Local Anesthetic Cardiac Toxicity Is Mediated by Cardiomyocyte Calcium Dynamics

Julia Plakhotnik, B.Sc., Libo Zhang, M.D., Ph.D., Marvin Estrada, R.V.T., John G. Coles, M.D., Per-Arne Lonnqvist, M.D., Ph.D., Jason T. Maynes, M.D., Ph.D.

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EDITOR’S PERSPECTIVE

What We Already Know about This Topic

- Use of local anesthetics for perioperative pain control can be complicated by cardiotoxicity that may result in serious cardiac complications such as cardiac arrest.
- There is variability in cardiac toxicity of local anesthetics, with increased cardiotoxicity encountered with longer-acting (e.g., bupivacaine) versus shorter-acting local anesthetics (e.g., ropivacaine). The mechanism for this differing propensity to cardiac toxicity is not well understood.

What This Article Tells Us That Is New

- This study used human induced pluripotent stem cell–derived cardiomyocytes and found significantly altered cardiomyocyte calcium dynamics with bupivacaine but not ropivacaine. Calcium supplementation restored normal cardiomyocyte rhythm to bupivacaine-treated tissue. Calcium channel blockade selectively worsened bupivacaine cardiotoxicity.
- This study used a rat model (female) of cardiac toxicity and found that pretreatment with calcium improved survival of bupivacaine-treated rats and reduced survival of ropivacaine-treated rats.

Through peripheral neuron sodium channel (e.g., Na\textsubscript{v}1.7) blockade, local anesthetics provide regional anesthesia within a nerve distribution, reducing perioperative systemic pain medication use. However, systemic anesthetic administration can occur via vascular uptake proximal to the anesthetized nerves, and/or inadvertent direct injection into blood vessels. This results in

ABSTRACT

Background: Long-lasting local anesthetic use for perioperative pain control is limited by possible cardiotoxicity (e.g., arrhythmias and contractile depression), potentially leading to cardiac arrest. Off-target cardiac sodium channel blockade is considered the canonical mechanism behind cardiotoxicity; however, it does not fully explain the observed toxicity variability between anesthetics. The authors hypothesize that more cardiotoxic anesthetics (e.g., bupivacaine) differentially perturb other important cardiomyocyte functions (e.g., calcium dynamics), which may be exploited to mitigate drug toxicity.

Methods: The authors investigated the effects of clinically relevant concentrations of racemic bupivacaine, levobupivacaine, or ropivacaine on human stem cell–derived cardiomyocyte tissue function. Contractility, rhythm, electro-mechanical coupling, field potential profile, and intracellular calcium dynamics were quantified using multielectrode arrays and optical imaging. Calcium flux differences between bupivacaine and ropivacaine were probed with pharmacologic calcium supplementation or blockade. In vitro findings were correlated in vivo using an anesthetic cardiotoxicity rat model (females; n = 5 per group).

Results: Bupivacaine more severely dysregulated calcium dynamics than ropivacaine in vivo (e.g., contraction calcium amplitude to 52±11% and calcium-mediated repolarization duration to 122±7% of ropivacaine effects, model estimate ± standard error). Calcium supplementation improved tissue contractility and restored normal beating rhythm (to 101±6%, and 101±26% of control, respectively) for bupivacaine-treated tissues, but not ropivacaine (e.g., contractility at 80±6% of control). Similarly, calcium pre-treatment mitigated anesthetic-induced arrhythmias and cardiac depression in rats, improving animal survival for bupivacaine by 8.3±2.4 min, but exacerbating ropivacaine adverse effects (reduced survival by 13.8±3.4 min and time to first arrhythmia by 12.0±2.9 min). Calcium channel blocker nifedipine coadministration with bupivacaine, but not ropivacaine, exacerbated cardiac toxicity, supporting the role of calcium flux in differentiating toxicity.

Conclusions: Our data illustrate differences in calcium dynamics between anesthetics and how calcium may mitigate bupivacaine cardiotoxicity. Moreover, our findings suggest that bupivacaine cardiotoxicity risk may be higher than for ropivacaine in a calcium deficiency context.

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local anesthetic systemic toxicity estimated at 0.27 times per 1,000 nerve blocks. Certain techniques, such as erector spinae plane block, may pose greater risks due to rapid systemic anesthetic absorption via the lymphatic. Toxicity typically presents with central nervous system and/or cardiovascular system symptoms, including seizures, arrhythmias, and contractile depression. The latter manifestations can lead to lethal cardiovascular collapse, observed in 5 to 10% of cases. To minimize patient risk, the clinically used anesthetic doses are limited. Concern over race-specific cardiotoxicity prompted development of less cardiotoxic long-acting anesthetics (e.g., ropivacaine, levobupivacaine). Although many anesthetics can cause cardiotoxicity, bupivacaine demonstrates a higher toxicity risk in human and animal studies and in clinical case reports. Current cardiotoxicity treatments are limited to specific cardiopulmonary support and intravenous lipid emulsion administration.

Preclinical investigations describe the Na\textsubscript{v}1.5 cardiac sodium channel blockade as the canonical cardiotoxicity mechanism due to Na\textsubscript{v}1.5 structural similarity to the intended target neuronal channels. However, human Na\textsubscript{v}1.5 in vitro binding studies show only modest channel affinity differences between bupivacaine and ropivacaine. Besides Na\textsubscript{v}1.5 binding, some anesthetics were shown to interact with cardiac L-type calcium channels and inhibit potassium repolarization currents. β-Adrenergic-mediated cAMP production blockade and mitochondrial dysfunction, including acylcarnitine exchange inhibition and direct effects on electron transport, were also noted, albeit at concentrations well above clinical utility. Anesthetic dose inconsistencies and model variability (including described proteomic and physiologic differences between animal models and humans) make it challenging to translate observed discrepancies in cardiotoxicity mechanisms into clinical utility. This issue applies more broadly to many pharmaceuticals demonstrating unexpected postmarket cardiotoxicity.

To assess cardiotoxicity risk and improve clinical translation, human induced pluripotent stem cell–derived cardiomyocytes can be used to supplement animal data. We and others have shown that these cells provide a cardiomyocyte model that exhibits a human physiologic ion channel and contractile protein profile, yielding expected responses to cardiotoxic drugs. To identify potential toxicity risks, we sought to elucidate and contrast the mechanisms involved in local anesthetic cardiotoxicity using stem cell–derived cardiomyocyte tissues. We hypothesized that accompanying canonical Na\textsubscript{v}1.5 blockade, bupivacaine and ropivacaine divergently perturb additional key cardiomyocyte processes, such as calcium dynamics, consistent with observed in vivo cardiotoxicity differences. These mechanisms could potentially be exploited for context-specific toxicity prevention. Therefore, we evaluated bupivacaine and ropivacaine impact on contractility and arrhythmogenesis in human-based tissues and a rat model, contrasting calcium supplementation effects. To further test the role of calcium flux in anesthetic-specific cardiotoxicity, we compared the effects of calcium supplementation or calcium channel blockade on cardiomyocyte function and intracellular calcium dynamics.

**Materials and Methods**

**Tissue Culture**

Human induced pluripotent stem cell–derived cardiomyocytes (iCell Cardiomyocytes; Cellular Dynamics International, USA) were thawed using Plating Medium (Cellular Dynamics) and cultured per manufacturer instructions in Maintenance Medium (Cellular Dynamics). Culture media contain galactose instead of glucose to encourage energy production through oxidative phosphorylation, promoting cardiomyocyte maturation. Microplates were coated with a mixture of gelatin (0.25 mg/ml), fibronectin (10 µg/ml), and laminin (5 µg/ml) before cell seeding.

**Multielectrode Array Recordings of Tissue Function**

Human induced pluripotent stem cell–derived cardiomyocytes were seeded onto 48-well Cardio ECR E-plates (Acea Biosciences, USA) at 45,000 cells/well. Cell contractility (impedance) and electrical activity (field potential) were simultaneously monitored using the xCELLigence RTCA Cardio ECR (Acea Biosciences). Beat interval deviation was calculated as the median absolute deviation of beat-to-beat intervals, corrected for beating rate as per Monfredi et al. After monolayer formation and stable beating (5 days), tissues were further matured by electrical stimulation (1, 1.5, and 2 Hz consecutively for 5 days each), then allowed rest to stabilize beating (at least 1 day) before drug treatment. Recordings (1-min duration, at 10- to 20-min intervals) were captured at baseline before drug treatment/cotreatment, then starting again at 5 h after drug treatment to minimize the disturbance of handling the tissues. For dose–response experiments, bupivacaine or ropivacaine were used at 0.1 to 9 µg/ml, or levobupivacaine at 0.1 to 6 µg/ml, corresponding to 0.3 to 27.7 µM bupivacaine, 0.3 to 28.9 µM ropivacaine, or 0.3 to 18.5 μM levobupivacaine; n = 6 to 9 measurements per three to four tissues per concentration. For calcium cotreatment experiments, bupivacaine or ropivacaine were used at 6 µg/ml (18.5 and 19.3 µM, respectively); n = 6 to 12 measurements per two to four tissues per concentration. For treatment causing beating arrest (all tissues at 9 µg/ml bupivacaine), the amplitude of either the impedance or the field potential noise signal was used in amplitude analysis to obtain a baseline value, but excluded from analysis of other metrics.
Functional Tissue Analysis Using Optical Imaging

Induced pluripotent stem cell–derived cardiomyocytes were seeded at 20,000 cells/well on microplates coated with a silicone polymer (Young’s modulus 5 kPa), mimicking healthy myocardial stiffness, and cultured for 4 weeks to allow tissue formation and maturation. Maturation of the calcium-handling sarcoplasmic reticulum structure was verified by immunostaining sample tissues for the sarcoendoplasmic reticulum calcium ATPase (Supplemental Figure S1, http://links.lww.com/ALN/C936). Videos were captured for 12 s using a Zeiss Axio Observer 7 epifluorescence microscope equipped with automated stage control (allowing multipoint acquisition) and environmental darkroom chamber (kept at 37°C, 5% CO₂). For label-free functional assessment, baseline videos were captured at 60 frames per second in brightfield immediately before drug or cotreatment addition, then again 5 h after treatment. Bupivacaine or ropivacaine were used at 2, 4, or 6 µg/ml, corresponding to 6.1, 12.3, or 18.5 µM bupivacaine or 6.4, 12.9, or 19.3 µM ropivacaine; n = 2 to 6 measurements per three to four tissues per group. Cotreatment with 6 µg/ml bupivacaine and 50 nM nifedipine caused beating arrest (all three tissues), which were excluded from further analysis. For assessment of intracellular calcium dynamics, tissues were loaded with FLIPR5 intracellular calcium dye (Molecular Devices, USA, diluted 1:3 in Cellular Dynamics Maintenance Medium) 5 h after treatment. Dye addition was staggered to match exposure time before imaging, with total dye incubation time of 45 min per well. Videos were captured at 45 frames per second in a fully automated fashion, ensuring equal dye incubation and light source exposure settings per sample per experiment (488 nm excitation, 525 nm emission). Bupivacaine or ropivacaine were used at both 2 and 6 µg/ml, and at 6 µg/ml for calcium cotreatment experiments; n = 76 to 132 measurements per three to four tissues per group. Nifedipine cotreatment calcium dynamics, because 6 µg/ml bupivacaine caused arrest, 4 µg/ml anesthetic dose was used; n = 10 to 36 measurements per four tissues per group. Videos were processed using fully automated, custom, laboratory-developed R and Python software, based on published algorithms to track tissue motion and calcium flux during beating cycles. FLIPR5 fluorescence was normalized to minimum fluorescence for each field of view (i.e., relaxation baseline), representing relative intracellular calcium intensity during beating (F/F₀).

In Vivo Rat Model

Following published anesthetic cardiotoxicity models, female Sprague-Dawley rats (300 to 400 g, purchased from Lab Animal Services, Hospital for Sick Children, Toronto) were housed two per cage in a controlled environment (12 h light-dark cycle), with free access to food and water. Experiments were performed at the same time of day during daylight hours. Animals were anesthetized (2% isoflurane), intubated via tracheostomy, and mechanically ventilated (10 ml/kg tidal volume at 65 breaths/min). Rectal temperature was maintained 36.5° to 37.5°C using underbody warming. Animals were randomized to three pretreatment groups per cage (10 mg/kg CaCl₂ in saline vs. saline), with the experimenter blinded to pretreatment, and assessed in sequential order. The pretreatment regimen was intravenously infused (24-gauge tail vein catheter) over 5 min, then after 5 min rest, anesthetics (1% bupivacaine or ropivacaine in saline) were infused until asystole (2 mg·kg⁻¹·min⁻¹, five animals per group). Blood oxygen saturation, arterial blood pressure (via 24-gauge catheter in the carotid artery) and 3-lead ECG were recorded every 2.5 to 5 min (Mindray PM9000Vet) after beginning of anesthetic infusion; n = 9 to 16 measurements per five animals per group. Arterial blood (0.3 ml) was sampled for blood gas and electrolytes once before pretreatment and once postasystole (i-STAT1 using CG8+ cartridges; Abbott, USA), and was not sampled in two rats due to an insufficient amount of blood obtained (excluded from analysis). Primary outcomes were the time to asystole (defined as absence of ECG activity for 10 s) and the time to first arrhythmia (defined as identifiable early or delayed afterdepolarizations, or irregular RR intervals lasting at least 2 s on the ECG, that were accompanied by irregular systolic intervals on the arterial blood pressure trace). Animals were terminated by isoflurane overdose.

Drugs

All drugs were purchased from Alfa Aesar (USA). Racemic bupivacaine, S-bupivacaine (levobupivacaine), and S-ropivacaine were used in all experiments, as specified.

Data Analysis and Statistics

Due to replicate measures and multiple predictor study designs, data were analyzed using mixed-effects linear (in the case of normally distributed data) or generalized linear (in the case of nonnormally distributed data) multiple regression in R version 4.1.1, including predictor (main effects) interaction terms and random error accounting for sample replication (as stated earlier and in figure legends). Anesthetic × cotreatment were the main effects in the in vitro studies; anesthetic × pretreatment × time were the main effects in the rat model, paired by cage to account for variation due to animal litters and day of procedure. Time-course rat hemodynamic parameters were fit via third-order polynomial with respect to time, with baseline pretreatment values as a covariate term. For in vitro tissue studies, data were normalized to baseline measurements per sample, then expressed as ratios of time-matched vehicle control tissues. All datapoints are shown in the figures, where possible, otherwise provided in the supplementary figures (http://links.lww.com/ALN/C936). No statistical power calculation was conducted before the study; sample size was based on previous experience. Data distribution was evaluated via Cullen and Fray plots (R package “fitdistrplus”). Models were built
using the R packages “lme4” (equal group variance) and “glmmTMB” (nonequal group variance) using restricted maximum likelihood. Model assumptions were tested by examining residual plots for normal distribution and homoscedasticity, and fit was evaluated by examining model estimates and errors in relation to input data. Results are presented as marginal model means and standard error, estimated using the “emmeans” R package to account for any sample size imbalance between groups. Post hoc comparisons were performed in “emmeans” via two-sided Welch’s t tests, and expressed as ratios to vehicle control group, then further comparisons made where shown in the figures. The 95% CIs and P values were adjusted for multiple comparisons (Dunnett when comparing to a control group; Tukey otherwise); significance was designated at P < 0.05 (P < 0.1 for interactions). Bootstrap CIs were generated using non-parametric bootstrap (R package “Hmisc”) with 1,000 resamples. In response to peer review, dose–response data were added—curves were generated by fitting a Hill function (variable slope, R package “drc”); estimated EC50 values are reported with standard error.

Study Approval

All experiments were approved by the Institutional Animal Care and Use Committee of the Hospital for Sick Children (Toronto, Canada) and performed in accordance with national, institutional, and ARRIVE 2.0 guidelines for animal care.

Results

Racemic Bupivacaine Inhibits Human Cardiomyocyte Tissue Contractility and Perturbs Integrated Ion Channel Activity

To differentiate how bupivacaine or ropivacaine may alter cardiomyocyte function and to define a toxicity threshold within typical clinically relevant serum concentrations (0.1 to 9 µg/ml), we evaluated drug effects on functional beating monolayers of human induced pluripotent stem cell–derived cardiomyocytes. For comparison with racemic bupivacaine, we additionally investigated levobupivacaine, the pure bupivacaine S-enantiomer with a lower clinical cardiotoxicity risk.5,8 Using a combined impedance and field potential multielectrode array, we measured tissue physical contraction (via tissue impedance) and captured changes to electric field potential, allowing for simultaneous evaluation of contraction, beat rate and rhythm, electromechanical coupling, and action potential profile25 (figs. 1A and 2A). At 2 µg/ml dose and below, consistent with previous in vivo data,27,29,31 we did not observe any significant changes from baseline to tissue physical contraction or beating rhythm, for any anesthetic. Our only observation was a small decrease in beating rate at 2 µg/ml bupivacaine (fig. 1).

Starting at 3 µg/ml, bupivacaine more strongly inhibited contractile amplitude, beating rate (beats/min EC50 7.3 ± 0.5 µM for bupivacaine and 11.7 ± 0.8 µM for ropivacaine), and induced dysrhythmic beating (beat interval deviation EC50 6.8 ± 0.9 µM for bupivacaine and 12.5 ± 3.6 µM for ropivacaine; fig. 1). The difference in contractile depression increased with higher doses (fig. 1B). At 6 µg/ml, bupivacaine more severely depressed contractile amplitude (to 55 ± 4% of control, P < 0.001, and 73 ± 6% of ropivacaine effect, P = 0.001), and induced 9.0 ± 5.1-fold higher beat interval deviation (dysrhythmia) than ropivacaine (P < 0.001). At 9 µg/ml, bupivacaine caused complete beating arrest of all tissues (noise–only impedance and field potential signal); however, ropivacaine effects were comparable to those at 6 µg/ml. Levobupivacaine effects were most similar to ropivacaine overall (fig. 1B; e.g., beat interval deviation P = 0.9999). Together, these results are consistent with increased bupivacaine cardiac contractile depression and arrhythmias observed clinically.2,6 For corroborative, we quantified the effects of bupivacaine and ropivacaine on stem cell–derived cardiomyocyte tissues cultured on soft substrates (5 kPa), to mimic physiologic myocardial stiffness.19,20 Similarly, we observed reductions in contractility measures (tissue displacement amplitude and contractile velocity) for both drugs above the subtoxic 2 µg/ml concentration (Supplemental Figure S2, http://links.lww.com/ALN/C936). The magnitude of these adverse effects was significantly larger for bupivacaine than for ropivacaine at both 4 and 6 µg/ml (e.g., bupivacaine decreased tissue contraction-phase velocity to 47 ± 8% of ropivacaine effect at 6 µg/ml anesthetic, P < 0.001).

To determine anesthetic effects on cardiomyocyte electromechanical coupling, we measured the coordinated timing of electrical excitation and cell contraction (excitation-contraction coupling time), via simultaneous capture of tissue impedance (physical contraction) and field potential (membrane potential) recordings (fig. 2A). All drugs prolonged the excitation-contraction coupling time at 6 µg/ml (bupivacaine to 144 ± 5%, ropivacaine to 125 ± 4%, and levobupivacaine to 128 ± 4% of control, P < 0.001 for all), indicative of impaired coordination between membrane depolarization and physical contraction. However, we observed markedly different field potential profiles between the anesthetics, as a measure of integrated channel activity23,34 (fig. 2). All drugs reduced total sodium spike amplitude, indicative of expected sodium current (Na+,1.5) inhibition during depolarization, but bupivacaine decreased sodium spike amplitude more than ropivacaine (EC50 7.5 ± 0.4 vs. 25.2 ± 5.1 µM; decrease to 13 ± 2% of control and 22 ± 4% of ropivacaine effect at 6 µg/ml, P < 0.001 for both). Interestingly, bupivacaine-treated tissues showed a more pronounced delay in early–phase repolarization compared to ropivacaine-treated tissues, indicative of greater perturbation of calcium currents (EC50 8.2 ± 0.5 vs. 15.9 ± 3.4 µM; increase to 131 ± 5% of...
control and 122 ± 7% of ropivacaine effect at 6 µg/ml, \( P < 0.001 \) for both). Comparable to ropivacaine, we did not observe a change in early-phase repolarization with levobupivacaine (fig. 2B). Last, we did not detect a measurable difference between the anesthetics in late-phase repolarization time, corresponding to potassium-mediated membrane repolarization (\( P > 0.16 \) at 6 µg/ml for all drugs). In general, levobupivacaine showed contractility, rhythm, and field potential effects most similar to ropivacaine (figs. 1 and 2), consistent with previous work and clinical toxicity observations.\(^{2,8} \) Thus, we focused on comparing bupivacaine to ropivacaine in the following experiments.

**Calcium Supplementation Rescues Bupivacaine-mediated Contractile Dysfunction**

Calcium supplementation can be used to improve cardiac inotropy; however, excess intracellular calcium can lead to arrhythmias.\(^{22,35} \) Because bupivacaine was
previously noted to antagonize L-type calcium channels, which is supported by our data, we tested the ability of moderate calcium supplementation to attenuate anesthetic toxicity at a dose showing quantifiable arrhythmias and contractile depression for both drugs (6 µg/ml anesthetic). Coadministration of...
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calcium chloride (final concentration 2.5 mM Ca$^{2+}$ vs. 2 mM Ca$^{2+}$ in media-only controls) corrected bupivacaine-induced changes to impedance amplitude (to 101 ± 6% of control, $P = 0.999$) and beating interval deviation (to 1.0 ± 0.3-fold of control, $P = 0.999$), indicating mitigation of contractile depression and arrhythmia (fig. 3 and Supplemental Figure S3, http://links.lww.com/ALN/C936). Conversely, cotreatment of ropivacaine tissues did not alter impedance amplitude (80 ± 6% of control, $P = 0.012$, and 109 ± 10% of ropivacaine, $P = 0.640$), and exacerbated beat interval deviation (to 17.8 ± 7.3-fold of control, $P < 0.001$, and 4.9 ± 2.7-fold of ropivacaine-only tissues, $P = 0.012$), indicating increased arrhythmic beating (fig. 3, A to C). Calcium supplementation corrected the delay in excitation-contraction coupling time caused by bupivacaine but worsened the change in ropivacaine-treated tissues (fig. 3E). Similar changes were observed in sodium spike amplitude and early-phase repolarization time, reflecting the divergent effects of the two anesthetics on cellular field potentials, and the ability of exogenous calcium to correct bupivacaine adverse alterations.

**Disruption of Intracellular Calcium Dynamics by Bupivacaine Is Partially Recovered by Calcium Supplementation**

Given the observed differences in calcium currents and the effects of calcium supplementation between the two anesthetics, we further investigated sarcolemmal calcium dynamics of anesthetic-treated stem cell–derived cardiomyocyte tissues via intracellular calcium imaging (fig. 4A). We did not observe any significant differences between the two drugs at the subtoxic 2 μg/ml concentration (Supplemental Figure S4, http://links.lww.com/ALN/C936).

However, at 6 μg/ml, bupivacaine more significantly affected all phases of calcium flux in the beating cycle than...
ropivacaine. Bupivacaine decreased peak calcium wave amplitude (to 44 ± 9% of control, $P < 0.001$, and 52 ± 11% of ropivacaine effect, $P = 0.002$) and prolonged contraction $T_{80}$ (time to reach 80% peak calcium intensity), indicating decreased peak cytosolic calcium and slowed release from the sarcoplasmic reticulum during contraction (fig. 4). Furthermore, bupivacaine prolonged relaxation $T_{20}$ (time to reach 20% of calcium decay) and increased the relaxation time constant tau ($2.8 ± 0.4$-fold of control, $P < 0.001$, and $1.5 ± 0.2$-fold of ropivacaine effect, $P = 0.006$), indicating slower kinetics in both early and late phases of sarcoplasmic calcium reuptake during relaxation (fig. 4). Similar results were observed at 4 μg/ml in subsequent experiments (fig. 5C).

We compared the ability of calcium supplementation to attenuate the adverse changes to calcium dynamics observed at the toxic 6 μg/ml anesthetic dose. The effects of calcium supplementation on sarcoplasmic reuptake did not differ between the two anesthetics (fig. 4C), but calcium effects on contraction dynamics (i.e., sarcoplasmic release) were distinct. In bupivacaine-treated tissues, calcium cotreatment increased peak calcium wave amplitude ($P = 0.026$ vs. bupivacaine-only), and reduced contraction $T_{80}$ ($P < 0.001$ vs. bupivacaine-only), indicating increased cytosolic calcium and speed of release from the sarcoplasm, partially mitigating the negative bupivacaine effects (fig. 4). Conversely, calcium did not affect peak wave amplitude ($P = 0.788$) or contraction $T_{80}$ ($P = 0.194$) in ropivacaine-treated tissues (fig. 4). These findings mirror the calcium supplementation effects on contraction amplitude, where calcium mitigated the bupivacaine contractile depression, but showed no effects in ropivacaine-treated tissues (fig. 3).

Overall, we found that both anesthetics lacked significant effects on cardiomyocyte function at subtoxic doses. However, above the toxic threshold, bupivacaine perturbed contractility, was more arrhythmogenic, and adversely affected calcium dynamics to a larger degree than ropivacaine. Calcium cotreatment mitigated the bupivacaine-induced changes, but exacerbated the negative effects of ropivacaine, highlighting the role of altered calcium flux in the different cardiotoxicity mechanisms between the two anesthetics (Supplemental Figure S5, http://links.lww.com/ALN/C936).

Exogenous Calcium Attenuates Bupivacaine Cardiotoxicity In Vivo

To validate our human induced pluripotent stem cell–derived cardiomyocyte tissue findings, we tested anesthetic effects in an in vivo rat model of anesthetic cardiotoxicity.36 Equal doses of bupivacaine or ropivacaine were continuously infused (intravenous) to gradually reach toxic serum concentrations, with continuous invasive arterial pressure...
and ECG monitoring until asystole (death). To evaluate the effects of calcium supplementation, intravenous calcium chloride pretreatment (10 mg/kg) was compared to saline pretreatment control for each anesthetic (fig. 6 and Supplemental Figures S6 and S7, http://links.lww.com/ALN/C936). The pretreatment model was chosen instead of cotreatment to promote delivery of a constant amount of calcium to each animal, despite any differences in survival.

Ropivacaine-treated animals survived longer than bupivacaine (time to asystole: -23.1 ± 5.0 min for bupivacaine and 52.1 ± 5.3 min for ropivacaine, corresponding to 46 ± 10 mg/kg bupivacaine and 104 ± 11 mg/kg ropivacaine, P = 0.002). Calcium pretreatment prolonged survival in bupivacaine-treated animals (+6 ± 2 ± 3.3 min, P = 0.007), but reduced survival with ropivacaine (-13.8 ± 3.4 min, P = 0.003; fig. 6A and Supplemental Figure S6A, http://links.lww.com/ALN/C936). At midpoint survival time (i.e., half-time to asystole, corresponding to 27 ± 2 mg/kg bupivacaine infused), we observed higher diastolic pressure (P = 0.012) and heart rate (P < 0.001) in calcium pre-treated bupivacaine animals, signifying partial mitigation of bupivacaine cardiotoxicity (fig. 6B and Supplemental Figure S6, http://links.lww.com/ALN/C936). However, calcium pretreatment showed no significant effects on blood pressure or heart rate in ropivacaine animals (P > 0.9). Interestingly, calcium supplementation paradoxically decreased terminal serum ionized calcium concentration in bupivacaine-treated animals but had the expected effect of increasing calcium in ropivacaine-treated animals (fig. 6C and Supplemental Figure S7, http://links.lww.com/ALN/C936).

L-Type Calcium Channel Blockade Selectively Worsens Bupivacaine Toxicity

Previous reports indicated that L-type calcium channel inhibition can exacerbate bupivacaine cardiotoxicity in preclinical models.38,39 Given our findings that bupivacaine adversely alters intracellular calcium dynamics, we sought to determine if bupivacaine effects are altered by calcium channel blockers by quantifying the effects of nifedipine on anesthetic-induced contractile depression and calcium dynamics in stem cell–derived cardiomyocyte tissues (fig. 5 and Supplemental Figures S8 to S10, http://links.lww.com/ALN/C936).

We found no significant adverse effects of nifedipine cotreatment (50 nM) with either anesthetic at the subtoxic anesthetic dose (2 μg/ml) on tissue contractility or beating rhythm (Supplemental Figure S9B, http://links.lww.com/ALN/C936). However, with higher doses of bupivacaine, the addition of nifedipine drastically reduced tissue displacement (to 5 ± 1% of 4 μg/ml bupivacaine-only...
tissues, $P < 0.001$), and increased beat interval deviation (14.5 ± 5.2-fold of 4 μg/ml bupivacaine-only tissues, $P < 0.001$), indicative of impaired contractility and increased arrhythmogenesis (fig. 5, A and B). At 6 μg/ml bupivacaine, nifedipine cotreatment completely arrested tissue beating (Supplemental Video 1, http://links.lww.com/ALN/C937).

Fig. 6. Calcium pretreatment attenuates bupivacaine toxicity in an in vivo rat model. Animal pretreatment (equivalent volume of saline or 10 mg/kg CaCl₂) was intravenously infused for 5 min, after which only bupivacaine or ropivacaine were infused (2 mg·kg⁻¹·min⁻¹) until asystole (death). n = 5 animals per group. (A and D) Time to first arrhythmia and time to asystole shown for each animal paired by cage, indicated by matched numbers and dashes (left). Calcium prolonged (improved) both metrics with bupivacaine but reduced (worsened) the times with ropivacaine. *$P < 0.05$ for calcium chloride pretreatment compared to saline controls (post hoc Welch’s t test of mixed-effects model estimates). (B) Time course analysis of anesthetic infusion and calcium supplementation on hemodynamic parameters, showing mitigation of bupivacaine adverse effects on diastolic blood pressure and heart rate with calcium pretreatment. Calcium had no effect on ropivacaine-induced hemodynamic changes. n = 5 animals per group; 9 to 16 measurements per animal; $P$ values shown for calcium chloride versus saline groups at midpoint survival time per anesthetic. (C) Relative effect of calcium pretreatment on change in blood ion concentrations (from preinfusion to postmortem) highlights differences in calcium flux between bupivacaine and ropivacaine, compared to sodium and potassium (no significant changes). (E) Poincaré plot of adjacent beat-to-beat intervals (RR1 and RR2) at midpoint survival time per anesthetic group, showing beat interval deviation between treatment groups as 90% confidence ellipses around the points. Higher point dispersion indicates increased beating irregularity. Calcium mitigated bupivacaine-related beating irregularity but exacerbated the adverse effect of ropivacaine.
At 4 μg/ml, nifedipine cotreatment perturbed sarcoplasmic calcium flux during contraction, decreasing peak calcium wave amplitude (to 78 ± 4% of bupivacaine-only tissues, P < 0.001; fig. 5C). Even at toxic anesthetic concentrations, nifedipine did not produce a measurable adverse effect on tissue contractility or intracellular calcium dynamics in ropivacaine-treated tissues (fig. 5, Supplemental Video 1). Interestingly, nifedipine cotreatment actually reduced beating irregularity, indicating partial mitigation of ropivacaine-induced arrhythmias at 6 μg/ml anesthetic (P = 0.035, fig. 5B). Furthermore, we observed an improved calcium relaxation rate constant tau in ropivacaine–nifedipine tissues, compared to nifedipine–only controls, signifying improved sarcoplasmic calcium reuptake due to the ropivacaine–nifedipine interaction (P = 0.003, fig. 5C).

Overall, nifedipine potentiated bupivacaine-mediated contractile depression, beating irregularity, and sarcotubal calcium dysfunction, while showing no adverse (and some beneficial) effects with ropivacaine. Our findings indicate that L-type calcium channel blockade produces a synergistic effect that exacerbates the adverse changes to cardiomyocyte function induced by toxic bupivacaine concentrations. This relationship does not exist between ropivacaine and nifedipine, and calcium blockade may partially mitigate ropivacaine toxicity. The divergent effect of calcium blockers between the two anesthetics further supports the role of calcium flux as key to their markedly different cardiotoxicities.

## Discussion

Pharmaceuticals exhibit unexpected postmarket cardiotoxicity, partially due to a deficit of preclinical models that reliably predict drug activity in the human heart.14-16 Testing on induced pluripotent stem cell–derived cardiomyocytes could improve drug safety by providing a native human cellular context, as illustrated by previous successful evaluations of ion channel modulators, arrhythmogens, and inotropes, showing parity to known in vivo effects.14-16,40 Supporting stem cell–derived cardiomyocyte model pertinence, we found no adverse effect of local anesthetics tested less than 2 μg/ml, agreeing with in vivo data.27,29 Above the toxicity threshold, at concentrations systemically achievable during routine anesthetic use,27,28,30,31 all drugs illustrated signs of toxicity, as expected given their known Na$_v$1.5 cardiac sodium channel inhibition. However, resulting from distinct cellular physiology changes, we observed different magnitudes of toxicity between bupivacaine and ropivacaine, consistent with clinically evident cardiotoxicity differences between these two drugs.

Conforming to the canonical mechanism of cardiotoxicity, we observed a markedly reduced sodium current spike amplitude with all drugs, although bupivacaine exhibited greater inhibition. Published studies showed small differences between anesthetic blockade of human Na$_v$1.5 in the clinically relevant inactive channel form (bupivacaine IC50 2.2 ± 0.2 μM vs. ropivacaine 2.7 ± 0.3 μM), concluding that other mechanisms are likely.5 Moreover, such isolated channel studies lack the native cardiac proteome context, where channel modulation by regulatory proteins and other nearby channels can alter drug effects.22,41 In isolated canine cardiomyocytes, used for their proposed electrophysiologic similarity to human cardiomyocytes, sodium current inhibition was the dominant effect observed for bupivacaine and ropivacaine.52 At concentrations similar to our study, bupivacaine induced a 2-fold stronger inhibition than ropivacaine, consistent with our data. Interestingly, the authors noted differences in the ropivacaine ion current inhibition profiles between artificial patch clamp and physiologic cardiomyocyte states, reiterating the importance of studying anesthetic toxicity in a native, contractile cellular context.

In contractile cardiomyocytes, the regulation of sodium and calcium flux is closely tied.22,41 In addition to Na$_v$1.5 blockade, bupivacaine was previously observed to inhibit calcium currents, intracellular calcium dynamics, and sarcotubal proteins (ryanodine receptor and sarcoplasmic reticulum calcium ATPase) more than ropivacaine in mammalian muscle preparations, but at concentrations significantly higher (more than 2,500 μg/ml) than the clinically relevant range.43,44 Closer to expected serum concentration (3 μg/ml), bupivacaine partially inhibited slow L-type calcium currents, but direct comparisons to ropivacaine were not investigated.11,36 Just above the toxicity threshold (at 3, 6 μg/ml), we noted that bupivacaine prolonged early (in the contractile cycle) cellular field potentials, primarily mediated by L-type calcium channels and ryanodine receptor sarcoplasmic calcium eflux, whereas ropivacaine or levobupivacaine showed negligible effects (fig. 2). Additionally, bupivacaine more strongly inhibited sarcoplasmic calcium release during contraction, and subsequent reuptake during relaxation, at both 4 and 6 μg/ml (figs. 4 and 5). This indicates inhibition of the sarcoplasmic reticulum calcium ATPase and the sodium–calcium exchanger (which help remove cytosolic calcium during relaxation).52 Thus, our data support calcium dynamics dysregulation being a key bupivacaine cardiotoxicity mediator. However, differences in binding modes at specific intracellular targets relevant to calcium flux remain to be elucidated. In rats, bupivacaine was observed to bind the dihydropyridine neuronal L-type calcium channel site 2-fold more potently than R- and S-ropivacaine.8 Such studies in human contractile cardiomyocytes, including R-ropivacaine and dextrobupivacaine, can further define molecular cardiotoxicity mechanistic differences, along with stereospecific effects, which we did not investigate. This would enhance our understanding of structure–activity relationships to therapeutic and cardiotoxic anesthetic effects, helping inform future drug design and reduce off-target toxicity.

Calcium modulation is fundamental to cardiomyocyte contractility and beating rhythm. Excess calcium (endogenous or exogenous) can lead to toxicity and arrhythmogenesis.52 Moderate calcium supplementation can be used for inotropic support during cardiac resuscitation and is particularly effective.
with baseline hypocalcemia. 38 We found that calcium supplementation mitigated bupivacaine cardiotoxic effects but had no effect (or exacerbated) ropivacaine functional decline in vitro (figs. 3 and 4). We observed similar effects of calcium supplementation in rats (fig. 6), underscoring the importance of calcium flux in cardiotoxicity mechanisms of different anesthetics. Previously, calcium supplementation reduced bupivacaine arrhythmias potentiated by high potassium buffer in isolated rat cardiomyocytes; however, calcium effects on anesthetic alone were not addressed. We found that intravenous calcium pretreatment delayed time to first arrhythmia and asystole, and mitigated diastolic blood pressure and heart rate decline in bupivacaine-treated animals. Ropivacaine effects were opposing, where exogenous calcium reduced survival and promoted arrhythmias. Thus, calcium could be used to attenuate bupivacaine, but not ropivacaine effects, illustrating how calcium preconditions may affect anesthetic-specific cardiotoxicity risk.

In mammalian ventricular muscle, bupivacaine was noted to lower myofilament calcium sensitivity more effectively than ropivacaine (i.e., more intracellular calcium required to generate equal force). 37,48 We observed, similar to other studies, that bupivacaine can inhibit sarcoplasmic calcium release (lower peak calcium wave amplitude). The combination of lower myofilament calcium sensitivity and reduced calcium cycling suggests that supplemental calcium might be beneficial in mitigating bupivacaine-induced contractile depression. This notion is supported by our in vitro and in vivo findings. In a mouse study of neuronal pain-sensing, pharmacologic calcium channel activation reduced bupivacaine analgesia, implying that increased intracellular calcium flux may interfere with the inhibitory channel effects of bupivacaine. 49 This finding mirrors the protective supplemental calcium effect on bupivacaine cardiotoxicity that we observed.

Calcium dysregulation represents a unique adverse bupivacaine effect, without parallel findings for ropivacaine at the concentrations studied. The divergent calcium supplementation effect on anesthetic toxicity highlights the need for anesthetic- and context-specific risk evaluation. Studies in mice indicate that calcium channel inhibitors can exacerbate bupivacaine lethality and enhance contractile depression in guinea pigs and dogs. 38,39 Specifically, experiments in guinea pig myocardium suggest that at drug doses similar to our study, bupivacaine and nifedipine interactions do not directly involve Na1.5 inhibition, but are due to synergistic block of L-type calcium channel. 39 Consistent with this, we did not observe any effects on calcium-related field potentials or intracellular calcium flux by either anesthetic, or any adverse nifedipine interactions, at subtoxic anesthetic doses. However, above the toxicity threshold, bupivacaine synergistically interacted with nifedipine to further depress contractility and increase arrhythmias. Ropivacaine was not previously compared to bupivacaine in this context. Our findings contrast bupivacaine, whereas nifedipine did not potentiate ropivacaine cardiotoxicity, instead improving sarcoplasmic calcium reuptake and beating regularity. The divergent nifedipine—anesthetic interaction suggests that calcium preconditions may affect toxicity risk and reiterates the importance of calcium flux in anesthetic cardiotoxicity.

Although stem cell–derived cardiomyocytes offer a sustainable human cardiomyocyte source, with demonstrated utility in disease modeling and drug development, 14,16,40 they initially exhibit a neonatal phenotype. Strategies exist to mature the cells (e.g., see Materials and Methods), but components of metabolism, sarcomere structure, and ion flux may lie at intermediate maturation states relative to adult human myocardium. 21,24 Other limitations include the lack of hemodynamic effects and coronary flow in our tissue model, where anesthetic concentrations remain static during treatment. The 5-h incubation may not represent immediate clinical cardiotoxicity; however, the timing is comparable to amide anesthetic block durations and elimination half-lives (i.e., slow perivascular absorption vs. acute toxicity). There may be discrepancies between media/serum and effect site (tissue) concentrations due to differences in biologic uptake, 37 and 11-fold higher liposolubility of bupivacaine and levobupivacaine than ropivacaine. 8 We did not adjust concentrations based on ropivacaine and bupivacaine lipid solubilities or anesthetic potencies. Due to ethical reasons, quantifying human therapeutic potency is challenging. This results in varying therapeutic potency differences reported for anesthetics that depend on nerve block type (e.g., between 2-fold higher for bupivacaine than ropivacaine in epidural labor analgesia, to equipotency in femoral nerve block). 8 This issue is further compounded by differences in drug liposolubility and potential tissue accumulation, and between myocardial and neuronal diffusion barriers. 8,37,50 However, overall trends indicate a~0.7:0.9:1.0 relative ratio for ropivacaine:levobupivacaine:ropivacaine therapeutic potency, 5,51 suggesting similarity to cardiotoxicity differences and similar toxicity risk at equipotent concentrations. Interestingly, we observed levobupivacaine effects comparable to ropivacaine, in particular, both lacked effects on calcium-related repolarization. Furthermore, nifedipine did not negatively interact with 6 μg/ml ropivacaine but was detrimental with 4 μg/ml bupivacaine (near equipotent concentrations). These findings support the unique bupivacaine effects on calcium dynamics. However, as we focused on select clinically relevant concentrations, additional concentrations can help more completely quantify the relationship to therapeutic anesthetic potency differences. Because clinical cardiotoxicity is difficult to predict, our calcium prophylaxis rat model is likely impractical. Moreover, we studied female rats due to animal availability; thus, we did not capture possible sex-specific differences.

Conclusions

We found that bupivacaine adversely altered cardiomyocyte calcium dynamics in a functional human-derived tissue model (fig. 7). The same effect was not observed with ropivacaine, where toxicity risk is lower. Calcium
supplementation prevented bupivacaine, but exacerbated ropivacaine, cardiotoxicity in vitro and in rats. Nifedipine coadministration worsened bupivacaine, but not ropivacaine, cardiotoxicity at the clinically relevant concentrations studied, suggesting that bupivacaine toxicity could be potentiated by adverse interactions with calcium channel blockers. Our data highlight the distinct role of calcium dynamics in the higher cardiotoxicity risk of bupivacaine and suggest that calcium modulation may mitigate bupivacaine detrimental effects. Our approach provides a model to understand the broader issue of postmarket cardiotoxicity demonstrated by other drugs, correlating in vitro testing to adverse in vivo activity.

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Competing Interests

The authors declare no conflicts of interest.

Correspondence

Address correspondence to Dr. Maynes, Hospital for Sick Children, 555 University Ave, Toronto, Canada M5G 1X8. jason.maynes@sickkids.ca. This article may be accessed for personal use at no charge through the Journal Web site, www.anesthesiology.org.
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A Prelude to Painless Surgery: Charles F. Heywood’s Invitation to W. T. G. Morton

Penned 2 days before surgical etherization’s public debut, an invitation (left) from house surgeon Charles F. Heywood, M.D. (1823 to 1893), to Boston dentist William T. G. Morton (1819 to 1868) requested that Morton administer his “preparation...to diminish the sensibility to pain” at the Massachusetts General Hospital on October 16, 1846. One of the few extant handwritten documents relating to “Ether Day,” the letter is held by the Massachusetts Historical Society, Boston, Massachusetts (W.T. G. Morton papers, call number: Ms. N-567, reproduced with permission). As evidence of his primacy in this discovery, Morton published a different transcript of this letter in the Statements volume submitted to the U.S. Congress in 1853, and later in Trials of a Public Benefactor (1859), his authorized biography. The advent of painless surgery, a great boon to humanity, would be tarnished almost immediately by the “Ether Controversy.” Now a precious historical document, Heywood’s letter served as a prologue to “Ether Day” and critical evidence in Morton’s ultimately futile claims for financial compensation. (Copyright © the American Society of Anesthesiologists’ Wood Library-Museum of Anesthesiology. www.woodlibrarymuseum.org)

Rajesh P. Hanida, M.B.Ch.B., F.A.N.Z.C.A., Sydney, New South Wales, Australia, and Melissa L. Coleman, M.D., Associate Professor, Department of Anesthesiology and Perioperative Medicine, Penn State College of Medicine, Hershey, Pennsylvania.