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ORIGINAL RESEARCH

Medicine Science International Medical Journal

Medicine Science 2018;7(4):891-7

Effects of perineural administration of phenytoin in combination with levobupivacaine in a rat sciatic nerve block

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Received 21 February 2018; Accepted 16 May 2018
Available online 05.10.2018 with doi:10.5455/medscience.2018.07.8899

Abstract
Peripheral nerve blocks are commonly preferred worldwide for the purposes of anesthesia application and postoperative analgesia. In this study, we investigated the effects of phenytoin which has a similar mechanism to local anesthetics in terms of the duration of analgesia and quality. The study was performed on 32 Sprague-Dawley male rats. Rats were randomly grouped into 4 groups. Group S: Sham group (n: 8); 0,2 ml saline perineural unilateral sciatic nerve. Group L: Perineural levobupivacaine (0,2 ml 0,5% levobupivacaine, n: 8); Group Ph: Perineural phenytoin (0,2 ml 62,5 mg / kg, n: 8); Group L + PH: Perineural phenytoin and levobupivacaine (0,2 ml 0,5% levobupivacaine + 62,5 mg / kg phenytoin, n: 8). Hot-plate and tail- flick tests were performed to measure acute thermal pain and histological changes were evaluated. The latency time at 30 minute in Group L+Ph were significantly increased when compared to the other groups during evaluation of the hot plate test. There was a significant difference in terms of latency time at 30 minute in Group L+Ph in the Tail Flick test and the latency time in Group L+Ph was longer when compared to the other groups ($p<0,05$) These results were obtained according to hot-plate and tail-flick tests and indicated that the analgesic quality. Perineural administration of phenytoin in combination with levobupivacaine did not affect the duration of the sensory and motor blockade at doses used in our study. However, phenytoin combined with levobupivacaine increased the duration and quality of the analgesia.

Keywords: Phenytoin, levobupivacaine, rat, sciatic nerve block

Introduction

Peripheral nerve blocks are commonly preferred worldwide for the purposes of anesthesia application and postoperative analgesia. Peripheral nerve blocks reduce opioid consumption, which has serious side effects. Single-dose peripheral nerve block with long-acting local anesthetics, such as bupivacaine, levobupivacaine or ropivacaine, maintains excellent analgesia for approximately 8-14 hours. Nevertheless, blocks administered in the early morning and mid-day may cause severe pain due to a loss of their effects late at night [1]. Many clinicians suggest taking opioids for analgesia to stop the pain that may occur during the night hours. Sleep disturbances, respiratory depression and other serious side effects caused by opioids may occur in these patients [2]. For this reason, long-acting local anesthetic adjuvants are added to increase the analgesic quality and duration of peripheral nerve blocks.

Levobupivacaine is a S-negative enantiomer of racemic bupivacaine and has similar potency to bupivacaine in nerve blocks. The combination of local anesthetics with clonidine, morphine or fentanyl is highly effective in the control of postoperative pain [3]. However, in addition to being a long-acting local anesthetic, levobupivacaine is known to have low lipid solubility, enhanced sensory blockade effect, less deep motor blockage, and less central nervous and cardiovascular system side effects compared to a racemic mixture [4].

Phenytoin is an antiepileptic drug that blocks voltage-sensitive and frequency-dependent sodium channels in neurons. Phenytoin stabilizes sodium channels in an inactive state [5]. This inhibitory effect is similar to local anesthetics. Phenytoin is an effective antiepileptic agent that is commonly used in all types of epilepsy, except absence epilepsy [6,7]. Phenytoin has no effect on the

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severity and duration of the action potential. Phenytoin limits the ability of high-frequency action potential by delaying the renewal of neurons. This function represses repetitive neuronal activity and prevents the distribution of seizure focus. At high concentrations, the neuronal refractory period is prolonged by preventing potassium excretion in the nerve (potassium channel blockage). However, previous studies have not examined the addition of perineural phenytoin to levobupivacaine on the effects of analgesia in rat sciatic blockage.

In this study, we extended the duration of analgesia by levobupivacaine, a long-acting local anesthetic, with phenytoin serving as an adjuvant, which has a similar mechanism to local anesthetics and provides a more comfortable period by extending the painless period after surgery and reducing drug consumption.

Materials and Methods

This experimental study was approved by the Animal Experiments Local Ethical Committee (2015/A-61). The study was performed on 32 Sprague-Dawley male rats weighing between 250-350 g from our Animal Research Center. Care for the experiments was performed in 4 rat cages (Euro type 3, polycarbonate stainless steel cage, 50 × 35 × 20 cm, 8 animals per cage) and were provided with rat chow and water ad libitum at 21-22° C room temperature, 12-hour light/dark cycle, before and during the study. The study was performed according to the guidelines of animal experiments reported by the National Health Research Institute.

Drug Administration

Normal saline and levobupivacaine (1%) or phenytoin solution was mixed together to obtained a final concentration of 0,5% levobupivacaine for administration to the perineural area. Phenytoin doses were determined by the weight of each rat. The pH value of phenytion was delivered to physiological values using Phosphate Buffered Saline (pH 7.2) (Phosphate Buffered Saline,BD, Heidelberg,Germany)

Experimental Design

Non-neurobehavioral rats were administered with ketamine (Ketalar; Pfizer, Istanbul, Turkey) and xylazine (Rompun; Bayer, Toronto, Canada) intraperitoneally at a dose of 10 mg/kg, respectively.

Rats were randomly grouped into 4 groups, in which each group consisted of 8 rats. Group S: Sham group (n: 8); the skin was closed after an injection of 0,2 ml saline perineural unilateral sciatic nerve. Group L: Perineural levobupivacaine (0,2 ml 0,5% levobupivacaine, n: 8); Group Ph: Perineural phenytoin (0,2 ml 62,5 mg / kg, n: 8); Group L + PH: Perineural phenytoin and levobupivacaine (0,2 ml 0,5% levobupivacaine + 62,5 mg / kg phenytoin, n: 8); and all study drugs were injected into the perineural zone.

Sciatic nerve injections were performed by an investigator who was blinded to the conditions of drug administration, and another investigator performed the neurobehavioral tests. Laboratory assistants prepared the drugs. After a lateral incision was performed along the thigh and the superficial fascia was separated, the sciatic nerve of the left hind limb was identified. The sciatic nerve was observed near the nerve's bifurcation. Next, 0,2 ml of prepared drug was administered perineurally using a 1-ml syringe. At the midpoint of the dissection, an unabsorbable muscle fascia suture was performed as a marker for the removal of the nerve. After the injection, tests were performed every 30 minutes.

After closing the skin in all groups, a paw withdrawal response was observed and recorded every 30 minutes until the sensory block of the test animal returned. Hot-plate and tail-flick tests were used to evaluate the analgesia. Histopathology of tissue was also examined for histopathological evaluation of the injection site.

Analgesia Measurement

Hot-plate and tail- flick tests were performed to measure acute thermal pain. These tests were used to evaluate thermal analgesia in rats.

Practice of the Hot-Plate Test

The surface of the Hot-Plate (Electro mag Instruments, Istanbul, Turkey), which could be controlled using a digital thermometer, was heated to obtain a stable temperature of 50°C. The time (in seconds) between shaking, claw licking, and plate-to-jump was determined to be the latency time when the rats were placed on the hot plate surface. To avoid tissue damage, the cut-off time was set to 60 seconds [8]. The preliminary response was reflected as the mean response times ensured 0 and 30 minutes before drug injection and was accepted as the normal response of the rats to heat effect. The latency time in the hot-plate test was recorded at 0, 30, 60, 90 and 120 minutes of drug administration.

Practice of the Tail-Flick Test

Anti-nociception and thermal analgesia were evaluated using the previously described radial Tail-Flick Test instrument (Type 812; Columbus Instruments) [9]. The rats were placed into a rigid plastic cage and non-related tests were performed in 15-seconds time intervals. The mean latency time of Tail-Flick, which represents the baseline latency time, was obtained from measurements of 3 tests before drug administration. Animals with a latency time of Tail-Flick changing between 2 and 5 seconds before drug administration were used in the experiments. Shortly after the baseline assessment, rats were injected with drugs or saline as described by the experimental protocol. The response of the rats were recorded at 0, 30, 60, 90 and 120 minutes after the procedure by performing pressure to the end of rat tail using an analgesometer. The cut-off time was established at 10 seconds to prevent tail injury. The duration of drug administration was performed as previously described [10,11].

Sensory and Motor Functions

The paw withdrawal response of the toes was used to evaluate sensory functions, while the force of the toes was applied laterally to the forceps. The forceps grip was limited to 1 second in order not to directly damage the claw tissue. The rats succeeded in pulling the paw tested in reaction to pain. Sensory reactions were defined according to withdrawal or dubbing and assessed as the following: the forceful paw withdrawal response after pinching (normal sensory function) was 0, moderate withdrawal was 1, lowest withdrawal was 2 and no response (complete sensory block) was 3. In addition, motor functions were evaluated using the following scale of 0-3; regular motor activation 0, regular dorsiflexion motion and moving with curved toes: 1, moderate dorsiflexion motion and moving with curved toes: 2, no dorsiflexion motion and moving

with curved toes: 3. Sensory and motor evaluations were measured at 30-minute intervals by another blinded researcher.

Histological Evaluation

The nerve tissues were obtained and fixed with 10% formaldehyde after the termination of the study. After fixation, tissues were embedded into the paraffin blocks after dehydration and clearing. Tissue sections were obtained at 4-µm thickness from paraffin blocks. Following the deparaffinization and rehydration process, sections were dyed with hematoxylene-eosin (H-E) and toluidine blue (TB). Stained sections were analyzed under Leica Q Win Image Analysis System (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

H-E stained sections were evaluated for fibrillar degeneration (shrinkage of axons, disruption of the myelin sheath, fibril loss), and edema in endonorium at 100× magnification. These histological changes were scored as none (0), little (1), intermediate (2) and severe (3). When sections stained with TB were evaluated, the total area was examined and mast cells were quantified in the endonorium. The number of mast cells per millimeter squared of area was obtained by dividing the total number of cells with the total area.

Statistical Analysis

The sample size value was determined in our study with statistical power analysis and was determined as a power of 0.80. The records were examined using a software program for Windows (IBM SPSS statistics version 23, IBM Corp, New York). The records were expressed as the minimum and maximum (median). The estimate of the standard distribution was supported using the Kolmogorov–Smirnov test. The Kruskal–Wallis H-test was performed without providing an estimate of normality. The Mann–Whitney U test with Bonferroni's correction was performed when multiple comparisons were required. All data were expressed as the median (minimum - maximum) and significance was established at $p<0.05$. For histological analysis, the statistical significance was detected at $p<0.01$, and similarly, the Mann-Whitney U test was preferred when multiple comparisons were required.

Results

Measurement of Analgesia

The latency times at 30 minutes in Group L+Ph were significantly increased compared to the other groups when the hot plate test was evaluated (Table 1). There was a significant difference in terms of latency times at 30 minutes in Group L+Ph in the Tail Flick test and the latency time in Group L+Ph was longer compared to the other groups (Table 2) ($p<0,05$). These results were obtained according to hot-plate and tail-flick tests and indicated that the analgesic quality and effect was better in Group L+Ph rats at 30 minutes compared with Group L rats.

Table 1. Hot-plate test latency time results

Time (min)	Group S (n : 8)	Group L (n : 8)	Group Ph (n : 8)	Group L+Ph (n : 8)	P value
			Median sec (minimum–maximum)		
Basal	14 (9-18)	11(6-17)	13(6-19)	12(9-20)	NS
30	15(7-25)	20(9-26)	14(7-19)	26(13-29)	0,018*
60	14(8-24)	14(6-19)	12(7-19)	17(9-23)	NS
90	13(9-20)	12(5-21)	11(4-20)	16(8-21)	NS
120	14(6-21)	13(7-21)	13(7-22)	14(11-20)	NS

NS: Not significant, Group S: sham, Group L: perineural levobupivacaine (0,2 mL 0,5% solution) and subcutaneous saline, Group Ph: perineural phenytoin (0,2 ml of 62,5 mg/kg), Group L+Ph: perineural levobupivacaine plus phenytoin (0,2 mL 0,5% levobupivacaine + 62,5 mg/kg phenytoin) and subcutaneous saline
*p<0.05, significantly difference

Table 2. Tail-flick latency time results

Time (min)	Group S (n : 8)	Group L (n : 8)	Group Ph (n : 8)	Group L+Ph (n ¼:8)	P value
			Median sec (minimum–maximum)		
Basal	9(5-10)	9(5-12)	8(5-11)	8(5-13)	NS
30	6(3-13)	6(4-11)	4(2-7)	8(4-11)	0,042*
60	7(5-12)	5(3-9)	4(2-8)	5(2-13)	NS
90	6(3-11)	4(3-7)	5(3-9)	6(3-13)	NS
120	6(2-8)	5(3-10)	4(2-7)	6(3-13)	NS

NS: Not significant, Group S: sham, Group L: perineural levobupivacaine (0,2 mL 0,5% solution) and subcutaneous saline, Group Ph: perineural phenytoin (0,2 ml of 62,5 mg/kg), Group L+Ph: perineural levobupivacaine plus phenytoin (0,2 mL 0,5% levobupivacaine + 62,5 mg/kg phenytoin) and subcutaneous saline
*p<0.05, significantly difference

Measurement of Sensory and Motor Functions

No complete sensory and motor blockade was observed in all groups. In particular, no significant difference between Group L and Group L+Ph was observed with regards to sensory and motor blockade. Scores at 30 minutes showed no significant difference between all groups (p>0,05).

Histological Findings

The histopathological scores and number of mast cells are shown at Table III and p-values for the histological evaluation are shown at Table IV. Sections stained with H-E and TB are shown in Figure 1 and 2.

1st day; in this study, in groups L and Ph, prominent degenerative changes, such as shrinkage of axons, disruption of the myelin sheath and loss of nerve fibril, were observed. Moreover, in group L, edema was observed in the endonorium. However, groups S and L+Ph also showed slight changes. As a result of histological scoring, there was a statistically significant difference between groups S and L in terms of both fibrillar degeneration and edema. In addition, there was a statistically significant difference between groups S and Ph in terms of fibrillar degeneration (p<0.05). Furthermore, there was a significant difference between groups L and L+Ph for both fibrillar degeneration and edema (p<0.05).

On the 14th day, compared to the 1st day of the study, the severity of histopathological changes increased in group Ph but decreased in other groups on the 14th day of the study. The lowest histopathological score of this study was observed in group S, and the highest score was observed in group Ph. Statistically significant differences were determined among group Ph and other groups (p<0.05). However, there was no statistically significant difference among groups L and L+Ph (p>0.05).

Histological changes on the 14th day in groups S and L were significantly decreased compared to groups S and L on the 1st day (p<0.05). In group Ph, the severity of degenerative changes on the 14th day significantly increased compared to group Ph on the 1st day (p<0.05). Lastly, histological changes on the 14th day in group L+Ph were similar to the 1st day (p<0.05).

Table 3. Histopathological scores and number of mast cells

		Degeneration	Edema	Number of Mast Cells
1st Day	Group S	1.0(0.0-2.0)	0.0 (0.0-2.0)	0.0 (0.0-18.1)
	Group L	2.0 (0.0-3.0)	2.0 (0.0-3.0)	0.0 (0.0-0.0)
	Group Ph	2.0 (0.0-2.0)	0.0 (0.0-2.0)	2.1 (0.0-6.7)
	Group L+Ph	0.0 (0.0-2.0)	0.0 (0.0-1.0)	4.4 (0.0-6.6)
14th day	Group S	0.0 (0.0-1.0)	0.0 (0.0-0.0)	0.0 (0.0-11.0)
	Group L	1.0 (0.0-3.0)	0.0 (0.0-1.0)	7.7 (5.7-13.8)
	Group Ph	3.0 (1.0-3.0)	2.0 (0.0-3.0)	0.0 (0.0-10.8)
	Group L+Ph	0.0 (0.0-2.0)	0.0 (0.0-1.0)	3.0 (0.0-14.3)

Group S: sham, Group L: perineural levobupivacaine (0,2 mL 0,5% solution) and subcutaneous saline, Group Ph: perineural phenytoin (0,2 ml of 62,5 mg/kg), Group L+Ph: perineural levobupivacaine plus phenytoin (0,2 mL 0,5% levobupivacaine + 62,5 mg/kg phenytoin) and subcutaneous saline

Table 4. p values for histological evaluation

		Degeneration	Edema	Number of
1st Day	Group S & Group L	0.001	0.001	0.140
	Group S & Group Ph	0.019	0.422	0.652
	Group S & Group L+Ph	0.131	0.429	0.428
	Group L & Group Ph	0.111	0.001	0.015
	Group L & Group L+Ph	0.001	0.001	0.004
	Group Ph & Group L+Ph	0.001	0.121	0.416
14th day **	Group S & Group L	0.007	0.152	0.043
	Group S & Group Ph	0.001	0.001	0.592
	Group S & Group L+Ph	0.018	0.004	0.397
	Group L & Group Ph	0.001	0.001	0.007
	Group L & Group L+Ph	0.651	0.062	0.195
	Group Ph & Group L+Ph	0.001	0.001	0.165
1th &14th day	Group S	0.001	0.009	0.833
	Group L	0.001	0.001	0.002
	Group Ph	0.004	0.001	0.259
	Group L+Ph	0.988	0.294	0.791

Group S: sham, Group L: perineural levobupivacaine (0,2 mL 0,5% solution) and subcutaneous saline, Group Ph: perineural phenytoin (0,2 ml of 62,5 mg/kg), Group L+Ph: perineural levobupivacaine plus phenytoin (0,2 mL 0,5% levobupivacaine + 62,5 mg/kg phenytoin) and subcutaneous saline
* Compared with each other, ** Compared with each other, *** Compared within themselves,

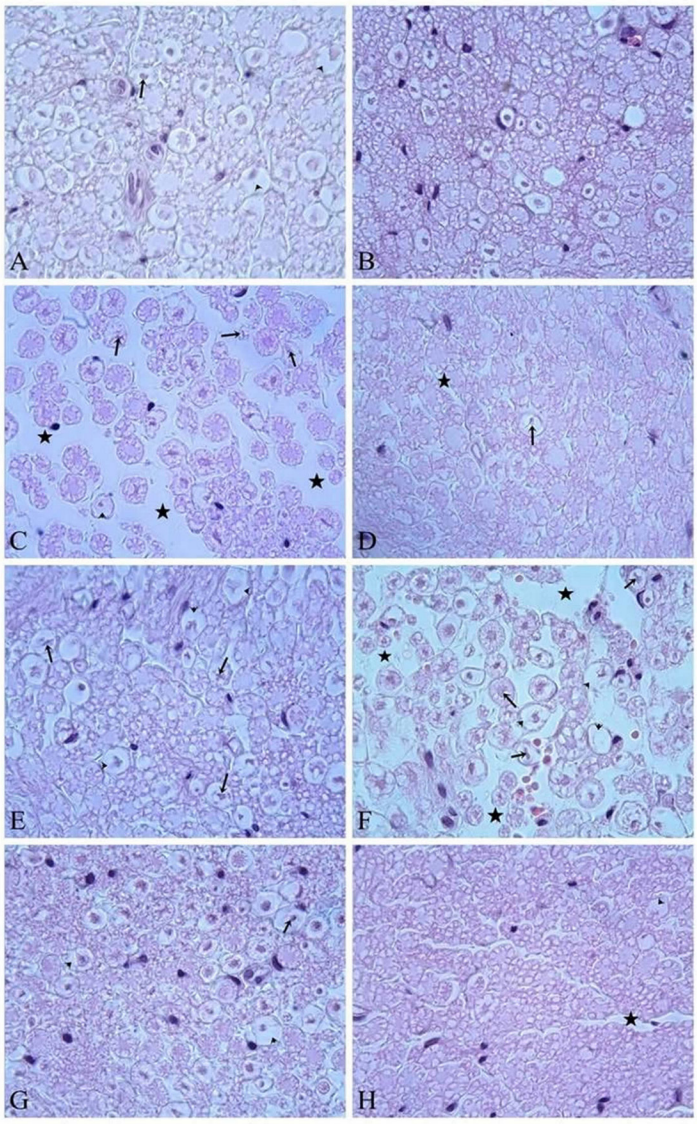

Figure 1. A (Group S, 1st day), C (Group L, 1st day), E (Group Ph, 1st day), G (Group L+Ph, 1st day) and B (Group S, 14th day), D (Group L, 14th day), F (Group Ph, 14th day), H (Group L+Ph, 14th day).
Arrow, arrowhead, and star represent the shrinkage of axons, disruption of the myelin sheath and edema in each image, respectively. H-E 100×.

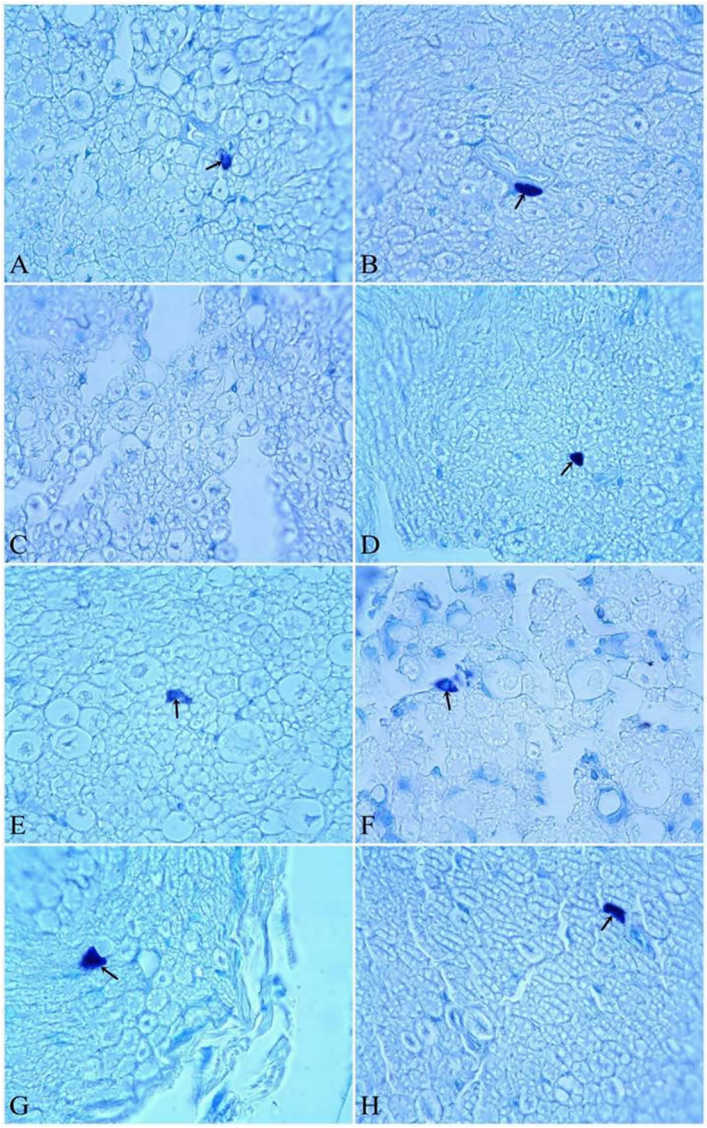

Figure 2. A (Group S, 1st day), C (Group L, 1st day), E (Group Ph, 1st day), G (Group L+Ph, 1st day) and B (Group S, 14th day), D (Group L, 14th day), F (Group Ph, 14th day), H (Group L+Ph, 14th day).
Arrow represents the mast cells in each image. Notice that there is no mast cell in B1 group. TB 100×.

Number of Mast Cells

On the 1st day, when each group was compared with each other in terms of the number of mast cells, it was shown that while there was a statistically significant difference among the groups L-Ph and groups L-L+Ph ($p<0.05$), there was no significant difference between the other pairs of groups ($p>0.05$).

On the 14th day, according to the cell number results, there was a statistically significant difference between groups S & L and groups L & Ph ($p<0.05$), but a significant difference was not observed between in other pairs of groups ($p>0.05$). When each of the groups of the 1st day and 14th day were compared, it was observed that there was a significant increase on the 14th day only in group L compared to group L on the 1st day ($p<0.05$). However, no difference was observed between the 1st day and 14th day in group L+Ph ($p>0.05$). In contrast, there was a decrease in the number of mast cells on the 14th day in groups S and Ph compared to S and Ph at 1st day, respectively ($p>0.05$). Group S at 14th day was nearly normal in appearance, except for slight fibrillar degeneration. The presence of more degenerative changes on the 14th day in group Ph was observed.

Discussion

The most important consequence of our study is the increase in analgesia quality in the first 30 minutes of sciatic block applied with levobupivacaine phenytoin combination at study doses as an adjuvant. In addition, histopathological changes in the group levobupivacaine + phenytoin are less than the phenytoin group compared to each other.

Local anesthetics are long preferred worldwide in the management of postoperative acute or chronic pain, as well as regional anesthesia and peripheral nerve blocks [12]. Many peripheral adjuvant agents are selected to increase the duration of the local anesthetic block, improve the quality of anesthesia, prevent toxicity, and initiate the operation more quickly [13]. Epinephrine, clonidine (inhibition

of voltage-gated sodium channels by perineural injection), buprenorphine (inhibition of voltage-gated sodium channels by perineural injection), dexamethasone (inhibition of C-fiber transmission) and midazolam are preferred as a single adjuvant or in combination in nerve blocks [14,15]. Kroin et al. [16] have shown that epinephrine is more potent when clonidine or epinephrine is added as an adjuvant, and clonidine substitution with epinephrine does not provide an additional benefit but may be used as an alternative in specific conditions. These researchers reported that the mechanism of action of clonidine is mediated via ion channel blockade, rather than via alpha-2 adrenoreceptors. Buvanendran et al. [17] reported that when they are combined with buprenorphine or dexamethasone, they reach the longest block in rat nerve block in combination with bupivacaine and clonidine [17]. Erdoğan et al. [2] reported that they applied a combination of levobupivacain and dexmedetomidine in rat sciatic nerve block and analgesic effects were better when assessed using the hot-plate test and tail-flick test when dexmedetomidine was added. However, there were no effects on sensory and motor blockade [2]. In addition, when dexmedetomidine was used at high doses, it caused analgesia and sedation via adrenoceptors in the head and spinal cord, which may cause some central effects due to absorption [18,19].

Levobupivacaine inhibits the transmission of nerve stimuli by blocking voltage sensitive ion channels expressed on neuron membranes. Local and reversible anesthesia is created by opening of the sodium channel, which prevents transmission of the action potential in the nerves, including sensory and motor activity and sympathetic activity. The effects on motor function are less than those on sensory function [3]. Phenytoin is an anti-epileptic drug that blocks sodium channels similar to local anesthetics. Activation of voltage-gated sodium channels is essential to pass the action potential between the axons. Anti-epileptic drugs suppress neuronal activation via a variety of mechanisms. Phenytoin is an anti-epileptic drug with strong evidence that it has a basic mechanism of action [5]. Phenytoin is an effective anti-epileptic agent that is commonly used in all types of epilepsy, except absence epilepsy [6,7]. This study was aimed to determine the benefits of phenytoin with regards to its adjuvant effect due to its similar effects to local anesthetics. The levobupivacaine phenytoin group showed increased analgesia quality compared to the other groups.

It was observed that clonidine, buprenorphine and dexamethasone adjuvants do not cause neurotoxicity due to local anesthesia but significantly developed neurotoxicity in combination with midazolam [15]. In this study, fibrillar degeneration was observed in the phenytoin group compared to other groups on the 1st day ($p<0.05$). Fibrillar degeneration and edema were also observed significantly more in group L+Ph than group L at 1st day. The severity of histopathological changes in group Ph increased on the 14th day compared to the 1st day but decreased in other groups. The lowest histopathological score of this study was observed in group S, and the highest score was observed in group Ph. Statistically significant differences were determined among group Ph and other groups ($p<0.05$). In group Ph, the severity of degenerative changes on the 14th day were significantly increased compared to group Ph on the 1st day ($p<0.05$). Thus, the application of phenytoin alone had a neurotoxic effect on the nerves. However, histological changes on the 14th day in group L+Ph were similar to the results obtained on the 1st day. Furthermore, when phenytoin was used alone, it had a neurotoxic effect on the nerves. Interestingly, when levobupicaine plus phenytoin were administered together, the neurotoxic effect was lower.

We would like to highlight the most important point of this study: phenytoin, which has a similar mechanism to local anesthetics and several adjuvants (such as clonidine and buprenorphine), has never been tested in peripheral nerve blocks. In this study, we observed that levobupivacaine in combination with phenytoin did not improve the duration and degree of sensory and motor blockade at the doses indicated in our study. However, levobupivacaine in combination with phenytoin improved the quality of analgesia in rats. Thus, we recommend that drugs combined with adjuvants can be used as peripheral nerve blocks to increase the quality of analgesia. However, the use of phenytoin as an adjuvant may be limited in terms of the histological outcome of the application according to our study. Phosphenytoin that was not toxic to tissue may be further studied for its effects on peripheral nerve block. Furthermore, our study may be serve as a guide for further studies in humans.

Limitations

Our study has some limitations. First, there was no knowledge regarding the systemic effects of phenytoin when administered perineurally. Due to the similarity of the mechanism of effect with local anesthetics, systemic effects must be studied for the emergence of the essential effect. Second, we used ketamine for anesthesia, and the analgesic effect of ketamine can affect our results. Anesthesia with inhaler anesthetics may be preferred to eliminate this limitation.

Conclusion

Perineural administration of phenytoin in combination with levobupivacaine did not affect the duration of the sensory and motor blockade at doses used in our study. However, phenytoin combined with levobupivacaine increased the duration and quality of the analgesia. Histological effects of phenytoin on tissue may limit the use of levobupivacaine as an adjuvant drug. Thus, further studies are needed to reduce the toxic histological effects of phenytoin on tissue and to achieve more effective results for analgesia. .

Competing interests
The authors declare that they have no competing interest

Acknowlegment
This study was supported by a grant from The Scientific Research Fund of Inonu University (No. 2016/71). The study was presented as oral presentation at 7th European Congress of Pharmacology, 2016. Manuscript was edited by native English speaker (American Journal of Experts) for proof-reading.

Ethical approval
Before the study, permissions were obtained from local ethical committee.

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