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Assessing the Use of 50% Enantiomeric Excess Bupivacaine-Loaded Microspheres after Sciatic Nerve Block in Rats

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Summary: Oliveira RM, Tanaka PP, Tenório SB – Assessing the Use of 50% Enantiomeric Excess Bupivacaine-Loaded Microspheres after Sciatic Nerve Block in Rats.

Background and objectives: To achieve better therapeutic benefits of local anesthetics in the control of postoperative pain through controlled-release carrier. The objective of this study was to compare the characteristics of sensory and motor blockade between microspheres without local anesthetic: racemic bupivacaine-loaded microspheres; 50% enantiomeric excess bupivacaine-loaded microspheres; and free 50% enantiomeric excess bupivacaine.

Methods: Wistar rats were distributed into four groups: A (Microsphere); B (S50-R50 bupivacaine-loaded microsphere); C (50% enantiomeric excess bupivacaine-loaded microsphere); and D (50% enantiomeric excess bupivacaine). Inhalation anesthesia was performed before the sciatic nerve block (2% halothane and 100% O_2). Sensorial blockade was measured by the time required for each rat to withdraw its paw from a hot plate at 56ºC (positive > 4 sec). Motor blockade was measured by the time between drug injection until recovery of a motor score of 2 on the established criterion.

Results: The sensory response was significantly more frequent in groups B, C, and D than in group A ($p < 0.001$). There were no statistically significant differences in the response to the sensory test in groups B, C, and D ($p > 0.05$). The response to the motor test was also significantly more frequent in groups B, C, and D than in group A ($p = 0.02$). A tendency to greater positivity in the motor test was more frequently found in groups B and D than in group C ($p = 0.10$).

Conclusions: Controlled-release of 50% enantiomeric excess bupivacaine-loaded microspheres showed similar results regarding analgesia and less motor blockade when compared to other anesthetic formulations.

Keywords: Pain, Postoperative; Microspheres; Bupivacaine; Rats, Wistar.

INTRODUCTION

Nerve conduction blocks are effective to provide postoperative analgesia. However, its usefulness is limited by the short duration of the effects of local anesthetics. The ideal local anesthetic should be long-acting and have low toxicity, properties that are not present in the drugs currently available [1]. New local anesthetics capable of promoting prolonged nerve blocks have been developed; however, they have not been accepted because of their high systemic toxicity [2,3].

With the help of catheters, continuous infusion of local anesthetics is possible, prolonging their action indefinitely; however, it is associated with increased risk of complications due to blood absorption. The addition of drugs that decrease the absorption of local anesthetics such as adrenaline, or potentiate its effects such as opioids does not bring benefits and contribute for the increased risk of toxicity and side effects of the drugs added [4-6]. Since the 1990s deposition system such as liposomes, cyclodextrins, and microspheres that release drugs slowly and continuously [7], prolonging the duration of their effects have been developed.

Among these systems, microspheres have an adequate profile to be used with local anesthetics. They are biodegradable and mechanically stable polymers whose diameter is small enough to allow encapsulated drugs to be transported until the nerve tissue through common needles [8-12]. Microspheres release the drug inside the body in small and controllable daily doses according to the way they were designed for several days.

Drug controlled-release systems offer several advantages when compared to conventional systems of drug administration, such as: a) greater efficacy, with progressive, controlled drug release from the matrix degradation; b) significant reduction of toxicity and stay longer in the circulation; c) variation in the composition and nature of vehicles and contrary to what one might expect there is no predominance of mechanisms of instability and drug decomposition (premature bio-inactivation); d) safe (without local inflammatory reactions) and con-

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venient (smaller number of doses) administration; e) directed to specific targets, without significant mobilization of bioactive species; and f) both hydrophilic and lipophilic substances may be incorporated.

Most amino-amide local anesthetics used clinically is a chiral compound. They have an asymmetrical carbon adjacent to the amine group and, therefore, they exist as isomers, which are the mirror image of each other. One can distinguish the dextrorotatory (D) and levorotatory (L) isomers. Fifty per cent enantiomeric excess bupivacaine is composed of an enantiomeric excess mixture of 75% levorotatory component and 25% dextrorotatory component. Stereoselectivity is important to reduce the cardiotoxicity of bupivacaine.

The objective of the present study was to compare the characteristics of the sensory and motor blockades between microspheres without local anesthetic; racemic bupivacaine-loaded microspheres; 50% enantiomeric excess bupivacaine-loaded microspheres; and free 50% enantiomeric excess bupivacaine.

MATERIALS AND METHODS

According to the Brazilian College of Animal Experimentation (COBEA) rules, male Wistar rats weighing between 200 g and 350 g were investigated after adaptation in a warm environment at 21°C and 55% humidity and with a day-night cycle to prevent changes in the circadian rhythm. Animals were randomly separated into four groups with eight rats each according to the solution used in the sciatic nerve:

Group A: microspheres without local anesthetic;
Group B: racemic bupivacaine-loaded microspheres;
Group C: 50% enantiomeric excess bupivacaine-loaded microspheres;
Group D: free 50% enantiomeric excess bupivacaine-loaded microspheres.

Under general anesthesia with halothane in oxygen through a mask and spontaneous ventilation, rats were placed in lateral decubitus and sciatic nerve block was performed, using as reference the groove between the head of the greater trochanter of the femur and the tuberosity of the ischium located by palpation. The nerve was identified by introducing into the groove a 24G Teflon needle (Stimuplex, B. Braun, Melsungen, Germany) connected to a nerve stimulator (DigiStim II, NeuroTechnology, Houston, TX).

The proximity of the needle tip to the sciatic nerve was confirmed by visible muscle contraction of the paw with a stimulus of 0.2 mA. The injection volume was 0.5 mL in an insulin syringe with a 3.175% solution of the local anesthetic in groups B and C. In groups A and D, the same volume of 0.5 mL containing only microspheres or local anesthetic was injected. The insulin syringe was connected to the puncture needle with the dead space filled with the study solution. Lyophilized microspheres were diluted in distilled water and mixed on maximum speed for 2 minutes before administration. The drugs were manipulated by another investigator. After the blockade, animals were placed in their cages. Microspheres with 50% enantiomeric excess bupivacaine were prepared based on the methodology described previously [13].

Assessment of sensory blockade [14]: sensory blockade was measured by the time required for each rat to withdraw its paw from a plate at 56°C. The hot plate is equipped with a diode that emits with a precision of ± 0.1°C. Besides the accuracy of the diode a thermometer was also used. Non-anesthetized intact rats withdraw their paws from the plate within 1-3 seconds. They were involved with a cloth placed above their hip to restrict the upper extremities and obstruct their vision. Rats were positioned to be with one posterior paw on the hot plate and the contralateral paw on a wooden block at room temperature. Posterior paws were exposed (first, the left and then the right) to the hot plate. Alternating sides, the contralateral paw worked as control to detect potential systemic analgesic effects or stress-induced analgesia. The latency for removal of each paw from the hot plate was recorded, alternating paws, allowing at least 15-second recovery time between each measurement. The experiment was finalized after 12 seconds when there was no reaction from the animal to the hot plate to avoid injuries or hyperalgesia, and the time was recorded; four seconds were considered a positive test.

Response to positioning [14]: When the rat is in normal resting position, the toes are flexed over the dorsum. Its ability to reposition the hindpaw and toes was evaluated. Rats were placed in pronation with their hindpaws stretched back (with the dorsum in contact with a firm surface). The response was evaluated according to the following criteria: a) the hindpaw returns to the original position with their claws opened (dorsiflexion or abduction and extension of the claws); b) the hindpaw returns to the original position, but the claws are closed (dorsiflexion and claws partially flexed and abducted); c) the hindpaw does not return completely to the original position (inability to open and extend the claws); and d) the hindpaw remains in the position it was placed and the claws are closed (motor blockade). The duration of the motor blockade was not measured. Assessment of the sensory blockade and presence of motor blockade were repeated daily at the same time. Results were considered positive when animals presented criteria c and d.

On the 2nd, 4th, 6th, and 8th days, one animal in each group was selected. After general anesthesia with halothane, cardiac puncture was performed and blood samples collected to evaluate the plasma concentration of local anesthetic. Killing was performed with intraperitoneal injection of 70 $mg.kg^{-1}$ of sodium thiopental soon after blood collection.

Statistical analysis: this is a prospective, longitudinal, and experimental study, which assessed the motor and sensorial blockade of 50% enantiomeric excess bupivacaine (S75-R25)-loaded microspheres. All data were obtained prospectively by the investigator evaluating the animals and recorded on the collection instrument elaborated by the author. Data were typed in an electronic spreadsheet (Excel), verified, and exported to the Statistica software.

Kruskal-Wallis Anova model was used to evaluate the difference in continuous measurements with asymmetric distribution (sensorial test) in the different groups. Bicaudal tests were used in all groups, considering that the differences could be distributed on both sides of the curve, with level of significance of 5%. The size of the sample was estimated considering a type I error of 5% (alpha) and type II error of 10% with a minimum estimated test power of 90%. Significant differences regarding the study characteristic comparing both local anesthetic solutions-loaded microspheres were not expected.

RESULTS

Assessment of sensory blockade showed that after infiltration of rat sciatic nerve with LA, an increase in pain threshold was observed with all anesthetic formulations investigated (groups B, C, and D), which was statistically different from the control group (A) ($p < 0.001$). However, comparisons between B, C, and D groups showed similar sensory blockade profiles ($p > 0.05$). Injection of LA-loaded microspheres in different concentrations did not induce an increase in the duration of analgesia and intensity of the effect when compared to free LA (Table I).

Assessment of motor blockade

Comparisons between anesthetic formulations demonstrated reversible loss of motor reflexes, indicating a smaller intensity of the motor blockade in group C (50% enantiomeric excess bupivacaine-loaded microspheres) (Table II).

Table I – Summary of the Sensory and Motor Tests and Plasma Concentrations of Local Anesthetics

Group	Day[1]	Sensory test[2]	Motor test[2]	Plasma concentration[3]
A	2nd	1	1	ND
	4th	1	1	ND
	6th	1	1	ND
	8th	1	1	ND
B	2nd	8	1	78 ng.mL^{-1}
	4th	1	1	ND
	6th	2	1	ND
	8th	1	1	ND
C	2nd	5	1	ND
	4th	2	1	255.70 ng.mL^{-1}
	6th	1	1	136.60 ng.mL^{-1}
	8th	1	1	81.6 ng.mL^{-1}
D	2nd	1	1	ND
	4th	1	1	ND
	6th	1	1	ND
	8th	1	1	ND

[1]: day of the animal's killing; [2]: tests measured in seconds; [3]: ND: non detected in concentrations > 50 ng.mL^{-1} of plasma.

Table II – Comparative Response of the Sensory Test in Groups A, B, C, and D

	Group A	Group B	Group C	Group D	p
1st day	1.00 (1.00-2.00	7.00 (1.00-12.00)	2,50 (1.00-12.00)	5.50 (1.00-12.00)	0.004*
2nd day	1.00 (1.00-2.00)	4.00 (1.00-12.00)	3.50 (1.00-10.00)	2.00 (1.00-12.00)	0.01*
3rd day	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-4.00)	1.00 (1.00-3.00)	0.03*
4th day	1.00 (1.00-2.00)	1.00 (1.00-2.00)	1.00 (1.00-2.00)	1.00 (1.00-2.00)	0.88
5th day	1.00 (1.00-2.00)	1.00 (1.00-2.00)	1.00 (1.00-2.00)	1.00 (1.00-1.00)	0.47
6th day	1.00 (1.00-1.00)	1.00 (1.00-2.00)	1.00 (1.00-2.00)	1.00 (1.00-1.00)	0.55
7th day	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1
8th day	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1

Kruskal-Wallis Anova; *: A B, C, D; differences between B, C, and D were not observed.

Assessment of drug concentration in plasma

Animals receiving 50% enantiomeric excess bupivacaine-loaded microspheres showed plasma concentrations above 50 mg.dL^{-1} up to the 8th day. Although plasma concentration of bupivacaine in group C was higher than the concentration of bupivacaine in group B, the differences observed in the sensory test (5 seconds for group C and 8 seconds for group B on the 2nd day) were not proportional to the drug plasma concentration. On the other hand, in group B, the moment the presence of bupivacaine was detected in the plasma interfered directly on the nerve blockade and it was proven by the increased sensitivity of the animal on the hot plate (Table III).

DISCUSSION

Evaluation of the risks and benefits of microspheres was the objective of this study. Microspheres are biodegradable polymers with 1 to 50 μm diameter that can incorporate several drugs. They can be differentiated by the type of polymer used, and the ones used in the present study received poly lactic-co-glycolic acid (PLGA) copolymer, which has the capacity of holding greater amount of drugs and prolong the duration of the effects [15]. Poly lactic-co-glycolic acid polymers are degraded to acid monomers (example: lactic and glycolic acids), being eliminated from the body as carbon dioxide and water [16,17]. Anesthetics incorporated into the microspheres should be lyophilized and later reconstituted in aqueous solution to be used. Due to the small diameter, microspheres can be introduced in the proximity of the nerve tissue through hypodermic needles [18], where the local anesthetic or another drug used spreads through the micropores and can produce prolonged pharmacological effects [19].

Wistar rats were used in this study for several reasons: they are commonly used in several experiments, facilitating comparison between several studies. They have short life cycle and genetic uniformity. Males were chosen because they have less hormone changes than females. These animals were maintained under conditions to minimize variables that could interfere with biological responses. The bioterium was maintained at mean temperatures of 21°C to avoid changes in environmental temperature that might lead to adaptation responses, such as behavioral, physiologic, and metabolic changes that could interfere with the results of the study. Humidity was maintained around 55% as rodents eliminate most of their body heat through the lungs, besides a drier environment facilitates pulmonary water evaporation.

The environment was ventilated to eliminate the ammonium produced from urine and feces nitrogen, another source of stress. Before the experiment, regular light-dark periods were created to synchronize their circadian cycle, because light intensity and photo-period (day length) influences metabolism and estrous cycle of animals, changing their biologic response. The bioterium was maintained in total isolation from natural light, allowing control of light intensity and, consequently, of the photo period. White fluorescent lights, which produce less heat, were used. As rodents have more acute hearing, the environment had low levels of noise to reduce stress [20].

Sciatic nerve block was performed in rats under general anesthesia with halothane, since punctures under anesthesia are more precise and the success rates are higher [21]. We considered that the needle was close enough to the sciatic nerve when there was motor response to currents of 0.2 mA from the peripheral nerve stimulator [22].

Withdrawal reflex in response to contact with a hot plate was used as the nociceptive test. This reflex involves contraction of the flexors muscle of the hips, knee, and ankle. It is a polysynaptic reflex induced by the nociceptive stimulation of the limb, and its latency, amplitude, and duration depend on the intensity of the stimulus. Very intense and frequent sensory stimuli could produce hyperalgesia, which could lead to misinterpretation because it reduces the sensitivity threshold of the nerve.

For this reason, the plate temperature and stimulus frequency were limited. The sciatic nerve was chosen due to its diameter and easy access, making puncture easier and

Table III –Comparative Response to the Motor Test in Groups A, B, C, and D

	Group A	Group B	Group C	Group D	p
1st day	08 (38.10%)	04 (19.05%)	05 (23.81%)	04 (19.05%)	0.05*
2nd day	16 (30.19%)	12 (22.64%)	15 (28.30%)	10 (18.87%)	0.03**
3rd day	14 (100.00%)	00 (0.0%)	00 (0.0%)	00 (0.0%)	< 0.01
4th day	12 (100.00%)	00 (0.0%)	00 (0.0%)	00 (0.0%)	< 0.01
5th day	10 (100.00%)	00 (0.0%)	00 (0.0%)	00 (0.0%)	< 0.01
6th day	08 (100.00%)	00 (0.0%)	00 (0.0%)	00 (0.0%)	< 0.01
7th day	06 (100.00%)	00 (0.0%)	00 (0.0%)	00 (0.0%)	< 0.01
8th day	04 (100.00%)	00 (0.0%)	00 (0.0%)	00 (0.0%)	< 0.01

Pearson's Chi-square test; *: A B, C, and D; **: A B, D; C D.

more precise [22,23]. Therefore, the sciatic nerve is the starting point for the study of local anesthetics in the intact animal, composing with *in vitro* investigations the requisite for the preclinical phase of new compounds before investigation phases in humans. Assessment of anesthetic efficacy was based on observation of the animal's behavior to nociceptive thermal stimuli, characterized by the fast exchange of feet support ("stamping"), licking gestures, biting, or raising one of the paws when placed on a hot surface at more than 50ºC. This model has been tested with good results in other investigations [12]. Note that, although the sensory innervation of the paw is mediated by the sciatic nerve, hip and knee flexion, which are needed to remove the paw from the hot plate, are mediated by the femoral nerve, which was not blocked.

Consequently, this test was specific to evaluate sensorial blockade. Under these conditions, more restricted paw stimulation was used. This evaluation differs from other methods used, such as immersion in hot water, when a larger area is stimulated and other sensory contributions occur, besides the possibility of inducing errors.

The temperature of 56ºC was chosen because it represents an intense stimulus and allows clear distinction between the sensorial blockade and more subtle analgesic effects. Rat sciatic nerve was localized with a peripheral nerve stimulator. This is the traditional and reliable method to localize peripheral nerves to guarantee that the local anesthetic, loaded into microspheres or as the free form, was deposited close to the sciatic nerve of the rats investigated eliminating technical failure.

In the present study differences in the duration of the sensory blockade or neurotoxicity of the microsphere-loaded or free local anesthetic were not observed. The latency for paw removal from the hot plate was similar in the groups who received free or microsphere-loaded bupivacaine. On the 3rd day the sensitivity to thermal stimulus of rats that had received bupivacaine did not differ from the control group estimating the duration of analgesia in the groups that received local anesthetic in 48 hours.

Duration of sensory blockade of this magnitude or greater has been described for bupivacaine-loaded microspheres, but not for the free enantiomeric mixture. As a rule the duration of free bupivacaine, when used to infiltrate peripheral nerves, does not exceed 24 hours. There are no explanations for the longer duration of the sensory blockade observed with the free enantiomeric mixture. Both experimental and clinical studies have demonstrated systematically that encapsulation of bupivacaine into microspheres prolongs the duration of the sensory effects of the local anesthetic. For example, the use of bupivacaine-loaded microspheres in rat sciatic nerve [24] caused sensory blockade that lasted between 10 hours and 5.5 days. Addition of dexamethasone to the local anesthetic in the microspheres prolonged the duration of the sensory blockade by up to 13-fold when compared to free bupivacaine. In volunteers, the duration of the intercostal block after the injection of bupivacaine-loaded spheres *versus* free bupivacaine, associated with dexamethasone or not, was significantly greater in the group that had received dexamethasone associated with bupivacaine in microspheres [26].

A potentiating effect of dexamethasone associated with bupivacaine microspheres in subcutaneous infiltration was observed in human volunteers [26]. In the present study, statistically significant differences were observed in the duration of motor blockade. The animals that received the enantiomeric mixture of bupivacaine-loaded microspheres (group C) had shorter duration of motor block than the other groups. This difference in duration of motor block of levorotatory forms of bupivacaine, which was also observed in other experimental and clinical studies [27,28], could be due to the greater fraction of the levorotatory component in the enantiomeric excess mixture of bupivacaine. In fact, 75% of the enantiomeric excess mixture of bupivacaine and 50% of bupivacaine are composed by the levorotatory component. But when one compares the duration of motor block of racemic bupivacaine-loaded microspheres or free, greater duration of motor block is observed with bupivacaine-loaded microspheres, according to a study with rabbits undergoing epidural anesthesia [29].

Some animals were killed to analyze the plasma concentration of the local anesthetic. Mean concentrations of the local anesthetic were greater in the group that received the enantiomeric mixture; however, this difference was not statistically significant. A study in sheep did not report clinically relevant plasma concentration of bupivacaine after injection in the brachial plexus. Histopathological analysis suggested the lack of significant differences between groups. Clinical changes were not observed, although one animal in the enantiomeric mixture group had seizures.

Plasma bupivacaine concentrations in this animal were four-fold below the central nervous system toxicity threshold in humans or rats [24], suggesting another cause for the seizures probably the stress during the experiment [30].

Despite differences between humans and rats regarding methods, doses, and volumes of the anesthetic solution, results under the conditions of this study revealed that levorotatory or racemic bupivacaine, loaded into microspheres or free, did not differed regarding the duration of the sensory blockade and pharmacokinetic parameters. This study also suggests enantiomeric bupivacaine causes shorter motor blockade.