ORIGINAL RESEARCH

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Myotoxic effects of levobupivacaine, bupivacaine and ropivacaine in a rat model

Abstract

Aim: The aim of this study is to histopathologically compare the myotoxic effects of a single injection of levobupivacaine, bupivacaine and ropivacaine in rat skeletal muscle.

Materials and Methods: Rats received intramuscular injections of 0.5% bupivacaine (Group B), 0.5% ropivacaine (Group R), 0.5% levobupivacaine (Group L), or 0.9% normal saline (Group SF) (30 rats/group). At two, 10 and 20 days, 10 rats from each group were sacrificed and muscle samples were examined for myotoxic effects using hematoxylin-eosin staining under a light microscope.

Results: Muscle damage in Groups B, L and R was similar qualitatively. In samples taken two days after injection, the muscle damage in Group B was maximal [Damage score: 3.0 (2.0-3.0)], Group R had less damage than Group B [damage score: 2.0 (2.0-3.0)] and the damage in Group L was minimal [Damage score: 1.0 (1.0-2.0)]. In muscle samples taken 10 days after injection, there was no significant difference in muscle damage scores among Groups B, R and L. In muscle samples taken 20 days after injection, regeneration was complete, and muscle mass was histologically normal for each of the three groups (B, L and R).

Conclusion: Levobupivacaine's myotoxic effect is qualitatively similar to that seen (and previously reported) with bupivacaine and ropivacaine. Levobupivacaine was found to be quantitatively less myotoxic than bupivacaine and ropivacaine after a single intramuscular injection, only two days after injection. Myonecrosis developed after a single intramuscular injection of local anesthetic but was completely regenerated by the 20th day after injection.

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A gradual increase in clinical issues related to the myotoxic effects of both new and existing local anesthetics has been reported in recent years [1-4]. Intramuscular (i.m.) injection of local anesthetics, depending on the dose and drug, can cause damage to striated muscle tissue and may result in myonecrosis [1,5,6]. Bupivacaine is a long-acting local anesthetic; it is also the most widely used agent in clinical use [7,8]. Bupivacaine is also known to be neurotoxic, cardiotoxic and myotoxic and has the highest potential negative impact [5,6,8,9]. Ropivacaine, which is a structural amide, is known to be less cardiotoxic and less myotoxic than bupivacaine [8-10].

Levobupivacaine, a pure S (-) enantiomer of bupivacaine, has been developed as a less cardiotoxic toxic agent, as it has been shown that racemic bupivacaine has similar cardiotoxicity to its R (+) enantiomers [8,9,11]. Bupivacaine-induced myotoxicity is associated with histological damage and alteration in mitochondrial metabolism and perturbations in calcium homeostasis [12,13], but a histopathological survey showing the myotoxic properties of levobupivacaine has not yet been published.

This study aims to compare the myotoxic effects of racemic bupivacaine (bupivacaine), ropivacaine and levobupivacaine using histopathological techniques.

Materials and Methods

This study was carried out at the Erciyes University, Clinical and Experimental Research Center (DEKAM), in accordance with guidelines of The Scientific and Technological Research Council of Turkey (TÜBİTAK), with the approval of the Erciyes University Animal Studies Ethics Board (Approval No: 01/257). Care of the animals conformed to the recommendation of the Helsinki Declaration. One hundred and twenty female Wistar-Albino rats (180-200 grams) were divided randomly into four groups. The first group (Group B, n = 30) received 100 µl of 0.5% bupivacaine, the second group (Group R, n = 30) received 100 µl 0.5% ropivacaine, the third group (Group L, n = 30) received 100 µl 0.5% levobupivacaine and the fourth group (group SF, n = 30) received 100 µl 0.9% normal saline. All drugs were delivered intramuscularly.

The right flexor digitorum superficialis muscles of rats were used for injection. The muscle was detected by hand and a 27G needle was used to slowly inject either 100 µL local anesthetic or 0.9% normal saline into each rat. To avoid intravascular injection, gentle aspiration was applied before each injection. The injected local anesthetics used in groups B and L were commercial preparations purchased from the following companies: 0.5% bupivacaine (Marcaine) was purchased from Astra Zeneca, England and 0.5% levobupivacaine (Chirocaine) was

purchased from Abbott, USA. Because ropivacaine (Naropin) was not available for purchase at a 0.5% concentration, the commercial preparation (1% Naropin, Astra Zeneca, England) was diluted at a 1:1 ratio with 0.9% normal saline.

After injection, each group of rats was put into separate cages. Optimum living conditions were provided to all rats. Rats were cared for in the same room with a 12 hour light/12 hour dark cycle, ambient temperature and identical feeding regimes.

Ten rats from each group were sacrificed under ketamine anesthesia using cervical dislocation two, 10 or 20 days after injection. The rats' extremities that had received the injections were separated from the rest of the body after the skin tissue was dissected. The specimens were numbered randomly then fixed in 10% formalin solution and sent to the Histology Department for histological examination.

Three hours after this process, the flexor digitorum superficialis muscles were separated from the extremities and placed into a 10% formalin solution. Twenty-four hours after being fixed in 10% formalin, tissues were dehydrated through an alcohol series (50%, 70%, 80%, 96% and 100%). Tissues were then passed through three different containers of xylene (15 minutes in the first, 30 minutes each in the second and third), leaving them transparent. They were then incubated in melted paraffin overnight and placed into paraffin blocks.

Twenty to 30 samples were removed from each rat and all samples were scanned microscopically to locate the injection area, then five were chosen for histological analysis. Five-micron thick sections were prepared from the paraffin blocks with a microtome (Leica RM 2155), stained with hematoxylin-eosin and then examined under a light microscope (Olympus BH-2). Microphotographs were taken from tissue sections and scoring was performed (Table 1) according to the damage size based on the Benoit, Yagiela and Ferrell scoring system [14] by a microscopist blinded to the identity of the group.

Statistical analysis of the study was performed using the computer program SPSS for Windows 13.0. Differences in the skeletal muscle injury scores of the groups were evaluated using a Kruskal-Wallis test. Differences were found in the median of each group with a Kruskal-Wallis test; therefore, each group was compared using a Mann-Whitney-u test for days two, 10

TABLE 1. Benoit-Yagiela-Ferrell Damage Scoring System

Score	Damage condition
0	No fibrillar damage
1	Localized and diffuse damage in places
2	Extensive necrosis
3	Destruction and necrosis in entire muscle mass

FIGURE 1. Light microscope view of skeletal muscle samples taken two days after 0.9% normal saline injection (SF). (H&E. X20, In the bottom right thumbnail X40), (N: normal muscle tissue).

and 20. Comparisons were done in duplicate. The required degree of significance was identified using The Holm's Bonferroni method ($\alpha/\text{group numbers} = 0.05/4 = 0.0125$).

Results

In the control group (Group SF), tissue samples taken on the 2nd, 10th and 20th days after injection had no histological lesions or inflammatory cell infiltration in skeletal muscle fibers or in the surrounding connective tissue elements (Fig. 1) [Damage score = 0.0 (0.0-0.00)]. In Groups B, L and R, the observed muscle damage was significantly different from the Group SF on days 2 and 10 after injection ($p < 0.01$, for each comparison) (Table 2).

In the bupivacaine group (Group B), necrosis was seen among the muscle cells and extensive inflammation was also observed at two days after injection. Extensive mononuclear leukocyte infiltration among myofibrils, necrobiotic changes and degeneration were observed in intact muscle cells [Damage score = 3.0 (2.0-3.0)]. In addition, septal areas had widespread inflammatory edema and a distinctive style of waves that reflected the contraction of myocytes (Fig. 2, B2). Myocyte precursors, also called satellite cells, were seen as myoblasts with a central core containing dense chromatin and acidophilic clear cytoplasm (Fig. 2, B2, small pictures).

In the levobupivacaine group (Group L), intact muscle fibers were observed among the muscle cells that underwent necrosis in tissue samples at 2 days after injection (Fig. 2, L2). Areas with common inflammatory cells, those with edema and

TABLE 2. Muscle damage scores following intramuscular injection with local anaesthetics

Groups	2 nd day	10 th day	20 th day
SF	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
B	3.0 (2.0-3.0)	0.5 (0.0-1.0)	0.0 (0.0-0.0)
L	1.0 (1.0-2.0)	1.0 (0.0-2.0)	0.0 (0.0-0.0)
R	2.0 (2.0-3.0)	1.0 (0.0-1.0)	0.0 (0.0-0.0)

[Med (min-max)]

SF, saline control; B, bupivacaine; L, levobupivacaine; and R, ropivacaine

those with dense inflammatory cells appeared to have undergone degeneration of muscle fibers [Damage score = 1.0 (1.0-2.0)]. In addition, some regeneration had occurred in those same areas, as shown by the presence of myocyte precursors (Fig. 2, L2, small pictures).

In the ropivacaine group (Group R) leukocyte infiltration was observed in robust-looking muscle fibers in areas where degeneration had occurred and myocyte structure had completely disappeared 2 days after injection [Damage score = 2.0 (2.0-3.0)] (Fig. 2, R2). Myocyte precursors, which indicate the beginning of regeneration, were widely seen (Fig. 2, R2, bottom right thumbnail).

The injury scores in Groups B, L and R were significantly different from SF group two days after injection ($p < 0.01$, for each comparison). Although Groups B, L and R had the same qualitative features of histopathological changes, the muscle damage in Group B was maximal, the damage in Group L was minimal, and Group R was between those two groups.

In Group B after 10 days, a conversion to normal muscle tissue was widely observed (Fig. 2, B10). Muscle cell nuclei were relatively round and dense, especially when myoblasts were integrated into myotubes and had changed into myocytes.

In Group L after 10 days, the tissue appeared nearly normal. Inflammatory cells were present in a very small area, and the scattered remains of infiltrating cells were present among the muscle fibers (Fig. 2, L10). Muscle fibers lay parallel to one another, and regenerative cells dominated the area (Fig. 2, L10, bottom right thumbnail). Mononuclear leukocytes were highlighted by the presence of eosinophilic leukocytes in the inflamed area (Fig. 2, L10, right above the small picture).

In Group R on day 10 after injection, the tissue had nearly normal-looking regenerated muscle fibers (Fig. 2, R10) and were dominated by regenerative cells (Fig. 2, R10, bottom right picture); in some places, small areas of remaining inflammatory degeneration were observed (Fig. 2, R10, top right picture).

FIGURE 2. Light microscope view of skeletal muscle samples taken two days after injection of bupivacaine 0.5% (B2), levobupivacaine 0.5% (L2), and ropivacaine 0.5% (R2); 10 days after injection of bupivacaine 0.5% (B10), levobupivacaine 0.5% (L10), and ropivacaine 0.5% (R10); 20 days after injection of bupivacaine 0.5% (B20), levobupivacaine 0.5% (L20), and ropivacaine 0.5% (R20). (H&E. X20, X40 thumbnail images). (N: normal muscle tissue, D: degenerative areas, I: inflammation regions, M: myocyte precursors (satellite cells), O: edematous areas, R: regeneration areas).

No statistically significant differences in terms of muscular damage scores among Groups B, R and L were present in samples taken 10 days after injection (Table 3).

In muscle tissue samples taken 20 days after injection, all three groups (B, L and R) showed a complete regeneration of muscle tissue, and a histological return to normal was observed (Fig. B20, L20 and R20). [Damage score = 0.0 (0.0-0.00)]. Therefore, no statistically significant difference among the groups was determined.

Blood vessels and nerve structures were not influenced histopathologically in any muscle tissue samples taken on days two, 10 or 20 in any of the groups.

All muscle damage scores are shown in Table 2.

The medians of the different groups from the Mann-Whitney-u test are shown in Table 3.

Discussion

Voluntary or involuntary i.m. injection of local anesthetics is often encountered during procedures, such as peripheral nerve

TABLE 3. Mann Whitney - u test: comparison of muscle damage following intramuscular injection with local anaesthetics

Groups compared	p values			Required p for significance (α / Group numbers)
	2 nd day p	10 th day p	20 th day p	
SF – R	0.00003*	0.0043*	1.000	<0.0125
SF – B	0.00003*	0.0012*	1.000	<0.0125
SF – L	0.00003*	0.005*	1.000	<0.0125
R – B	0.009*	0.66	1.000	<0.0125
R – L	0.0012*	0.80	1.000	<0.0125
B – L	0.0002*	0.52	1.000	<0.0125

*The Holm's Bonferroni method, with $p < 0.0125$ according to the degree of significance.

SF, saline control; B, bupivacaine; L, levobupivacaine; and R, ropivacaine

blocks, wound infiltration, trigger point injections, and peri-/retrobulbar blocks [1-5,15-20]. Many experimental and clinical studies have investigated the myotoxic effects of local anesthetic agents [21,22]. Clinical concentrations of local anesthetics were all found to be myotoxic [1,5,23]. Some reports in the literature did not consider the rare and reversible myotoxic effects of local anesthetics to be important [1,5,6]; however, in clinical practice not all cases are reversible [4]. Myonecrosis and serious muscle function disorders following the injection of a local anesthetic have been reported in a growing number of cases [1,2,18, 24].

Although the specific mechanisms of the myotoxic effects of local anesthetics are not fully understood, researchers [12,25-30] believe that these effects are caused by an increase in the level of intracellular Ca^{++} . The myotoxic effect of the local anesthetic tetradoxin [25] supports this view because tetradoxin has no direct impact on intramuscular Ca^{++} but does show an effect by inhibiting sarcolemmal Na^+ channels. In addition, sarcolemmal morphological structures remain strong in muscle tissues that are exposed to local anesthetics for a long time. This indicates that fiber degeneration occurs intracellularly and does not happen due to the destruction of external barriers [1]. Bupivacaine has the most myotoxic effects of all tested local anesthetics, and it is also known to increase ryanodine receptor activity. Depending on the increased ryanodine receptor activity, it also increases Ca^{++} release from the sarcoplasmic reticulum and reduces Ca^{++} re-uptake, both of which are known to cause muscle damage [14,25-30]. Although it has been reported that all of these local anesthetics increase Ca^{++} permeability in the sarcoplasmic reticulum, this is also related to the lipophilicity of the local anesthetic agent [1]. Koman and Lokuta [28] have shown that bupivacaine and tetracaine, which have lipid-soluble structures, do not have similar effects on the sarcoplasmic reticulum, ryanodine receptors and intracellular Ca^{++} release. Also, Nouette-Gaulain *et al.* [13] have demonstrated that levobupivacaine has a marked effect on

Ca^{++} homeostasis. Levobupivacaine was expected to have a myotoxic effect similar to that observed for bupivacaine because the two anesthetics exert similar effects on intracellular Ca^{++} homeostasis; interestingly, levobupivacaine was found to have a less myotoxic effect than bupivacaine or ropivacaine. Further studies are needed to explain the observation of a lower myotoxic effect of levobupivacaine and with the impact on intracellular Ca^{++} homeostasis.

Changes in muscle tissue after the injection of local anesthetic agents are nonspecific and uniform [1]. The damage to the skeletal muscle depends on the local anesthetic used, although the underlying mechanisms are not clear. Pathologic change in the muscle can include fiber vacuolization and, myocyte edema, a total disintegration of intracellular structures and myonecrosis, leading to a full spectrum of necrobiotic changes [1,5,10]. These changes are not specific to local anesthetic agents. This disease morphology can be found after traumatic injury, including pressure injury, which causes cellular damage in within the skeletal muscle [1,5,10]. The volume of the drug used in intramuscular injection is important for predicting the amount of muscle damage that will occur. High injection volumes can cause damage to muscle tissue via pressure effects [1, 5,10]. In this study, all local anesthetics and 0.9% normal saline were given at a volume of 100 μ L. It has been reported previously that this volume does not cause muscle damage [5,6]. Previous studies have investigated whether needles themselves cause muscle damage or myonecrosis when no drugs are administered. In previous studies, no myonecrosis was found when needles only pricked the tissue [5,6]; therefore, this study did not reinvestigate the effects of a traumatic needle-stick.

It has been reported that the myotoxic effects of repetitive injections and the continuous infusion of local anesthetics are worse than a single injection [1,24]. Zink *et al.* [10] investigated the effects of bupivacaine and ropivacaine on acute myotoxicity by applying a local anesthetic infusion to the femoral nerve of a pig for six hours using a catheter. Kytta *et al.* [31]

investigated the role of bupivacaine on myotoxicity using repeated bupivacaine injections around the sciatic nerve of rats. Amaniti *et al.* [6] investigated the myotoxic effect of ropivacaine using a single injection of local anesthetic in rats. In clinical practice, intraoperative infiltration into the surgical field, peripheral nerve blocks and processes to treat pain in outpatients are commonly used methods and are generally administered using a single dose injection of local anesthetic agents [1, 2,32]. Therefore, a single-dose i.m. injection was used in this study.

In studies where bupivacaine was used, hypercontractive myofibrils were observed a few minutes after i.m. injection, followed by lytic degeneration of the sarcoplasmic reticulum in the skeletal muscle within hours and, eventually, myocyte edema and necrosis [1,5,10]. Amaniti *et al.* [6] examined the myotoxic effects of ropivacaine in rats. They found that fiber degeneration, necrosis, infiltration of phagocytic cells in necrotic areas and unaffected fibers were observed among phagocytes on the second day after injection. Researchers have reported that infiltration into necrotic areas occurs because of phagocytic cells that remove dead debris [1,5]. Zink *et al.* [10] investigated the effects of bupivacaine and ropivacaine on acute myotoxic conditions and saw that both local anesthetic agents caused similar qualitative histopathological changes. In another study, Zink *et al.* [24] investigated the long-term myotoxic effects of bupivacaine and ropivacaine in pigs by applying a peripheral nerve block with a continuous infusion technique. That study observed comparable histopathologic findings with bupivacaine and ropivacaine on day 7, in agreement with the findings of Amaniti *et al.* [6]. In the present study, the first histopathological examination was performed on skeletal muscle samples 48 hours after i.m. injection of the rats. After the second day of bupivacaine injection, a wavy appearance of myocytes was seen, reflecting contraction. Common necrobiotic changes, phagocytic infiltration, and, in some places, unaffected fiber bundles were also observed within necrotic areas. These histopathological findings were qualitatively similar in muscle samples from the levobupivacaine- and ropivacaine-injected groups. Previous studies had found that the myotoxic effects of different local anesthetics were qualitatively similar [1,5,6,10]. In the present study, the myotoxic effects of levobupivacaine were qualitatively similar to other local anesthetics.

It has been reported that nerves, vasculature and connective tissue are not affected by myotoxic damage caused by local anesthetics [1]. Foster *et al.* [5] examined the myotoxic effects of procaine, tetracain, lidocaine and bupivacaine with a single i.m. injection in rats and found that nerves, vasculature and

connective tissue were not involved in the damage formed in the muscle tissue due to these agents. Similarly, Amaniti *et al.* [6] reported that the rat muscle tissue damage associated with local anesthetics did not affect nerves, connective tissue or blood vessels. Hogan *et al.* [2] found vein, nerve and connective tissue structures to be normal in myonecrotic sternocleidomastoid muscle biopsy samples after the use of local anesthetic agents. In this study, after injection of bupivacaine, ropivacaine or levobupivacaine, muscle tissue damage was observed, but blood vessel, nerve and connective tissue damage was not observed.

Previous research has shown that the myotoxic effects of local anesthetics are not influenced by myocyte precursors (satellite cells or myoblasts) [1,5,6,10]. This strongly suggests that satellite cells play a key role in tissue regeneration [1]. Zink *et al.* [24] investigated the myotoxic effects of bupivacaine and ropivacaine and found that seven days after injection, satellite cells were observed, along with the necrobiotic changes. Previous studies [1,5,6] reported that after the exposure of muscle tissue to myotoxic agents for 24-48 hours, satellite cells were activated and regeneration was completed by 4-6 weeks. Hogan *et al.* [2] placed a catheter in the interscalene area and injected repeated doses of bupivacaine and epinephrine into the sternocleidomastoid muscles of patients. Histopathological myotoxicity due to local anesthetic agents was found to require a minimum of three months for complete remission. In this study, precursors of myocytes, or satellite cells, were seen in tissue samples taken two days after injection with bupivacaine, ropivacaine or levobupivacaine. The formation of new myocytes from satellite cells and the remains of degenerated myocytes were seen in tissue samples taken 10 days after injection. As reported in previous studies, regeneration started before the 2nd day after injection and continued until the 10th day. Although previous experimental studies [1] showed that regeneration was completed in 4-6 weeks, we observed almost normal muscle tissue samples after 20 days. In the above-mentioned studies, Kytta *et al.* [31] applied recurrent bupivacaine injection around the sciatic rat nerve and Zink *et al.* [24] applied continuous infusion of a local anesthetic around the femoral pig nerve. In the present study, a single i.m. injection was given to rats. Thus, the reason for the earlier regeneration seen in this study could be attributed to the methodological differences between the three studies. In a study that investigated the potential myotoxic effects of ropivacaine and the time-dependent changes of possible damage, Amaniti *et al.* [6] reported that muscle damage was corrected by day 30; however, Amaniti evaluated tissue samples only at 2, 4, 7 and 30 days after injection.

Amaniti *et al.* [6] reported the myotoxic effect of ropivacaine in rats was dose-dependent and Zink *et al.* [10,24] showed that bupivacaine is more myotoxic than ropivacaine at both 7 and 28 days. In this study, when the skeletal muscle tissue samples collected on day 2 were examined histopathologically, it was seen that bupivacaine caused significantly greater myotoxic damage than ropivacaine ($p < 0.01$) and levobupivacaine, which had not been previously investigated, caused less myotoxicity than either bupivacaine or ropivacaine ($p < 0.01$). By the 10th day, no statistically significant differences were observed among the bupivacaine-, ropivacaine- and levobupivacaine-injected groups.

In this study, we were limited to conducting histopathologic examinations using light microscopy. Recent research related to the myotoxic effect of local anesthetics have been published by Zink *et al.* [10,24] in 2003 and 2005 and in 2006 by Amaniti *et al.* [6]. In these studies, all local anesthetic agents were compared in ways similar to those in the present study. In terms of myotoxic effects, they were scored according to their appearance under light microscopy, and selected samples in which myotoxic effects were densely observed were examined using an electron microscope. The goal of this research was to compare the myotoxic effect of levobupivacaine, which had not yet been investigated in a direct comparison, with racemic bupivacaine and ropivacaine. Zink *et al.* [10,24] had already compared bupivacaine and ropivacaine in two different studies, and Amaniti *et al.* [6] had also compared two different concentrations of ropivacaine for the same purposes. In addition to investigating mechanisms at the intracellular level, they used an electron microscope. Our research reveals that levobupivacaine has a less myotoxic effect [damage score: 1.0(1.0-2.0)] than bupivacaine [damage score: 3.0(2.0-3.0)] ($p < 0.01$). To determine the mechanism of levobupivacaine function on an intracellular level and whether it causes apoptosis in a similar manner to bupivacaine, further research is required. The myotoxic effects of local anesthetics could possibly be investigated at the level of cell organelles using an electron microscope.

In conclusion, the muscle damage in Groups B, L and R was qualitatively similar. In muscle samples taken two days after injection, muscle damage was maximal in Group B, smaller in Group R and minimal in Group L ($p < 0.01$, for each comparison). In muscle samples taken 10 days after injection, there were no significant differences in muscle damage scores between any of the groups. In muscle samples taken 20 days after injection, we observed that regeneration had been completed, and the muscle mass had become histologically normal in each of the three groups (B, L and R).

In this study, levobupivacaine was found to have a smaller myotoxic effect than bupivacaine and ropivacaine, but only when assessed shortly after injection (two days). Qualitatively, the myotoxic effect of levobupivacaine is nonspecific and uniform, as are other local anesthetics. Myonecrosis due to local anesthetics had completely regenerated 20 days after a single dose injection in rats.

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