RESEARCH ARTICLE

HISTOMORPHOMETRIC PARAMETERS AS INDICATORS OF NERVE TISSUE DAMAGE AFTER PERINEURAL AND INTRANEURAL APPLICATION OF LIPOSOMAL BUPIVACAINE IN RAT MODEL

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ABSTRACT

Liposomal bupivacaine is a long-acting local anesthetic agent and it is thought to be one of the safest local anesthetics. However, there has been detailing possible neurotoxic effects. At present, the exact molecular mechanism of liposomal bupivacaine-mediated neurotoxicity is unknown. We postulated that intraneural injection of 1.33% liposomal bupivacaine resulted in greater nerve injury than perineural injection, and this would be proved by objective quantitative histological analysis.

A rat sciatic nerve block model was used. The study was conducted in accordance with the principles of laboratory animal care and was approved by the Laboratory Animal Care and Use Committee. Thirty adult Wistar rats of both sexes were studied. After induction of general anesthesia, the sciatic nerve was exposed unilaterally. Sciatic nerves were randomly assigned by the method of sealed envelopes to recieve: 2 mL perineurally 1.33% liposomal bupivacaine, 2 mL intraneurally 1.33% liposomal bupivacaine, 2 mL perineurally saline or 2 mL intraneurally saline. Quantitative histological examination was followed to determine the potential damage to nerve tissue. All intraneural injections showed significantly smaller number of nerve fibers ($p<0.001$). There was no statistical significance in myelin thickness ($p>0.005$) and nerve fiber diameter ($p>0.005$) between the groups. Intraneurally administrated liposomal bupivacaine showed reduction in axon diameter ($p<0.005$) comparing to perineurally administrated liuposomal bupivacaine and saline.

Keywords: Liposomal bupivacaine, peripheral nerve blocks, neurotoxicity

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INTRODUCTION

Pain is a protective mechanism that has an adaptive value, and the inability to feel pain is associated with early mortality from accidental injuries or joint damage (Shuang and Leigh, 2022; Sandhu et al., 2021). However, the pain that came as a result of surgical intervention is not desirable. The benefits of optimal control of postoperative pain include: improved cardiac, respiratory and gastrointestinal functions, fewer thromboembolic complications, demonstrated longer duration of arterial grafts, fewer septic complications, lower incidence of developing chronic pain conditions, and reduced mortality in high-risk patients (den Bandt, 2019).

The application of local anaesthetic in the connective tissue around the nerve will cause a loss of sensation and / or paralysis in the area that the nerve innervates. Peripheral nerve stem injury results in varying degrees of injury and nerve fascicles. The decisive factor that determines the degree of injury and recovery after injury is axonal injury. After a peripheral nerve suffers an injury, complex pathophysiological, morphological, and metabolic changes occur at the site of injury. These complex changes also occur within the body of the nerve cell, but also proximal and distal to the site of nerve injury (Kadioglu, 2004; Park et al., 2019; Suaid Hen, 2022).

Liposomal bupivacaine is a prolonged-release formulation of bupivacaine, designed to allow drug diffusion for up to 72 h after a single application at the end of surgery (Sandhu et al., 2021). To date, the only approved indications for the clinical use of liposomal bupivacaine are postoperative wound infiltration after various surgical procedures and interscalenic blockade of the brachial plexus for postoperative analgesia. There is currently no experimental study in the world that would define the possible neurotoxic effect of liposomal bupivacaine. The aim of our study is to define the safety profile of liposomal bupivacaine using quantitative histological analysis of nerve tissue after perineural and intraneural application (Malik et al., 2017).

MATERIAL AND METHODS

After approval of the Ethics Committee of the Faculty of Medicine and Veterinary Medicine of the University of Sarajevo (02-3-4-2819/17) and by the Principles of Care and Preservation of Laboratory Animals (Bethesda, 1985), 30 adult Wistar rats of both sexes with an average weight of 300 g were included in the study. The animals were introduced to general anaesthesia by intraperitoneal injection of Nembutal - sodium pentobarbital (50 mg / kg), respecting the rules of strict asepsis. An incision was then made on the skin and gluteal muscle to access the sciatic nerve. All procedures were performed between 08.00 and 14.00.

Total of 60 nerves (30 rats) were randomly assigned to one of four groups (15 per group) using a computer-generated sequence with sealed envelopes. The first group received an injection of 4 ml liposomal bupivacaine 1.3% perineurally, the second group received 4 ml liposomal bupivacaine 1.3% intraneurally, the third group (control) received 4 ml saline intraneurally, whereas the fourth group (control) received 4 ml saline perineurally. The injections were performed under direct vision. Perineural injections were applied on the right side, while intraneural injections were applied on the left side. For perineural injections, the needle bevel was placed outside the external epineurium to inject liposomal bupivacaine around the nerve, whereas for intraneural injections, the needle was inserted under the external epineurium.

Insulated 27-G, 5-cm-long, long-beveled nerveblock needles (Terumo Europe NV, Leuven, Belgium) were used. Drugs were injected by an automated infusion pump (PHD 2000; HarvardApparatus, Holliston, MA, USA) at a 4 ml/min speed. Data were acquired with an in-line manometer (PG5000; PSI Tronics Technologies, Inc., Tulare, CA, USA) coupled to the computer via an analog-digital conversion board (DAQ card 6023; National Instruments, Austin, TX) and placed proximal to the needle in line with a non-distensible high-durometer polyvinylchloride injection tubing (2.1-m arterial pressure tubing, Abbott Critical Care Systems; Abbott Laboratories,

North Chicago, IL, USA). After application, the wounds were closed with a stitch.

After monitoring the animals for 3 days, the animals were euthanized with an overdose of sodium pentobarbital and potassium chloride. Samples of the examined nerves were then excised bilaterally (site of application, 1 cm proximal and distal to the site of application). After fixation in 10% formalin and dehydration in ethylene alcohol, the samples were treated with xylene as an intermediate and embedded in paraffin. Then, the tissue blocks were cut with a microtome (Leica „RM 2165) into incisions 3 - 4 µm thick. The sections were then passed through a series of alcohol of decreasing concentration and stained with the Hematoxylin Eosin method. After obtaining the appropriate sections and preparations, they were subjected to quantitative histological analysis, by light microscopy. Quantitative histomorphological analysis of all samples was performed by an experienced pathologist who did not know from which group of experimental animals the samples originated. Nerve fibrehistomorphometry was performed using a light microscope (Eclipse E400, Nikon) with a digital camera installed and a computer on which image processing and analysis software was installed (Modular software for interactive image processing and analysis - ELLIPSE Version 2, 0, 8, 1). Half of the preparations were selected from each group. Nerve field samples were selected as described by Geuna et al. (Geuna et al., 2001). To avoid potential errors in histomorphometry, we first divided the nerve into 12 large fields and then each large into 9 smaller ones. Only 1 of the 9 smaller fields were randomly selected (medium). In order to overcome the "marginal effect", a method based on the counting of fibres whose cross sections covered the upper and lower boundary of the observed field, was used (Geuna et al., 2004).

The parameters we determined in each selected field were: total number of nerve fibers (N), diameter of nerve fibers (D), axon diameter and myelin thickness, which was calculated by subtracting axonal diameter from the total nerve fiber diameter.

Statistical analysis

Total of 60 nerves were required to obtain relevant results to detect a significant difference in the proportion of nerve injury between intraneural and perineural injections $\alpha=0.05$. Statistical analysis was performed using SPSS program (Statistical Package for Social Sciences) version 19.0 (Chicago, IL, USA). For histomorphometry, a statistical comparison of the quantitative data was subjected to a one-way ANOVA test. The P value <0.05 was considered statistically significant. The results are expressed through the methods of standard descriptive statistics: mean ($\bar{X}$), standard deviation (SD), standard error of the mean (SEM), median (Me) and percentiles (25th, 50th and 75th), minimum (Min.) and maximum (Max.) value, absolute frequency (N) and relative frequency (%).

The Shapiro - Wilk test was used to estimate the normal distribution of continuous variables. The significance of the difference for the independent continuous variables that did not follow the normal distribution was tested by the Kruskal-Wallis test if there were more than two examined groups. The nonparametric Mann - Whitney U test was used to examine the differences between the two groups in cases where the data distribution deviated from the normal, while the Friedman test was used to examine the difference between repeated measurements within the group. Student's t-test for independent samples was used to examine the difference between the two groups. In order to examine the difference in the results of repeated measurements, the ANOVA multiple comparison test with post-hoc Bonferroni correction was used. The P value <0.05 was considered statistically significant.

RESULTS

All animals successfully completed the experiment and had uneventful post-surgical recovery and weight gain. There were no signs of local or systemic infection in any of the animals.

There were statistically significant differences in number of nerve fibers between intraneural liposomal bupivacaine and perineural saline

(p=0.002), and between perinaural and intraneural saline (p=0.039).

There were statistically significant differences in axon diameter between perineural and intraneural applied liposomal bupivacaine (p =0.017), and between intraneural liposomal bupivacaine and perineural saline (p=0.0001).

The groups did not differ in myelin thickness values and nerve fiber diameter (p>0.05).

Table 1 Number of nerve fibres in each group

Group	N		SD	SEM	95% CI*		Min.	Max.
					Lower limit	Upper limit		
l.b.intraneural	15.000	1.402	0.327	0.084	1.221	1.583	0.832	2.032
l.b.perineural	15.000	1.624	0.316	0.082	1.449	1.799	0.949	2.229
s.intraneural	15.000	1.521	0.394	0.102	1.303	1.739	0.999	2.506
s.perineural	15.000	1.870	0.311	0.080	1.697	2.042	1.416	2.458

l.-liposomal bupivacaine
s.-saline

Table 2 Differences in the number of nerve fibres between each group

(I) group	(J) group	Mean difference (I-J)	SEM	p	95% CI	
					Lower limit	Upper limit
l.b.intraneural	l.b.perineural	-0.222	0.124	0.463	-0.560	0.116
	s. intraneural	-0.119	0.124	1.000	-0.457	0.219
	s. perineural	-0.468(*)	0.124	**0.002**	-0.806	-0.130
l.b.perineural	l.b.intraneural	0.222	0.124	0.463	-0.116	0.560
	s. intraneural	0.104	0.124	1.000	-0.234	0.442
	s.perineural	-0.245	0.124	0.312	-0.583	0.093
s. intraneural	l.b.intraneural	0.119	0.124	1.000	-0.219	0.457
	l.b.perineural	-0.104	0.124	1.000	-0.442	0.234
	s. perineural	-0.349(*)	0.124	**0.039**	-0.687	-0.011
s. perineural	l.b.intraneural	0.468(*)	0.124	**0.002**	0.130	0.806
	l.b.perineural	0.245	0.124	0.312	-0.093	0.583
	s. intraneural	0.349(*)	0.124	**0.039**	0.011	0.687

l.-liposomal bupivacaine
s.-saline

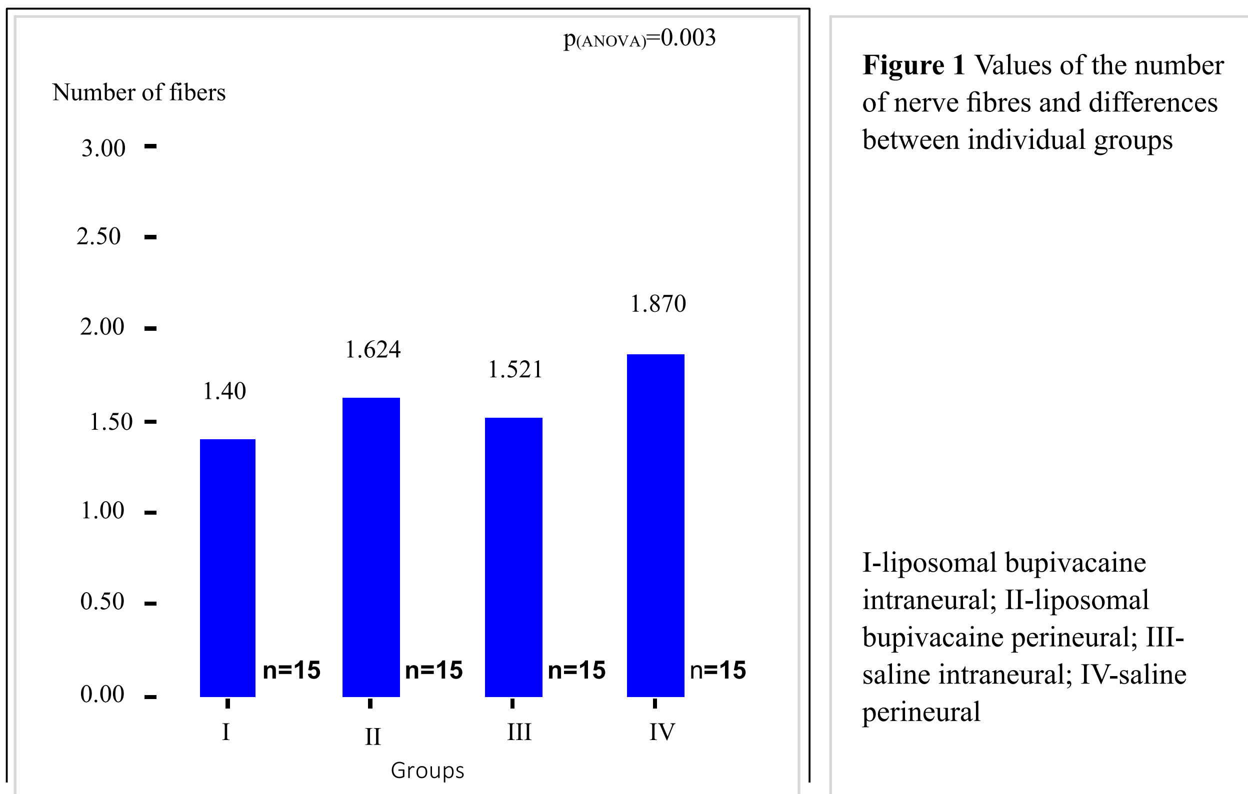

Figure 1 Values of the number of nerve fibres and differences between individual groups

I-liposomal bupivacaine intraneural; II-liposomal bupivacaine perineural; III-saline intraneural; IV-saline perineural

Table 2 Nerve fibre diameter

Group	N	*	SD	SEM	95% CI*		Min.	Max.
					Lower limit	Upper limit		
l.b.intraneural	15.000	6.168	0.471	0.122	5.907	6.429	5.491	6.906
l.b.perineural	15.000	6.359	0.370	0.096	6.154	6.564	5.901	7.339
s. intraneural	15.000	6.603	0.934	0.241	6.085	7.120	5.734	8.683
s. perineural	15.000	6.427	0.575	0.149	6.108	6.745	4.943	7.230

l.-liposomal bupivacaine
s.-saline

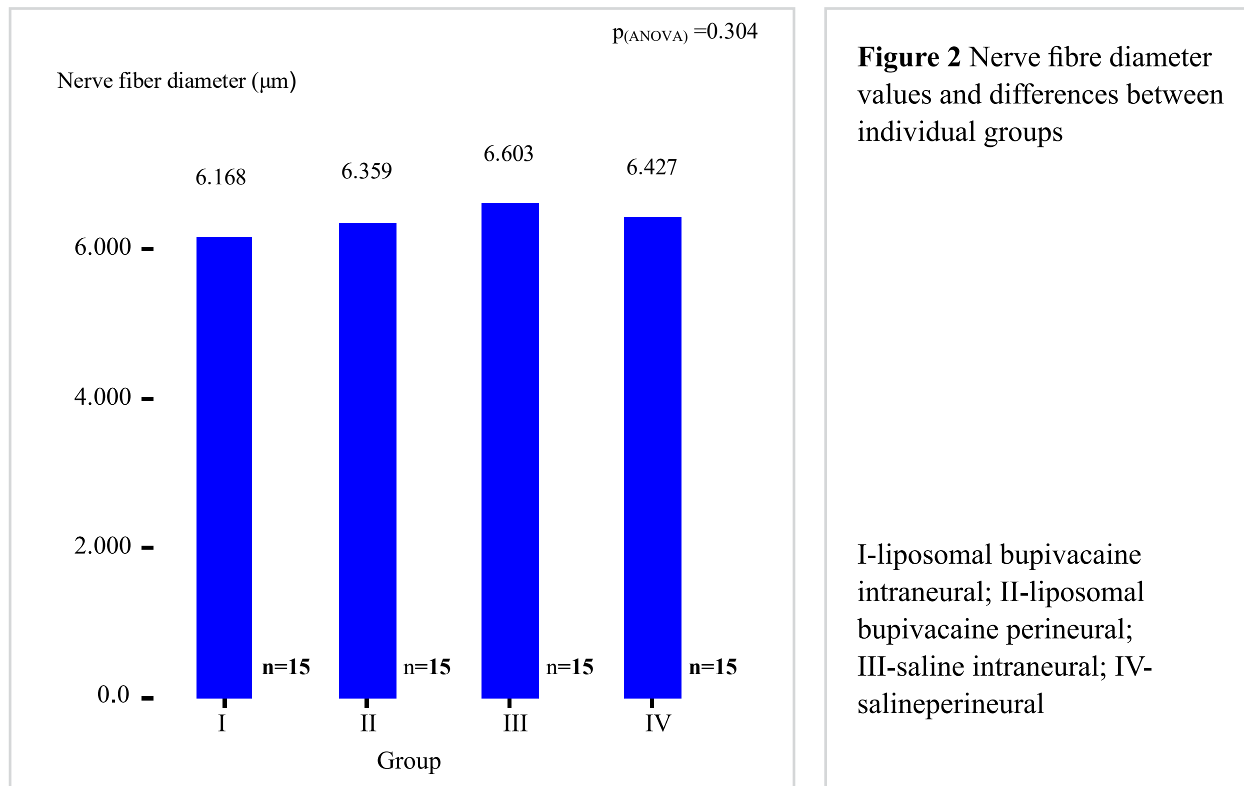

Figure 2 Nerve fibre diameter values and differences between individual groups

I-liposomal bupivacaine intraneural; II-liposomal bupivacaine perineural; III-saline intraneural; IV-salineperineural

Table 3 Axon diameter

Group	N		SD	SEM	95% CI*		Min.	Max.
					Lower limit	Upper limit		
l.b.intraneural	15.000	2.030	0.253	0.065	1.889	2.170	1.545	2.491
l.b.perineural	15.000	2.324	0.240	0.062	2.191	2.457	1.790	2.655
s.intraneural	15.000	2.482	0.613	0.158	2.142	2.821	1.809	3.933
s.perineural	15.000	2.549	0.323	0.083	2.370	2.728	1.956	3.005

l.-liposomal bupivacaine
s.-saline

Table 4 Differences in axon diameter between each group

(I) group	(J) group	Mean difference (I-J)	SEM	p	95% CI	
					Lower limit	Upper limit
l.b.intraneural	l.b.perineural	-0.294(*)	0.090	**0.017**	-0.548	-0.040
	s.intraneural	-0.452	0.171	0.089	-0.952	0.048
	s.perineural	-0.519(*)	0.106	**0.0001**	-0.819	-0.219
l.b.perineural	l.b.intraneural	0.294(*)	0.090	**0.017**	0.040	0.548
	fiz.intraneural	-0.158	0.170	0.920	-0.655	0.340
	fiz.perineural	-0.225	0.104	0.208	-0.520	0.070
s.intraneural	l.b.intraneural	0.452	0.171	0.089	-0.048	0.952
	l.b.perineural	0.158	0.170	0.920	-0.340	0.655
	fiz.perineural	-0.067	0.179	0.999	-0.583	0.448
s.perineural	l.b.intraneural	0.519(*)	0.106	**0.0001**	0.219	0.819
	l.b.perineural	0.225	0.104	0.208	-0.070	0.520
	fiz.intraneural	0.067	0.179	0.999	-0.448	0.583

l.-liposomal bupivacaine
s.-saline

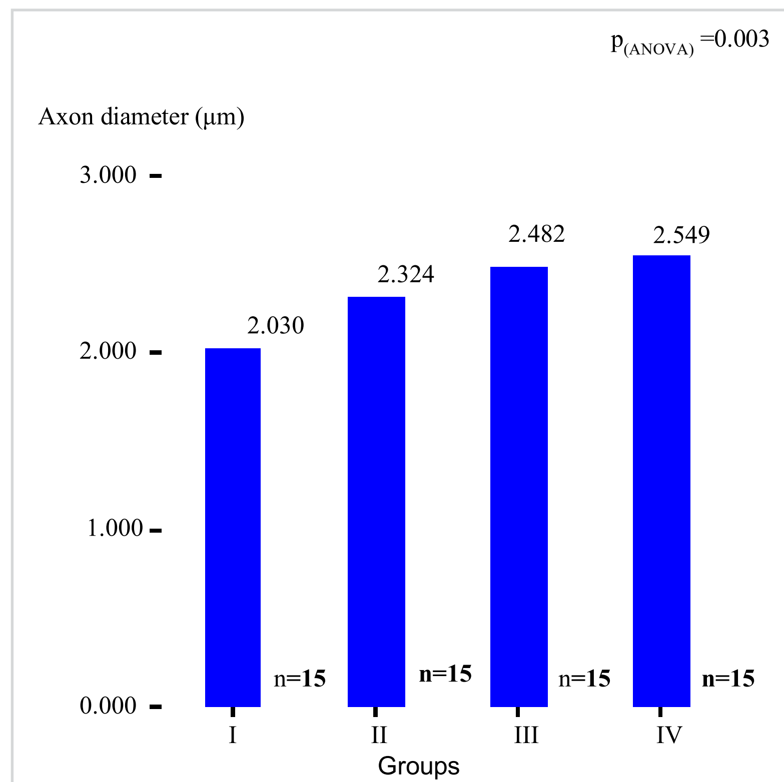

Figure 3 Axon diameter values and differences between individual groups

I-liposomal bupivacaine intraneural; II-liposomal bupivacaine perineural; III-saline intraneural; IV-saline perineural

Table 4 Myelin thickness

Group	N		SD	SEM	95% CI*		Min.	Max.
					Lower limit	Upper limit		
l.b.intraneural	15.000	2.039	0.212	0.055	1.921	2.156	1.675	2.306
l.b.perineural	15.000	2.025	0.183	0.047	1.924	2.126	1.830	2.373
s.intraneural	15.000	2.046	0.216	0.056	1.926	2.165	1.757	2.689
s.perineural	15.000	1.951	0.216	0.056	1.831	2.071	1.513	2.356

l.-liposomal bupivacaine
s.-saline

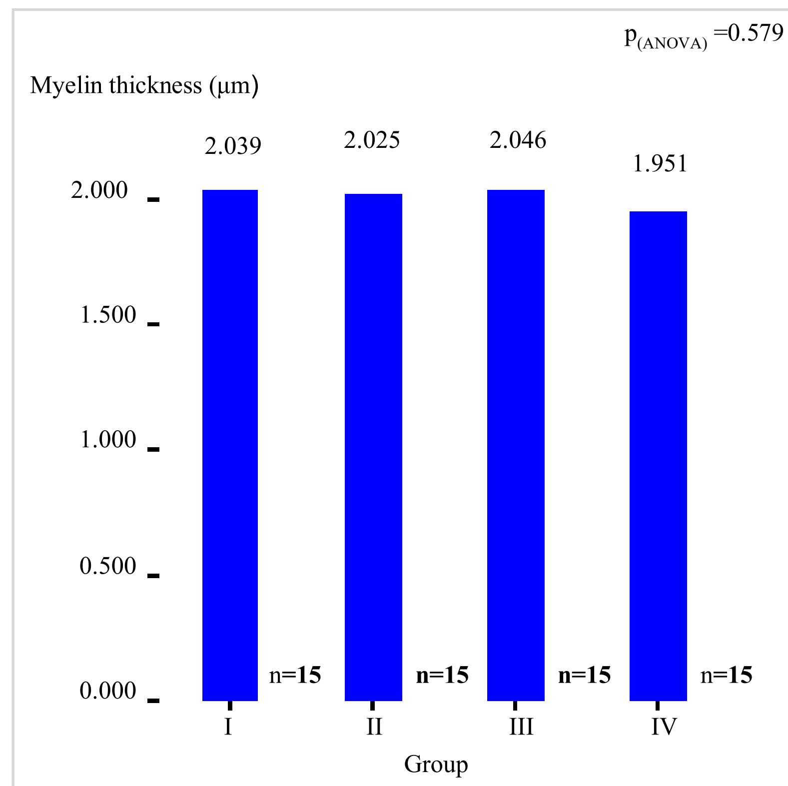

Figure 4 Myelin thickness values and differences between groups

I-liposomal bupivacaine intraneural; II-liposomal bupivacaine perineural; III-saline intraneural; IV-salineperineural

DISCUSSION AND CONCLUSION

Morphoquantitative stereological assessment of nerve fibres is a major research task in a number of biomedical disciplines, including neuroanatomy, neuropathology, neurosurgery, and reconstructive microsurgery. Nerve fibre histomorphometry has been shown to be particularly useful in studies of development, aging, regeneration, neurotoxicity, and various pathological conditions (Costa et al., 2013). Changes in the number of nerve fibres, especially the myelinated ones, are important for the assessment of various pathological nerve conditions, such as intoxication with some drugs. The use of histomorphometry in our study allowed us to objectively quantify the found structural changes in the nerves and identify minimal morphological differences in the examined groups.

In our study, we used an objective method of field selection on a nerve trunk, the Geuna method, which allowed us to have all fields within the nerves have equal chances of selection, that fibers had systemic distribution in different nerve areas, and ultimately had subjectivity in selection fields removed, so that we were given the most efficient approach to obtain relevant results of our study. The results of our study gave us a clear insight into the degree of nerve damage depending on the type of solution applied and the method of application.

The results of our study showed that there was a statistically significant difference in the number of fibres in the groups where liposomal bupivacaine was administered intraneurally and in the group where saline was applied perineurally. Decreased fibre number need not to be due solely to the neurotoxic effects of liposomal bupivacaine, as the same statistically significant difference in fibre number was found between the groups with intraneurally and perineurally administered saline. This finding may indicate that these lesions were due to mechanical trauma during intraneural application, because we found no difference in nerve fibre diameter and myelin thickness between the tested groups. The results of our study correlate well with the number of fibres after perineural and intraneural administration of traditional, already present formulations of local anaesthetics, which were found not to be neurotoxic after perineural administration in clinically permitted doses (Farber et al., 2013; Hasanbegović et al., 2013).

No statistically significant difference in nerve fibre and myelin thickness was found between the groups, which correlates with the results of Damjanovska et al. (2019), but also with the results of Zel et al. (2019), after subarachnoid application of liposomal bupivacaine and saline. However, the results of Damjanovska et al. show slightly higher absolute values of fibre thickness. We believe that this is due to the anatomical difference in fibre size between the nerve fibre of pigs and rats.

The statistically significant difference in axon diameter between intraneurally and perineurally applied liposomal bupivacaine may indicate a selective toxic effect of liposomal bupivacaine on nerve fibres. Perineurally administered liposomal bupivacaine has not shown significant differences comparing to saline in any of the histological parameters monitored so far, indicating its good safety profile.

Compared with the obtained histomorphometric results in our study, Damjanovska et al. found no differences in any parameters measured by histomorphometry after administration of liposomal bupivacaine intraneurally, ordinary bupivacaine HCl solution and saline (Damjanovska, 2015; Damjanovska, 2019). Fibre density and diameter and the ratio of axon diameter to myelin thickness were without significant differences between the examined groups.

Nevertheless, the Whitlock et al. showed similar changes and significantly reduced nerve fibre density after intraneural injection of ropivacaine in rat nerves, similar to our results. However, rats used in our and in the Whitlock study, because of their usually unifascicular structure, are more susceptible to injury than the polyfascicular nerve of pigs used in the Damjanovska study (Damjanovska, 2019; Whitlock et al., 2010).

In our study, the sciatic nerve was surgically exposed and isolated from the surrounding

connective tissue under direct visual monitoring. There is a possibility that this surgical isolation makes the nerve more susceptible to injury, because in everyday clinical practice the nerves are much more mobile in the surrounding tissue and there is less chance that the needle will actually penetrate the nerve during nerve blockade. The use of the results of our study in everyday clinical practice in performing peripheral nerve blockades would be important for patients and for the health system in general. Determining the number of myelinated nerve fibres and the thickness of individual nerve fibre ultrastructure provided us with a detailed and objective insight into the actual condition of the examined nerves after perineural and intraneural application of different test solutions. The size and number of nerve fibres provided a good basis for comparing fibres within and between experimental groups. Liposomal bupivacaine, compared to other short-acting anaesthetics, is a good choice for perioperative and intraoperative regional anaesthesia and analgesia. Providing a longer duration of anaesthesia shown in clinical studies in humans, liposomal bupivacaine is a superior, better and more comfortable choice for the patient, because it is adequately administered and devoid of neurotoxic effects.

In the course of numerous experimental sciatic nerve injuries, it has been shown that there are numerous deficiencies in the assessment of the neurological functions of laboratory animals. All this led to a greater interest in quantitative histological analysis, which proved to be an objective, reliable method to assess the venous toxic effect of local anesthetics on nerves. After the injury, the animal had its back extremity paralyzed and often bit its own limb, which could have led to the amputation of the toes on the injured extremity. This behavior and joint stiffness reduce the reliability of functional tests, such as assessment of sciatic nerve function. In some cases, the researcher may be willing to exclude the animal from the research, due to ethical reasons and the welfare of the animal itself. Henceforth, in awake animals it is very difficult to assess the function of the sciatic nerve after injury or it is very limited (Navarro, 2016). In our study, quantitative histological analysis enabled us to objectively assess and quantitatively describe the microstructures of nerve fibers, their size, and the parts of the endoneural space that occurred during the research.

Rats and mice are the most frequently used laboratory animals for evaluating the toxic effects of local anesthetics. However, when rats or mice are used in such research, we must be aware of the distinct differences that exist in the structure of the sciatic nerve of laboratory animals and the human sciatic nerve: (1) the voids that occur during the application of anesthetics are smaller than those that occur during sciatic nerve injuries in humans; (2) axonal regeneration occurs more rapidly in rats than in humans; (3) after the occurrence of an injury in rats, recovery is usually complete after some time, which is not the case in humans (Kaplan et al., 2015).

On the basis of these preclinical data, we conclude that liposomal bupivacaine poses no risk beyond that of classical local anaesthetics that are commonly used in everyday clinical practice. Morphometry enabled us to describe structural changes after intraneural and perineural application of 1.33% liposomal bupivacaine in quantitative terms and in particular revealed to us minimal morphological differences between the states of function.

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HISTOMORFOMETRIJSKI PARAMETRI KAO INDIKATORI OŠTEĆENJA NERVNOG TKIVA NAKON PERINEURALNE I INTRANEURALNE APLIKACIJE LIPOZOMALNOG BUPIVAKAINA NA MODELU ŠTAKORA

SAŽETAK

Lipozomalni bupivakain je dugodjelujući lokalni anestetik koji se smatra jednim od najsigurnijih lokalnih anestetika za koji su, međutim, opisani i mogući neurotoksični efekti. Trenutno nije poznat tačan molekularni mehanizam neurotoksičnosti posredovane lipozomalnim bupivakainom. Pretpostavili smo da intraneuralna injekcija 1.33% lipozomalnog bupivakaina rezultira većim oštećenjem nerva u odnosu na perineuralnu injekciju, što smo i dokazali objektivnom kvantitativnom histološkom analizom. Kao model je korištena blokada ishijadikusa kod štakora. Istraživanje je izvedeno u skladu sa principima zaštite laboratorijskih životinja i odobreno je od Odbora za zaštitu i korištenje laboratorijskih životinja. Istraživanje je obuhvatilo trideset odraslih Wistar štakora obaju spolova. Nakon uvođenja u opću anesteziju, unilateralno je isprepariran ishijadični nerv kojemu je randomizacijom metodom zapečaćenih koverti za aplikaciju dodijeljeno: 2 ml 1.33% lipozomalnog bupivakaina perineuralno, 2 ml 1.33% lipozomalnog bupivakaina intraneuralno, 2 ml fiziološke otopine perineuralno ili 2 ml fiziološke otopine intraneuralno. Nakon toga je izvršen kvantitativni histološki pregled kako bi se odredilo moguće oštećenje nervnog tkiva. Kod svih intraneuralnih injekcija je postojao signifikantno manji broj nervnih vlakana ($p<0.001$). Nije postojala statistički signifikantna razlika među grupama u debljini mijelina ($p>0.005$) i prečniku nervnih vlakana ($p>0.005$). Intraneuralno apliciran lipozomalni bupivakain je pokazao smanjenje prečnika aksona ($p<0.005$) u odnosu na perineuralno apliciran lipozomalni bupivakain i fiziološku otopinu.

Ključne riječi: Blokada perifernog nerva, liposomalni bupivakain, neurotoksičnost