Abstract

Purpose  Bupivacaine-induced myotoxicity is associated with mitochondrial bioenergetic alterations. The impact of the duration of bupivacaine treatment on mitochondrial energy production remains undetermined. Here, we assessed, in vivo, the alteration of mitochondrial metabolism following different durations of bupivacaine exposure (40, 56, or 112 hr) that correspond to 5, 7, or 14 repeated injections of 0.25% bupivacaine, respectively.

Methods  Rats were divided randomly into seven different groups: one control group (no catheter); three groups with normal saline injections (1 mL kg⁻¹ every eight hours) via a femoral nerve catheter for 40, 56, and 112 hr, respectively; and three groups with 0.25% bupivacaine injections (1 mL kg⁻¹) every eight hours via a femoral nerve catheter for 40, 56, and 112 hr. Psoas and gracilis muscle samples located within the bupivacaine infusion-diffusion space were investigated. To estimate mitochondrial respiratory capacity, the protein content of the mitochondrial respiratory chain apparatus was evaluated by measuring citrate synthase activity. To measure mitochondrial respiratory function, adenosine diphosphate-stimulated oxygen consumption was measured by polarography in saponin-skinned muscle fibres using glutamate-malate or succinate as energy substrates.

Results  In psoas and gracilis muscles, saline solution had no effect on the two mitochondrial parameters. Bupivacaine induced a significant decrease in the citrate synthase activity.
Conclusion

La gravité de la myotoxicité induite par bupivacaine est étroitement liée à la durée d’exposition à la bupivacaine dans les fibres musculaires situées à proximité de la pointe du cathéter.

Methods

This study, including care of the animals involved, was conducted according to the official edict presented by the French Ministry of Agriculture (Paris, France), in keeping with the recommendations of the Helsinki Declaration, and with approval of the local institutional animal care and use committee. All experiments were conducted in an authorized laboratory and under the supervision of an authorized researcher (Nouette-Gaulain).
Chemicals

Plain 0.25% bupivacaine hydrochloride (7.5 mM) was purchased from Astra Zeneca (Rueil-Malmaison, France) for rat administration. All other reagents were purchased from Sigma-Aldrich (Saint-Louis, MO, USA).

Rat model

Experiments were conducted on adult male Wistar rats, 10–12 weeks old weighing 200-240 g. The rats were housed in a regulated facility with a 12-hr light/12-hr dark cycle; they were fed with chow and were allowed free access to tap water. After anesthesia with intraperitoneal pentobarbital sodium 40 mg/kg, a 20G plexus catheter with a 0.9 mm outer diameter (Pajunk, Geisingen, Germany) was inserted under the inguinal ligament near the left femoral nerve sheath, as previously described.6

In the first phase of the experiment, 12 rats were used for measurement of bupivacaine accumulation in muscle. Then, 41 rats were divided into seven different groups: 1) the first group (n = 5) was the control group (rats without catheter); 2) three groups (n = 6 per group) received normal saline injections (1 mL·kg⁻¹ with a delay of eight hours between injections) via the femoral nerve catheter, for total durations of 40, 56, and 112 hr, i.e., five, seven, or 14 injections, respectively; and 3) three groups (n = 6 per group) received 0.25% bupivacaine injections (1 mL·kg⁻¹, with a delay of eight hours between injections) via the femoral nerve catheter, for total durations of 40, 56, and 112 hr, i.e., five, seven, or 14 injections, respectively.

In the bupivacaine groups, a decrease in pinprick sensation was induced in the cutaneous distribution of the femoral nerve within an hour after each injection, but there was not a complete motor blockade. The rats were killed by cervical dislocation eight hours after the last perineural injection when bupivacaine concentration in psoas muscles was below the threshold of detection, i.e., <0.3 μg·g⁻¹ of tissue.6

Measurement of bupivacaine accumulation in rat muscle

To confirm the presence of bupivacaine in the gracilis muscle, residual bupivacaine concentrations in the gracilis muscle were measured at one and eight hours after the seventh injection, as published in a previous study for psoas muscle.6 However, in the case of this experiment, the rats were sacrificed one hour (n = 6) or eight hours (n = 6) after the seventh injection, and a large piece of gracilis muscle was removed to measure bupivacaine concentrations. These concentrations were determined using a high performance chromatographic (HPLC) method, as described previously.6 Briefly, standard tissue samples containing known amounts of bupivacaine were prepared by spiking homogenized tissues from 1 mg·mL⁻¹ methanol stock solutions of bupivacaine to yield concentrations from 100-1,000 ng·mL⁻¹. The tissue samples were washed in cold isotonic buffer and were then blotted, weighed, and quickly frozen at −80°C. Before analysis, the tissue samples were diluted (1/2; weight of solute per volume [w/v]) and homogenized in a physiological buffer with a mixer. Pentacaine 100 μL (internal standard, at 5 μg·mL⁻¹ in distilled water) and NaOH 1 N 200 μL were added to 500 μL of homogenized tissue. The mixture was extracted with 6 mL of ethylacetate by rotative shaking for 20 min. After centrifugation, the HPLC system consisted of a constant flow pump, M 510, a 717 plus autoinjector (Waters Corp, Milford, MA, USA), a UV 1,000 ultraviolet model detector, and a Chromjet integrator (Thermoquest, San Jose, CA, USA). The chromatographic separation was performed at room temperature on an XTerra RP 18 analytical column (Waters, Saint Quentin en Yvelines, France) (150 mm × 4.6 mm; 5 μm particle size). The mobile phase consisted of a binary mixture (17/83, volume of solute per volume of solvent [v/v]) of acetonitrile and potassium dihydrogen phosphate buffer (0.01 M, adjusted pH at 2.1 with concentrated orthophosphoric acid) with a 2 mL·min⁻¹ flow rate. Compounds were chromatographed at 210 nm within 12 min. For bupivacaine determination, intraday precision ranged from 3.27–5.05%, and interday precision ranged from 8.1–12.8% with less than 11% bias. The lower limit of quantification was 100 ng·mL⁻¹ (300 ng·g⁻¹ of tissue).

Bioenergetics investigations: adenosine diphosphate-stimulated oxygen consumption and citrate synthase activity

Eight hours after the last injection, psoas and gracilis muscles were quickly dissected adjacent to the femoral nerve and placed in a normoxic (i.e., equilibrated with air) cooled (4°C) relaxing solution (solution 1: 10 mM EGTA, 3 mM Mg²⁺, 20 mM taurine, 0.5 mM dithiothreitol, 5 mM adenosine triphosphate (ATP), 15 mM phosphocreatine, 20 mM imidazole, and 0.1 M K⁺-2-[N-morpholino]ethane sulfonic acid, pH 7.2). To assess mitochondrial respiration in situ, we used a permeabilized muscle fibre technique routinely used for the biochemical diagnostic of OXPHOS disorders.8 Bundles of 2-5 mg fibres were excised from the surface of the muscle and then permeabilized in solution I added with saponin 50 μg·mL⁻¹. Each time, the bundle was then washed twice for ten minutes in solution 2 (10 mM EGTA, 3 mM Mg²⁺, 20 mM taurine, 0.5 mM dithiothreitol, 3 mM phosphate, 1 mg·mL⁻¹ fatty acid-free bovine
serum albumin, 20 mM imidazole, and 0.1 M K\(^+\). Bupivacaine mitochondrial toxicity and time 839

glycolysis or adenylate kinase), and ATP hydrolysis. After cuvette to inhibit extramitochondrial ATP synthesis (via l

mM EDTA, pH 7.2. The homogenate was then cen-

225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl,

group size to six animals to allow for exclusion due to catheter displacement.

Results

Rat analgesia protocol

Forty-eight rats were anesthetized and no self-mutilation was observed following catheter placement. Another five rats without catheter were investigated as control, for a total of 53 rats. The catheters were inserted into perimysial conjunctive tissue and between muscle fibres without destruction, and they reached the vicinity of the femoral nerve where bupivacaine was released. Two rats with catheter displacement were excluded from our analysis (one in saline 112-hr protocol and one in the group required to measure bupivacaine concentration 8 hr after the seventh injection). Bupivacaine muscle concentration in gracilis muscle was 8.06 (0.5, 62.4) \(\mu g \cdot g^{-1}\) of tissue one hour after the seventh injection (\(n = 6\) rats). It decreased
below the detection threshold (< 300 ng.g−1 of tissue) eight hours after the seventh injection (n = 5 rats).

Citrate synthase activity

Repeated saline injections had no significant effect on citrate synthase activity on psoas (r² = 0.00; P = 0.68 with linear regression) or on gracilis muscle (r² = 0.00; P = 0.67). Bupivacaine induced a significant decrease of the citrate synthase activity with time, both in psoas (r² = 0.74; P < 0.001) and in gracilis muscle (r² = 0.52; P < 0.001) (Fig. 1).

Adenosine-stimulated oxygen consumption

Similarly, we observed a significant decrease in adenosine diphosphate-stimulated oxygen consumption in the bupivacaine groups (Fig. 2). After repeated injections of bupivacaine with glutamate as substrate, adenosine diphosphate-stimulated oxygen consumption was decreased in psoas muscle by 30%, 41%, and 75% of the control value after the 40th, the 56th, and the 112th hr, respectively (r² = 0.84; P < 0.001). In the same way, bupivacaine with glutamate induced a decrease of adenosine diphosphate-stimulated oxygen consumption in gracilis muscle by 21%, 66%, and 76% of the control value after the 40th, the 56th, and the 112th hr, respectively (r² = 0.72; P < 0.001). Similar findings were observed in both muscles with succinate as substrate. The duration of bupivacaine protocol triggered a reduction of adenosine diphosphate-stimulated oxygen consumption in muscle that was closely linked to a decrease in citrate synthase activity. This time-dependent effect caused by bupivacaine was observed without distinction between both muscles (psoas and gracilis).

Discussion

In this study, we evaluated the influence of the duration of the bupivacaine exposure on the severity of mitochondrial inhibition. We used a rat model of perineural analgesia, validated in previous studies, that permitted the evaluation of the toxicity of protocols similar to those used in clinical practice. In the present work, we observed that the mitochondrial adenosine diphosphate-stimulated oxygen consumption and citrate synthase activity were significantly reduced in rat muscle exposed to bupivacaine compared with rat muscle injected with a saline solution. Energy transduction requires the consumption of oxygen by the respiratory chain to oxidize the reduced equivalents (nicotinamide adenine dinucleotide [NADH] and 1,5-dihydro-flavin adenine dinucleotide [FADH2]) produced by the intermediary metabolism (Krebs cycle, beta-oxidation) during the degradation of the nutrients. Depending on the energy demand by the cell and the energy supply, the mitochondrial respiratory chain can work at low regime (resting-state or state 4) where oxygen consumption is low, ATP synthesis does not occur, and NADH oxidation serves to maintain the membrane potential at high values. When the need for cellular ATP is increased, the concentration of ADP is elevated and stimulates the F1-F0ATPsynthase, which in turn decreases the membrane potential and further activates mitochondrial respiration. In these conditions of high respiratory rate (state 3, phosphorylating), ATP is produced. In our study, we measured the adenosine diphosphate-stimulated oxygen consumption, which gives a measure of the capacity of mitochondria to produce energy; this capacity is reduced following exposure to bupivacaine. This capacity also depends on the content of mitochondria per cell, and this parameter can be evaluated by different means. In particular, the amount of mitochondrial respiratory chain complex can

![Fig. 1](image-url) Effects of repeated injections of bupivacaine or saline on citrate synthase activity vs time in psoas (A) and gracilis (B) muscle. Each symbol represents an animal in the saline (open circle) and bupivacaine (dark circle) groups. Results of linear regression analysis were expressed as r² and P values for the saline and bupivacaine groups. Six different rats were investigated in each group (except in the saline 112-hr protocol, n = 5 rats per group).
be evaluated by measuring the activity of the citrate synthase, an enzyme located within the mitochondrial matrix. Previous studies in rat tissues demonstrated that the activity of citrate synthase is proportional to the protein content of respiratory chain complexes measured by Western blot. In our study, we measured the citrate synthase activity to understand whether the observed changes in mitochondrial oxygen consumption could be attributed to changes in the mitochondrial content.

We compared the capacity for mitochondrial energy production in the muscle of rats submitted to three protocols (40, 56, or 112-hr protocol duration), including repeated injections of 0.25% bupivacaine 1 mL kg⁻¹. This concentration of bupivacaine was chosen from a previous study that indicated moderate mitochondrial toxicity and muscle structural alterations.

Our observations indicate that the observed decline in mitochondrial adenosine diphosphate-stimulated oxygen consumption was explained by two additive mechanisms: 1) the inhibition of oxidative phosphorylation (as measured by the reduction in adenosine diphosphate-stimulated oxygen consumption); and 2) the reduction of the muscle mitochondrial content (as measured by the reduction of citrate synthase activity and previous studies that validated the use of this marker to follow the respiratory chain content of various tissues). For instance, the citrate synthase activity decreased by 50% in rats subjected to bupivacaine injections for 112 hr, and mitochondrial adenosine diphosphate-stimulated oxygen consumption measured in situ was further reduced by 80% when compared with the control group. In a previous study, we discussed the different modes of bupivacaine inhibition of mitochondrial energy metabolism, including 1) the specific inhibition of mitochondrial respiratory chain complex I (as observed on isolated mitochondria); 2) OXPHOS uncoupling; 3) the specific inhibition of the mitochondrial F1-F0-ATP synthase; 4) the decrease of mitochondrial membrane electric potential; 5) the fragmentation of the mitochondrial network; and 6) the possible onset of mitoptosis. In this study, we identified a novel mechanism, i.e., the strong reduction of the respiratory chain protein content that can be observed from long-lasting exposure to bupivacaine. Our study helps to delineate the spectrum of bupivacaine mitochondrial toxicity as the alteration of mitochondrial energy production progresses from respiratory chain kinetic and thermodynamic inhibition (reduction of the state 3 respiration) to more global organelle membrane alterations and to the ultimate reduction of mitochondrial respiratory chain content (>40 hr exposure to bupivacaine). This decrease in citrate synthase activity could be linked to the appearance of morphological signs of mitochondrial autophagy (mitophagy) induced by high doses (>5 mM) of bupivacaine. This decrease following injections of high concentrations of bupivacaine in rat muscle could be closely associated with disjoined fibres, interstitial edema, infiltrating cells, and a wide range of morphological fibre abnormalities ranging from absent, to focal, to moderate, to extreme.

The above discussed bupivacaine-induced mitochondrial inhibition showed a time dependency on the duration of bupivacaine exposure in psoas muscle. Interestingly, this effect was also observed in the gracilis muscle. Our findings confirmed the presence of bupivacaine in gracilis...
muscle. A mean bupivacaine concentration of 30 μM was measured in psoas muscle one hour after the seventh injection, and bupivacaine concentration in gracilis muscle was not significantly different. However, it was not possible, in the strict diffusion space, to obtain a precise measurement of the local concentration of bupivacaine in each muscle due to the limited sensitivity of the high-performance liquid chromatography system that required a larger biopsy. Thus, our findings seem to demonstrate that bupivacaine induced a marked focal degeneration of skeletal muscle tissue that was time-dependent for muscle fibres close to the nerve catheter.17,18

We have previously described this rat model and validated its use for mitochondrial investigations in prior studies.1,2,15 No complete motor blockade was observed with this protocol, and only a decrease in pinprick sensation was induced.6 We have arbitrarily chosen two other times, i.e., just prior to 40 hr and a long time after 112 hr. Although continuous or basal-bolus infusion is recommended in clinical practice, a bolus repeated every eight hours is better adapted to our experimentation on rats.19,20

Bupivacaine-induced myotoxicity is time-dependent for muscle fibres close to the nerve catheter and is concentration-dependent on mitochondria in vitro.4,21 This finding suggests the need for clarifying analgesia protocols with optimal duration of protocol and probably optimal concentration of local anesthetic, as investigated for ropivacaine.22 Chemical properties of local anesthetics are also important parameters for the occurrence of ultrastructural alterations in muscle,18 but similar effects are not yet described in mitochondria toxicity in vivo.6

In conclusion, our data suggest that doses of bupivacaine routinely used in clinical practice alter mitochondrial metabolism in rat muscle. The strong dependency of bupivacaine-induced myotoxicity on the duration of the treatment indicates that analgesia protocol should be optimized according to this parameter.

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Conflict of interest None declared.

References


