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HISTOMORPHOMETRIC DIFFERENCES BETWEEN PERINEURAL AND INTRANEURAL APPLICATION OF 0.75% ROPIVACAINE IN WISTAR RATS

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Background: Ropivacaine is a long-acting amide local anaesthetic agent and first produced as a pure enantiomer. Ropivacaine is thought to be one of the safest local anesthetics however, there have been several studies detailing possible neurotoxic effects. At present, the exact molecular mechanism of ropivacaine-mediated neurotoxicity is unknown. We postulated that intraneural injection of 0,5% ropivacaine results in greater nerve injury then perineural injection, and this would be proved by objective quantitative histological analysis

Methods: We used quantitative histomorphometric analyses to evaluate the neurotoxic effect of 0.5% ropivacaine after intraneural and perineural injection of sciatic nerve in Wistar rats. 10 Wistar rats (20 nerves) were studied. After general anesthesia, the sciatic nerves (n=20) were exposed bilaterally. Under direct vision, a 27-gauge needle was placed perineurally (n=10) or intraneurally (n=10) and 4 ml of 0.5% ropivacaine was injected using automated pump at speed 3 ml/min. Manometer wes used to measure injection pressure. Three days after application, animals were euthanized and nerves samples were taken for histomorphometric analysis.

Results: All intraneural injections showed significantly smaller number of nerve fibers (p<0,001), smaller nerve fiber (p<0,002) and axon diameter (p<0,001) and myelin thickness (p<0,002) compared with perineurally administarated 0.5% ropivacaine. Maximum achieved injection pressures were 20,3 psi (intraneural) vs 6,7 psi (perineural) (p=0.02).

Conclusion: Intraneural injection of 0,5% ropivacaine resulted in an indistiguishable quantitative histologic changes as compared to the perineural injection. Ropivacaine administered intraneuraly is neurotoxic, similar to other local anesthetics.

Keywords: ropivacaine, peripheral nerve blocks, neurotoxicity.

INTRODUCTION

Ropivacaine is a long-acting aminoamide local anesthetic structurally related to bupivacaine and mepivacaine [1]. One of the most important properties of a long-acting local anaesthetic is to reversibly inhibit the nerve impulses, thus causing a prolonged sensory or motor blockade appropriate for anaesthesia in different types of surgeries [2]. Various controlled clinical studies have demonstrated that ropivacaine is a suitable choice for peripheral nerve block [3, 4]. Animal studies have shown that 0.5–1 % ropivacaine consistently produces effective sensory and motor anaesthesia in sciatic nerve and brachial plexus block [5]. Peripheral nerve block is employed for anaesthesia for different kind of surgery, and the onset and spread of local anaesthetic is influenced by the site of injection. [6] In lower limb surgeries where sciatic or combined femoral and sciatic block was given for knee, ankle, or foot procedures, ropivacaine 0.75% (25 ml) had a significantly faster onset of sensory and motor block than 25 ml bupivacaine 0.5% [7].

Nerve damage after regional anesthesia is appropriately regarded as major complication. One causative factor that has been the subject of intense discussion involves the direct intraneural injection of local anesthetics [8]. Ropivacaine has been documented to be less cardiotoxic and neurotoxic when compared to other local anesthetics (LA) [9]. Ropivacaine is thought to be one of the safest LAs for pain relief, however, its efficiency is low, resulting in the utilization of high concentrations of LAs to relieve pain completely [10,11]. Previous studies have reported considerable neurotoxicities associated with ropivacaine, particularly at high concentrations [12, 13]. However, the molecular mechanisms through which LAs induce neurotoxicity remain poorly understood. Apoptosis, necrotic cell death and protein kinase B (Akt) signaling pathways may be involved [14]. Injury during peripheral nerve blocks is relatively

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uncommon, but potentially devastating complication. Recent studies emphasized that location of needle insertion in relationship to the fascicles may be the predominant factor that determines the risk for nerve injury [15, 16, 17].

The current study was designed to investigate potentional neurotoxic effect of 0.5% ropivacaine on the sciatic nerve after perineural and intraneural injection, using quantitative histomorphometric analysis.

MATERIAL AND METHODS

After animal care Ethics committee approval of the Medical faculty University of Sarajevo, 10 adult Wistar rats both sexes (300-350 g, 3 months old) were used in experimental designed type of the study. Animals were housed in central animal care facility. Food and water were provided ad libitum. Animals were monitored for appropriate post-surgical recovery. The study was conducted in accordance with the Principles of Laboratory Animal Care [18]. The animals were anesthetized with an intraperitoneal injection of pentobarbital 50 mg/kg. The sciatic nerve was surgically exposed bilaterally to insert a 27 G needle (Terumo Europe NV, Leuven, Belgium) intraneurally on one side and perineurally on the contralateral side. For perineural injections needle was placed within the epineural tissue but outside the perineurium, while for intraneural injections the needle was placed intraneurally inside the perineurium. An automated injection pump (PHD 2000 Harvard Apparatus, Holliston, MA) administered 4 ml of 0,5% ropivacaine at a speed of 3 ml/min. The achieved pressure during the application was registered by manometer (PG 5000: PSI – Tronics Technologies Inc, Tulare, CA). The manometer was used to distinguish intraneural intrafascicular injection and intraneural extrafascicular injection. If the pressure achieved was above 10 psi, injection was considered intraneural intrafascicular. The pressure data were analysed by software package (BioBench version 1.2, National Institutes, Austin, TX). After application the wounds were closed with a stitch.

On the third day after ropivacaine administration, rats were euthanized and nerve samples with surrounding tissue were sampled. Tissue samples were fixed in 4% formalin, then processed according to standard protocol, embedded in paraffin, and cut into 3-micrometer-thick slices. After staining according to standard HE method, 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.

Quantitative histological analysis was performed through histomorphometry of myelinated nerve fibers. On all nerve samples, histomorphometry was performed using a Videoplan Image Processing System (Carl Zeiss, Jena Germany), consisting of a camera and a digital graphic board coupled to a Laborlux-S light microscope (Leitz, Wetzlar, Germany). Graphic board was coupled to IBM 286 PC computer. From each group, half of the preparations were randomly selected. We first divide the whole nerve into 12 large fields, and then each large field into 9 smaller fields. Only 1 of the 9 smaller fields was randomly selected (middle in our study). To overcome the „marginal effect", a procedure based on counting the nerve fiber tips was used. Thus, each nerve peak can appear in only one histologic field and each fiber has the same possibility of being included in the selection. The parameters we determined in each selected field were: total number of nerve fibers (N), diameter of nerve fibers (D), axon diameter and diameter of myelin. Myelin thickness was calculated by subtracting axonal thickness from the total nerve fiber diameter.

Total of 60 nerves were required to obtain relevant results to detect a significant difference in the proportion of nerve injury between intraneural and perineual injections $\alpha=0.05$. Statistical analysis was performed using SPSS program, version 11.5. 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.

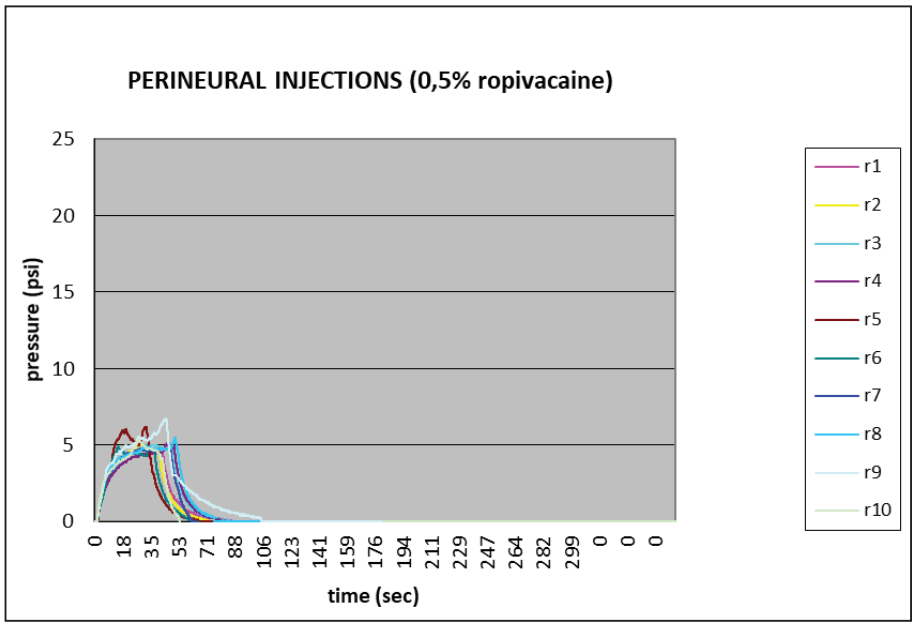
RESULTS

The maximum pressure achieved in rats during intraneural applications of 0.5% ropivacaine was 20,3 psi and the minimum pressure achieved in intraneural injections was 9.4 psi. The maximum pressure in all perineural applications of 0.5% ropivacaine was 6,7 psi, and minimum was 4,5 psi.

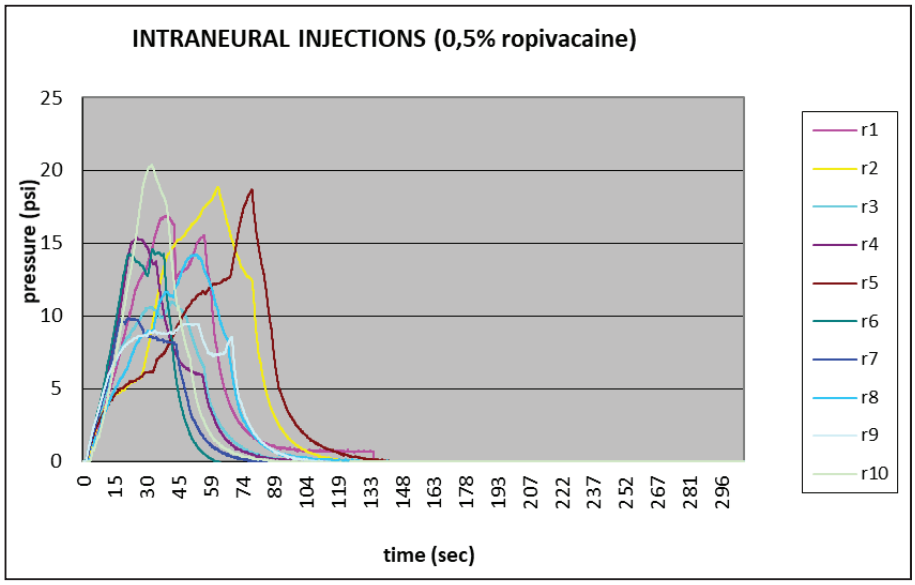
The difference between mean value between intra and perineural injections with 0.5% ropivacaine (with 95% safety interval) was statistically significant ($t=3,14$; $df=6$; $p=0,02$) (Table I, Graphic I, Graphic II).

Table I. Mean injection pressure for perineural and intraneural injections

Injection pressure (psi)	Intraneural 0.5% ropivacaine	Perineural 0.5% ropivacaine
Rat 1	16.866	4.554
Rat 2	18.868	5.305
Rat 3	11.010	5.004
Rat 4	15.364	5.155
Rat 5	18.668	6.206
Rat 6	14.564	4.904
Rat 7	9.859	4.804
Rat 8	14.213	5.455
Rat 9	9.459	6.706
Rat 10	20.369	5.305
± SD	15,1±3,8	5,3±0,6



Graphic I. Perineural application of 0.5% ropivacaine in rats



Graphic II. Intraneural application of 0.5% ropivacaine in rats

The morphometric evaluation of the total number of nerve fibers (N) at intraneural injections was significantly lower than that in the perineural group, (p<0,001 for all comparisons). (Table II, Figure I).

Table II. Histomorphometric analysis of total number of nerve fiber

Total number of nerve fiber (N)	Intraneural 0.5% ropivacaine	Perineural 0.5% ropivacaine
Rat 1	5.768	7.768
Rat 2	5.777	7.777
Rat 3	5.675	8.675
Rat 4	5.442	8.442
Rat 5	6.221	8.991
Rat 6	5.267	8.267
Rat 7	8.956	8.956
Rat 8	4.867	9.867
Rat 9	5.065	8.065
Rat 10	5.124	7.124
± SD	5.916 ±1.16	8.393± 0.77

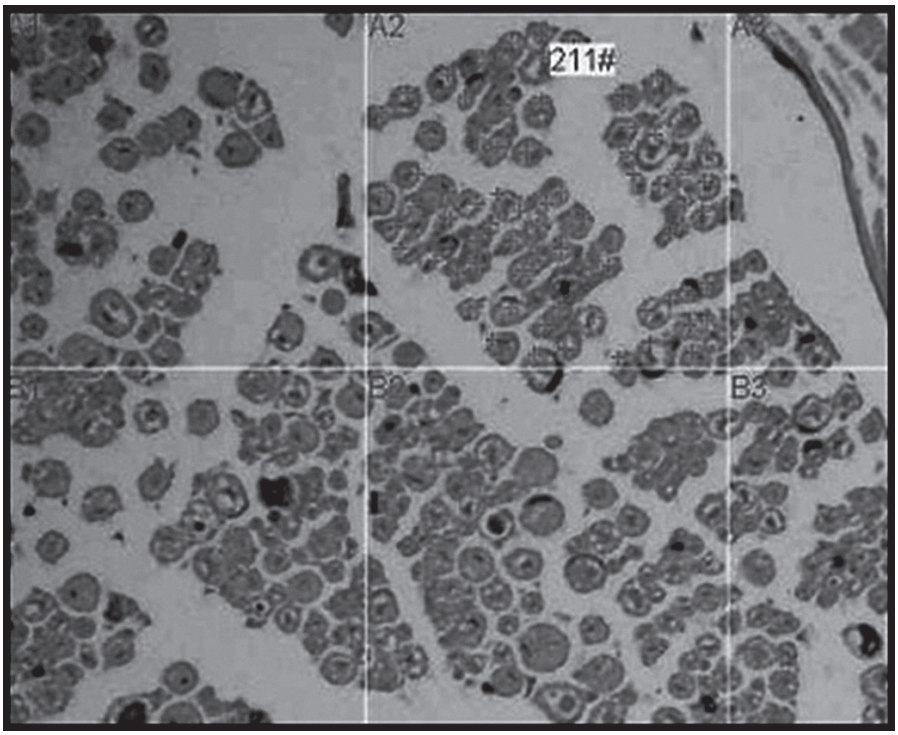


Figure I. An example of histomorphometric evaluation of the number of nerve fibers in a selected field sample

The morphometric analysis of nerve fiber circular diameter in intraneural injections was significantly higher than in the perineural group, (p<0,002 for all comparisons) (Tabel III, Figure II).

Table III. Histomorphometric analysis of circular diameter of nerve fibers

Circular diameter of nerve fibers (μm)	Intraneural 0.5% ropivacaine	Perineural 0.5% ropivacaine
Rat 1	8.96	6.76
Rat 2	8.75	6.77
Rat 3	8.65	7.65
Rat 4	8.45	7.42
Rat 5	9.75	7.71
Rat 6	8.25	7.25
Rat 7	7.46	7.86
Rat 8	8.42	7.82
Rat 9	8.54	7.55
Rat 10	9.14	7.12
± SD	8,63 ±0,59	7.39± 0.40

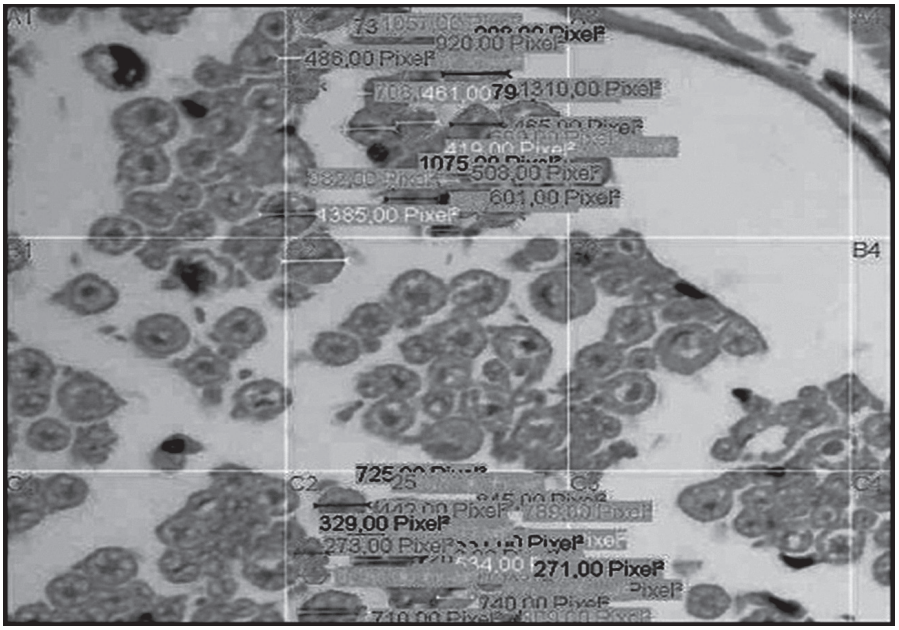


Figure II. An example of histomorphometric evaluation of the circular diameter of nerve fibers in a selected field sample

The histomorphometric analysis of the circular diameter of the axon was similar to the histomorphometric evaluation of the circular diameter of the nerve fibers. The circular axon diameters in the intraneural group were significantly higher than in the perineural group, ($p<0,001$ for all comparisons) (Table IV, Figure III).

Table IV. Histomorphometric analysis of circular diameter of axons

Circular diameter of axons (μm)	Intraneural 0.5% ropivacaine	Perineural 0.5% ropivacaine
Rat 1	7.16	4.16
Rat 2	7.37	4.37
Rat 3	6.67	4.65
Rat 4	6.47	5.45
Rat 5	6.51	4.71
Rat 6	7.25	5.25
Rat 7	4.85	4.85
Rat 8	6.82	4.82
Rat 9	6.75	5.75
Rat 10	6.45	5.15
± SD	6.63± 0.70	4.91± 0.48

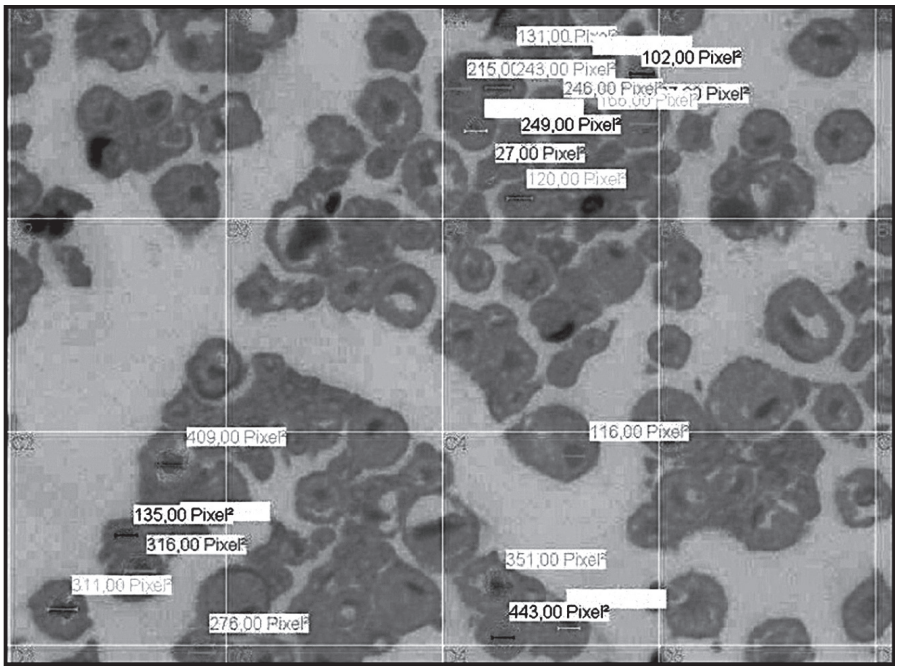


Figure III. An example of histomorphometric evaluation of the circular diameter of axons in a selected field sample

Myelin thickness was significantly reduced in the intraneural group compared with the perineural group, ($p<0,002$ for all comparisons) (Table V).

Table V. Histomorphometric analysis of myelin thickness

Myelin thickness (μm)	Intraneural 0.5% ropivacaine	Perineural 0.5% ropivacaine
Rat 1	0.9	1.3
Rat 2	0.69	1.2
Rat 3	0.99	1.5
Rat 4	0.99	0.98
Rat 5	1.62	1.5
Rat 6	0.5	1
Rat 7	1.3	1.5
Rat 8	0.8	0.5
Rat 9	0.89	0.9
Rat 10	1.34	0.98
± SD	1.00 ±0.33	1.13±0.32

DISSCUSION

Local anesthetic neurotoxicity is a rare but catastrophic occurance during peripheral nerve blocks [19]. The mechanism of the occurance of neurological sequelae after intraneural injection of local anesthetic is not completely understood. Because neurological injuries after peripheral nerve blocks are so rare, it is extremely difficult to obtain reliable and consistent data about their incidence. Some retrospective studies estimate an incidence of 0.5-1.0% [20].

0.5% ropivacaine is the most suitable choice of local anesthetic for combined peripheral nerve block, providing an onset similar to mepivacaine and prolonged postoperative analgesia, with a more adequate volume of local anesthetic solution available to the anesthesiologist for block placement. Recently, the use of highly concentrated solutions of local anesthetic for regional anesthesia has prompted some concerns because of the theoretical risk for direct local neurotoxicity [21]. In vitro and in vivo studies on ropivacaine failed to produce evidence of direct neurotoxicity, [22, 23, 24] but results of Casati et al., showed that the use of 1% ropivacaine does not affect the complete recovery of neurologic function after combined sciatic-femoral nerve block [25]. Werdehausen reported that high concentrations of ropivacaine result in neurotoxicity in specific cell lines [26].

Most of the studies performed to date have been based on the examination of the potential neurotoxicity of ropivacaine through the examination of neurological functions and the assessment of recovery of neurological functions after application or qualitative histological analysis [27]. This type of examination may be subject to the subjectivity of the examiners when assessing a neurological deficit or qualitative description of the histological changes found. Therefore, in our study, we used quantitative morphometry, which provided us with more information about individual structural parts of nerves. The obtained quantitative parameters provide important information on the consequences of various physiological, pathological and experimental states of the nervous structures and is one of the main indicators of acute or chronic changes in the nerves or successful repair of peripheral nerves.

In our study all intraneural injections combined with high injection pressure resulted in a reduction in total fiber count, nerve fiber and axon swelling, and reduction of the myelin sheath. All this confirms the occurrence of acute neurotoxic effect of the examined nerves and nerve fibers. As expected, in the demyelination process, the thickness of the myelin was significantly reduced by intraneural administration of 0.5% ropivacain followed by high injection pressure. Generally speaking, the morphometric changes affecting myelin are of a greater degree than those affecting the axon. Histomorphometric analysis of the nerves after perineural administration of 0.5% ropivacaine, which was not followed by an increase in injection pressure, showed that there were no morphological abnormalities, confirming the visual impression obtained by qualitative analysis. The number and density of nerve fibers is the main parameter that is evaluated in nerve research. Changes in the number of nerve fibers (especially myelinated ones) are important for the investigation of the effects of various pathological conditions on the nerve sturcture, such as mercury intoxication or zinc deficiency [28, 29]. In our study, an intraneural injection that was associated with low injection pressure did not result in histologic evidence of nerve damage. Histomorphometric analysis showed that there were no morphological abnormalities, nor a significant difference between intraneural injections followed by lower injection pressure and perineural injections followed by low injection pressure. The possible reason is that the needle was inserted intraneurally but between the fascicles. The intrafascular needle placement is very difficult to secure even under direct visual guidance. Peripheral nerves possess natural

defense mechanisms. This refers first to the relatively resistant membrane of the perineurium, which is difficult to penetrate due to its elasticity, mobility and adaptation to external force. This model of intraneural extrafascicular injection offers an explanation for the relatively common clinical scenario when, after some nerve blocks, the unusual rapid onset of deep blocks lasts longer than expected. This is because intaneural extrafascicular injection leads to close exposure of the fascicles to high concentrations and doses of local anesthetic. However, permanent histologic lesions do not develop because the local anesthetic is deposited outside the fascicles and such blockade slowly ceases after injection without evidence of histologic disorder.

The study of the morphology of nerve fibers is one of the pillars for the investigation of peripheral nerves in various experimental conditions, such as examining neurotoxic effect of local anesthetic as in our study. Mornjakovic et al. used monitoring injection pressure to distingues intrafascicular from extrafacicular injection as in our study. But in their study nerve injury was assesed by qualitative histological analysis [17]. Quantitative histomorphometry is more objective indicator of nerve injury in comparing to qualitative histologic analysis. Because achieved data are quantified, numerical and objective informations on the injured nerve structures. To get as much as possible objective data in our study we used systemic random sampling for nerve fiber stereology. The units were the single sampling boxes on a given nerve cross-section and after the starting box was selected by chance, the following boxes were selected by systematically jumping at a given distance from the former box.

The computer image analysis used in our study (made up of microscopes, digital cameras, and computers with appropriate image analysis software) enabled the numerical objectification of the most subtle changes unavailable to visual inspection, and provided objective quantification of the required parameters instead of subjective assessment. And manometer helped us to make difference between intraneural intrafascicular and intraneural extrafascicular injection.

Different downsides resulting from experimental injury to the sciatic nerve and assessment of neurologic functions by specially designed tests for laboratory animals, led to increasing interest in the quantitative histomorphometry as more objective and reliable alternative of assessment of neurotoxicity of local anesthetics. At first, injury to the sciatic nerve results in a paralysis of the hind limb and, often, in automutilation behavior, such as biting and self-amputation of denervated toes and paw areas by the subjected animal. Longer lasting paralysis (>4 weeks in the rat) often leads to joint contractures and stiffness. Automutilation behavior and joint contractures reduce the reliability of functional tests, such as estimation of the sciatic function index or, in severe cases, will lead to exclusion of the respective animal from a study for ethical and animal welfare reasons. Furthermore, possibilities for evaluation functional recovery of motor skills after sciatic nerve lesion in the awaken animal are rather limited or need considerable efforts to be realized [30].

Rat model, as in our study, is the most commonly used in vivo model for research of neurotoxic effects of local aneasthetic. But when using rodents (mice and rats) as animal models for assesing neurotoxic effect of local anesthetic, we must be aware of the immanent differences to mankind: (1) gaps that can be produced during injectons are shorter than those commonly found in human nerve lesions; (2) axonal regeneration rate is faster than in humans; (3) recovery is often complete, while in humans it is often incomplete [31].

CONCLUSION

Morphometry enabled us to describe structural changes after intarneural and perineural application of 0,5% ropivacaine in quantitative terms and in particular revealed us minimal morphological differences between states of function. Ropivacaine administered perineurally is suitable for intraoperative and for postoperative regional anesthesia and analgesia.

Conflict of interest
None declared.

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