Local Neurotoxicity and Myotoxicity Evaluation of Cyclodextrin Complexes of Bupivacaine and Ropivacaine

Cintia Maria Saia Cereda, PhD,* Giovana Radomille Tofoli, PhD,† Luiz Gabriel Maturana, MSc,‡ Amauri Pierucci, PhD,† Lazaro Alessandro Soares Nunes, PhD,* Michelle Franz-Montan, PhD,* Alexandre Leite Rodrigues de Oliveira, PhD,§ Sarah Arana, PhD,‖ Daniele Ribeiro de Araujo, PhD,¶ and Eneida de Paula, PhD*

BACKGROUND: Bupivacaine (BVC) and ropivacaine (RVC) are local anesthetics widely used in surgical procedures. In previous studies, inclusion complexes of BVC or RVC in hydroxypropyl-β-cyclodextrin (HP-β-CD) increased differential nervous blockade, compared to the plain anesthetic solutions. In this study we evaluated the local neural and muscular toxicity of these new formulations containing 0.5% BVC or RVC complexed with HP-β-CD (BVC_{HP-β-CD} and RVC_{HP-β-CD}).

METHODS: Schwann cell viability was assessed by determination of mitochondrial dehydrogenase activity, and histopathological evaluation of the rat sciatic nerve was used to identify local neurotoxic effects (48 hours and 7 days after the treatments). Evaluations of serum creatine kinase levels and the histopathology of rat gastrocnemius muscle (48 hours after treatment) were also performed.

RESULTS: Schwann cell toxicity evaluations revealed no significant differences between complexed and plain local anesthetic formulations. However, use of the complexed local anesthetics reduced serum creatine kinase levels 5.5-fold, relative to the plain formulations. The differences were significant at \( P < 0.05 \) (BVC) and \( P < 0.01 \) (RVC). The histopathological muscle evaluation showed that differences between groups treated with local anesthetics (BVC or RVC) and their respective complexed formulations (BVC_{HP-β-CD} or RVC_{HP-β-CD}) were significant \( (P < 0.05) \).

CONCLUSIONS: We concluded that the new formulations presented a lower myotoxicity and a similar cytotoxic effect when compared to plain local anesthetic solutions. (Anesth Analg 2012;115:1234–41)

Bupivacaine (BVC) and ropivacaine (RVC) are local anesthetics widely used in surgical procedures worldwide.\(^1,2\) In current clinical practice there is a need for local anesthetics whose action is long-lasting and which present low systemic uptake to minimize any toxic side effects.\(^3,4\) Drug delivery systems can be used to prolong the duration of action and reduce the toxicity of local anesthetics, since because they enable the controlled release of the anesthetic molecules. Local anesthetics carried in drug delivery systems such as cyclodextrins have been used as potential new formulations for pain treatment.\(^5,6\)

In previous studies by other workers\(^7,8\) and by our own research group,\(^9,11\) the inclusion of BVC or RVC in hydroxypropyl-β-cyclodextrin (HP-β-CD) increased the anesthetic effect after injection in the subarachnoid space or after sciatic nerve blockade, compared to plain solutions at the same concentration. However, for the development of new pharmaceutical formulations, such as the BVC-hydroxypropyl-β-cyclodextrin inclusion complex (BVC_{HP-β-CD}) or the RVC-hydroxypropyl-β-cyclodextrin inclusion complex (RVC_{HP-β-CD}), an assessment of toxicity is essential to provide the fundamental information required to ensure their safe application.

The aims of this study were therefore to: (1) evaluate the cytotoxicity of the new local anesthetic formulations, BVC_{HP-β-CD} and RVC_{HP-β-CD}, in primary Schwann cell cultures; (2) evaluate their local neurotoxicity by histopathological analysis of rat sciatic nerve after intraneural injection; and
(3) assess their local myotoxicity by observing changes in serum creatine kinase (CK) levels, and by histopathological assessment of the gastrocnemius muscle, after IM injection of the formulations.

**METHODS**

**Animal Care Committee**

The animals used in this study were newborn and adult (200 to 250 g) male Wistar (Unib: WH) rats obtained from (Centro Multidisciplinar para Investigação Biológica, State University of Campinas - UNICAMP, Brazil) certified by the International Council for Laboratory Animals Sciences (ICLAS). The rats were housed five per cage, with a 12:12 hour light-dark cycle, at 23 ± 2°C and with free access to water and food throughout the study. All experiments were approved by the Institutional Committee for Ethics in Animal Research of the State University of Campinas (Protocol numbers 1322-1, 1637–1 and 1983–1), which follows the recommendations of the International Council for Laboratory Animals Sciences Guide for the Care and Use of Laboratory Animals.

**Chemicals**

BVC hydrochloride and RVC hydrochloride were donated by Cristália Prod. Quim. Farm. Ltda. (Itapira, SP, Brazil) and HP-β-CD (Kleptose HP®) was obtained from Roquette Serv. Tech. Lab. (Lestrem, France). HEPES buffer, lidocaine hydrochloride, urethane, α-chloralose, 3,4,5-trimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin, and 4,6-diamidino-2-phenylindole dihydrochloride were purchased from Sigma Chem. Corp. (St. Louis, MO, USA). Polyclonal anti-S100 antibody was obtained from DAKO (Glostrup, Denmark), and Dubelco’s Modified Eagle’s Medium (DMEM), collagenase, and trypsin were obtained from Nutricell (Campinas, SP, Brazil). Embed 812 resin and osmium tetroxide were purchased from Electron Microscopy Sciences (Hatfield, PA, USA). Tolyuidine blue and glutaraldehyde were acquired from Merck KGaA (Darmstadt, Germany). All other reagents were of analytical grade.

**Preparation of Local Anesthetic Inclusion Complexes**

The inclusion complexes were produced by mixing equimolar (1:1 molar ratio) amounts of HP-β-CD and either BVC or RVC in deionized water, at room temperature (25 ± 1°C) for 24 hours. After reaching equilibrium, with complete dissolusion, the solution was freeze-dried (Labconco Freezone 4.5 freeze dry system) and stored at -20°C before further use.10 Immediately before use, 20 mM HEPES in 154 mM NaCl buffer was added to the solid complexes to obtain a solution, the solution was freeze-dried (Labconco Freezone 4.5 freeze dry system) and stored at -20°C before further use.10

**Histopathological Analysis**

For the surgical procedures, the animals were anesthetized with urethane (1 g/kg i.p.) plus α-chloralose (50 mg/kg −1), injected intraperitoneally. The sciatic nerves were exposed by lateral incision of the thighs and retraction of the superficial fascia and muscle, so that the underlying connective tissue layer, containing the sciatic nerve, was not damaged.18 After exposure of the sciatic nerve, a 30-gauge needle was introduced parallel to the direction of the nerve fibers, and the rats of each group (n = 12 per group) were intraneurally treated with separate 0.1 mL doses of BVC, RVC, BVC<sub>HP-β-CD</sub>, RVC<sub>HP-β-CD</sub>, HP-β-CD, 0.9% NaCl (negative control), or 15% lidocaine (positive control). The sciatic nerve was excised after either 48 hours (n = 6 per group) or 7 days (n = 6 per group). The samples were fixed by immersion for 24 hours in 2.5% phosphate-buffered glutaraldehyde (the chemical fixative of choice for nervous

**Neurotoxicity Evaluation**

**Cell Culture and Cytotoxicity Assay: Sciatic Nerve Schwann Cells**

Purified cultures of the Schwann cells used for the cytotoxicity tests were prepared according to Hammarberg et al.12 and Pierucci et al.13 The neonatal rats were deeply anesthetized by hypothermia. Adequate anesthesia was ascertained by the absence of any pain reflex, which was tested by carefully pinching the forelimb paws with a fine forceps.14 Dislocation of the neck was then performed. Sciatic nerves complete with the nerve sheaths were dissected from the newborn rats. The nerves were then aseptically dissected out from the epineurium and surrounding tissue.

The fragmented nerves were incubated in 0.05% collagenase (30 min, 37°C), followed by incubation in 0.15% trypsin (15 minutes). The cell mixture was recovered by centrifugation (250 × g, 10 minutes) in 3% bovine serum albumin BSA and resuspended in DMEM containing 10% fetal bovine serum supplement together with glucose, insulin-like nerve growth factor, pituitary extract, forskolin, and antibiotics (penicillin and streptomycin). The cells were seeded into a plastic cell culture dish with 96 wells (Corning-Costar Co., Cambridge, MA) and cultured for 2 days (37°C, 5% CO2). To evaluate the purity of the Schwann cell culture by the binding of anti-S100 antibody, cultures were fixed using 4% paraformaldehyde in phosphate buffer for 10 minutes (pH 7.4, 37°C), rinsed in phosphate buffered saline (37°C), and incubated with antirabbit Cy3 conjugated antibody for 45 minutes. The observations were performed using a Nikon Eclipse TS100 microscope equipped with a fluorescence analysis attachment (Nikon Instruments Inc., Melville, NY).

Cell viability was assessed by determination of mitochondrial dehydrogenase activity15–17 (n = 6 replicates for each 1 of the 3 different experiments). After 24-hour exposure to different concentrations of the tested formulations, the medium containing 0.5 mg mL<sup>-1</sup> MTT was changed in each well of the plates. The medium was removed after MTT incubation for 3 hours at 37°C, and 100 μL of ethanol was added to dissolve the formazan crystals. The absorbance of the resulting purple solution was determined spectrophotometrically at 570 nm (BioTek Instruments Inc., Winooski, VT).15–17 The results (mean ± SD) were expressed as a percentage of the value obtained for the untreated control and the inhibition concentration for a 50% reduction of the cell number per culture (IC50) was calculated for each tested formulation. The IC50 values were determined by nonlinear regression analysis using a sigmoidal concentration-response equation of individual experiments using Origin 6.0 (MicrocalTM Software, Inc., Northampton, MA).

**For the surgical procedures, the animals were anesthetized with urethane (1 g/kg i.p.) plus α-chloralose (50 mg/kg −1), injected intraperitoneally. The sciatic nerves were exposed by lateral incision of the thighs and retraction of the superficial fascia and muscle, so that the underlying connective tissue layer, containing the sciatic nerve, was not damaged.**18 After exposure of the sciatic nerve, a 30-gauge needle was introduced parallel to the direction of the nerve fibers, and the rats of each group (n = 12 per group) were intraneurally treated with separate 0.1 mL doses of BVC, RVC, BVC<sub>HP-β-CD</sub>, RVC<sub>HP-β-CD</sub>, HP-β-CD, 0.9% NaCl (negative control), or 15% lidocaine (positive control). The sciatic nerve was excised after either 48 hours (n = 6 per group) or 7 days (n = 6 per group). The samples were fixed by immersion for 24 hours in 2.5% phosphate-buffered glutaraldehyde (the chemical fixative of choice for nervous

**Histopathological Analysis**

For the surgical procedures, the animals were anesthetized with urethane (1 g/kg i.p.) plus α-chloralose (50 mg/kg −1), injected intraperitoneally. The sciatic nerves were exposed by lateral incision of the thighs and retraction of the superficial fascia and muscle, so that the underlying connective tissue layer, containing the sciatic nerve, was not damaged.18 After exposure of the sciatic nerve, a 30-gauge needle was introduced parallel to the direction of the nerve fibers, and the rats of each group (n = 12 per group) were intraneurally treated with separate 0.1 mL doses of BVC, RVC, BVC<sub>HP-β-CD</sub>, RVC<sub>HP-β-CD</sub>, HP-β-CD, 0.9% NaCl (negative control), or 15% lidocaine (positive control). The sciatic nerve was excised after either 48 hours (n = 6 per group) or 7 days (n = 6 per group). The samples were fixed by immersion for 24 hours in 2.5% phosphate-buffered glutaraldehyde (the chemical fixative of choice for nervous

**Neurotoxicity Evaluation**

**Cell Culture and Cytotoxicity Assay: Sciatic Nerve Schwann Cells**

Purified cultures of the Schwann cells used for the cytotoxicity tests were prepared according to Hammarberg et al.12 and Pierucci et al.13 The neonatal rats were deeply anesthetized by hypothermia. Adequate anesthesia was ascertained by the absence of any pain reflex, which was tested by carefully pinching the forelimb paws with a fine forceps.14 Dislocation of the neck was then performed. Sciatic nerves complete with the nerve sheaths were dissected from the newborn rats. The nerves were then aseptically dissected out from the epineurium and surrounding tissue.

The fragmented nerves were incubated in 0.05% collagenase (30 min, 37°C), followed by incubation in 0.15% trypsin (15 minutes). The cell mixture was recovered by centrifugation (250 × g, 10 minutes) in 3% bovine serum albumin BSA and resuspended in DMEM containing 10% fetal bovine serum supplement together with glucose, insulin-like nerve growth factor, pituitary extract, forskolin, and antibiotics (penicillin and streptomycin). The cells were seeded into a plastic cell culture dish with 96 wells (Corning-Costar Co., Cambridge, MA) and cultured for 2 days (37°C, 5% CO2). To evaluate the purity of the Schwann cell culture by the binding of anti-S100 antibody, cultures were fixed using 4% paraformaldehyde in phosphate buffer for 10 minutes (pH 7.4, 37°C), rinsed in phosphate buffered saline (37°C), and incubated with antirabbit Cy3 conjugated antibody for 45 minutes. The observations were performed using a Nikon Eclipse TS100 microscope equipped with a fluorescence analysis attachment (Nikon Instruments Inc., Melville, NY).

Cell viability was assessed by determination of mitochondrial dehydrogenase activity15–17 (n = 6 replicates for each 1 of the 3 different experiments). After 24-hour exposure to different concentrations of the tested formulations, the medium containing 0.5 mg mL<sup>-1</sup> MTT was changed in each well of the plates. The medium was removed after MTT incubation for 3 hours at 37°C, and 100 μL of ethanol was added to dissolve the formazan crystals. The absorbance of the resulting purple solution was determined spectrophotometrically at 570 nm (BioTek Instruments Inc., Winooski, VT).15–17 The results (mean ± SD) were expressed as a percentage of the value obtained for the untreated control and the inhibition concentration for a 50% reduction of the cell number per culture (IC50) was calculated for each tested formulation. The IC50 values were determined by nonlinear regression analysis using a sigmoidal concentration-response equation of individual experiments using Origin 6.0 (Microcal™ Software, Inc., Northampton, MA).
system tissue). Osmium tetroxide (1%) was used as a secondary fixative, because it binds to lipids, enhancing contrast in light microscopy.21

After dehydration, the specimens were embedded in Embed 812 resin, cut into 1 μm-thick semithin sections (6 different depths) using a microtome (Model RM2145, Leica Microsystems, Wetzlar, Germany) and stained with toluidine blue. The nerve samples underwent histological analysis using a light microscope (Model Eclipse E-800, Nikon Instruments, Melville, NY) fitted with a video camera (Cool SNAP-Pro color, Media Cybernetics, Bethesda, MD) to measure axonal swelling and neuronal degeneration. An injury score22 of 0 to 4 was assigned for each slide (0 = normal; 1 = minimal; 2 = slight; 3 = moderate; 4 = marked). The final results were expressed as medians with 25th and 75th percentiles. The images were codified and 2 individuals blinded to treatment conditions evaluated the images according to the qualitative score previously described.

**Myotoxicity Assessment**

**Determination of Serum Creatine Kinase Levels in Rats**

Animals (n = 6 to 7 per group) were treated by injection (0.1 mL) of the vehicles (0.9% NaCl or HP-β-CD) and the plain or complexed local anesthetics (0.5% BVC or RVC) into the gastrocnemius muscle. Two hours after administration,23 the animals were killed under intraperitoneal anesthesia with urethane and α-chloralose (1 g/kg and 50 mg/kg, respectively). Blood samples were collected by cardiac puncture and centrifuged at 1500 x g for 10 minutes. The serum was analyzed by spectrophotometry at 340 nm (Autolab, Boehringer, Mannheim, Germany), using a commercially available kit (CK-NAC, Wiener Lab, Rosario, Argentina).

**Histopathological Analysis**

Histopathological analysis of the rat gastrocnemius muscle (n = 6 per group) was performed after IM administration of the tested formulations. The samples were fixed by immersion in 10% formalin solution (48 hours). After fixation, the samples were dehydrated and embedded in paraffin. Cross-sections (5 μm) were obtained at 5 different depths using the microtome and stained with hematoxylin and eosin. The sample analyses were performed using light microscopy, as described above, with observation of the presence of fiber destruction or myonecrosis. A score of 0 to 3 was assigned for each slide: 0 = no fiber damage, 1 = localized and/or sparsely scattered fiber destruction, 2 = more extensive necrosis after major connective tissue planes and involving numerous muscle fascicles, and 3 = destruction of essentially the entire muscle mass in the eye-field.22-25 The images were codified and 2 individuals blinded to treatment conditions evaluated the images according to the qualitative score previously described.

**Statistical Analysis**

Comparisons performed by 1-way ANOVA, with the post hoc Tukey-Kramer multiple comparisons test (used to compare all pairs), were used in the evaluation of serum CK levels analysis. The unpaired t-test was used to evaluate differences in the Schwann cell viability analysis. Differences in muscle damage between treatment groups were examined for significance by using Mann-Whitney test. The results of neural histopathological evaluation were submitted to the WMWoods analysis to observe differences between the neural damage evoked by the tested formulations. All samples were randomly selected from the population. Each subject (or each experimental unit) was sampled independently of the rest. The 1-way ANOVA and unpaired t-test assume that the data are sampled from population that follow Gaussian distribution. The Mann-Whitney test does not assume that data follow a Gaussian distribution. Statistical significance was defined as P < 0.05.26 Data were analyzed using the GraphPad Instat program, version 3.0 (GraphPad Software, San Diego, CA). The WMWoods analysis is available from Dr. Ralph O’Brien (ObrienRalph@gmail.com) with the WMWoods function in the RStudio Software (R version 2.14.2© 2012 The R Foundation for Statistical Computing, ISBN 3-900051–07-0).

**RESULTS**

**Neurotoxicity Evaluation**

**Cell Culture and Cytotoxicity Assay Using Sciatic Nerve Schwann Cells**

The purity of the primary Schwann cell culture used was confirmed by binding with the anti-S-100 antibody associated with 4,6-diamidino-2-phenylindole dihydrochloride cytochemistry. Different concentrations, determined by aqueous solubility at pH 7.4 (8 mM for RVC and 4 mM for BVC),31 were used to evaluate in vitro cytotoxicity evoked by RVC and BVC. BVC formulations, both plain and complexed with HP-β-CD, were tested at concentrations ranging from 0.4 mM to 4 mM. The formulations of RVC, whose aqueous solubility31 is higher than BVC’s, were tested at concentrations ranging from 0.8 mM to 8 mM. The results (Fig. 1) showed no statistically significant differences (unpaired t-test) between cultures treated either with the plain solutions and their respective complexed formulations: RVC (IC50 = 3.67 ± 0.45) versus RVC<sub>HP-β-CD</sub> (IC50 = 3.98 ± 0.05) (P = 0.22, 95% confidence interval = -0.87 to 0.25) and BVC (IC50 = 1.42 ± 0.14) versus BVC<sub>HP-β-CD</sub>.
At a concentration of 2 mM, both the plain and complexed formulations of BVC caused a decrease of about 80% in the viability of neural cells. On the other hand, the viability curves obtained after treatment with both plain and complexed RVC in 4 mM showed a decrease of 70% in primary Schwann cell viability. In the comparison between RVC and BVC, statistical analysis showed significant differences (unpaired t-test, P < 0.001, 95% confidence interval = -2.83 to -1.67).

**Histopathological Evaluation**

Results for the groups evaluated at 48 hours and 7 days after intraneurale injection of the formulations tested (RVC, BVC, RVC–HP–CD, BVC–HP–CD, HP–β–CD, 0.9% NaCl, and lidocaine) are summarized in Table 1.

Histopathological images of the sciatic nerves did not reveal any morphological tissue changes, such as axonal swelling, neuronal degeneration, or signs of cell infiltration, after application of the plain (0.5%) or complexed local anesthetic formulations (0.5%) (Fig. 2, C–F) and after application of 0.9% NaCl or HP–β–CD (Fig. 2, A and B). However, the statistical analysis (WMWoods analysis) between the groups (Table 2) showed that, although P > 0.05, the odds ratios are very wide and, consequently, it is not possible to conclude that there is no difference. In addition, lidocaine at high concentration (15%), used as a positive control, induced local neurotoxicity, evidenced by signs of endoneurial edema and total axonal degeneration (Fig. 2, G).

**Myotoxicity Evaluation**

**Evaluation of Serum Creatine Kinase Levels in Rats**

The plain formulations of RVC and BVC (0.5%) caused a 5.5-fold increase in serum CK levels, leading to cell lysis and leakage of CK to the blood, confirming the myotoxicity of the local anesthetics (Fig. 3). The inclusion complexes (RVC–HP–CD and BVC–HP–CD at 0.5%) induced increases in the serum levels of this enzyme (3.3- and 3.8-fold, respectively) that were significantly lower than those obtained after treatment with the plain anesthetic formulations, indicating protection against local anesthetic myotoxicity.

**Histopathological Analysis**

There was no sign of muscle damage or myonecrosis in the group injected with 0.9% NaCl, and the same result was obtained for animals treated with the HP–β–CD vehicle. However, the animals treated with the local anesthetic formulations showed varying degrees of muscle fiber damage (Fig. 4). The median (25th–75th) score values were 0.0 (0.0–0.0) for the 0.9% NaCl group and for the HP–β–CD group. For BVC, BVC–HP–CD, RVC, and RVC–HP–CD, the corresponding values were 1.9 (1.65–2.0), 1.4 (1.4–1.55), 1.4 (1.4–1.4), and 0.9 (0.8–1.0), respectively. When comparing BVC or RVC groups and their respective

**Table 1. Median (25th–75th) of the Score Values (0 to 4) Obtained From the Histopathological Evaluation of the Rat Sciatic Nerve, 48 H and 7 Days After the Treatments**

<table>
<thead>
<tr>
<th>Tested groups</th>
<th>Median scores (25th–75th)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 h</td>
</tr>
<tr>
<td>0.9% NaCl</td>
<td>0.33 (0.33–0.38)</td>
</tr>
<tr>
<td>HP–β–CD</td>
<td>0.70 (0.42–0.70)</td>
</tr>
<tr>
<td>BVC (0.5%)</td>
<td>0.60 (0.46–0.70)</td>
</tr>
<tr>
<td>BVC–HP–CD (0.5%)</td>
<td>0.66 (0.45–0.83)</td>
</tr>
<tr>
<td>RVC (0.5%)</td>
<td>0.50 (0.44–0.62)</td>
</tr>
<tr>
<td>RVC–HP–CD (0.5%)</td>
<td>0.50 (0.33–0.66)</td>
</tr>
<tr>
<td>LDC (15%) (positive control)</td>
<td>2.65 (2.2–3.2)</td>
</tr>
</tbody>
</table>

Local Anesthetics in Cyclodextrin: Toxicity Assays

<table>
<thead>
<tr>
<th>Treatments</th>
<th>WMWoods</th>
<th>95% confidence interval (2-sided)</th>
<th>P (2-sided)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RVC × RVC&lt;sub&gt;HP-β-CD&lt;/sub&gt; (48 h)</td>
<td>0.31</td>
<td>[0.08–1.34]</td>
<td>0.13</td>
</tr>
<tr>
<td>RVC × RVC&lt;sub&gt;HP-β-CD&lt;/sub&gt; (7 days)</td>
<td>0.76</td>
<td>[0.22–2.67]</td>
<td>0.69</td>
</tr>
<tr>
<td>BVC × BVC&lt;sub&gt;HP-β-CD&lt;/sub&gt; (48 h)</td>
<td>1.25</td>
<td>[0.36–4.32]</td>
<td>0.75</td>
</tr>
<tr>
<td>BVC × BVC&lt;sub&gt;HP-β-CD&lt;/sub&gt; (7 days)</td>
<td>0.31</td>
<td>[0.08–1.34]</td>
<td>0.13</td>
</tr>
<tr>
<td>HP-β-CD × 0.9% NaCl (7 days)</td>
<td>0.38</td>
<td>[0.10–1.55]</td>
<td>0.20</td>
</tr>
<tr>
<td>HP-β-CD × 0.9% NaCl (7 days)</td>
<td>0.80</td>
<td>[0.23–2.81]</td>
<td>0.75</td>
</tr>
</tbody>
</table>

RVC = ropivacaine; RVC<sub>HP-β-CD</sub> = hydroxypropyl-β-cyclodextrin ropivacaine; BVC = bupivacaine; BVC<sub>HP-β-CD</sub> = hydroxypropyl-β-cyclodextrin bupivacaine; HP-β-CD = hydroxypropyl-β-cyclodextrin; 0.9% NaCl = saline solution.

Table 2. Statistical Analysis (WMWoods) of Rat Sciatic Nerve Histopathological Evaluation After Treatments

**DISCUSSION**

RVC and BVC are chemically related amino-amide local anesthetics, widely used for regional and surgical anesthesia as well as for the relief of postoperative and labor pain. One of the most important aspects of the development of new RVC or BVC formulations is the possibility of achieving prolonged analgesic effects using a small local dose that minimizes plasma concentrations of the anesthetic.

Many different systems have been developed for this purpose, using carriers such as liposomes, microspheres, and nanocapsules and cyclodextrins. The studies reported in the literature suggest that complexation of local anesthetics with cyclodextrins prolongs the duration and enhances the intensity of the pharmacological effects of the anesthetics. Local anesthetics agents such as BVC and RVC are relatively hydrophobic ionizable amines that undergo partitioning into the lipid membrane phase, as a requirement to their action at the sodium channel. Moreover lipophilic molecules such as BVC and RVC have the highest potencies and systemic toxicities among the clinically used local anesthetics. It is therefore reasonable to think that membrane partitioning could be responsible for some of the toxic effects caused by these molecules.

Various methodologies have been used to evaluate the cytotoxicity induced by drugs and carriers, including colorimetric assays (involving uptake or exclusion of a dye), enzymatic dosages and lyses of red blood cells. The cytotoxic evaluation of a drug, as well as of its carrier, is vital to establish therapeutic potential.

In this study, in the sciatic nerve Schwann cells cytotoxicity assay, we reported a lower cytotoxicity of RVC, compared to BVC. This can be accounted by the lower hydrophobicity of RVC, which results in reduced partitioning into membranes and hence a lower lytic effect. This hypothesis is supported by the results of Werdehausen et al. who found that local anesthetics (including RVC and BVC) induced apoptosis in a neuroblastoma cell line, and that toxicity correlated with lipophilicity. Zink et al. showed that BVC decreased the uptake and increased the release of calcium from the sarcoplasmic.
reticulum. This led to an increase in the intracellular concentration of this ion, contributing to the occurrence of toxic effects in skeletal muscle. The local myotoxic effect of BVC in skeletal muscle mitochondria of rats was investigated by Irwin et al., who observed a concentration-dependent mitochondrial depolarization caused by BVC. This depolarization increased membrane permeability, which led to cell death. Acute and chronic myotoxic effects of BVC and RVC, after peripheral nerve block, were evaluated by Zink et al. Local anesthetics were administered by continuous injection for 6 hours, and muscle tissues were collected and processed after 1 hour, 7 days, and 28 days from the end of the injection, showing that RVC’s myotoxic potential is more moderate than that of BVC. Amaniti et al. found that RVC administered in a single IM dose (at concentrations of 0.5% and 0.75%) caused dose-dependent damage in rat muscle. A combination of BVC and tetrodotoxin, a potent sodium channel blocker, administered in rats, has been found to cause increased duration of neural blockade without a consequent increase in myotoxicity. Investigation of a sustained release formulation of BVC in microparticles (polymeric microspheres and lipid–protein–sugar particles) detected local myotoxicity after sciatic blockade in rats. Here, results of the morphological analyses and measurements of serum CK levels showed that RVC-HP-β-CD and BVC-HP-β-CD formulations were less toxic to muscle cells than plain local anesthetic formulations at the same concentration (0.5%). Furthermore, the HP-β-CD vehicle neither reduced the viability of neural cells nor induced myotoxic effects. These findings are supported by studies reported in the literature that have shown that HP-β-CD is a well-tolerated vehicle in animals and humans.

Previous studies have reported that RVC and BVC, at clinical concentrations, do not cause local neurotoxicity. Bouaziz et al. showed that there were no histopathological changes in the sciatic nerve of rats resulting from the direct deposition of RVC or levobupivacaine (at concentrations of 0.25% to 0.75%), 48 hours after treatment. It has also been found that RVC (0.2% to 0.75%) did not cause neuropathological changes in rats over periods from 1 to 67 days. Sakura et al. tested the local anesthetics lidocaine and BVC in equimolar concentrations in rats, which were higher than the doses used clinically, in the spinal cord of rats, and observed that, at this high concentration, lidocaine showed neurotoxic effects, while although BVC, even at high concentrations, did not cause nerve changes. In our current study work, the histopathological images of rat sciatic nerves after treatments did not show morphological changes. However, the statistical analysis does not allow concluding that there are no significant differences between the groups. The small sample size is a limitation of this study.

It is also important to emphasize the need to perform other tests to complete an evaluation of toxicity of these new formulations with HP-β-CD. Studies in animals about the toxicity of β-cyclodextrin (β-CD) and their derivatives indicated that nephrotoxicity may be considered as an important toxic effect. Renal changes induced by β-CD occur especially in the proximal tubule and are accompanied by vacuolation, cell disintegration, and mineralization. Thus, an important next step to evaluate the new HP-β-CD formulations toxicity is a nephrotoxicity assessment.

In conclusion, our results indicate that the vehicle, HP-β-CD, did not reduce Schwann cell viability. It was also found that the new inclusion complex formulations, RVC-HP-β-CD and BVC-HP-β-CD, showed cytotoxicities equivalent to those of equipotent plain anesthetic solutions. In the case of myotoxicity, the inclusion complexes of RVC and BVC presented lower local toxicity to muscle cells, compared to plain local anesthetic formulations.

DISCLOSURES

Name: Cintia Maria Saia Cereda, PhD.
Contribution: This author helped design the study, analyze the data, and write the manuscript.
Attestation: Cintia Maria Saia Cereda has seen the original study data, reviewed the analysis of the data, approved the final manuscript, and is the author responsible for archiving the study files.
Name: Giovana Radomille Tofoli, PhD.
Contribution: This author helped conduct the study.
Attestation: Giovana Radomille Tofoli has seen the original study data and approved the final manuscript.
Name: Luiz Gabriel Maturana, MSc.
Contribution: This author helped conduct the study.
Attestation: Luiz Gabriel Maturana has seen the original study data and approved the final manuscript.
Name: Amauri Pierucci, PhD.
Contribution: This author helped conduct the study.
Attestation: Amauri Pierucci has seen the original study data and approved the final manuscript.
Name: Lazaro Alessandro Soares Nunes, PhD.
Contribution: This author helped conduct the study and data collection.
Attestation: Lazaro Alessandro Soares Nunes has seen the original study data and approved the final manuscript.
Name: Michelle Franz-Montan, PhD.
Contribution: This author helped write the manuscript.
Attestation: Michelle Franz-Montan has seen the original study data and approved the final manuscript.
Name: Alexandre Leite Rodrigues de Oliveira, PhD.
Contribution: This author helped design the study.
Attestation: Alexandre Leite Rodrigues de Oliveira has seen the original study data and approved the final manuscript.
Name: Sarah Arana, PhD.
Contribution: This author helped design the study and analyze the data.
Attestation: Sarah Arana has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.
Name: Daniele Ribeiro de Araujo, PhD.
Contribution: This author helped design the study, analyze the data, and write the manuscript.
Attestation: Daniele Ribeiro de Araujo has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.
Name: Eneida de Paula, PhD.
Contribution: This author helped analyze the data and write the manuscript.
Attestation: Eneida de Paula has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.

This manuscript was handled by: Terese T. Horlocker, MD.
Local Anesthetics in Cyclodextrin: Toxicity Assays

ACKNOWLEDGMENTS

The authors are grateful to Dr. Ralph O'Brien for providing material and guidance for WMWoods analysis. The authors also thank Luis H. S. Cereda for his kind contribution with this manuscript.

REFERENCES

10. Araujo DR, Braga AFA, Moraes CM, Fraceto LF, de Paula E. Complexation of 50% enantiomer excess (S75-R25) bupivacaine with cyclodextrins and spinal block anesthesia in rats. Rev Bras Anestesiol 2006;56:495–06.
45. Padera RF, Tse JY, Bellas E, Kohane DS. Tetrodotoxin for prolonged local anesthesia with minimal myotoxicity. Muscle Nerve 2006;34:747–53