Light and Electron Microscopic Evaluation of Continuous Perineural Levobupivacaine Infusion Effects on Peripheral Nerve and Muscle Tissues in Infant and Adult Rats

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Abstract

Objective: Peripheral nerve and muscle damage is one of the risks of continuous peripheral nerve blockade. The aim of this study was to evaluate the tissue histopathological effects of levobupivacaine in infant and adult rats.

Methods: This study was performed in 10 infant and 10 adult rats under ketamine anesthesia. A first bolus dose of levobupivacaine followed by 3 h of infusion was applied to the right sciatic nerve area and 0.9% sodium chloride solution was administered to the left sciatic nerve area to both groups. Removal of the sciatic nerve and the muscle tissues was done on the first hour and first week in three rats, respectively, while in the fourth week in the four other rats in both groups. The nerve and muscle tissues were analyzed by light and electron microscopy separately.

Results: Light microscopy revealed in all periods various degrees of axonal degeneration and mast cell and epineural cell infiltration in the nerve tissues. Damage was the same between groups. The myonecrosis and inflammatory cell infiltration on muscle tissue were more profound in the levobupivacaine group. More granulation tissue was produced on the fourth week in infant than adult rats. Electron microscopy showed myelin degeneration, edema of myofibrils and mitochondrions, and effacement of cristae in mitochondria. Apoptosis was observed in the muscle tissue of infant rats.

Conclusion: There were no differences between the neurotoxic effects of 0.9% sodium chloride and levobupivacaine infusions on either infant or adult rats. In adult rats, myotoxicity caused by levobupivacaine was later healed compared to that by 0.9% sodium chloride infusion and in infant rats, muscle damage was permanent with levobupivacaine.

Keywords: Peripheral nerve block, levobupivacaine, neurotoxicity, myotoxicity, light microscopy, electron microscopy

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Levobupivakain'in Perinöral Sürekli Infüzyonunun Infant ve Erişkin Ratlarda Periferik Sinir ve Kas Dokusuna Etkilerinin Işık ve Elektron Mikroskopisi ile Değerlendirilmesi

Öz

Amaç: Periferik sinir ve kas hasarı, sürekli periferik sinir bloğu tekniğinin risklerinden birisidir. Bu çalışmada levobupivakainin histopatolojik etkilerini infant ve erişkin ratlarda değerlendirmeyi amaçladık.

Yöntemler: Bu çalışmada ketamine anestezisi altında 10 infant ve 10 erişkin olmak üzere 20 adet rat ile çalışıldı. Ratların sağ siyatik bölgelerine levobupivakain, sol siyatik sinir bölgelerine ise 0,9% sodium klorid bolus doz sonrası üç saat süreyle infüzyon ile uygulandı. Birinci gün ve birinci hafta her gruptan üçer ratın, dördüncü haftada her gruptan dörder ratın siyatik sinirleri ve kas dokuları çıkarıldı. Periferik sinir ve kas dokusu ışık ve elektron mikroskobunda incelendi.

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Bulgular: Işık mikroskopisi değerlendirmesi sonucunda, sinir dokusunda çeşitli düzeylerde akson dejenerasyonu, sinirde mast hücresi ve epinöral hücre infiltrasyonu saptandı, gruplararası hasar benzerdi. Kas dokusunda miyonekroz ve iltihabi hücre infiltrasyonu levobupivakain grubunda daha belirgindi. Kas dokusunda dördüncü haftada infant ratlarda oluşan granülasyon dokusu erişkinden daha fazlaydı. Elektron mikroskobunda, miyelin dejenerasyon, miyofibril ve mitokondride ödem ve kristalarda silinme saptandı. İnfant ratların kas dokularında apopitoz gözlendi.

Sonuç: Levobupivakain ve 0,9% sodium chloride solüsyonunun, infant ve erişkin ratlarda nörotoksik etkileri benzerdir. Levobupivakainin neden olduğu miyonekroz daha geç iyileşmekte ve infant ratlarda kaslarda kalıcı hasara neden olmaktadır.

Anahtar Sözcükler: Periferik sinir bloğu, levobupivakain, nörotoksisite, miyotoksisite, ışık mikroskopisi, elektron mikroskopisi

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Continuous peripheral nerve block is an effective and safe technique for anesthesia and postoperative analgesia. The drug preferred in this technique should be less toxic to reduce side effects. Especially when using long-acting local anesthetics, peripheral nerve and muscle damage has been declared as a risk [1]. Levobupivacaine, an S-isomer of bupivacaine, has been used with the same clinical effects as bupivacaine and significantly with fewer neurotoxic and cardiotoxic side effects [2].

In our study, we aimed to evaluate early and late histopathological changes in the sciatic nerve and biceps femoris muscle in infant and adult rats under a light and an electron microscope during continuous levobupivacaine infusion.

Material and Methods

This study was conducted after obtaining approval from the Istanbul University Cerrahpaşa School of Medicine Experimental Animal Ethics Committee, in compliance with the "Guide for Use and Care of Laboratory Animals."

This study was performed in infant (GI) and adult (GII) rats. Levobupivacaine was injected to the right (GI R and GII R) and 0.9% sodium chloride solution to the left (GI L and GII L) sciatic nerve to each group (GI and GII) rats with a bolus dose and 3 h of continuous infusion.

Animal model: Ten 3–4 weeks old infant Sprague-Dawley rats weighing 50–60 g, and ten 8–10 weeks old adult Sprague-Dawley rats weighing 200–300 g, all male, were included in each group. Their core temperatures were preserved by heating the environment. Under ketamine hydrochloride (Ketalar, Eczacıbaşı, Turkey) (50 mg kg^{-1} intraperitoneally) anesthesia, 24 G IV catheters were inserted into their sciatic nerve tracks by an open surgical approach and were stabilized with a tape (Figure 1, 2). Surgery was performed with aseptic techniques, and 0.5 mg mL^{-1} levobupivacaine and 0.9% sodium chloride solution were infused (Baxter Colleague 3, Baxter Healthcare Corporation Medication Delivery Division Deerfield, IL 60015, USA) into the right and left sciatic areas, respectively. Animals were anesthetized for the whole duration of the infusion with xylazine hydrochloride 3 mg kg^{-1} (Rompun 2%, Bayer, Germany).

Bolus doses of 0.5 mL levobupivacaine solution at an infusion rate of 0.1 mL h^{-1} were administered to the right sciatic area of infant rats (GI R). For the adult rats, 1 mL bolus levobupivacaine was administered and the infusion rate was 0.2 mL h^{-1} (GII R). The same procedure was applied to the rats of each group with saline to their left sciatic nerves (GI L and GII L).

After the bolus doses, hindlimb withdrawal responses of rats to pinch stimuli were recorded as present, re-

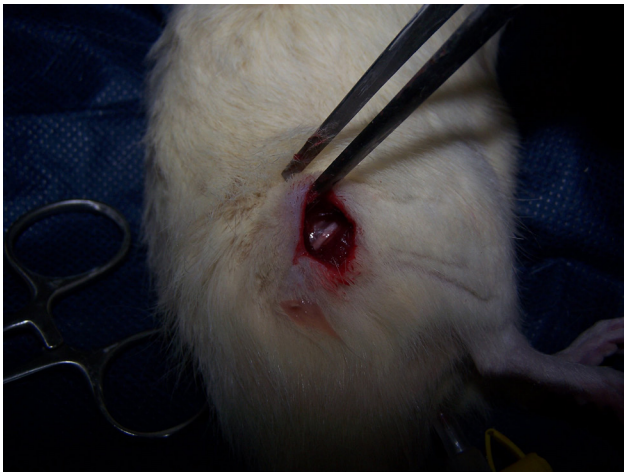

Figure 1. Sciatic nerve locations of an adult rat

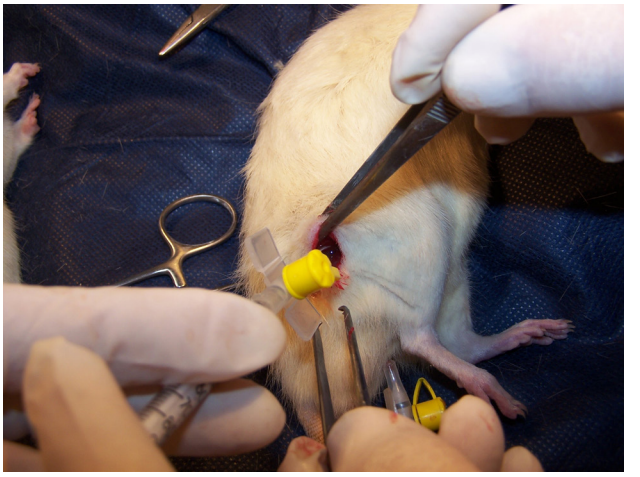

Figure 2. Catheters into both sciatic nerves of a rat

duced, or absent. The hindlimb withdrawal response to pinch stimuli was also recorded every hour during the infusions. At the end of the infusion, we have evaluated the time required for the first step and the proper walking of the rats. Before sacrificing the rats, under ketamine anesthesia, the sciatic nerves and muscle tissues of each group rats (GI and GII) were removed on the first day (n=3), first week (n=3), and fourth week (n=4). The tissues were separated into two parts for the histopathological evaluation. One part was immersed in glutaraldehyde solution (for electron microscopic evaluation) in 0.1 M phosphate buffered saline (pH 7.4) and the second part was immersed in 10% formaldehyde solution (for light microscopic evaluation) and stored at 4 °C. Examinations were performed by a neuropathologist and a histologist blinded for the solutions infused.

Light microscopic study: Nerve and muscle samples were fixed with 10% formalin in phosphate buffered saline solution and dehydrated in graded alcohol series prior to embedding in paraffin blocks. They were cut with a microtome in 3 µm sections. Hematoxylin-eosin and toluidine blue stained slides were examined using a light microscope (40× magnification) according to predefined criteria.

Nerve tissue evaluation criteria: The criteria for evaluation of nerve tissue include axonal degeneration, mast cell and epineural cell infiltration, and fibrosis. The sections were inspected at 40× magnification for mast cells, and the numbers of mast cells in the 10× fields were counted.

Muscle tissue evaluation criteria: Myonecrosis, inflammatory cell infiltration, and granulation tissue and nucleus regeneration are the evaluation criteria for muscle tissue.

We determined degeneration point for each criterion and then calculated the total degeneration scores (Table 1, 2).

Electron microscopic study: For electron microscopic investigation, cross-sectioned slices, each 1 mm wide, were obtained from the biceps femoris muscles. Tissue specimens sized about 1×1 mm were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde solution in 0.1 M sodium cacodylate buffer for a night and then post fixed with 0.1% osmium tetraoxide solution in the same buffer. After a routine dehydration process in graded alcohol series and propylene oxide, specimens were embedded in Epon-812 embedding media. Semithin (1 µm) and thin (0.5 µm) cross-sections of tissue samples were cut using an ultramicrotome (Reichert, Austria OM-u3), from prepared blocks using a glass knife. The sections were passed through 100 mesh copper grids and contrasted with a lead ci-

Table 1. Nerve tissue evaluation criteria under a light microscope

Nerve	Absent	Mild	Moderate	Severe
Axonal degeneration score	0	1	2	3
Mast cell (at the nerve) score	0	1 (1–2 cells)	2 (3–4 cells)	3 (4+ cells)
Epineural cell score	0	1 (1–10 cells)	2 (11–20 cells)	3 (21+ cells)
Fibrosis score	0	Present	x	x
		1		
Total	0 (0)	1 (1–3)	2 (4–6)	3 (7+)

Table 2. Muscle tissue evaluation criteria under a light microscope

Muscle	Absent	Mild	Moderate	Severe
Myonecrosis	0	1	2	3
Inflammatory cell score	0 (0 cell)	1 (1–2 cells)	2 (3–4 cells)	3 (4+ cells)
Granulation tissue	0	Present	x	x
		1		
Regeneration nucleus	0	Present	x	x
		1		
Total	0 (0)	1 (1–3)	2 (4–6)	3 (7+)

trate-uranyl acetate solution. The structural differences in both the experiment and control groups were evaluated in ultra-thin sections using a Jeol-JEM 1011 transmission electron microscope (Jeol Global, Japan).

Results

After the bolus levobupivacaine doses and infusions, the pulling away response to the painful stimulus of the right foot was reduced compared to the left foot in all rats, and dropped foot was observed. After the infusion was completed, the response was observed again in approximately 37.5 (maximum 45, minimum 30) min in infant rats (GI R) and 54 (maximum 75, minimum 30) min in adult rats (GII R), and the rats started to stagger. There were no signs of wound infection in the animals after several weeks.

Light microscopy results

Under a light microscope, there was no difference between the first day, first week, and fourth week sciatic nerve examination of infant and adult rats except for that on the first day, there was denser epineural cell infiltration in the adult rats exposed to levobupivacaine.

Under the light microscope, there was more damage to the muscle tissue exposed to levobupivacaine in infant and adult rats.

There was no difference between levobupivacaine and 0.9% sodium chloride solution groups in respect to neurotoxicity in both groups. Only on the first day of 0.9% sodium chloride solution administration, axonal degeneration and inflammatory cell infiltration in the sciatic nerve were more profound in the adult rats, whereas epineural cell infiltration was more profound in the infant rats. Muscle tissue toxicity results were the same in infant and adult rats on the first day and first week. At the end of the fourth week, the GI R group was affected more than the GII R group, and they had higher damage points (Table 3, 4; Figure 3-6).

Electron microscopy results

The nerve tissue findings for the three time periods had the same properties in the infant rat groups (GI R and GI L). These properties were the disintegration of myelin lamellae and nerve fiber degeneration that can be seen as vacuoles. Schwann cell proliferation was higher in the fourth week group.

The structural changes in the muscle tissues of infant rats were not different in levobupivacaine and 0.9% sodium chloride solution groups. In both the groups, after 24 h, myofibrillar degeneration and enlargement of perimyofibrillar space were seen because of intracellular edema, sarcosomal edema, effacement of cristae, and muscle degeneration. These signs fit the first stage of reversible cell damage, hydropic degeneration.

Table 3. Nerve tissue light microscopy results

Neurotoxicity score (first day)	**0**	**1–3**	**4–6**	**7+**
GI L (n=3)	0	2	1	0
GI R (n=3)	0	1	2	0
GII L (n=3)	0	1	2	0
GII R (n=3)	0	0	2	1
Neurotoxicity score (first week)	0	1–3	4–6	7+
GI L (n=3)	0	1	2	0
GI R (n=3)	0	3	0	0
GII L (n=3)	0	0	3	0
GII R (n=3)	0	1	2	0
Neurotoxicity score (fourth week)	0	1–3	4–6	7+
GI L (n=4)	1	3	0	0
GI R (n=4)	0	3	1	0
GII L (n=4)	0	2	2	0
GII R (n=4)	0	0	4	0

GI L: infant 0.9% sodium chloride solution; GI R: infant levobupivacaine; GII L: adult 0.9% sodium chloride solution; GII R: adult levobupivacaine

Table 4. Muscle tissue light microscopy results.

Myotoxicity (first day)	**0**	**1–3**	**4–6**	**7+**
GI L (n=3)	1	2	0	0
GI R (n=3)	0	2	1	0
GII L (n=3)	0	3	0	0
GII R (n=3)	0	2	1	0
Myotoxicity (first week)	0	1–3	4–6	7+
GI L (n=3)	2	1	0	0
GI R (n=3)	0	2	1	0
GII L (n=3)	0	3	0	0
GII R (n=3)	0	3	0	0
Myotoxicity (fourth week)	0	1–3	4–6	7+
GI L (n=4)	1	3	0	0
GI R (n=4)	0	2	2	0
GII L (n=4)	3	1	0	0
GII R (n=4)	1	3	0	0

GI L: infant 0.9% sodium chloride solution; GI R: infant levobupivacaine; GII L: adult 0.9% sodium chloride solution; GII R: adult levobupivacaine

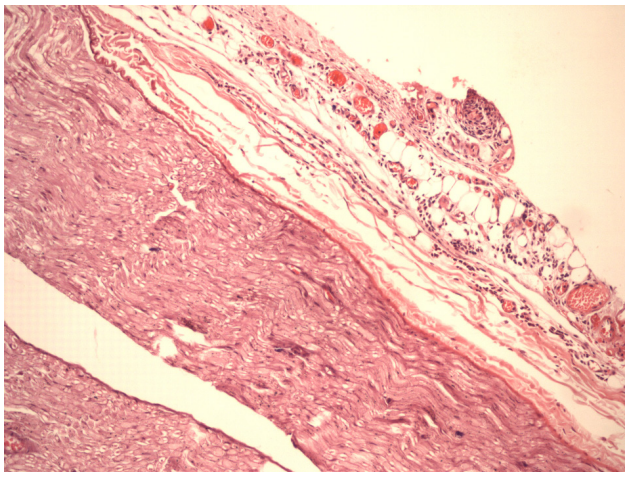

Figure 3. Sciatic nerve of an adult rat on the first day of levobupivacaine infusion. Epineural inflammatory cell infiltration (neutrophil, lymphocyte, and eosinophil) (100× hematoxylin-eosin)

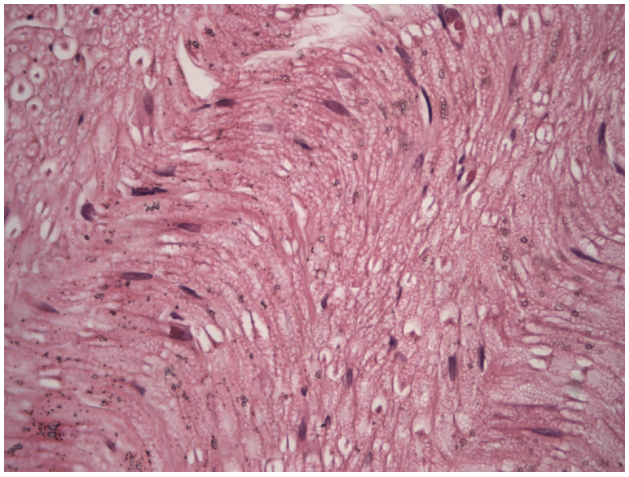

Figure 4. Sciatic nerve of an infant rat at the fourth week of levobupivacaine infusion. Mild axonal degeneration (400× hematoxylin-eosin)

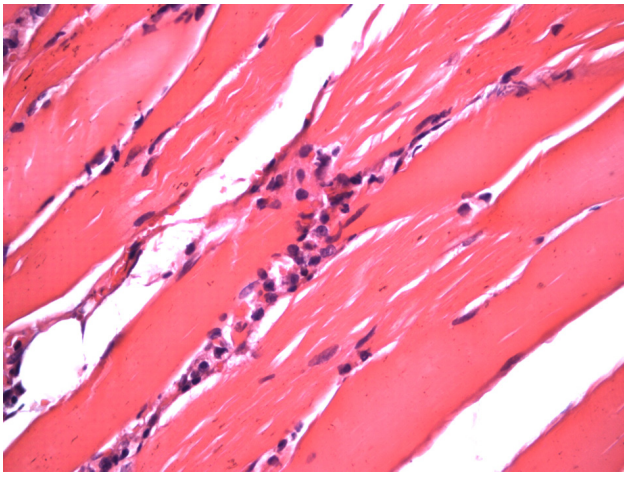

Figure 5. Muscle tissue of an adult rat on the first day of levobupivacaine infusion. Intense neutrophil infiltration (400× hematoxylin-eosin)

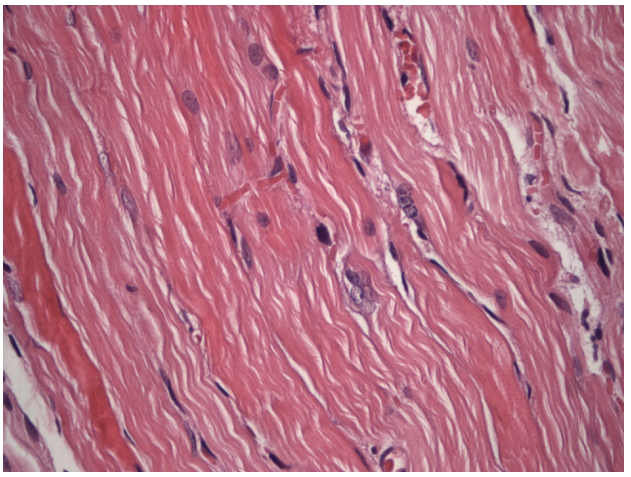

Figure 6. Muscle tissue of an infant rat on the fourth week of levobupivacaine infusion. Dual nucleus and plenty of nucleus formation (400× hematoxylin-eosin)

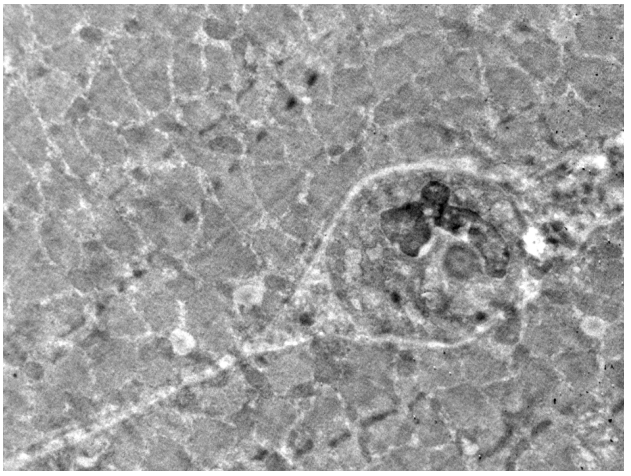

Figure 7. Muscle tissue of an infant rat at the first week of levobupivacaine infusion. Lipid vacuoles, densely looped chromatin in cell nuclei (electron microscopy; lead citrate-uranyl acetate)

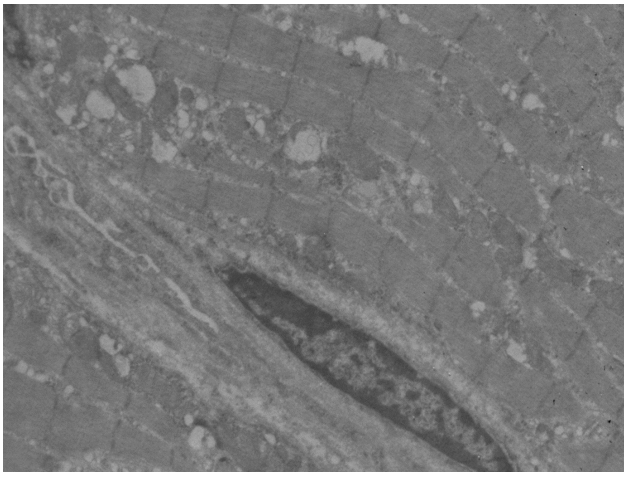

Figure 8. Muscle tissue of an infant rat at the fourth week of levobupivacaine infusion. Myofibrillolysis and vacuolization (electron microscopy; lead citrate-uranyl acetate)

The first-week group had lipid vacuoles, densely looped chromatin in cell nuclei, and apoptosis in addition to other degeneration signs. We thought the degeneration had progressed and was characterized with cell loss, thus being irreversible (Figure 7).

In the fourth week, the levobupivacaine group had sarcosomal edema, multilamellar inclusions, enlargement of perimyofibrillar space, decrease of glycogen granules, dilatation of sarcoplasmic reticulum cisternae, disorganized Z disks, macrophage in muscle fibers, degradation of triad structure, and myofibrillolysis. Permanent morphologic changes at the tissue level were observed (Figure 8).

Nerve tissue changes were in the form of myelin degeneration instead of axonal changes in the adult rat groups (GII R and GII L). There was no difference between muscle tissues of levobupivacaine and 0.9% sodium chloride solution groups, but in the fourth week, sarcoplasmic vacuoles and effacement of Z disks were observed in the levobupivacaine group.

Discussion

Many studies have investigated the neurotoxicity and myotoxicity related to peripheral nerve blockade [3-15]. Besides the trauma of the application, the local anesthetics and their different volumes and concentrations can cause damage at various levels [3].

In our study, we gave a 0.5 mg mL^{-1} levobupivacaine solution (total dose 0.8 mL) to infant rats and a 1.6 mL solution to adult rats.

Many studies have tried to show the mechanism of neurotoxicity caused by local anesthetics [4, 5]. Kitagawa et al. [4] found that high clinical concentrations of local anesthetics are molecular aggregants and behave as detergents. As a result, they damage the nerve membrane and cause irreversible neurotoxicity.

Bouaziz et al. [5] showed that, once used topically, all concentrations of levobupivacaine and ropivacaine cause a decrease in the nerve blood flow in the rat sciatic nerve. However, they could not find significant histological changes after 48 h.

Many local anesthetics were evaluated for neurotoxicity and some of them were found to be neurotoxic [6, 8-12], while some were not [12].

Selander et al. [6] studied the intrafascicular or topical usage of bupivacaine on the sciatic nerve of rabbits and found axonal degenerations of the 0.5% bupivacaine and normal groups to be the same, but increasing the bupivacaine concentration and adding adrenaline can augment the damage.

Inas et al. [7] found all local anesthetics to be neurotoxic in their study on dorsal root ganglions of chicken embryos, and they declared mepivacaine safer than lidocaine, bupivacaine, and ropivacaine.

Helm et al. [8] showed that bupivacaine and normal saline caused the same axonal damage and demyelination on the rabbit sciatic nerve. They showed that the damage was observed due to mechanical trauma by the catheter used.

Gozil et al. [9] examined the toxic effects of lidocaine and bupivacaine on rabbit facial nerve and found degeneration in the second and fourth weeks with lidocaine, bupivacaine, and 0.9% sodium chloride solution. Regeneration began in the sixth week with 0.9% sodium chloride solution and eighth week with lidocaine and bupivacaine. In addition, the regeneration was faster in the tissue exposed to bupivacaine than the tissue exposed to lidocaine.

Tamie et al. [10] showed that lidocaine was more neurotoxic than bupivacaine when administered intrathecally to rats.

Jean-Marc Mallinousky et al. [11] declared that ropivacaine was not neurotoxic in rabbits when given intrathecally.

Boogaerts et al. [12] studied neurotoxicity and motor blockade of the liposomal bupivacaine injected into the brachial plexus of rabbits and observed weak perineural inflammation under a light microscope after second and seventh days. Under an electron microscope, they did not see any differences between myelinated and demyelinated nerves. They pointed out that liposomal bupivacaine has no neurotoxic effects.

We have encountered only one study on the peripheral nerve toxicity of levobupivacaine in related literature [5]. In our study, light microscopy results revealed that the neurotoxicity of levobupivacaine is not different from the neurotoxicity of normal saline. This result matched with the studies of Selander et al. [6], Helm et al. [8], and Gozil et al. [9]. In our study, the similarity between the neurotoxicity levels of levobupivacaine and 0.9% sodium chloride solution that we found gave us the suggestion that the damage was caused by mechanical trauma by the catheter and the volume compression effect. The difference seen regarding that more axonal degeneration in the nerve tissue of adult rats and more perineural cell infiltration in the nerve tissue was found in infant rats might be caused by excess volume calculated from the weight. There was no comparison involving levobupivacaine in the literature. Our results showed that levobupivacaine had the same toxic effects on sciatic nerves of adult and infant rats. First day results matched with the early phase of degeneration, first week results with degeneration survival, and fourth week results with the beginning of regeneration. Under an electron microscope, disintegration of myelin lamellae and axonal degeneration that can be seen as vacuoles were seen in all groups, except that Schwann cell proliferation was higher in

the group sacrificed in the fourth week. In the adult rats, myelin degeneration was observed more than axonal degeneration. This might be encountered to the reversibility of the neurotoxicity.

There are also many studies about the myotoxicity of local anesthetics [3, 13-15]. Zink et al. [3] published a review about the myotoxicity of local anesthetics. They observed interstitial and myoseptal edema in the skeletal muscle histopathologically; after 24–48 h, phagocyte cells appeared and 4–6 weeks later, regeneration began. They declared that all local anesthetics are myotoxic at clinical concentrations. They determined that muscle damage differed according to dose, volume, or method of administration. Toxic effects were seen principally in the muscle fibers. Apoptosis played a role in the myotoxicity of bupivacaine.

Again, Zink et al. [13] examined the effect of bupivacaine and ropivacaine on the Ca^{+2} regulation of murine skeletal muscle fiber and found the increase in intracellular Ca^{+2} concentrations to be responsible for the myotoxicity of the local anesthetic. Stereoselectivity was declared to be a principal mechanism in intracellular Ca^{+2} regulations.

Pere et al. [14] determined the local myotoxicity of bupivacaine in rabbits after supraclavicular blockade; bupivacaine caused more muscle damage than 0.9% sodium chloride solution, but it was reversible and after one week, muscle fiber regeneration began.

Calguner et al. [15] examined the effects of lidocaine and bupivacaine on mimic muscles when applied to the facial nerve of rabbits. Atrophic changes were seen in 2–6 weeks and regeneration in 6–8 weeks. Lidocaine was found to be more myotoxic.

Zink et al. [13] examined the myotoxicity of bupivacaine and ropivacaine after continuous peripheral block in pigs and found both local anesthetics responsible for calcific myonecrosis and scar tissue formation, and bupivacaine was observed to be more toxic.

In this study, we found levobupivacaine to be more myotoxic than 0.9% sodium chloride solution. Although the damage points were less in the 0.9% sodium chloride solution group, damage was observed in that group too. The mechanical trauma might be responsible for myotoxicity, as it is in neurotoxicity. In addition, under an electron microscope, we found apoptosis in one week infant rats, which supports Zink et al.'s [13] study. In the fourth week group, levobupivacaine caused more damage than 0.9% sodium chloride solution; this might be explained either for a need of a longer healing period or a time depending repairmen of a more profound damage. In the infant and adult rats, first day and first week myotoxicity results were the same, but in the fourth week, there was more damage in infant rats than adult rats. Under an electron microscope, although there was evidence that showed reversible degeneration in adult rats, myotoxicity in infant rats brought permanent morphological changes.

The neurotoxicity of levobupivacaine was the same as that of 0.9% sodium chloride solution, in both infant and adult rats. In terms of myotoxicity, damage caused by levobupivacaine was healed later in adult rats and permanent muscle damage could be observed in infant rats, contrary to adult rats.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of Istanbul University-Cerrahpaşa, Cerrahpaşa School of Medicine Experimental Animal.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - Y.T., G.T.; Design - Y.T., G.T., G.K.; Supervision - Y.T., G.T., G.K.; Resource - Y.T.; Materials - Y.T., G.T., G.K.; Data Collection and/or Processing - Y.T., G.T., A.K.G.; Analysis and/or Interpretation - Y.T., G.T., A.K.G., E.F.A., Ö.K.D.; Literature Search - Y.T., G.T., A.K.G., E.F.A., Ö.K.D.; Writing - Y.T., G.T., A.K.G., E.F.A., Ö.K.D.; Critical Reviews - Y.T., G.K.

Conflict of Interest: The authors have no conflicts of interest to declare.

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