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Local Anesthetic Schwann Cell Toxicity is Time and Concentration-Dependent

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Abstract

Background—Peripheral nerve blocks (PNB's) with local anesthetics (LA's) are commonly performed to provide surgical anesthesia or postoperative analgesia. Nerve injury resulting in persistent numbness or weakness is a potentially serious complication. LA's have previously been shown to damage neuronal and Schwann cells *via* several mechanisms. We sought to test the hypothesis that LA's are toxic to Schwann cells and that the degree of toxicity is directly related to the concentration of LA and duration of exposure. Intraneural injection of LA's has been shown to produce nerve injury. We sought to test the hypothesis that a prolonged extraneural infusion of LA can also produce injury.

Materials and Methods—Schwann cells cultured from neonatal rat sciatic nerves were incubated with LA's at different concentrations (10, 100, 500 and 1000 μ M), and each concentration was assessed for toxicity after 4, 24, 48 and 72 hours of exposure. LA's tested were lidocaine, mepivacaine, chloroprocaine, ropivacaine and bupivacaine. Cell death was assessed by LDH release measured by optical density.

In a separate experiment, a microcatheter was placed along the sciatic nerves of Sprague-Dawley rats. Rats were randomly assigned to receive either 0.9% saline (n = 8) or bupivacaine (0.5% n = 4, 0.75% n = 4) *via* the perineural catheters for 72 hours. The rats were then euthanized and their nerves sectioned and stained for analysis. Sections were stained for myelin and with an anti-macrophage (CD68) antibody.

Results—None of the LA's tested produced significant Schwann cell death at very low concentrations (10mM, or 0.0003%) even after prolonged exposure. With prolonged exposure (48 or 72 hours) to high concentrations (1000 μ M, or 0.03%), all of the LA's tested produced significant Schwann cell death (increased LDH release relative to control as measured by optical density 0.384–0.974, all *p*-values < 0.001). Only bupivacaine produced significant cell death (0.482, *p* < 0.001) after prolonged exposure to low concentrations (100 μ M, or 0.003%). At

intermediate concentrations (500 μM , or 0.015%), cell death was more widespread with bupivacaine (0.768, $p < 0.001$) and ropivacaine (0.675, $p < 0.001$) than the other agents (0.204–0.368, all p -values < 0.001).

Prolonged extraneural exposure of rat sciatic nerves to bupivacaine caused significant demyelination and infiltration of nerves with inflammatory cells.

Conclusions—LA's induce Schwann cell death in a time and concentration-dependent manner. Bupivacaine and ropivacaine have greater toxicity at intermediate concentrations, and prolonged exposure to bupivacaine produces significant toxicity even at low concentrations. Brief exposure to high concentrations of bupivacaine damages Schwann cells. Prolonged extraneural infusion of bupivacaine results in nerve injury.

Anesthesiologists commonly use local anesthetics (LA's) to perform peripheral nerve blocks (PNB's) for surgical anesthesia or to provide analgesia for surgical procedures or other painful conditions. PNB's have been shown to allow superior early recovery compared to general anesthesia (decreased pain, nausea, vomiting, time to discharge home, rate of unplanned admission) (^{1–4}), provide better pain control than systemic opioid analgesics (⁵), and facilitate aggressive rehabilitation, improving long-term functional outcomes (^{6–9}). However, PNB's may result in nerve damage (^{10–13}). The effects are usually minor and resolve spontaneously in a few days or weeks. Unfortunately, these injuries are occasionally severe and may be permanent and debilitating (^{14, 15}). Such injuries may be multifactorial, though LA cytotoxicity is likely a major contributing factor (¹⁶). An improved understanding of the effects of LA's on nerve cells may lead to strategies to decrease the risk of nerve injury following PNB's.

In previous studies injection of LA's within the sciatic nerve sheath have been found to cause demyelination, Schwann cell death, and infiltration of inflammatory cells (^{17–20}). More recent *in vitro* studies have demonstrated that clinically used LA's act to uncouple oxidative phosphorylation in mitochondria isolated from rat liver cells (²¹). Micro-array analysis showed that bupivacaine exposure increased expression of cDNA for heat shock protein 70 (HSP70), c-jun, c-fos genes and decreased expression of c-myc and poly-ADP ribose polymerase (PARP) in a promyelocytic leukemia cell line (²²). Lidocaine, bupivacaine, and ropivacaine have been shown to induce apoptosis in neuronal cells *in vitro* through the activation of enzymes p38 MAPK, ERK and JNK (^{23, 24}). LA's (including procaine, mepivacaine, lidocaine, chloroprocaine, ropivacaine, and bupivacaine) have also been shown to cause necrosis of neuroblastoma cells *in vitro*, as well as apoptosis *via* activation of caspase (²⁵). Previous *in vitro* studies showed that bupivacaine induced apoptosis in Schwann cells through the generation of reactive oxygen species (ROS) (²⁶). Unlike this study however, we used primary rat Schwann cells in our experiments.

The overall goal of the current study was to test the hypothesis that LA's cause injury to Schwann cells. Schwann cells are an important component of the peripheral nervous system. They ensheath the axon and play an important part in axonal growth and regeneration, myelination, and normal electrophysiological conductivity. Therefore, damage to Schwann cells is likely to have a direct effect on the conductivity of the axon and may cause pathologic changes in neurons or delay the recovery of nerve conduction beyond the pharmacologic duration of action of the local anesthetic (^{27–29}).

We used a primary Schwann cell culture model to assess the effects of commonly used LA's for up to 72 hours of exposure *in vitro*. We also used an *in vivo* model in which LA's were infused for 72 hours *via* a catheter placed in close proximity to the sciatic nerve in rats.

Materials and Methods

Experimental protocols were approved by the Institutional Animal Care and Use Committee and conform to the National Institutes of Health guidelines for the care and use of animals in research.

Chemicals

The LA clinical solutions used in our experiments were purchased from Hospira (Lake Forest IL, USA). The LA powders, cytosine arabinoside, guinea pig complement, anti-S100 antibody, and anti-Thy 1.1 antibody were purchased from Sigma-Aldrich (St. Louis MO, USA). The bovine pituitary extract was purchased from Upstate (Lake Placid NY, USA). The forskolin was purchased from Calbiochem (Gibbstown NJ, USA), and the anti-macrophage antibody was purchased from Chemicon (Temecula CA, USA).

Cell Culture

Primary Schwann cells were cultured from neonatal rat sciatic nerves by methods published previously^(30, 31). Sciatic nerves harvested from 2–3 day-old Sprague-Dawley rat pups (Charles River Laboratories; Wilmington MA, USA) were dissociated enzymatically and grown in Dulbecco modified Eagle's medium (DMEM) with 10% fetal bovine serum containing 10 μ M cytosine arabinoside on poly-L-ornithine coated six-well plates. Following a 48 hour incubation period, the cells were grown in DMEM and 10% fetal bovine serum (FBS) with 2 μ M forskolin and 10 μ g/ml bovine pituitary extract. After cell confluence, the cells were trypsinized and incubated for 1 hour at 4°C in DMEM and fetal bovine serum containing monoclonal anti-Thy1.1 antibody. We then removed the unbound antibody by replacing the medium with DMEM containing guinea pig complement (3:1). The cells were then incubated for 1 hour at 37°C. The cells were removed by centrifugation, resuspended in DMEM and fetal bovine serum with 2 μ M forskolin and 10 μ g/ml bovine pituitary extracts, and plated onto poly-L-ornithine coated 96-well plates for the experiment. For all of the experiments, only cells from the first or second passage were used.

To assess the purity of the culture, the cells were fixed in 10% formalin for 15 minutes and washed in phosphate buffered saline (PBS) three times for five minutes each cycle. Cells were then incubated for 20 minutes in 3% Triton x-100 (Sigma) in PBS. To mark the Schwann cells, they were again washed in PBS and incubated overnight with anti-S100 polyclonal antibody (diluted 1:1000 in 2% goat serum in PBS). The cells were then washed again in PBS and incubated for 2 hours at 37°C with fluorescein isothiocyanate (FITC, Vector Laboratories Inc; Burlingame CA, USA) conjugated to a goat anti-rabbit antibody (diluted 1:100 2% goat serum in PBS). To mark the fibroblasts, the cells were washed again in PBS and incubated for 2 hours at 37°C with an anti-Thy1.1 monoclonal antibody (diluted 1:500 2% goat serum in PBS). The cells were washed in PBS again, and incubated for 2 hours at 37°C with CY³ conjugated to a goat anti-mouse antibody (1:380 2% goat serum in PBS, Vector). After a final washing with PBS, the cells were mounted on slides and cover-slipped (Figure 1).

Local Anesthetic Treatment and Cell Death Assay

The cells were incubated with solutions of several LA's at different concentrations (10 μ M or 0.0003%, 100 μ M or 0.003%, 500 μ M or 0.015%, and 1000 μ M or 0.03%) in DMEM with 3% FBS. Each concentration was assessed for toxicity at each of four different durations of exposure (4, 24, 48, and 72 hours). LA's tested were bupivacaine, ropivacaine, lidocaine, chloroprocaine, and mepivacaine. Clinically available solutions and generic laboratory preparations made from powder and water were tested. Cell death was indicated by lactate dehydrogenase (LDH) release in the cell culture medium as measured by optical density

(³²). Background optical density was subtracted from each sample to give control samples a value of zero. Live cells were also stained with calcein-AM.

Animal Surgery

We placed microcatheters adjacent to the left sciatic nerves of adult male Sprague-Dawley rats (250–300g) under direct vision using an open approach (technique described below). The rats were then randomly divided into three experimental groups. One group (n = 8) received an infusion of 0.9% saline vehicle via the microcatheter for 72 hours, and the other two groups received an infusion of either 0.5% (n = 4) or 0.75% (n = 4) bupivacaine. We selected bupivacaine as it was the most toxic LA in our in vitro experiments (see Results section below).

To place the microcatheters, the rats were anesthetized with isoflurane (4 to 5% in induction chamber; 1.5 to 2% for maintenance *via* snout mask) in oxygen enriched air. The sciatic nerve of the rats' left hind paw was isolated proximal to the trifurcation, and a sterile microcatheter (tubing PE50) was placed through the gluteal muscle, in close proximity to the nerve, while taking great caution not to manipulate the nerve. Each catheter was then exteriorized by tunneling it subcutaneously to the posterior base of the rat's neck and using a swivel adaptor attached to the rat's skin. Incisions were closed with sterile sutures. The catheters were then connected to minipumps (microdialysis pumps purchased from CMA Inc; North Chelmsford MA, USA) for continuous infusions at a rate of 100 μ L/hour for 72 hours post -implantation. At the end of the treatment period, the rats were anesthetized with 5% isoflurane and tissue fixed under deep anesthesia *via* perfusion with 4% paraformaldehyde.

Tissue Processing

To conduct histological and immunohistochemical examination of the nerves, the left sciatic nerves were removed, post-fixed overnight in the same fixative solution used for perfusion (4% buffered paraformaldehyde), and embedded in paraffin. The nerves were then cut into 6 μ m-thick longitudinal sections for analysis. In preliminary animals, sections were stained with hematoxylin and eosin, however, the myelin stain was much more sensitive for the identification of demyelination, and was thus used for comparisons between groups.

Myelin Stain (Eriochrome)

The sections of nerve were deparaffinized in histoclear and rehydrated in a series of graded ethanol solutions. The sections were then washed with water and stained for 1 hour with eriochrome working solution (end concentrations of 0.16% eriochrome and 0.8% ferric alum). The sections were then washed for 15 minutes with water and differentiated in 10% ferric alum until the background was decolorized. After this they were differentiated in borax-ferricyanide solution, washed again, and counterstained with 0.1% cresyl violet. Finally, the sections were dehydrated and placed under a cover-slip with mounting media. Sections were examined for evidence of myelin injury, including disruption of normal architecture, myelin globules, and loss of myelin staining. Sections were coded with random numbers and scored on a scale of 0 (normal) to 3. The sections with the greatest amount of myelin injury were assigned the maximum score of 3. A score of 1 indicated mild, focal disruption of the myelin; a score of 2 indicated myelin injury more widespread or severe than 1, but less than 3 (some histologically normal myelin still present). A score of 3 indicated multiple findings consistent with myelin damage (see above) throughout the entire section and an absence of histologically normal myelin. Chi-square analysis was used to evaluate statistical significance of myelin injury since multiple animals had a score of 0, precluding analysis based on rank.

Immunohistochemistry

The sections were deparaffinized and rehydrated as above and rinsed in PBS. Sections were heated in citrate buffer (pH 6.0) in a steamer for 30 minutes at 100°C to intensify staining. After peroxidase quenching, they were washed three times with PBS. Sections were incubated with anti-macrophage (CD68) antibody (1:200, Chemicon) for 90 minutes. After incubation with a biotinylated anti-mouse immunoglobulin for 30 minutes, a Streptavidin conjugated to horseradish peroxidase (HRP) was applied. Sections were developed with a DAB (3,3'-diaminobenzidine) peroxidase substrate kit (Vector), and counterstained with hematoxylin. Coded sections were evaluated for the presence and number of endoneurial macrophages and were scored on a scale of 0 to 3. Similar to the myelin scoring, the sections with the greatest numbers of endoneurial macrophages were assigned the maximum score of 3 (Table 2). A score of 1 indicated only rare endoneurial CD68 –positive macrophages; a score of 2 indicated an intermediate number of macrophages. A score of 3 indicated a large number of endoneurial macrophages. After the initial scoring, the still-coded sections were force-ranked in order of increasing severity by comparing individual sections with the same score to each other (³³).

Statistical Analysis

Cell death data were tested for statistical significance using a one-way ANOVA (SigmaStat, version 3.1; Systat Software, Inc; San Jose CA, USA). Myelin stains were tested for statistical significance by Chi-square analysis using GraphPad Prism 4.0c for Macintosh, GraphPad Software, San Diego CA, USA. The numbers of endoneurial macrophages were tested for significance by Mann-Whitney U test using GraphPad Prism 4.0c for Macintosh, GraphPad Software, San Diego CA, USA. *P*-values of less than 0.05 were considered statistically significant.

Results

Effects of Local Anesthetics on Schwann Cells

All of the LA's we tested induced Schwann cell death in a time and concentration-dependant manner. The results of our experiments on the effects of LA's on Schwann cells *in vitro* are shown in Figures 2 and 3. Figure 2 shows representative Schwann cell cultures exposed to increasing concentrations of bupivacaine for 24 hours. Live cells are stained with calcein-AM. The cultures treated with 500μM and 1000μM bupivacaine show significantly fewer live cells, indicating significant cell death with prolonged exposure to these concentrations.

We conducted similar experiments for several other LA's commonly used in clinical practice. For these experiments LDH release was used as a marker of Schwann cell death. For the results of these experiments (Figure 3), LDH release (relative to control, measured by optical density) is measured on the *y*-axis and exposure time on the *x*-axis. Results are displayed for each concentration (Figure 3) to compare the relative toxicity of the various LA's at each time point. At 10μM (0.0003%), none of the LA's induced significant cell death. At 100μM (0.003%), none of the LA's induced significant cell death with 24 hours of exposure. Only bupivacaine induced significant cell death after 48 hours (0.482, *p* < 0.001), and both bupivacaine (0.222, *p* < 0.001) and ropivacaine (0.145, *p* 0.048) induced significant cell death with 72 hours of exposure. At 500μM (0.015%) and 1000μM (0.03%), only bupivacaine (500μM 0.482, *p* < 0.001, 1000μM 0.994, *p* < 0.001) and ropivacaine (500μM 0.261, *p* < 0.001, 1000μM 0.526, *p* < 0.001) induced significant cell death with 24 hours of exposure, however all of the LA's induced significant cell death with 48 or 72 hours of exposure (Table 1).

Effects of Continuous Infusions of Bupivacaine on Rat Sciatic Nerves

The effects of prolonged exposure of the sciatic nerves of rats to continuous infusions of bupivacaine are shown in Figures 4 and 5. Myelin stains were available for 4 nerves exposed to saline and 8 exposed to bupivacaine. All 4 saline-exposed nerves had normal myelin staining, while in the bupivacaine exposed groups 2 nerves were normal and 8 had evidence of myelin damage, with varying degrees of disruption of the normal myelin architecture, formation of myelin globules, vacuolization, and focal loss of myelin staining (Figure 4). Chi-square analysis evaluating the presence or absence of myelin damage did not show significant differences between groups. In our preliminary studies we also stained sections using an antibody to neurofilaments to look for axonal injury. No axonal abnormalities were identified and this stain was not performed on the remaining animals. Sections stained with an anti-macrophage antibody (CD68) were evaluated for the presence and number of macrophages. Nerves exposed to infusions of bupivacaine 0.5 or 0.75% (n = 11) for 72 hours were compared to nerves exposed to saline (n = 6). The nerves exposed to bupivacaine demonstrated focal to extensive infiltration by large, plump macrophages, while those exposed to the saline showed only rare, small, CD68-positive cells, consistent with resting histiocytes (Figure 5). Sections were scored then ranked in order of increasing numbers of macrophages (Table 2). The ranking based on number of endoneurial macrophages was greater in bupivacaine exposed nerves than in saline exposed nerves (Mann-Whitney U test, $p = 0.039$). There were no differences between the 0.5% and 0.75% bupivacaine doses.

Discussion

We have shown that several LA's commonly used in clinical practice cause Schwann cell death in a time and concentration dependent manner, and that prolonged exposure of rat sciatic nerves to bupivacaine causes myelin damage and infiltration of nerves by macrophages. Previous studies have demonstrated toxic effects of LA's on neurons and Schwann cells *via* several mechanisms. LA's have been shown to directly induce apoptosis in Schwann cells *via* the generation of ROS ⁽²⁶⁾ and in neuronal cells through activation of the p38 mitogen-activated protein kinase (MAPK) system (lidocaine, ropivacaine, bupivacaine ³⁴). MAPK inhibition has been shown to reduce LA neurotoxicity both *in vitro* and *in vivo* ^(23, 24). Bupivacaine also directly uncouples mitochondrial oxidative phosphorylation in leukemia cells ⁽²¹⁾, leads to caspase activation in neuroblastoma cells, and indirectly affects mitochondria by up-regulating transcription of apoptosis-related genes such as HSP70, c-jun, and c-fos and by down-regulating transcription of c-myc and PARP ⁽²²⁾. LA's have also been shown to decrease nerve blood flow, indicating an ischemic mechanism may contribute to LA-induced nerve damage *in vivo* ⁽³⁵⁻³⁷⁾.

Though Park *et al* found that bupivacaine (and to a lesser degree levobupivacaine) induced apoptosis in Schwann cells, and that the toxicity was directly related to the concentration and duration of exposure, they did not observe toxicity associated with other local anesthetics ⁽²⁶⁾. In their study, the effects of LA's were measured only after an 18-hour incubation period, and toxicity was only associated with high (1000 μ M) concentrations of LA. Our findings are consistent with theirs as none of the LA's other than bupivacaine produced significant cell death at 24 hours. Perhaps if Park *et al* had assessed cytotoxicity after longer incubation periods, they would have found that the other LA's they studied (lidocaine, mepivacaine, procaine, and ropivacaine) were also capable of producing cell damage. In contrast with their findings, we found that ropivacaine did produce cell death at higher concentrations (500 and 1000 μ M). It is possible that they could have observed cytotoxicity after 24 hours, even if it was not evident after 18 hours. The findings of our study are especially important as the use of continuous PNB's is becoming more commonplace. Our findings suggest that any LA could be associated with nerve injury at concentrations common in clinical practice given a long enough duration of exposure.

Ours is the first study examining the effects of local anesthetics on primary Schwann cells rather than immortalized cells from established tumor cell lines. Neoplastic cells have been shown to differ from primary cells directly harvested from peripheral nerves in numerous ways. Expression of ion channels involved in regulation of cell proliferation is abnormal in neoplastic Schwann cells⁽³⁸⁾. Schwann cells from tumor cell lines display abnormal cell-surface ligands implicated in cell migration, cell-matrix interaction and tumor progression such as CD44⁽³⁹⁾. Because we have used cells harvested directly from rat peripheral nerves, our study may more accurately reflect the effects of local anesthetics on normal nerves *in vivo*.

Earlier studies have demonstrated demyelination and infiltration of nerves by inflammatory cells after a single injection of LA (chloroprocaine, tetracaine, etidocaine or mepivacaine) around the sciatic nerve in rats^(17–19, 40), or following an injection of bupivacaine within the sciatic nerve of pigs⁽²⁰⁾ and dogs^(19, 41). However, ours is the first experiment specifically evaluating the effects of a prolonged infusion of LA around a nerve. Because of growing interest in the use of perineural catheters for postoperative pain control⁽⁴²⁾, it is important to determine what long-term effects a prolonged perineural infusion of local anesthetic might have on target nerves. Our finding of nerve infiltration by macrophages may indicate that an inflammatory mechanism contributes to nerve injury in clinical settings, as previous studies have noted infiltration by neutrophils and degranulation of mast cells within nerves after exposure to LA's^(17, 18, 20). Also, the presence of macrophages could be the result (rather than the cause) of nerve injury as macrophages may help clear cellular debris in peripheral nerves if the phagocytic capacity of surviving Schwann cells is not sufficient^(28, 29). As we did not observe axonal damage using immunohistochemical techniques to stain for neurofilaments, it is likely that the myelin damage is due to direct Schwann cell injury, rather than secondary to axonal injury.

In order to understand the implications of these results for the clinical practice of regional anesthesia, one must consider several factors. One such factor is the relative potency of the different LA's. In clinical practice, lower concentrations (0.5% or 0.75%) of bupivacaine or ropivacaine produce similar effects as more concentrated solutions of chloroprocaine (2 or 3%), lidocaine (1.5 or 2%) and mepivacaine (1.5%). In our experiments, if the effects of chloroprocaine, lidocaine or mepivacaine at a concentration of 100μM are compared to those of bupivacaine or ropivacaine at 500μM, the amount of LDH release is similar. This suggests that bupivacaine and ropivacaine are not necessarily more neurotoxic than other LA's at clinically equipotent concentrations. It is not clear from our data if one LA is safer than another. Indeed some previous studies have shown lidocaine to be especially toxic⁽⁴³⁾ while others have shown bupivacaine to be the most toxic^(25, 26). It seems likely that the mechanism of injury is not specific to a particular LA however, as all of the LA's we tested did cause cell death.

Another factor that must be considered is that the actual concentration of LA within the nerve after perineural administration of local anesthetic solution is not known. Though the concentrations we have used in our *in vitro* experiments are very low (1000mM = 0.03%), the Schwann cells in culture were directly exposed to these concentrations of LA's. It is possible that the concentrations of LA's within nerves in clinical scenarios are higher or lower than the concentrations we have used in this study. Because severe injury following PNB's is rare^(12, 13) even after intraneural injection or infusion of LA^(44, 45), and because injection of LA within the perineurium correlates with a high likelihood of severe injury⁽⁴¹⁾, it is possible that the perineurium's function as a blood/nerve barrier protects neurons and Schwann cells from direct exposure to high concentrations of LA's. However, studies showing subperineurial edema following perineural administration of LA's^(46, 47) suggest that this barrier system is disrupted by LA's, contributing to nerve fiber injury. It is also

possible that sub-clinical nerve injury in the setting of PNB is common, and that other contributing factors (patient or procedure-related) are necessary to produce a severe injury. It is possible that prolonged perineural infusion of LA increases the risk of nerve damage, but further studies on the time course of histologic changes within nerves during prolonged LA exposure are still needed.

Conclusions

Local anesthetics cause Schwann cell death *in vitro* in a time and concentration-dependent manner. All LA's may produce significant cell death given sufficient concentration and duration of exposure. Prolonged exposure of nerves *in vivo* to continuous infusions of bupivacaine causes damage to myelin and infiltration and activation of macrophages.

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Figure 1.

Schwann cell cultures were assessed for purity by immunofluorescence. Cultures were stained with an anti-S100 antibody to mark the Schwann cells and an anti-Thy1.1 antibody to mark the fibroblasts. The merged slide shows that the cultured cells are highly bound by the anti-S100 antibody but remain largely unbound by the anti-Thy1.1 antibody, indicating that the cultures contain more than 95% Schwann cells.

Figure 2. Schwann cell cultures exposed to different concentrations of bupivacaine for 24 hours. Live cells are stained with calcein-AM. Schwann cell death is significant in cells exposed to bupivacaine concentrations of 500 μM and 1000 μM relative to control ($p < 0.001$).

Figure 3.

Toxicity of local anesthetic agents by concentration (10 μM = 0.0003%, 100 μM = 0.003%, 500 μM = 0.015% and 1000 μM = 0.03%). LDH release relative to control was used as an indicator of cell death (* = $p < 0.05$).

A) Nerves exposed to saline or 0.5% bupivacaine

B) Nerves exposed to saline or 0.75% bupivacaine

Figure 4.

Sections of sciatic nerves stained for myelin following continuous exposure to saline or bupivacaine for 72 hours. All 4 saline nerves had normal myelin staining (left panels in A and B), while in the bupivacaine exposed groups there was loss of the normal myelin staining pattern. A shows more severe injury than B, with patchy loss of myelin staining. Both A and B show irregular staining, myelin globules (darker stained, globular structures) and vacuoles. (Chi-square analysis not significant). (Magnification bar in each micrograph = 50 microns.)

A) Nerves exposed to saline or 0.5% bupivacaine

B) Nerves exposed to saline or 0.75% bupivacaine

Figure 5. Sections of sciatic nerves stained with an anti-macrophage (CD68) antibody following continuous exposure to saline or bupivacaine for 72 hours. The number of endoneurial macrophages was greater in bupivacaine exposed nerves than in saline exposed nerves (Mann-Whitney U test, $p = 0.039$). (Magnification bar in each micrograph = 50 microns.)

Table 1

Toxicity of local anesthetic agents relative to concentration and duration of exposure. Agents associated with significant toxicity are listed for each time point/concentration. C = chloroprocaine, L = lidocaine, M = mepivacaine, R = ropivacaine, B = bupivacaine.

Duration of exposure 10mM (hours):	100mM	500mM	1000mM
4			
24		R, B	R, B
48	B	C, L, M, R, B	C, L, M, R, B
72	R, B	C, L, M, R, B	C, L, M, R, B

Table 2

Rat peripheral nerve sections scored on the number of endoneurial CD68-positive macrophages and ranked in ascending order.

Group	Score	Rank
Saline		
1	1	3
2	1	8
3	1	4
4	2	10
5	2	9
6	1	2
Bupivacaine 0.5%		
1	2	12
2	3	17
3	3	13
4	3	16
5	3	14
6	1	1
Bupivacaine 0.75%		
1	2	11
2	3	15
3	1	5
4	1	7
5	1	6