

Analysis of the Acute Cytotoxic Potential of Bupivacaine and 50% Enantiomeric Excess Bupivacaine (S75-R25) Incorporated into Microspheres in Rat Sciatic Nerves

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Summary: Tanaka PP, Torres MF, Tenorio SB – Analysis of the Acute Cytotoxic Potential of Bupivacaine and 50% Enantiomeric Excess Bupivacaine (S75-R25) Incorporated into Microspheres in Rat Sciatic Nerves.

Background and objectives: The duration of Local Anesthetic (LA) effects can be expanded by its incorporation into systems of sustained release microspheres. However, the possibility that LA sustained release systems are neurotoxic has not received due attention in literature. The objective of this study was to investigate the effects of pure microspheres of poly(lactic-co-glycolic acid), filled with 50% enantiomeric excess bupivacaine or bupivacaine (BP), as well as the effects of 50% enantiomeric excess bupivacaine in the sciatic nerve of Wistar rats.

Methods: The rats were allocated into four groups according to the evaluation time (two, four, six, and eight days) and nominated according to the injected solution on the sciatic nerve: Microspheres with 50% Enantiomeric excess Bupivacaine (MEB), Microspheres with Bupivacaine (MB), Pure Microspheres (PM), and 50% Enantiomeric excess Bupivacaine (EB).

Results: In semi-fine histologic sections, no regular homogeneous distribution of collagen fibers in the endoneurium or degenerative changes of axons and myelin sheaths were observed. In ultrathin sections, we found myelinated axons and normal Remak fibers with axoplasm showing homogeneous distribution of neurofilaments and microtubules. Histomorphometric analysis of axons revealed no significant difference between the axon diameters of the studied groups.

Keywords: Anesthetics, Local/toxicity; Bupivacaine; Microscopy, Electron, Transmission; Microspheres; Rats; Stereoisomerism.

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INTRODUCTION

Administration of Local Anesthetic (LA) directly on nerves or nerve trunks to block the perception of pain by the patient is an effective technique. The physiological mechanisms of neurotoxicity caused by LA have been related to inhibition of axonal transport, destabilization of the axonal cytoskeleton, axonal degeneration, and neural ischemia ¹. However, few studies have described the mechanism responsible for the injury and if injury is caused directly or indirectly by LA.

Some authors ² have shown that higher concentrations of lidocaine and bupivacaine (BP) promote longer-lasting blockade of the sciatic nerves of rats; however, these concentrations are clearly neurotoxic in histopathological analysis.

These results suggest the existence of a relationship between prolonged anesthesia and neurotoxic damage.

Studies have investigated the pharmacokinetics and pharmacodynamics of acute intoxication caused by LA and, although there are reports in literature on neurotoxicity ¹, there are no in vivo experimental studies on the use of Microspheres with 50% Enantiomeric excess Bupivacaine (MEB) (S75-R25), as well as on the morphological evaluation of this anesthetic neurotoxic effects by Optical Microscopy (OM) or Transmission Electron Microscopy (TEM).

This study aimed to investigate the cytotoxic effects on the sciatic nerve of Wistar rats of pure microspheres of poly(lactic-co-glycolic acid), filled with 50% Enantiomeric excess Bupivacaine (EB) (S75-R25) and BP, as well as 50% EB (S75-R25).

METHODS

The research population consisted of 16 male Wistar rats, approximately three months old and mean weight of 300 g, from the Animal Facility of the Universidade Positivo. Throughout the study period the animals were maintained in a room (average temperature 22°C) with a photoperiod of 12 hours and free access to water and food. This project was approved by the Animal Experimentation Ethics Committee at the Universidade Positivo.

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The experiments were conducted in a quiet environment and performed by the same investigator who was blinded to the drug injected. The rats were allocated into 4 groups of 4 animals named according to the injected solution on the sciatic nerve: Microspheres with 50% Enantiomeric excess Bupivacaine (MEB), microspheres with bupivacaine (MB), pure microspheres (PM), and 50% Enantiomeric excess Bupivacaine (EB).

After anesthetizing the rats by evaporation of halothane (2%-4%) in oxygen via face mask, manipulation could be performed for injecting the solution directly on the sciatic nerve.

On 2, 4, 6, and 8 days after application of the solutions, the animals were euthanized by intraperitoneal injection of thiopental 2.5%; then the proximal end of the sciatic nerve was dissected and fixed in 3% glutaraldehyde of 0.4 M cacodylate buffer and sent for processing at the Electron Microscopy Center at Universidade Federal do Paraná (UFPR).

The neurotoxic potential of the drug was evaluated by OM and TEM to investigate: demyelination, axonal degeneration, and signs of inflammation evidenced by infiltration of inflammatory cells.

The semi-fine sections (0.5 μm) were stained with toluidine blue and the fields with good image quality were photographed and subjected to histomorphometry using *Image*

Pro Plus version 4.5. the study measured the diameter of 10 axons per field photographed, with a total registration of the diameter of 100 axons per nerve. The ultrathin sections (70 nm) were stained with 2% uranyl acetate and Reynolds solution for later analysis using JEOL JEM - 1200 EX II at the Electron Microscopy Center, UFPR.

The sciatic nerves results obtained by histomorphometry were compared two by two using the nonparametric Mann-Whitney test. The significance level was 0.05, and corrected by Bonferroni. As six comparisons of the groups were performed two by two, the differences in which the p-value was lower than 0.008 were considered significant.

RESULTS

This study presents a systematic analysis of the toxicological effects on the peripheral nervous system between groups and solutions applied to the sciatic nerve in Wistar rats. TEM was used for ultrastructural analysis of the rats' sciatic nerve in search of evidence on the neurotoxic effect of LA and microspheres. Ultrastructural analysis was performed together with the histomorphometry under light microscopy for differences between the diameters of axons that could be interpreted as neurotoxicological changes.

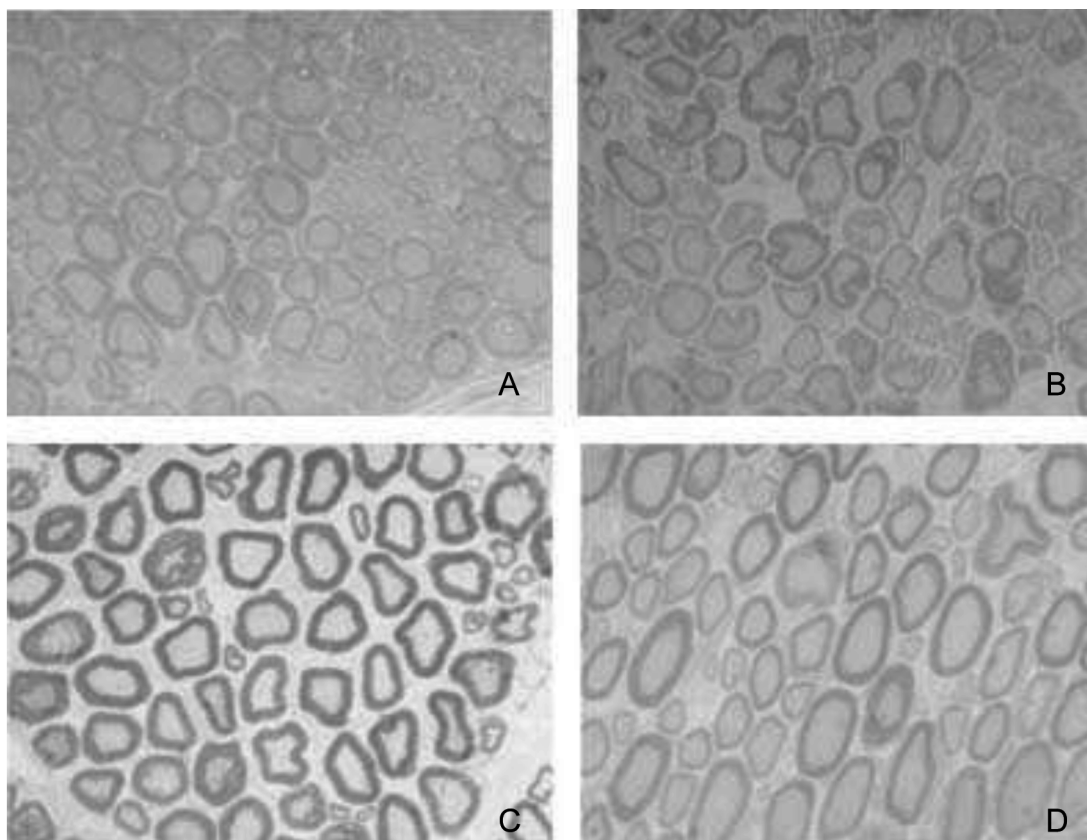


Figure 1 – Semi-fine Sections of Nerves Blocked with Microspheres Containing 50% Enantiomeric Excess Bupivacaine. Note the morphological characteristics of normal axons at this magnification. Axons are sectioned transversely and obliquely. The changes observed in some myelin sheaths are consistent with artifact of tissue processing. A: Mouse 30, second postoperative day; B: Mouse 4, fourth postoperative day; C: Mouse 11, sixth postoperative day; D: Mouse 23, eighth postoperative day. Staining: toluidine blue (100x objective).

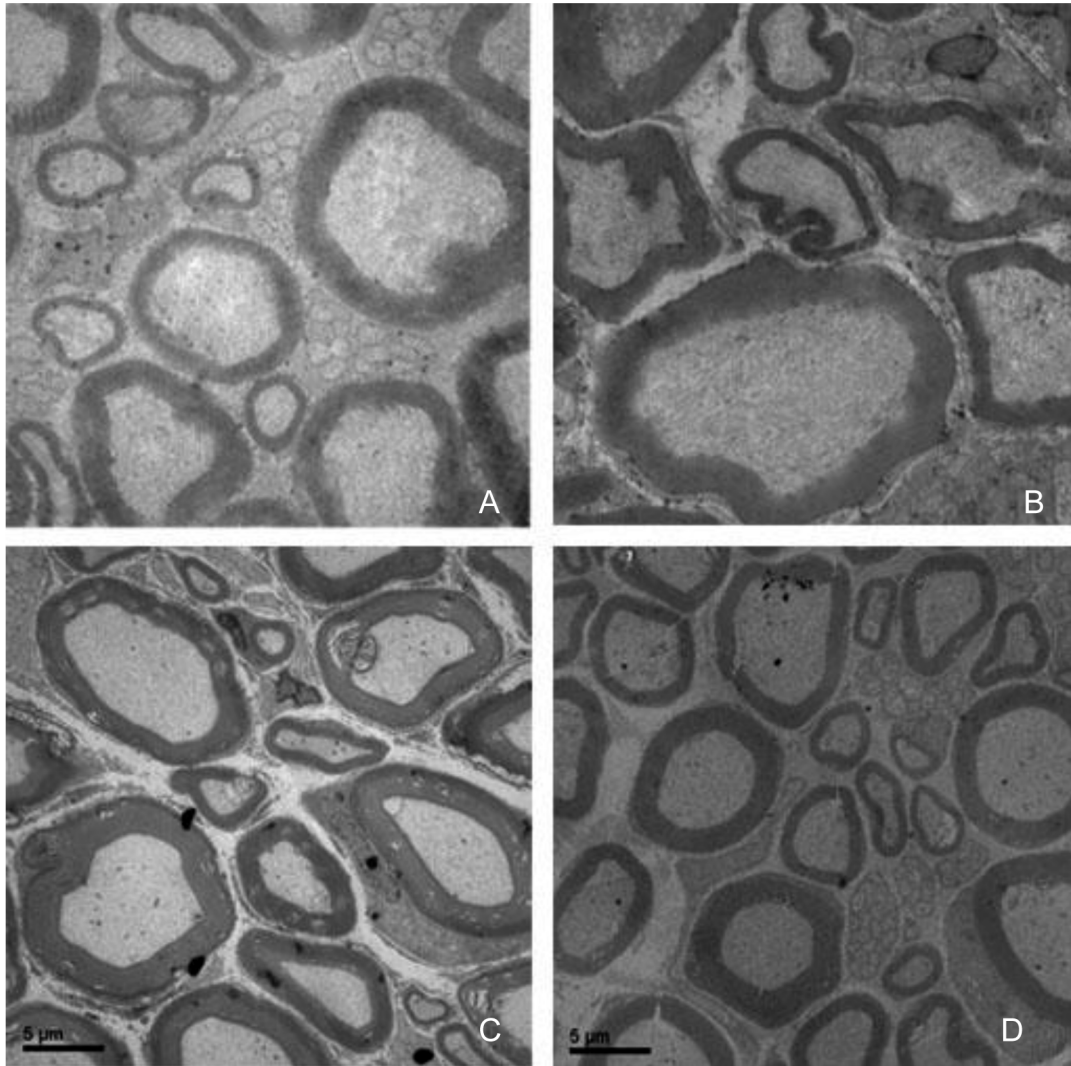


Figure 2 – Electron Microscopy of Nerves Anesthetized with Microspheres Containing 50% Excess Enantiomeric Bupivacaine. NOTE: Myelinated axons and fibers of Remak are seen (*) and in one of them the nucleus of Schwann cell is seen (N). Collagen bundles are seen between nerve fibers. Some of the myelin sheaths present lamellae deformation due to artifact of tissue processing. Microtubules and neurofilaments are seen evenly distributed within the axon. A: Rat 2, second postoperative day. B: Rat 9, fourth postoperative day. C: Mouse 14, sixth postoperative day. D: Mouse 31, eighth postoperative day.

In semi-fine sections of all groups and at all times studied (2, 4, 6, and 8 days) fibers of normal aspect were seen within the fascicles (Figure 1). Small diameter axons with a thin myelin sheath and larger axons with thick myelin sheaths were also found. There was a regular homogeneous distribution of collagen fibers in the endoneurium. Degenerative changes of axons and myelin sheaths were not observed. Mast cells were observed in several semi-fine sections. Inflammatory cells in endoneurium or perineurium were not observed. The disarranged aspect of some myelin sheaths was interpreted as an artifact of tissue processing.

In ultrathin sections in all groups at all times (2, 4, 6, and 8 days), myelinated axons of normal aspect and axons unmyelinated involved by Schwann cells in structures designated as fibers of Remak were found (Figure 2). The

axoplasm contained a homogeneous distribution of neurofilaments with microtubules and occasional mitochondria. In some sections, nuclei of Schwann cells associated with the internodes of myelin and fibers of Remak were visualized. Fibroblasts and mast cells were found in the endoneurium. In all sections examined collagen fibers in contact with the basement membrane of Schwann cells were clearly visible.

The semi-fine sections of sciatic nerves of each rat were photographed and processed for histomorphometric analysis using the program *Image Pro Plus* version 4.5. In each sciatic nerve, the diameters of 100 axons were recorded, and the selection criteria were the color and central position of axons in the field photographed. For statistical analysis, it was considered the mean of the 100 measurements for each rat.

The null hypothesis of equal results in both groups was tested by comparison versus the alternative hypothesis of different results. Table I presents the minimum, medium, and maximum diameter of axons, considering the 100 measurements for each rat. It is noted that one of the rats in group M had the lowest mean of the group. It is also noticed that the M group showed a greater dispersion of results.

Table II shows the p-values of two by two group comparisons. We tested the null hypothesis of equal results in the two groups under comparison versus the alternative hypothesis of different results.

The toxicity of BP for cardiovascular and central nervous systems led to the research of new LA with similar blockade profile and less toxicity, which resulted in new preparations as the EB. In a study assessing the efficacy of sciatic nerve blockade in mice³, the enantiomeric mixture of BP (S75-R25) was considered an alternative for the development of controlled release formulations that are safer and more effective than BP (S50-R50).

Researches of controlled release LA formulations have shown promising results. Despite major advances in the development of these formulations, studies are needed to evaluate the local neurotoxicity in order to ensure their safe use in clinical routine⁴.

The small sample size (n = 16) was due to several factors: firstly, the limitations arising from the new animal welfare guidelines and secondly from the financial constraints that a study using this methodology requires.

Table I – Descriptive Analysis of Mean Diameter of 100 Measurements of Sciatic Nerve Axons of Rats in Different Groups

Group	N	Diameter (μm)				
		Mean	Median	Minimum	Maximum	SD
MEB	4	4.63	4.62	4.26	5.02	0.31
EB	4	5.33	5.21	5.00	5.91	0.42
MB	4	4.56	4.69	3.71	5.15	0.61
PM	3	3.96	4.56	1.90	4.83	1.39

MEB: rats given extraneural injections of microspheres with 50% enantiomeric excess bupivacaine (S75-R25); EB: rats given extraneural injections of (S75-R25) alone; MB: rats given extraneural injections of microspheres with bupivacaine; PM: rats given extraneural injections of pure microspheres; n: number of rats per group; μm : micrometers.

Table II – P-values of Group Comparisons Two by Two

Groups compared	p-value*
MEB x EB	0.057
MEB x MB	0.886
MEB x PM	0.886
EB x MB	0.114
EB x PM	0.029
MB x PM	0.686

MEB: rats given extraneural injections of microspheres with 50% enantiomeric excess bupivacaine (S75-R25); EB: rats given extraneural injections of (S75-R25) alone; MB: rats given extraneural injections of microspheres with bupivacaine; PM: rats given extraneural injections of pure microspheres.

* Nonparametric Mann-Whitney test, p < 0.008.

The sciatic nerve is the starting point for the in vivo study of LA, composing with the in vitro investigations the requirements of the pre-clinical phase of new compounds, before the stages of research in humans⁵. The sciatic nerve of rat is a mammal experimental model well-established for searches on peripheral nerve toxicology⁶.

The injection method was appropriate because, after the recovery of anesthetized limb proprioceptive function, the animals showed no motor or sensory sequelae. These goals were part of a study conducted in parallel in the same group of animals⁷.

It is imperative that a drug be tested for peripheral neurotoxicity using established neuropathological techniques with higher sensitivity and resolution than the paraffin preparations stained with hematoxylin and eosin. Although useful for the assessment of more evident histological changes, OM may obscure recent changes among the axons, Schwann cells, and myelin that indicate neurotoxicological mechanisms⁸.

Evidence of structural changes induced by drugs depends on ultrastructural analysis. The choice of study through analysis of semi-fine and ultrafine sections can be considered the most appropriate because often the clinical and histological changes do not correspond to or are very subtle; thus only ultrastructural study allows the observation of discrete lesions⁹⁻¹⁰. Given the need for a careful analysis in the search for axonal and demyelination damage, this study presents an evaluation of the ultrastructure of sciatic nerves.

Electron microscopy was indicated as a more sensitive test able to differentiate ultrastructural neurotoxic lesions². When evaluating the neurotoxic effects of six lipid formulations of different deposits consisting of a mixture of BP and lidocaine on rat sciatic nerve, tumefaction axonal, neuronal degeneration, and demyelination in nerves anesthetized with 64% BP and lidocaine at a proportion of 4:1 were observed through OM. Despite these findings, it was concluded that BP can be used as active compound of deposit formulations and suggested that its neurotoxic potential must be assessed by more sensitive tests such as TEM.

None of the samples of this study showed signs of neurotoxicity when analyzed by both OM and TEM.

The pathological process indicative of neurotoxicity most mentioned in literature is Wallerian degeneration, which was first described by Augustus Waller in 1850. It is a complex process triggered by axon damage and results in the degeneration of the axon and its supporting cells, group known as nervous fiber. Initially, there is a grouping of organelles and other structures in the axoplasm, with increased swelling and disintegration of the axon. Myelin disintegrates and is phagocytized by Schwann cells and macrophages, which are recruited in large numbers in response to increased tumor necrosis factor and proinflammatory proteins⁸.

An increased permeability of blood-nerve barrier caused by LA can enhance the osmolarity of interstitial fluid, leading to reduced size axons or changes in concentration gradients of inorganic ions and, consequently, to decreased nerve conduction¹.

In this study, it was considered the hypothesis that the presence of the microspheres and slow release of LA would cause change in permeability of local blood vessels, making them more permeable and allowing the extravasation of osmotically active proteins that are not normally found near the endoneurium and could attract water thus limiting the space of axons and endoneurial cells. In TEM images, however, distension of the intercellular and interaxonal space was not seen in the endoneurium, which would characterize the extravasation of plasma.

Some authors¹¹ implanted matrix with microspheres in subcutaneous tissue of Wistar rats and observed that the microspheres began to degrade 15 days after the implant application. However, in the present study, no microsphere was observed over the sciatic nerves studied, indicating that the deposited microspheres were already degraded two days after the application. One reason for this inconsistency lies in the matrix used in the production of microspheres.

Contrary to the myelin sheath instability demonstrated through TEM and x-ray by Mateu et al.¹² after tetracaine application on isolated optic and sciatic nerves of rats, in this study there were no changes in cells of Schwann, such as vacuolization and cytoplasmic disintegration and cytoplasmic lipid droplets accumulation indicative of lipid metabolism interruption.

The possibility of an inflammatory reaction caused by the surgical procedure was excluded from this study, as the application of the MEB was by transcutaneous injection.

There were no structural changes in the study after extra-neural application of microspheres containing BP and EB. In another study¹³ investigating the neurotoxicological effects of amitriptyline and BP in rat sciatic nerve, structural changes weren't found either. Samples taken 1, 3, 5, and 7 days after drug applications were analyzed by OM and showed intact epineurium and endoneurium, organized nerve fibers, few inflammatory cells, and no significant Wallerian degeneration.

The LA used in the present study, alone or incorporated into microspheres, as well as the microspheres did not induce histological or ultrastructural changes in sciatic nerves evaluated. In the obtained images the nerve structures (fibers, cells, and extracellular space) showed no changes: the axons showed homogeneous distribution of the cytoskeleton components (microtubules and microfilaments), indicating that the diffusion of molecules occurred normally. Schwann cells showed no signs of intoxication that could be visualized as cytoplasmic edema and sheath rejection. However, it should be pointed out that Schwann cells are very resistant elements that recover quickly from an injury regardless of the offending agent¹⁴. The rats euthanized at the evaluated times (2, 4, 6, and 8 days post-injection) showed no changes in these cells.

Myelin sheaths were normal with eventual changes explained as resulting from artifact of tissue processing. The peripheral nervous system, although with characteristics different from those of central nervous system (notably by the presence of extracellular collagenic matrix in the first) is a delicate tissue. This means that manipulation can induce artifacts

in fascicles and sheaths, which complicates image interpretation¹⁵.

In many semi-fine and ultrafine sections fibroblasts and collagen fibers were seen, which characterizes the normal morphology of the nerve. Occasional mast cells filled with granules were also seen. Mast cells proliferate in any type of damage to the peripheral nerves, although the real cause of this proliferation and the role it would play are still unknown¹⁴. There was no proliferation of these cells in the analyzed sections.

Presence of inflammatory cells, which would be abundant in case of injury to axons or myelin, was not observed in any of the sections¹⁶. The absence of visible changes in ultrastructure by day 8 post-injection suggests that there will be no changes when using the same anesthetics on long-term basis.

However, the results obtained with a drug should be carefully extrapolated to another drug even if they are structurally similar¹⁷. Moreover, multiple factors interfere with the release of LA by polymeric microsphere, such as the nature and molecular weight of polymer.

Considering that one of the signs of accidental intraneural injection is the resistance to needle insertion or increased pressure required for LA injection, the hypothesis that this type of technical failure have occurred in this experiment can be discarded, as the anesthesiologist did not report such occurrences. Furthermore, no sample of this study showed evidence of Wallerian degeneration of the fibers within the fascicles and perineurium. Only high-pressure injections could cause detectable neurological deficit and histological damage to the fascicles.

Histomorphometry was performed to measure possible changes in the diameter of axons, taking into account that the beginning of the Wallerian degeneration process causes edema and subsequent axonal disintegration.

Histomorphometric analysis of axons revealed a trend toward significant difference between EB and PM groups due to the difficulty in obtaining homogeneous cross-sections of axons (visible in selected images). Note that in some semi-fine sections, axonal guidance was oblique, leading to a distortion in measurement results. One way to minimize the consequences of a biased analysis due to different axon presentations would be the individual study of the relationship between axon diameter and myelin sheath thickness.

In this study, acute application of microspheres with BP and EB on sciatic nerve of rats had no deleterious effects on nerves. Similarly, PM and EB caused no ultrastructural changes in the nerves studied.

CONCLUSION

None of the groups had histopathological changes of axons and myelin, indicating absence of inflammation. In conclusion, under the conditions of this study, the application of microspheres with BP and EB on sciatic nerve of Wistar rats does not cause direct neurotoxic changes.

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