ± 6) (de Araujo et al 2004; Cereda et al 2004, 2006),

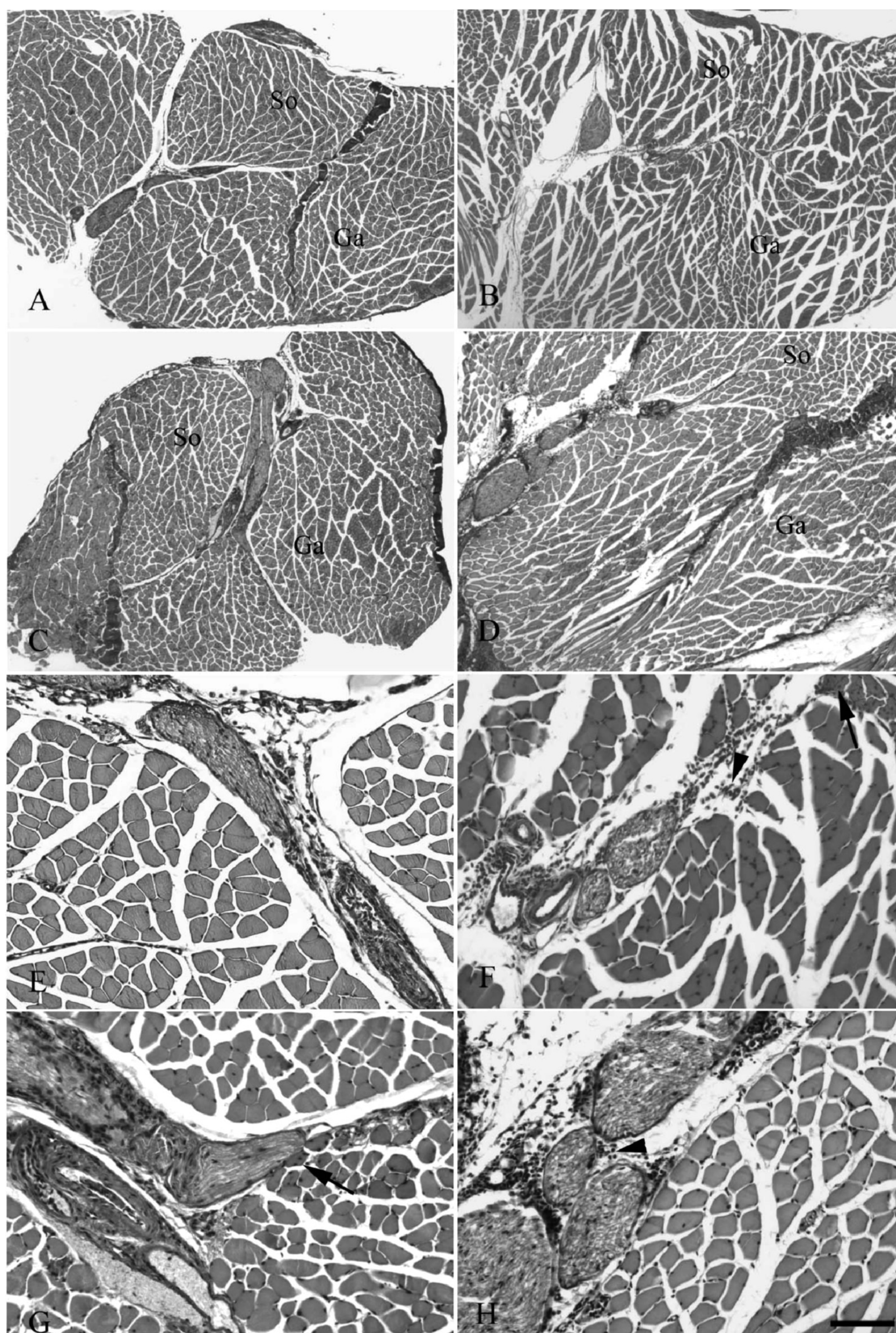

Figure 4 Transverse sections of mouse gastrocnemius (Ga) and soleus (So) muscles, in controls (A and E) and mice treated with LUV 5 mM (B and F), RVC 0.5% (C and G) or RVC_{LUV} 0.5% (D and H), showing normal muscle fibres and a few cells with central nuclei (arrows). Some inflammatory cells were also observed (arrowhead). Scale bar, 100 μm (A, B, C, D); 400 μm (E, F, G, H) (n = 6 or 7/group).

revealed that RVC (partition coefficient value 132 ± 26) has a relatively high hydrophobic character in relation to the other linear (lidocaine, prilocaine) and cyclic (mepivacaine) amino-amides, as expected by the length of the alkyl chain substitution (propyl) at its piperidine ring. Thus, the lipophilicity also justifies the differences in terms of anaesthesia (potency, toxicity and duration of action) since RVC is known to produce a sensory blockade profile similar to that of bupivacaine (Mizogami et al 2002).

The partition coefficient value of RVC corresponded to an encapsulation efficiency of approximately 24%, obtained from the liposome preparation method used here. Reports in the literature have described different procedures, such as freeze–thaw and dehydration–rehydration, showing increased

encapsulation efficiency (Grant et al 2001, 2003, 2004). In fact, to increase the encapsulation efficiency, to improve the liposomal stability and to extend the duration of this formulation, we have tested the liposome preparation by spray-drying in a pilot scale, showing promising results for other local anaesthetics such as lidocaine (Almeida 2008). Besides, we also focused our attention on the interaction with membrane lipid components, since this formulation presents approximately 40% mol cholesterol, reflecting the membrane lipid composition of nerve cells (Mizogami et al 2002). In this manner, we believe that by enhancing the encapsulation efficiency or changing the lipid composition or the molar ratios of the liposomal constituents and controlling the liposomal size (to avoid fast clearance or delayed onset) as well as the release rate, it will be possible to obtain a prolonged analgesic effect associated with lower cytotoxicity.

Concerning the in-vitro assay, a previous study reported that among the amino-amide local anaesthetics, bupivacaine significantly induced Schwann cell death, but this effect was not evoked by RVC (ranging from 0.001 to 1 mM concentration) (Park et al 2005). In our study, the cytotoxicity of RVC was assessed at higher concentrations (4.02, 8.04 and 16.08 mM) than those, to attain the clinical concentrations. One of the most important considerations about this toxicity model is that RVC induced cytotoxic effects in a dose-dependent manner, when used at these higher concentrations, and cellular protective effects were observed after RVC was encapsulated into liposomes.

Regarding myotoxicity, it is well-described that intramuscular injections of local anaesthetics regularly result in striated muscle damage and myonecrosis, with a drug-specific and dose-dependent rate of toxicity (Zink & Graf 2004). Although a variety of local anaesthetics used in clinical practice have been studied, few data are available about the recently introduced local anaesthetic ropivacaine.

Previous studies showed that the acute myotoxicity evoked by ropivacaine is less severe than that seen with bupivacaine after continuous peripheral nerve block (Zink et al 2003, 2005). In addition, another study evaluated the influence of two different concentrations of ropivacaine used in clinical practice, reporting that the muscle damage was reversible and also occurred in a dose-dependent manner after single intramuscular injection (Amaniti et al 2006).

The histopathologic changes and time course of skeletal muscle injury after local anaesthetic administration appear to be rather uniform and non-specific. In general, these morphologic alterations are characterized by hypercontracted myofibrils, followed by lytic degeneration of sarcoplasmic reticulum and by myocyte oedema and necrosis over the next 1–2 days. The Ca^{2+} released from the sarcoplasmic reticulum of skeletal muscle fibres and simultaneous inhibition of the Ca^{2+} reuptake is considered the major pathomechanism in local anaesthetic myotoxicity, especially for ropivacaine and bupivacaine (Zink et al 2005).

In this study, no obvious signs of cell damage were observed and there was no evidence of necrotic material around the area of the injection. This three days post-injection study revealed only the presence of sparse inflammatory cells in one of the mice treated with the vehicle, plain and liposomal RVC at the higher local anaesthetic concentration (0.5%), suggesting no adverse reaction upon application of the formulations, at this condition.

Even the skeletal muscle injuries are reversible within a few weeks and frequently remain clinically inapparent, limiting the detection of their clinical impact; all local anaesthetics that have been examined are myotoxic in clinical concentrations, with a drug-specific and dose-dependent rate of toxicity (Foster & Carlson 1980; Zink & Graf 2004). For this reason, further studies are necessary to evaluate the consequences of long-term exposure to this liposomal system and the reversibility of its effects on skeletal muscle.

Conclusions

Despite the differences between this study and the real clinical conditions, we showed an in-vitro–in-vivo evaluation of a liposomal system for RVC. Encapsulation into the liposomes provided an improvement in analgesic effect and a decrease in cytotoxicity of RVC in comparison with its plain solution. Thus, we suggest the liposomal system RVC_{LUV} as a potential new formulation, since RVC is a new and safe long-acting local anaesthetic agent.

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