ORIGINAL ARTICLE

Effect of ropivacaine infiltration on muscle regeneration: a morphometric analysis

Kapoukranidou D[1], Amaniti E[2], Kalpidis J[1], Karakoulas K[2], Papazisis G[3], Albani M[1], Kouvelas D[3]

[1]Department of Physiology
[2]Department of Anaesthesia and Critical Care
[3]Department of Pharmacology
School of Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece

Abstract

Background/aim: Local anaesthetic myotoxicity is a well described phenomenon resulting in reversible muscle damage. Considering that in previous studies microscopic images were evaluated without quantification of morphologic characteristics, the aim of the present study was evaluate muscle regeneration after local anaesthetic infiltration.

Materials and Methods: Wistar rats underwent injection of the left tibialis anterior muscle with ropivacaine (0.75%, group HC or 0.375%, group LC), while the contralateral muscle was injected with saline (group SL). Six weeks later, the muscles were dissected, stained using acid ATPase and examined under light microscope coupled with a computer imaging system for morphometric analysis. Sections were evaluated regarding the content of different muscle fibre types (type I, IIa and IIb), fibre cross-section area and perimeter.

Results: Groups were comparable regarding the ratio of different muscle fibre types. Regenerated type I fibres of both HC and LC groups had significant greater mean cross-sectional area and perimeter, compared to SL fibres. No signs of necrosis or inflammation were observed. Type IIa and IIb fibres didn't show significant differences.

Conclusions: Regenerated muscles, following local anaesthetic application, showed long-term morphological differences, which could lead to impaired function. Further studies are needed, in order to clarify the underlying cellular mechanisms and the subsequent possible functional impairment. Hippokratia 2013; 17 (4): 351-354.

Keywords: Myotoxicity, ropivacaine, regeneration, morphometry, rat

Corresponding Author: Ekaterini Amaniti MD, PhD, Lecturer of Anaesthesia, Department of Anaesthesia and Critical Care, School of Medicine, Aristotle University of Thessaloniki, tel +302310994862, fax +302310994860, e-mail: amanitik@gmail.com

Introduction

Myotoxicity of local anaesthetics is a well-described phenomenon since 1959[1]. Local anaesthetics have been proven to cause reversible myonecrosis in clinical concentrations[2-6]. Muscle damage has been observed few hours after the injection and was characterized by disruption and condensation of myofilaments, lytic degeneration of the sarcoplasmic reticulum and mitochondria and pycnotic changes of the myonuclei, with pathologically condensed chromatin[7]. However, sarcolemma remains undamaged, indicating an intracellular pathologic process. Alterations of Ca^{2+} homeostasis, induced by local anaesthetics, in combination with enhanced oxidative stress, have both been found to be major causes of myonecrosis. Interestingly, the damage is limited to muscle fibres and has a sparing effect to the precursor "satellite cells". The latter permits the full recovery of the muscle[6-8].

Previous studies, examining histopathologic changes, using both light and electron microscopy noticed that muscle damage had a reversible course and muscles had fully recovered after a period of 4-6 weeks[9,10]. Similar results of muscle regeneration appeared after muscle infiltration with clinical doses of ropivacaine[5]. However, these conclusions rose from studies evaluating microscopic images without simultaneous quantification of morphologic characteristics.

The aim of the present study wasto evaluate muscle regeneration after ropivacaine injection, based on morphometric criteria, compared to muscles injected with normal saline. Regenerated muscles were studied regarding various types of muscle fibres and their morphologic characteristics, as cross-sectional area and perimeter.

Material and methods

All experiments were conducted in accordance with the European Communities Council Directive of November 24 1986 (86/609/EEC) and the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) and were approved by the Animal Care and Use Committee of the National Veterinary Institute and the Ethical Committee of the School of Medicine, Aristotle University of Thessaloniki.

Sixteen male Wistar Rats, weighting 160-190g, were used in the study. All experimental animals housed two individuals per cage, on a 12/12 h light/dark cycle (lights on, at 7 am) at a controlled temperature of 21±1 °C with low noise and humidity, throughout the whole study. Animals had ad libitum access to food and water.

Animals were anaesthetized with chloral hydrate 4.5% w/v (1 ml/kg) and were assigned into two groups. Group HC (High Concentration of ropivacaine) animals (n=8) underwent injections of left tibialis anterior muscle with 100 μl of ropivacaine 0.75% w/v (Astra Zeneca®, Sweden), while group LC (Low Concentration of ropivacaine) animals (n=8) underwent injections of the left tibialis anterior muscles with 100μl of ropivacaine 0.375% w/v. Injections in HC group were made using the commercial preparation of ropivacaine 0.75% (Astra Zeneca®, Sweden). The solution of ropivacaine 0.375% w/v was prepared by mixing equal volumes of the commercially available ropivacaine 0.75% with saline (Braun Melsungen AC®, Germany). The right tibialis anterior muscles of both HC and LC animals were injected with 100 μl of saline (Braun Melsungen AC®, Germany), consisting group SL (Saline) and used as control (n=16).

Intramuscular punctures were performed with 7G needle in all groups. The needle penetrated the distal part of the muscle, reached the tibia perpendicularly and then it was advanced longitudinally for 3 mm, across the long axis of the muscle, pointing to its median part. After aspiration, the solution was slowly injected, distributing the local anaesthetic along the belly of the muscle. After completion of the injections, rats were left to recover and then they were put back in their home cages.

Six weeks later, the animals were sacrificed with a lethal dose of chloral hydrate 4.5% w/v. Next, the right and left tibialis anterior muscles were carefully dissected, immersed in melting isopentane, frozen in liquid nitrogen and stored in -80°C. Muscles were then cut in a cryomicrotome (Microm 505 E) in transverse sections of 10 μm thick. Two transverse sections were obtained from the belly of every dissected muscle. The sections were stained with acid myosin-ATPase (pH = 4.6) and examined under light microscope (Zeiss Axioscope).

Then the sections were subjected to morphometric analysis. For this purpose, the image obtained from the light microscope was transferred to a PC, equipped with special image analysis software (KS 300 Imaging System Release 3.0). The image field transferred to the PC screen (optical field) composed of 768 x 576 pixels. At a magnification of 100x (using eyepiece 10x and objective lens 10x), each optical field corresponded to an area of 837 x 628 μm.

Eight optical fields, which followed a meander configuration in the centre of the belly, were examined in every transverse section. Provided that two transverse sections were cut from the belly of each muscle examined, a total of sixteen optical fields were evaluated in each muscle. All muscle fibres, appearing in cross-section in these sixteen optical fields, were examined, regarding the type of muscle fibres, the fibre cross-sectional area and its perimeter. For the estimation of fibre type composition, fibre types were distinguished by the differential staining resulting from pre-incubation at pH 4.6. The method distinguishes type IIa (light staining) fibres from type I (dark staining) and type IIb (medium staining) fibres[11]. Moreover, in order to avoid false results in qualification and quantification of each experimental group muscle fibre types, all observations were made only in the central area of the muscle belly, avoiding the poles and the superficial areas, thus minimizing conflicting results due to regional differences in muscle fibre type composition.

Data were analyzed using SPSS statistical package 13.0 (SPSS Inc., Chicago, IL, USA). Differences among groups regarding muscle fibre types, cross-sectional area and cross-sectional perimeter were analyzed using ANOVA, while pairwise comparisons were made using Student's t-test for independent variables. Differences between groups, regarding animals' weight, were evaluated using Student's t-test for independent variables. The level of statistical significance was set at $p<0.05$.

Results

All experimental animal groups were comparable, regarding animals' weight ($p>0.05$). Under light microscope, type I muscle fibres of groups HC and LC had hypertrophic appearance compared to type I SL group fibres. However none of the sections examined presented

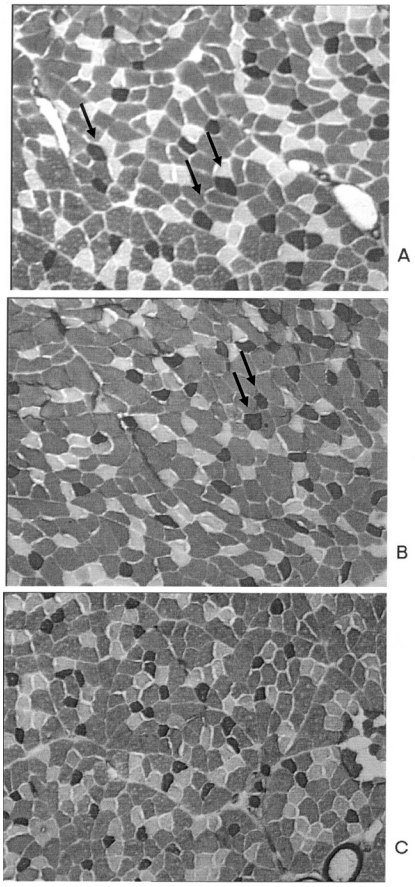

Figure 1: Muscles stained with myosin-ATPase (after preincubation at pH=4.6). Type I fibres are stained dark, type IIb are the most lightly stained, while type IIa fibres are characterized by an intermediate appearance. Type I fibres of groups HC and LC have a hypertrophied appearance, compared to SL group (arrows). **A:** HC fibre, **B:** LC fibre, **C:** SL fibre. Acid ATPase, 100x.
HC: High Concentration of ropivacaine group, LC: Low Concentration of ropivacaine group, SL: Saline group.

any signs of necrosis or inflammation (Figure 1). The percentages of various types of muscle fibres among groups showed no significant differences (Figure 2).

Mean cross-sectional areas and standard deviations of all fibre types among groups are shown in Figure 3. Statistical analysis revealed greater cross-sectional area for type I fibres in both HC and LC groups, compared to SL group, while type IIa and IIb fibre comparisons among groups did not reveal any differences. Regarding HC vs. LC comparisons for type I fibres, no differences

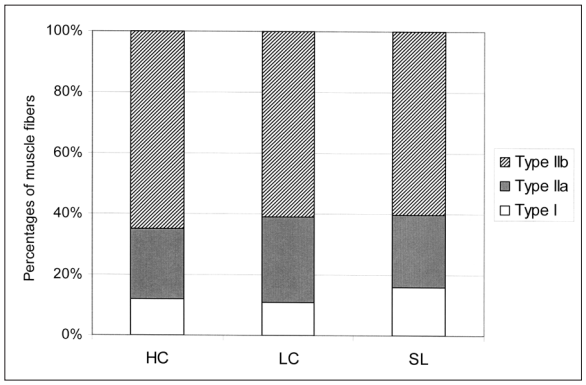

Figure 2: Percentages of various muscle fibre types (type I-IIa-IIb) among groups.
High Concentration of ropivacaine group, LC: Low Concentration of ropivacaine group, SL: Saline group.

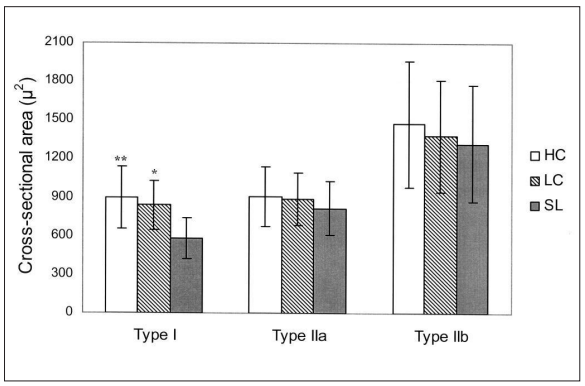

Figure 3: Mean cross sectional areas and standard deviations of different muscle fibre types among groups.
High Concentration of ropivacaine group, LC: Low Concentration of ropivacaine group, SL: Saline group, **: p=0.000 for HC vs. SL group, p=0.015 for LC vs. LS

were observed.

Pairwise comparisons of mean cross-sectional perimeters and standard deviations of all fibre types revealed significant greater perimeter of type I fibre in group HC and LC muscles, compared to SL group. Differences between type IIa and IIb fibre perimeters were not observed (Figure 4).

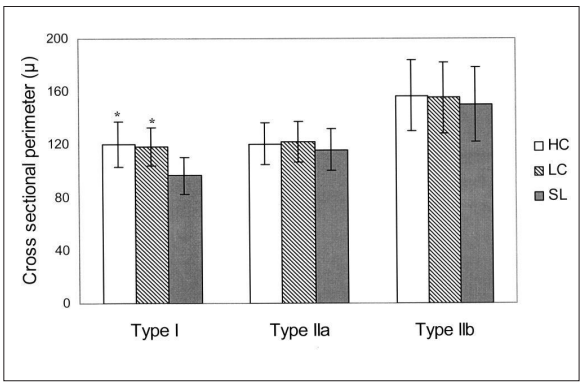

Figure 4: Mean cross sectional perimeters and standard deviations of different muscle fibre types among groups.
High Concentration of ropivacaine group, LC: Low Concentration of ropivacaine group, SL: Saline group, *: p=0.014 for HC vs. SL group, p=0.039 for LC vs. SL

Discussion

Skeletal muscles are composed of different muscle fibres, showing specific physiological and biochemical properties. Type I fibres are characterized by slow twitch and resistance to fatigue. Type IIb fibres behave with fast twitch and easy fatigability and type IIa fibres show an intermediate profile. Depending on their function, innervation, humoral factors and exercise, different muscles exhibit diverse percentages of various muscle fibres. Furthermore, regional differences in various fibre types have been observed[11].

After a period of six weeks following local anaesthetic infiltration, ropivacaine injected muscles demonstrated ratios of various muscle fibre types comparable to control muscles. Therefore, myotoxic insult of ropivacaine and subsequent regeneration process didn't seem to induce changes in fibre type composition. However, evaluation of the mechanisms of muscle damage has showed intracellular Ca^{2+} homeostasis derangement and previous histopathologic studies have confirmed isolated damage in muscle fibres with preservation of neural structures. Hence, absence of alterations of regenerated muscle, expressed as muscle type fibre composition, should be anticipated[7,9]. Analysis of muscle fibre composition of extensor digitorum longus, after bupivacaine injection, has revealed an increase of type I fibres compared to control muscles, observed until 180 days after injection[12]. Based on the above data, different mechanisms must be considered, in order to explain discrepancy in the results between Rosenblatt and Woods work[12] and the present study.

Despite rather uniform muscle damage, previous studies have demonstrated differences among various local anaesthetics, regarding the magnitude of necrosis[6,13]. Hence, bupivacaine tended to evoke the most serious muscle injury, while ropivacaine has been shown to cause slightly less severe damage. Authors concluded that differences in lipophilicity between the two local anaesthetics are mainly responsible for the different extent of muscle lesions while within a time period of 28 days, muscle regeneration had been completed[13]. However, the present study revealed that ropivacaine, in the same doses used by Zink and colleagues[13] didn't lead to alteration in the ratio of different muscle fibre types. In another study however, bupivacaine actually did alter this ratio[12], suggesting differences in the mechanism of toxicity between the two local anaesthetics and its long-term impact on muscle regeneration.

It is well known that muscle innervation is a predominant determinant in muscle fibre type composition[14]. However, both bupivacaine and ropivacaine have been proven to be neurotoxic, in equipotent concentrations[15]. Moreover, apart from muscle fibres, bupivacaine injection has also shown to affect neuromuscular junction and significantly delay its recovery in comparison to muscle fibre regeneration[16]. Bupivacaine has also been found to influence neurotrophic factors expression, during experimental muscle damage[17]. According to these data, bupivacaine may induce more prolonged perturbations in nerve impulses to the regenerating muscle than ropivacaine[14] and different mechanisms in nerve-induced muscle regeneration between the two local anaesthetics could explain the observed differences in muscle plasticity.

The present study revealed ropivacaine induced morphologic transformation of regenerated type I fibres, compared to control animals. Both HC and LC rats showed type I fibre hypertrophy, expressed as greater cross-sectional area and perimeter. These findings are in accordance with previous studies. Rosenblatt and Woods measured an increased cross-sectional area of type I fibre, 40-60 days after injection, whereas at the same interval period, type II fibres showed no differences in comparison with the control group. Authors considered these changes as permanent since type I fibres remained hypertrophic up to 180 days after injection[12]. On the other hand, the local anaesthetic concentration of the solution didn't seem to influence this process since no differences between HC and LC were recorded, despite the fact that ropivacaine generally produces less severe damage[5]. Selected type I muscle fibre hypertrophy is a quite rare entity and few data are available in the literature about the underlying mechanisms of this morphologic variation. Consequently, further studies are needed in order to clarify the underlying cellular mechanisms and the subsequent possible functional impairment.

Despite extensive use of muscle infiltration, either for pain control or for peripheral nerve blockade, the clinical significance of muscle toxicity is still debated. While some consider it as a factual adverse effect with long term consequences, others claim that it is a temporary and completely reversible damage on the muscle functionality in the process of an effective anaesthetic intervention[13]. The goal of eliminating it either by the use of toxicity-modifying pharmaceutical agents or by the designing of more appropriate molecular structures of local anaesthetics remains challenging and aspiring. The present study contributes to the histopathologic details of this damage that could hopefully lead to the delineation of the corresponding mechanisms of muscle toxicity.

Possible limitations of this study are related to the time course of muscle regeneration examination. The present study examined regenerated muscle fibres only six weeks after injection. This time point was chosen based on previous studies, suggesting "full recovery" of the degenerated muscle six weeks after injection. Provided that intact muscle fibres are prerequisite for morphometric analysis, no other time points, earlier than six weeks after injection, were chosen during study design. However, further studies are possibly necessary in order to clarify the regenerated muscle fibre characteristics beyond this time point.

In conclusion, under light microscope, regenerated rat muscles after infiltration with myotoxic doses of ropivacaine showed long-term hypertrophy of type I muscle fibres, despite generally "normal appearance". However, regenerated muscles are characterized by comparable ratio of various muscle fibre types with control ones, suggesting that despite its profound myotoxic effect, ropivacaine failed to influence muscle plasticity.

Conflict of interest

Authors declare no conflict of interest regarding this paper.

References

1. Brun A. Effect of procaine, carbocaine and xylocaine on cutaneous muscle in rabbits and mice. Acta Anaesthesiol Scand. 1959; 3: 59-73.
2 Hall-Craggs EC. Early ultrastructural changes in skeletal muscle exposed to local anesthetic bupivacaine (Marcaine). Br J Exp Pathol. 1980; 61: 139-149.
3 Basson MD, Carlson BM. Myotoxicity of single and repeated injections of mepivacaine (Carbocaine) in the rat. Anesth Analg. 1980; 59: 275-282.
4 Komai H, Lokuta AJ. Interaction of bupivacaine and tetracaine with the sarcoplasmic reticulum Ca2+ release channel of skeletal and cardiac muscles. Anesthesiology. 1999; 90: 835-843.
5 Amaniti E, Drampa F, Kouzi-Koliakos K, Kapoukranidou D, Pourzitaki C, Tsalie E, et al. Ropivacaine myotoxicity after single intramuscular injection in rats. Eur J Anaesthesiol. 2005; 23: 130-135.
6. Yildiz K, Efesoy SN, Ozdamar S, Yay A, Bicer C, Aksu R, et al. Myotoxic effects of levobupivacaine, bupivacaine and ropivacaine in a rat model. Clin Invest Med. 2011; 34: E273.
7. Zink W, Graf BM. Local anesthetic myotoxicity. Reg Anesth Pain Med. 2004; 29: 333-340.
8. McLoon LK, Nguyen LT, Wirtschafter J. Time course of the regenerative response in bupivacaine injured orbicularis oculi muscle. Cell Tissue Res. 1998; 294: 439-447.
9. Foster AH, Carlson BM. Myotoxicity of local anesthetics and regeneration of the damaged muscle fibers. Anesth Analg. 1980; 59: 727-736.
10. Kyttä J, Heinonen E, Rosenberg PH, Wahlström T, Gripenberg J, Huopaniemi T. Effects of repeated bupivacaine administration on sciatic nerve and surrounding muscle tissue in rats. Acta Anaesthesiol Scand. 1986; 30: 625-629.
11 Dubowitz V, Sewry CA. The normal muscle. Dubowitz V, Sewry CA (eds), Muscle biopsy. A practical approach. 3rd ed. Saunders, Elsevier, Philadelphia, 2007, 28-54.
12 Rosenblatt JD, Woods RI. Hypertrophy of rat extensor digitorum longus muscle injected with bupivacaine. A sequential histochemical, immunohistochemical, histological and morphometric study. J Anat. 1992; 181: 11-27.
13 Zink W, Bohl JR, Hacke N, Sinner B, Martin E, Graf BM. The long term myotoxic effects of bupivacaine and ropivacaine after continuous peripheral nerve blocks. Anesth Analg. 2005; 101: 548-554.
14 Pette D, Staron RS. Transitions of muscle fiber phenotypic profiles. Histochem Cell Biol. 2001; 115: 359-372.
15 Kasaba, T, Onizuka S, Takasaki M. Procaine and mepivacaine have less toxicity in vitro than other clinically used local anesthetics. Anesth Analg. 2003; 97: 85-90.
16 Nishizawa T, Tamaki H, Kasuga N, Takekura H. Degeneration and regeneration of neuromuscular junction architecture in rat skeletal muscle fibers damaged by bupivacaine hydrochloride. J Muscle Res Cell Motil. 2003; 24: 527-537.
17 Sakuma K, Watanabe K, Sano M, Uramoto I, Nakano H, Li YJ, et al. A possible role for BDNF, NT-4 and TrkB in the spinal cord and muscle of rat subjected to mechanical overload, bupivacaine