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Comparative effects of levobupivacaine and racemic bupivacaine on excitotoxic neuronal death in culture and *N*-methyl-D-aspartate-induced seizures in mice

Concetta Marganella [a,c], Valeria Bruno [a,b], Francesco Matrisciano [a], Carlo Reale [c], Ferdinando Nicoletti [a,b], Daniela Melchiorri [a,*]

[a] *Department of Human Physiology and Pharmacology, University of Rome "La Sapienza", Ple. A. Moro 5, 00185, Rome, Italy*
[b] *I.N.M. Neuromed, Pozzilli, University of Rome, "La Sapienza", Italy*
[c] *Department of Urology, University of Rome, "La Sapienza", Italy*

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Abstract

We compared the neurotoxic profile of racemic bupivacaine and levobupivacaine in: (i) a mouse model of *N*-methyl-D-aspartate (NMDA)-induced seizures and (ii) in an in vitro model of excitotoxic cell death. When used at high doses (36 mg/kg) both bupivacaine and levobupivacaine reduced the latency to NMDA-induced seizures and increased seizure severity. However, levobupivacaine-treated animals underwent less severe seizures as compared with bupivacaine-treated animals. Lower doses of levobupivacaine and bupivacaine had opposite effects on NMDA-induced seizures. At doses of 5 mg/kg, levobupivacaine increased the latency to partial seizures and prevented the occurrence of generalized seizures, whereas bupivacaine decreased the latency to partial seizures and did not influence the development of generalized seizures. In in vitro experiments, we exposed primary cultures of mouse cortical cells, containing both neurons and astrocytes, to 100 μM NMDA for 10 min for the induction of excitotoxic neuronal death. This treatment killed 70–80% of the neuronal population, as assessed 24 h after the excitotoxic pulse. In this particular model, both levobupivacaine and bupivacaine were neuroprotective against NMDA toxicity. However, neuroprotection by levobupivacaine was seen at lower concentrations (with respect to bupivacaine) and was maintained at concentrations of 3 mM, which are much higher than the plasma security threshold for the drug in vivo. In contrast, no protection against NMDA toxicity was detected when 3 mM concentrations of bupivacaine were applied to the cultures. Our data show a better neurotoxic profile of levobupivacaine as compared to racemic bupivacaine, and are indicative of a safer profile of levobupivacaine in clinical practice.

Keywords: Bupivacaine; Levobupivacaine; Neurotoxicity; NMDA; Mice mixed cultures of cortical neurons

1. Introduction

Bupivacaine is a local anaesthetic commonly used in dental, ophthalmologic, and simple surgical procedures. Its widespread use is derived from its potency, long half-life and its tendency to provide more sensory than motor block. The clinical use of bupivacaine is hampered by the occurrence of severe adverse effects ensuing from the systemic absorption of the drug, such as cardiotoxic and neurotoxic effects (Graf et al., 2002; Mather and Chang, 2001; Simon et al., 2002; Gristwood, 2002; Heavner, 2002). Bupivacaine toxicity results from the inhibition of voltage-gated sodium channels and several types of potassium channels (Clarkson and Hondeghem, 1985; Olschewski et al., 1999; Longobardo et al., 2001; Friederich et al., 2004). Neurotoxicity depends on the impact of the drug on excitatory and inhibitory processes in the central nervous system (CNS). Bupivacaine induces generalized seizures by depressing the activity of inhibitory neurons and by inhibiting potassium channels in excitatory

* Corresponding author. Tel.: +39 06 49912969; fax: +39 06 4450307.
E-mail address: daniela.melchiorri@uniroma1.it (D. Melchiorri).

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neurons (Graf et al., 2002). The neurotoxic and cardiodotoxic (increased QTc interval, arrhythmias, and hypotension) effects of bupivacaine have been ascribed to the racemic nature of the drug, and, in particular, to the presence of the D (+) isomer in the commercial preparation. Electrophysiological studies show that the D (+) isomer of bupivacaine is a more potent and faster blocker of voltage-gated Na^+ channels, and inactivates several subtypes of ATP-dependent and voltage-dependent K^+ channels (Kv) with higher affinity than the L (−) isomer (Arias, 2002; Franqueza et al., 1997; Kawano et al., 2004). The recent development of the L(−)-enantiomer of bupivacaine, levobupivacaine, holds the potential of a better safety profile. However, while several studies show that levobupivacaine treatment is associated with a lower risk of cardiovascular toxicity (Mather and Chang, 2001; Morrison et al., 2000; Gristwood, 2002), there are only a few studies that compare the neurotoxic profile of levobupivacaine and bupivacaine. In sheeps, levobupivacane induces less CNS toxicity than racemic bupivacaine (Ladd and Mather, 2000; Santos and DeArmas, 2001), and higher doses of levobupivacaine are required for the induction of motor seizures, as compared with racemic bupivacaine (Huang et al., 1998); in addition, levobupivacaine induces lesser electroencephalography (EEG) abnormalities than bupivacaine in human volunteers (Arias, 2002) and causes less apnea and death when infused in rats (Srinivasa et al., 2003). How these drugs affect mechanisms of neuronal excitation under physiological and pathological conditions is unknown. Here, we have examined the comparative effects of racemic bupivacaine and levobupivacaine in an in vitro model of excitotoxic death (mixed cultures of mouse cortical cells challenged with NMDA) and in an in vivo model of chemically induced seizures, i.e. NMDA-induced motor seizures in mice.

2. Materials and methods

2.1. Materials

Levobupivacaine was bought from Chirocaine; Purdue Pharma LP (Stamford, CT, USA), bupivacaine and all other drugs were bought from Sigma (Milano, Italy).

2.2. In vivo studies

Animal studies were performed according to the European Community guidelines for the use of experimental animals. CD1 mice, 30–35 g body weight, were used. We induced epileptic seizures by injecting mice with NMDA. This model is widely used to test the activity of anticonvulsant drugs (Sofia et al., 1994; Velisek and Mares, 1995; Villetti et al., 2001; De Sarro et al., 2003). Valproate in particular is protective against NMDA-induced seizures (Czuczwar et al., 1985; Kabova et al., 1999). Mice were divided into 6 groups (6 animals per group). All groups received one injection of NMDA (100 mg/kg, i.p.) with or without an injection of bupivacaine or levobupivacaine (both administered i.p. either a high dose, 36 mg/kg, or at a low dose, 5 mg/kg). All drugs were dissolved in saline (pH adjusted to 7.4 with NaOH). Mice were examined for the following 2 h by an observer who was unaware of the treatment. In NMDA-treated mice, seizures developed through a sequence of paroxysmal scratching, hypermotility and circling, tonic–clonic convulsions and, occasionally, death. The following semi-quantitative scale was used for the examination of seizure severity: 0=no response; 1=excessive grooming+paroxysmal scratching; 2=mild hypermotility; 3=extensive hypermotility and circling; 4=forepaw clonus+tail hypertonus; 5=generalized tonic–clonic convulsions; 6="status epilepticus" and death. The latency (in min) for partial (excessive grooming, scratching or hypermotility) or generalized (clonic or tonic–clonic convulsions) seizures was also determined.

2.3. In vitro studies

Mixed cortical cell cultures containing both neurons and astrocytes were prepared from fetal mice at 14–16 days of gestation, as described by Rose et al. (1992). Briefly, dissociated cortical cells were plated in 15 ml multiwell vessels (Falcon Primaria, Lincoln Park, NY) on a layer of confluent astrocytes, using a plating medium of MEM-Eagle's salts (supplied glutamine-free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, glutamine (2 mM), glucose (21 mM), and $NaHCO_3$ (25 mM). After 3–5 days in vitro, non-neuronal cell division was halted by a 1–3 day exposure to 10 μM cytosine arabinoside, and cultures were shifted to a maintenance medium identical to plating medium, but lacking fetal bovine serum. For the induction of excitotoxic neuronal death, cultures were exposed to a 10 min pulse with NMDA (100 μM) at room temperature in a solution containing (in mM): 120 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 1.8 $CaCl2_2$, 20 HEPES and 15 glucose (pH 7.4). Afterwards, cultures were extensively washed and incubated at 37 °C for the following 24 h in MEM-Eagles medium supplemented with 15.8 mM $NaHCO_3$ and 25 mM glucose. When present, bupivacaine and levobupivacaine (both at concentrations ranging from 1 to 1000 μM) were either combined with NMDA or applied to the cultures after the NMDA pulse. In the latter case, drugs were maintained in the medium for the following 24 h. Excitotoxic neuronal death was examined by trypan blue staining. Stained neurons were counted from 3 random fields per well with a phase contrast microscopy at 100–400×.

3. Results

3.1. In vivo experiments

We compared the pro-convulsant effect of levobupivacaine and racemic bupivacaine in mice co-injected with NMDA. All control mice injected with NMDA (100 mg/kg i.p.) showed generalized tonic–clonic convulsions, but none of them reached scores 5 or 6 of seizure severity (generalized tonic–clonic convlusions, status epilepsicus, and death) during the 2-h observation period (see Tables 1 and 2). Bupivacaine and leobupivacaine were combined with NMDA either at low or high doses (5 or 36 mg/kg, i.p., respectively). High doses of both racemic bupivacaine and levobupivacaine potentiated NMDA-induced seizures in mice (Tables 1 and 2; Fig. 1). This was expected because doses of 36 mg/kg are close to the

Table 1
Effects of low or high doses of levobupivacaine and racemic bupivacaine on the latency of NMDA-induced seizures in mice

Latency to:	Partial seizures (min) (scores 1 to 4)	Generalized seizures (min) (scores 5 to 6)
NMDA	12±0.8	20±3
+*Bupivacaine*		
5 mg/kg	5±0.7[a]	26±2
36 mg/kg	2.5±0.3[a]	12±3[a]
+*Levobupivacaine*		
5 mg/kg	13±0.4	No seizures
36 mg/kg	2±0.4[a]	13±2[a]

Values are the means+S.E.M. of 12 values from 2 individual experiments.
[a] $P<0.05$ vs. controls (one-way ANOVA+Fisher's probability at the least significant difference, PLSD).

reported median convulsant dose (CD_{50}) for bupivacaine in mice (de Jong and Bonin, 1980). However, the percentage of mice with extremely severe seizures was higher in mice treated with NMDA plus bupivacaine than in mice treated with NMDA plus levobupivacaine (Table 2). These data are of some clinical interest as the anaesthetic potency of levobupivacaine is considered equivalent to that of racemic bupivacaine (Lyons et al., 1998; Vladimirov et al., 2000). Fifty percent of mice treated with levobupivacaine plus NMDA, and 70% of mice treated with bupivacaine plus NMDA died during the observation period (Table 2). When injected at lower doses (5 mg/kg), levobupivacaine did not affect the latency of partial seizures (Table 1), but reduced the number of mice showing forepaw clonus and tail hypertonus (score 4) (Table 2), although it produced only a trend to a reduction in the total seizure score, as compared with mice treated with NMDA alone (Fig. 1). In contrast, 5 mg/kg of racemic bupivacaine decreased the latency to partial seizures (Table 1), and increased the number of mice showing severe seizures (scores 4 to 6) as compared with mice treated with NMDA alone or NMDA+5 mg/kg of levobupivacaine (Table 2). One out of 6 mice died within the group treated with 5 mg/kg of bupivacaine and NMDA, whereas no deaths were observed in the group treated with NMDA and 5 mg/kg of levobupivacaine. The total seizure score of mice treated with NMDA+5 mg/kg of bupivacaine was significantly higher than the score of mice treated with NMDA+5 mg/kg of levobupivacaine (Fig. 1).

Table 2
Comparative effects of low and high doses of levobupivacaine and racemic bupivacaine on NMDA-induced seizures in mice

	Seizure severity score				
	2	3	4	5	6
NMDA (100 mg/kg)	–	6/12	6/12	–	–
+levobupivacaine, 5 mg/kg	2/12	8/12	2/12	–	–
+bupivacaine, 5 mg/kg	–	–	7/12	4/12	1/12
+levobupivacaine, 36 mg/kg	–	–	3/12	3/12	6/12
+bupivacaine, 36 mg/kg	–	–	1/12	3/12	8/12

Mice were examined for 2 h following drug injections by an observer who was unaware of the treatment.

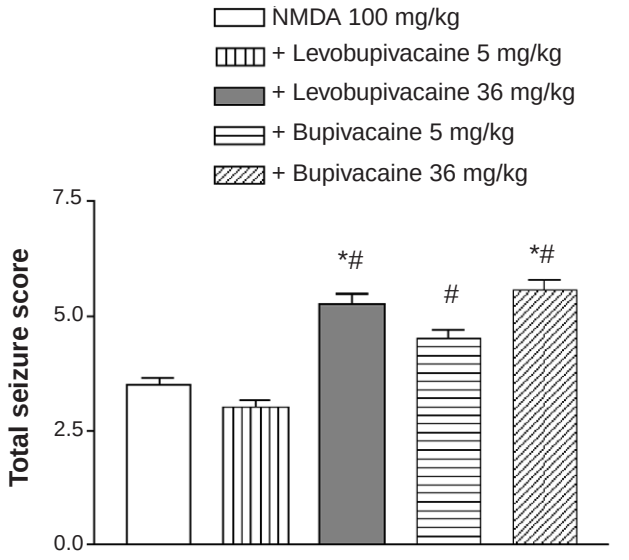

Fig. 1. Comparative effects of low and high doses of levobupivacaine and bupivacaine on total seizure score in mice treated with NMDA. Means± S.E.M. of 12 values from 2 individual experiments are shown. For statistical analysis, we have calculated the medians with 25th and 75th percentiles; then, the Kruskal–Wallis non-parametric ANOVA was used followed by the Dunns test to isolate the differences. $P<0.05$ vs. NMDA alone (*) or vs. levobupivacaine, 5 mg/kg (#).

3.2. In vitro experiments

We also examined the comparative effect of levobupivacaine and bupivacaine in an in vitro model of excitotoxic neuronal death, i.e. in mixed cultures of mouse cortical cells challenged with NMDA. These cultures, in which cortical neurons are plated over a monolayer of astrocytes, represent one of the most validated models for the study of excitotoxicity in vitro because they express functional NMDA receptors and carry the advantage of preserving the physiological interplay between neurons and astrocytes (Rose et al., 1992). Exposure of cultures to 100 μM NMDA for 10 min killed 70–80% of the neuronal population, as assessed by trypan blue staining 24 h later. In this in vitro model, both levobupivacaine and bupivacaine were neuroprotective rather than neurotoxic, and reduced NMDA toxicity when applied to the cultures either during or immediately after the excitotoxic pulse (Fig. 2A,B). However, levobupivacaine showed a greater potency as a neuroprotectant (i.e. it was active at lower concentrations as compared to bupivacaine), and was still protective at concentrations as high as 3 mM, which are well above the plasma security threshold for the drug in vivo (Arias, 2002). In contrast, no protection against NMDA toxicity was observed with 3 mM bupivacaine (Fig. 2A,B).

4. Discussion

Bupivacaine, a local anaesthetic, possesses steroisomeric properties that have been related to the cardiovascular and neurological toxicity of the drug. Within the last few years, the L (−) isomer of bupivacaine, levobupivacaine, has been introduced into clinical practice in the attempt of reducing the side effects of the racemic formulation. Induction of

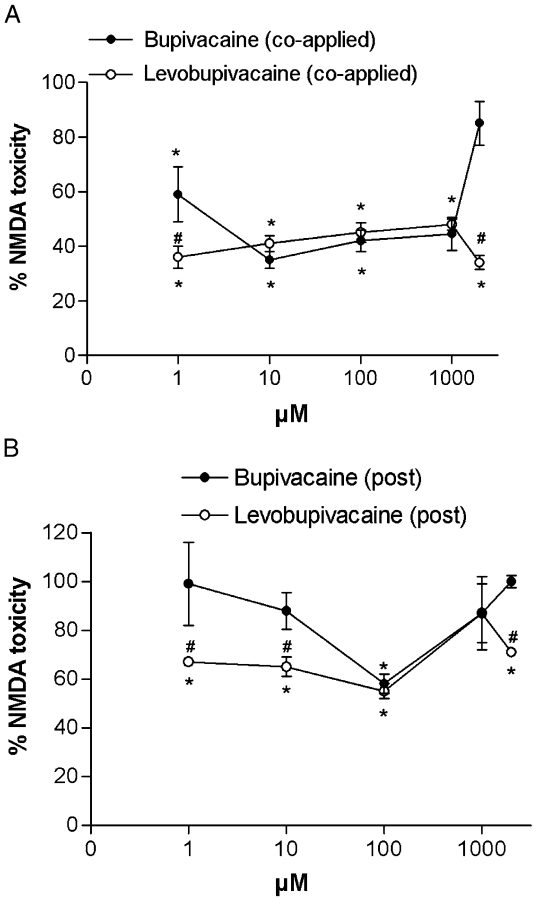

Fig. 2. Comparative effects of racemic bupivacaine and levobupivacaine on NMDA neurotoxicity in mixed cultures of mouse cortical cells. Drugs were applied to the cultures either for 10 min in combination with 100 μM NMDA (A) or immediately after the NMDA pulse (B). In latter case, drugs were maintained in the medium for 24 h. In control cultures, the 10 min pulse with NMDA killed about 75–80% of the neuronal population. This value was set as 100% of NMDA toxicity. Values are the means+S.E.M. of 6–8 determinations from 2 individual experiments. $P<0.05$ (one-way ANOVA and Fisher's PLSD) vs. or NMDA alone (*) or racemic bupivacaine+NMDA (#).

seizures is one of the major concerns related to the neurotoxic adverse effects of local anaesthetics. This apparent paradoxical effect results from mechanisms of disinhibition, due to the blockade of voltage-sensitive Na^+ on inhibitory γ-aminobutirric acid (GABAergic) neurons, or from a direct stimulation of excitatory neurons resulting from the blockade of several types of K^+ channels. Electrophysiological studies have shown that the D (+) isomer of bupivacaine is a more potent and faster blocker of voltage-gated Na^+ channels than the L (−) isomer (Arias, 2002), and inactivates selective subtypes of ATP-dependent and voltage-dependent $K^+(K_v)$ channels, with greater potency than the L (−) isomer (Kawano et al., 2004; Franqueza et al., 1997). Our results with the NMDA model of secondarily generalized seizures support the hypothesis of a lower neurotoxicity of levobupivacaine with respect to racemic bupivacaine. The trend to a protection against generalized seizures observed with 5 mg/kg of levobupivacaine suggests that the drug, at this relatively low dose, reduces the activity of excitatory neurons in the CNS. In contrast, the reduced latency to partial NMDA seizures observed with 5 mg/kg of racemic bupivacaine is consistent with the ability of the D (+) isomer to inhibit several types of K^+ channels. Among these, voltage-gated delayed rectifier-type channels, including $K_v1.5$, and -2.1, are widely expressed in the CNS and mediate membrane repolarizarion in neurons (Pan et al., 2004). Bupivacaine stereoselectively blocks $K_v1.5$ subtype of delayed rectifier-type K channels, with the D (+) enantiomer showing a much greater potency (about 7-fold) than the L (−) enantiomer (Franqueza et al., 1997; Luzhkov et al., 2003). Blockade of these K^+ channels by the D (+) isomer may prolong membrane depolarisation, thus facilitating the activation of NMDA receptors because the Mg^{2+} blockade of the NMDA channel is voltage-sensitive. This would account for the pro-convulsant effect of 5 mg/kg of racemic bupivacaine combined with NMDA in mice. At higher doses (36 mg/kg) both levobupivacaine and racemic bipivacaine enhanced NMDA seizures, but seizure severity was higher in the group treated with racemic bupivacaine. It is likely that disinhibition prevails with high doses of L (−) and D (+) bupivacaine, but that the D (+) isomer produces an additional excitation resulting from the blockade of K^+ channel. The more favourable profile of levobupivacaine was confirmed by data obtained with the in vitro model of excitotoxic neuronal death, in which both levobupivacaine and racemic bupivacaine were neuroprotective rather than neurotoxic. This unexpected finding might reflect the neuronal composition of our cell cultures, in which inhibitory GABAergic neurons are only a minority (about 10%) of the total neuronal population, as assessed by glutamate decarboxilase-65 (GAD-65) immunostaining (Battaglia et al. 2001). Thus, in these cultures the L (−) isomer of bupivacaine might preferentially reduce the activity of excitatory neurons, thus reducing the extent of neurotoxicity. This action, once again, might be counterbalanced by the inhibition of K^+ channels produced by the D (+) isomer with two consequences: (i) the lower potency of racemic bupivacaine (with respect to leveobupivacaine) in protecting neurons against NMDA toxicity; and (ii) the loss of neuroprotection observed when very high doses of bupivacaine (3 mM) were applied to the cultures. Contrary to our expectation, levobupivacaine maintained its neuroprotective activity even at the dose of 3 mM, suggesting that the nature of drug is primarily neuroprotective when the population of inhibitory neurons is low (a situation that, however, does not occur in vivo). Taken collectively, our data supports the hypothesis that levobupivacaine and racemic bupivacaine differ substantially in their safety profile, with the former being less pro-convulsant in vivo and consistently neuroprotective in a cellular model of excitotoxic neuronal death in which mechanisms of disinhibition are unlikely to occur. This should not give the wrong impression that levobupivacaine

is totally devoid of central toxicity because there are case reports that the drug may also induce seizure activity (Crews and Rothman, 2003).

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