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ORIGINAL ARTICLE

Myotoxicity of local anesthetics is equivalent in individuals with and without predisposition to malignant hyperthermia

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Abstract

Purpose Malignant hyperthermia (MH) is an inherited muscle disorder caused by abnormal elevations of intracellular calcium (Ca^{2+}) in skeletal muscle. There are several reports of myotoxicity caused by local anesthetics, and the increased intracellular Ca^{2+} is considered to be an important cause. However, there is insufficient evidence regarding myotoxicity in MH-susceptible individuals when large doses of local anesthetics are administered. This study investigated the effect of MH predisposition on myotoxicity.

Methods Human skeletal muscle samples were obtained from 22 individuals to determine susceptibility to MH, and were evaluated according to whether their Ca^{2+}-induced Ca^{2+} release (CICR) rates were accelerated or not. This study was performed using surplus muscle that remained after the CICR rate test. We calculated the 50% effective concentration (EC_{50}) values of three local anesthetics, namely lidocaine, levobupivacaine, and ropivacaine using the ratiometric dye Fura-2 AM. Significance was tested using the unpaired *t* test.

Results In the accelerated and unaccelerated groups, respectively, the mean ± SD of the EC_{50} values were 1.52 ± 0.72 and 1.75 ± 0.37 mM for lidocaine ($p = 0.42$), 0.72 ± 0.36 and 0.79 ± 0.46 mM for levobupivacaine ($p = 0.68$), and 1.21 ± 0.35 and 1.62 ± 0.57 mM for ropivacaine ($p = 0.06$). These values were similar in individuals with and without MH predisposition.

Conclusion The myotoxicity of local anesthetics was equivalent in individuals with and without predisposition to MH.

Keywords Myotoxicity · Local anesthetics · Malignant hyperthermia

Introduction

Malignant hyperthermia (MH) is an inherited muscle disorder induced by volatile anesthetics and depolarizing muscle relaxants. Its main characteristic is abnormal elevation of intracellular Ca^{2+} concentrations in skeletal muscle, a phenomenon that is closely linked to dysfunction of ryanodine receptor 1 (RYR1), and a Ca^{2+}-releasing channel in the sarcoplasmic reticulum (SR) of skeletal muscle [1, 2]. The in vitro contracture test in Europe and the caffeine–halothane contracture test in North America have been the gold standards for the diagnosis of MH. By contrast, in Japan, the diagnosis of MH predisposition is based on the Ca^{2+}-induced Ca^{2+} release (CICR) rate test, which is used to assess the Ca^{2+} release ability of RYR1.

There are several reports of myotoxicity caused by local anesthetics. Zink et al. reported that when 8.7 mM bupivacaine and 13.7 mM ropivacaine were continuously infused through femoral nerve catheters (8 mL/h) over 6 h in piglets, both local anesthetics produced irreversible skeletal muscle damage [3]. Furthermore, Neal et al. reported that three individuals who received adductor canal block with 63 mM lidocaine or 53 mM mepivacaine bolus followed by 6.6 mM ropivacaine at 8 mL/h developed progressive, profound weakness of the quadriceps muscles [4]. One of the primary underlying mechanisms of myotoxicity by local anesthetics is the elevation of intracellular Ca^{2+} [5]. Since

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myotoxicity of local anesthetics is suppressed by dantrolene and enhanced by caffeine [6], it is possible that RYR1 is involved in this process. In general, local anesthetics are safe to use in MH patients [7, 8]; however, there is insufficient evidence on myotoxicity in individuals predisposed to MH when large doses of local anesthetics are given, such as during peripheral nerve blocks. This lack of data might be due to the rarity of MH and the fact that it is, therefore, difficult to conduct clinical research in this area.

It has been reported that CICR is promoted by local anesthetics in the skeletal muscle of normal rats and guinea pigs [9, 10]. To date, no studies have assessed intracellular Ca^{2+} kinetics following the application of local anesthetics to human skeletal muscle cells derived from persons predisposed to MH. The skeletal muscles in these individuals may have disordered Ca^{2+} regulation, and may, therefore, exhibit elevated Ca^{2+} concentrations following lower doses of local anesthetics than in healthy individuals.

This study was performed to investigate the effect of local anesthetics on Ca^{2+} dynamics and myotoxicity in skeletal muscle cells derived from individuals predisposed to MH.

Methods

This study was approved by the ethics committee of Hiroshima University. Subjects were referred to our facility for advanced assessment because they or their families had MH or MH-like symptoms. We obtained each participant's written informed consent prior to implementing the experimental research protocol described below. In addition, all personal information, including genetic information, was managed according to the regulations established by the university.

Human skeletal muscle specimens were obtained by biopsy of the quadriceps or biceps brachii muscles to determine susceptibility to MH. 22 individuals underwent muscle biopsy, and the CICR rate test was performed according to the protocol developed by Endo et al. [11, 12]. Information on the results was offered to all participants.

In brief, chemically stripped muscle fibers were obtained from biopsied muscle tissue using saponin, and fibers were treated with varying concentrations of Ca^{2+} (0, 0.3, 1.0, 3.0, and 10.0 μM). The tension of each specimen was measured using a force transducer, and CICR rates were measured. Acceleration of CICR rates was determined according to our previous studies [13]. The mean normal CICR values were determined from 12 individuals with negative in vitro contracture tests (IVCT; EMHG protocol) and caffeine–halothane contracture tests (CHCT; North American MH Group protocol). An accelerated CICR rate was defined by a CICR value 2 standard deviations (SD) above the mean of normal individuals, and indicated a predisposition to developing MH. Characteristics of the individuals and the results of the CICR rate test are shown in Table 1.

Preparation of myotubes

This study was performed using surplus muscle remaining after the CICR rate test. The skeletal muscle cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) supplemented with 10% heat-inactivated bovine calf serum (FBS; Sigma-Aldrich, USA) containing 1% ampicillin sodium salt, kanamycin sulfate (Sigma-Aldrich, USA), and amphotericin B (Invitrogen, USA), in 25-cm^2 cell culture flasks (Corning; Corning, NY, USA) at 37 °C under 5% CO_2. The medium was changed every 3 days. After 2 or 3 weeks in culture, the cells were collected with trypsin and plated on 35-mm glass-bottom culture dishes. They were cultivated for 7–10 days in DMEM supplemented with 10% FBS and then for 5–7 days in the same medium supplemented with 2% FBS. Myotubes were confirmed by morphological identification of fusiform-shaped multinuclear cells (Fig. 1).

Ca^{2+} imaging of myotubes

Myotubes were washed in Hank's balanced salt solution (HBSS) containing 130 mM NaCl, 5.4 mM KCl, 20 mM HEPES, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 5.5 mM glucose (pH 7.4). The myotubes were loaded with 5.0 μM Fura-2 AM (Dojindo Molecular Technologies, Tokyo, Japan) in HBSS for 1 h at room temperature (24–26 °C), and then washed with HBSS. Measurements were performed after cells were incubated in HBSS for 30 min.

The cells were excited at 340 and 380 nm. Fluorescence emissions of Fura-2 AM were observed at 510 nm using a fluorescence microscope (Nikon, Tokyo, Japan) at 5-s intervals to evaluate intracellular Ca^{2+} changes. Images were acquired using a cooled high-speed digital video camera (ORCA-AG; Hamamatsu Photonics, Hamamatsu, Japan).

Each solution was added to one side of the culture dish and aspirated from the opposite side for 2 min at a rate of 1.2 mL/min at 37 °C, then washed out 2 min before addition of the next chemicals. Chemical-induced changes in Fura-2 AM fluorescence were measured using various chemical concentrations, and the 340/380 nm signal ratio was calculated using a Ca^{2+} imaging system (Aquacosmos 2.5; Hamamatsu Photonics).

Caffeine, 4-chloro-m-cresol (4-CmC)

To identify myotube characteristics, we examined their responses to caffeine and 4-CmC, both of which are pure RYR1-stimulating agents. HBSS containing 10 mM caffeine was added to the culture dish. Next, caffeine was tested

Table 1 Biometric data, indications, and results of calcium-induced calcium release test

Individual no.	Sex	Age (years)	Indications for CICR testing	Results of CICR testing
1	F	16	MH (CGS 63, rank 6)	Accelerated
2	M	10	Suspected of NMD	Accelerated
3	M	41	High serum creatine kinase	Accelerated
4	F	39	MH family	Accelerated
5	M	62	MH family	Accelerated
6	M	37	MH	Accelerated
7	M	52	MH	Accelerated
8	M	55	Post-operative MH	Accelerated
9	F	34	MH family	Accelerated
10	F	41	Post-operative MH	Accelerated
11	M	56	MH	Accelerated
12	F	44	MH (CGS 53, rank 6)	Accelerated
13	F	25	MH (CGS 8, rank 2)	Accelerated
14	M	18	MH (CGS 48, rank 5)	Accelerated
15	M	44	High serum creatine kinase	Unaccelerated
16	M	53	Suspected of MH	Unaccelerated
17	F	63	MH family	Unaccelerated
18	M	30	MH family	Unaccelerated
19	F	44	MH family	Unaccelerated
20	M	44	High serum potassium	Unaccelerated
21	M	40	Rhabdomyolysis	Unaccelerated
22	M	47	MH family	Unaccelerated

CGS clinical grading scale, *CICR* Ca^{2+}-induced Ca^{2+} release, MH: malignant hyperthermia; *NMD* neuromuscular disease

Each CGS (the rank) indicates the likelihood of malignant hyperthermia; 0 (rank 1) is "almost never", 3–9 (rank 2) is "unlikely", 10–19 (rank 3) is "somewhat less than likely", 20–34 (rank 4) is "somewhat greater than likely", 35–49 (rank 5) is "very likely", and 50+ (rank 6) is "almost certain"

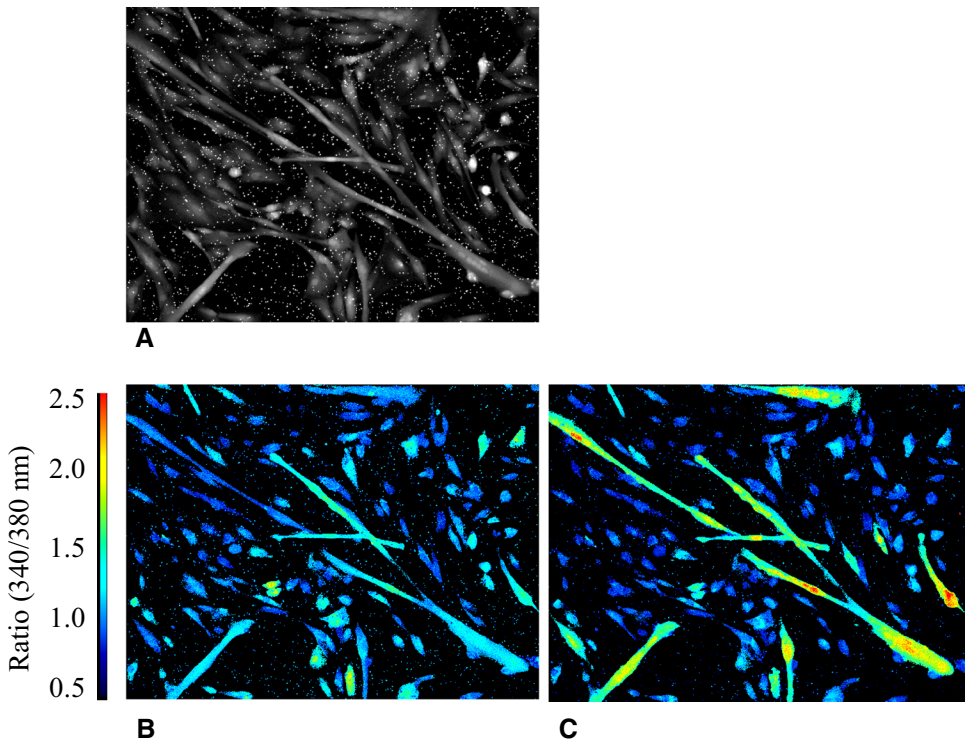

Fig. 1 Myotubes loaded with fura-2 AM. Multinuclear myotubes at an excitation wave length of 340 nm (**a**), the ratio of wavelength of 340 nm and 380 nm before (**b**) and after (**c**) application of 10 mM caffeine

incrementally at 0.25, 0.5, 1.0, 2.5, 5.0, 10, and 20 mM, and 4-CmC was tested incrementally at 3.0, 10, 30, 100, 300, 500, and 1000 μM.

Local anesthetics

Only the cells that demonstrated RYR1 expression, defined by increased Ca^{2+} concentrations in response to 10 mM caffeine, were used for experiments. HBSS containing incremental levels of lidocaine dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) (0.1, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 mM) was added to the dish. This measurement was performed within 90 min after washing away the excess Fura-2 AM. In the same way, levobupivacaine (0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.5, and 2.0 mM) and ropivacaine (0.1, 0.25, 0.5, 1.0, 2.0, and 4.0 mM) were perfused and the respective changes in Fura-2 AM fluorescence were measured.

Data analysis

Changes in ratios were calculated as the difference between the maximal response and the preceding baseline. To trace dose–response curves for caffeine, 4-CmC, and each local anesthetic, the data were normalized to the maximal response of each cell. Then, the half-maximal effective concentration (EC_{50}) was calculated from the acquired dose–response curves. All data were analyzed using PRISM 7.0 (GraphPad Software, San Diego, USA). The EC_{50} was measured in each cell and used as a summary measure, and we compared the EC_{50} value of the accelerated group with that of the unaccelerated group. Significance was tested via *t* test, and a *p* value of <0.05 was considered to be statistically significant.

Results

Responses to caffeine and 4-CmC

In all cells, intracellular Ca^{2+} concentrations increased as the concentrations of loaded caffeine and 4-CmC increased. Each EC_{50} is shown in Table 2. The EC_{50}s of the cells in the accelerated group were all lower than the cutoff value determined by Kobayashi et al. [14].

The EC_{50} values for caffeine were 3.11 ± 0.54 mM in the accelerated group and 4.85 ± 0.64 mM in the unaccelerated group ($p < 0.0001$). The EC_{50} values for 4-CmC were 144.8 ± 30.7 μM in the accelerated group and 249.8 ± 34.3 μM in the unaccelerated group ($p < 0.0001$) (Fig. 2).

EC_{50}s for local anesthetics in myotubes

Figure 3 shows representative traces of Ca^{2+} responses to incremental doses of lidocaine in myotubes, and Fig. 4 shows the dose–response curves of Ca^{2+} release in myotubes following exposure to local anesthetics. The responses of both the accelerated and unaccelerated groups increased as the concentration of each anesthetic was increased. There were no differences in mean EC_{50} for lidocaine, levobupivacaine, and ropivacaine between accelerated and unaccelerated groups (Fig. 5a).

In addition, the pH of each local anesthetic solution was within the range of 7.226–7.696. Ca^{2+} concentrations did not increase following the addition of HBSS adjusted to the same pH.

Discussion

All EC_{50} values of each of the three local anesthetics applied to the accelerated and unaccelerated groups were lower than the physiological concentrations, and the values were not statistically different between the groups. This indicated that the local anesthetics increase the intracellular Ca^{2+} concentration in MH-susceptible individuals and non-MH-susceptible individuals, but the degree of myotoxicity of local anesthetics was equivalent in individuals with and without predisposition to malignant hyperthermia. Therefore, we consider that extra caution is not warranted when using local anesthetics in MH-susceptible individuals.

Increased intracellular Ca^{2+} concentrations caused by local anesthetics

Regardless of the presence or absence of MH predisposition (corresponding to the accelerated and unaccelerated groups, respectively), similar increases were seen in Ca^{2+} concentrations following myotube treatment with each local anesthetic. These increases occurred at concentrations of 0.25 mM for lidocaine, 0.2 mM for levobupivacaine, and 0.25 mM for ropivacaine. The EC_{50} for levobupivacaine was the lowest, at 0.72 mM, consistent with previous reports that bupivacaine showed higher skeletal muscle toxicity than other local anesthetics [15, 16]. In general, the concentrations of local anesthetics used clinically are 0.5–2% (21–85 mM) for lidocaine, 0.25–0.75% (7.9–23 mM) for levobupivacaine, and 0.2–1% (6.6–33 mM) for ropivacaine. There have been reports of partial myotoxicity in animals following the use of local anesthetics at high concentrations or over long periods and it is considered that necrotization of skeletal muscles and the apoptosis through mitochondrial pathways are involved in [3, 17]. In humans, muscular strength

Table 2 EC_{50}s for local anesthetics and ryanodine receptor 1 activators (caffeine and 4-CmC)

Individual no.	Local anesthetics			Pure ryanodine receptor 1 activators	
	Lidocaine (mM)	Levobupivacaine (mM)	Ropivacaine (mM)	Caffeine (mM)	4-CmC (μM)
1	1.24	0.84	1.12	2.84	133
2	1.71	0.99	1.26	3.02	161
3	3.50	1.10	0.85	3.65	150
4	2.12	0.78	1.03	3.40	150
5	2.20	0.40	1.40	2.55	131
6	1.50	0.12	1.51	3.60	157
7	1.42	0.73	0.38	2.30	90
8	1.33	0.45	0.95	2.59	148
9	0.64	0.22	1.16	3.60	191
10	0.95	0.50	1.22	3.60	85
11	1.25	1.24	1.21	3.46	135
12	1.36	1.15	1.44	3.39	145
13	0.72	1.00	1.50	3.43	193
14	1.34	0.49	1.87	2.10	161
15	2.09	1.74	0.81	5.93	225
16	1.28	0.55	2.13	4.91	225
17	1.45	0.84	1.45	5.50	306
18	1.72	0.69	1.74	3.83	259
19	2.13	0.49	1.11	4.57	235
20	2.21	0.33	1.61	4.81	237
21	1.32			4.78	296
22	1.77	0.92	2.46	4.46	216

The EC_{50}s of all cells in the CICR-accelerated group were lower than the cutoff value determined by Kobayashi et al. [12]

EC_{50} 50% effective concentration, 4-CmC: 4-chloro-m-cresol

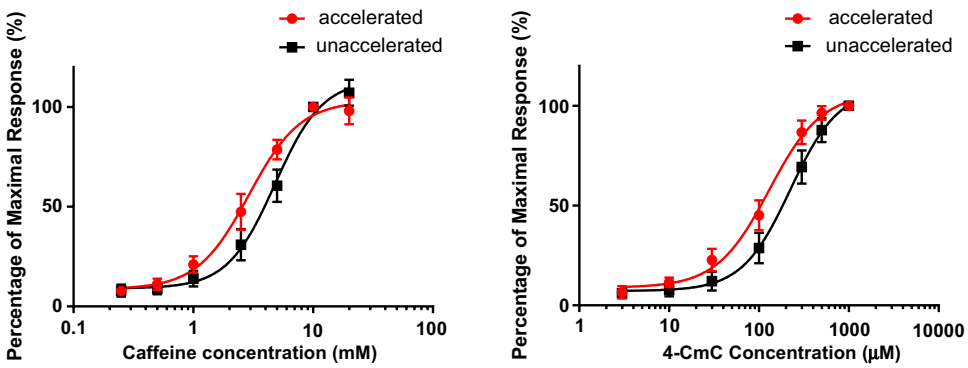

Fig. 2 Caffeine and 4-chloro-m-cresol dose–response curves of Ca^{2+} release in myotubes. Data are mean ± SD of the Ca^{2+}-induced Ca^{2+} release accelerated (circle) and unaccelerated (square) groups

decreased with prolonged administration [4]. However, skeletal muscle disorders have also often occurred during the acute phase, though these have resolved with time [18, 19], and clinically, skeletal muscle toxicity is rarely a problem in healthy persons [20]. This study showed that when local anesthetics were administered intramuscularly, Ca^{2+} concentrations increased locally and skeletal muscle cells were destroyed. However, since the increases in Ca^{2+} concentrations were not significantly different between the accelerated and unaccelerated groups, there should be no clinically meaningful difference in myotoxicity caused by local anesthetics between MH-predisposed persons and those who are healthy.

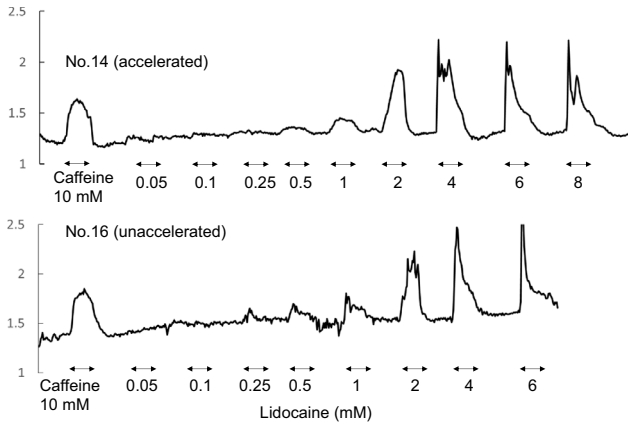

Fig. 3 Intracellular Ca^{2+} concentrations in myotubes. Intracellular Ca^{2+} concentration increased with lidocaine ≥ 0.25 mM

Mechanisms underlying elevated intracellular Ca^{2+} concentrations following administration of local anesthetics

In myotubes, the EC_{50} of each local anesthetic did not differ significantly between the CICR-accelerated group and those of the unaccelerated group. There are several Ca^{2+} regulatory mechanisms in human skeletal muscle cells. RYR1 is closely related to the L-type voltage-dependent Ca^{2+} channel (DHPR) on the cell membrane and regulates Ca^{2+} release from the sarcoplasmic reticulum [21, 22]. Previous stydies have reported that not only the RYR1 [23, 24], but also the voltage-dependent calcium channels [25] are involved in the calcium induce of local anesthetics. In myotubes, there were significant differences in the EC_{50}s of caffeine and 4-CmC but no significant differences in the EC_{50}s of local anesthetics between the accelerated and unaccelerated CICR groups. If local anesthetics act only on RYR1, there should be significant differences in the EC_{50}s of local anesthetics such as caffeine and 4-CmC. Relatedly, Ca^{2+} inflow into skeletal muscle is mediated by store-operated channels, mechanosensitive channels, and other mechanisms [26, 27]. The elevated intracellular Ca^{2+} concentrations following local anesthetic administration in this study were also observed using Ca^{2+}-free perfusate (data not shown). Therefore, while release of Ca^{2+} from the SR is the main cause of increased intracellular Ca^{2+} concentrations, it is certainly possible that the above inflow mechanisms interact with other processes outside the cell. We found that in myotubes there were no significant differences in the EC_{50}s of local anesthetics between the accelerated and unaccelerated CICR groups, probably because the mechanism whereby local anesthetics raise intracellular Ca^{2+} levels involves not only RYR1 but also various Ca^{2+} regulatory processes. Further investigations are required.

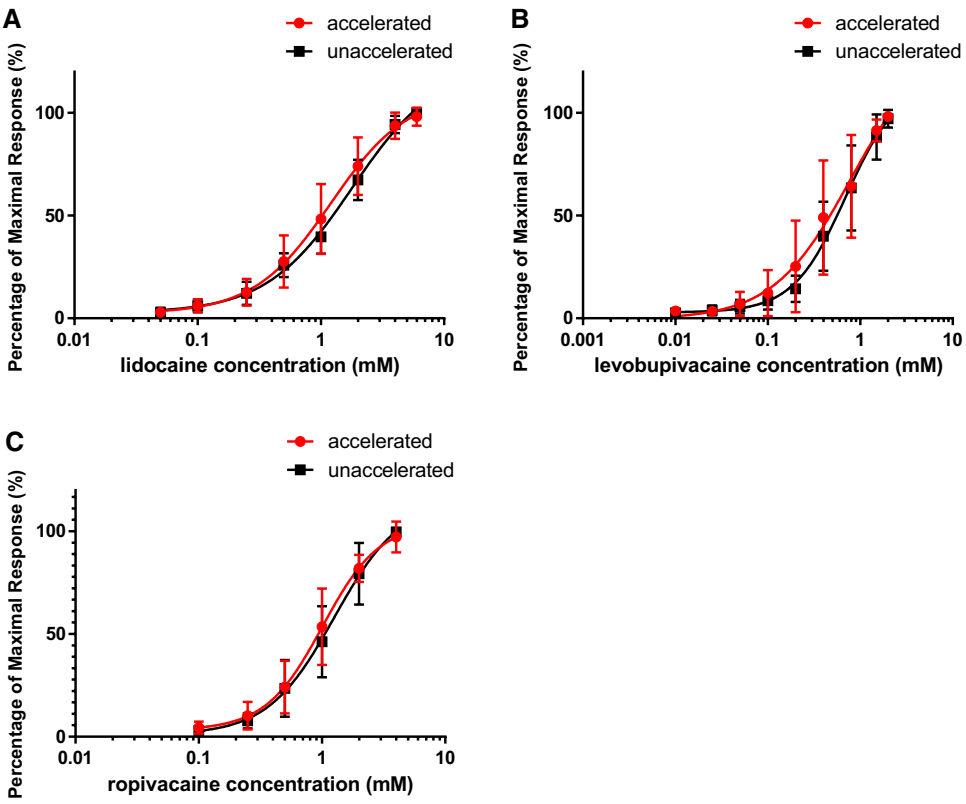

Fig. 4 Local anesthetic dose–response curves for Ca^{2+} release in myotubes. Data are mean ± SD of the Ca^{2+}-induced Ca^{2+} release in accelerated (circle) and unaccelerated (square) groups with lidocaine (**a**), levobupivacaine (**b**), and ropivacaine (**c**)

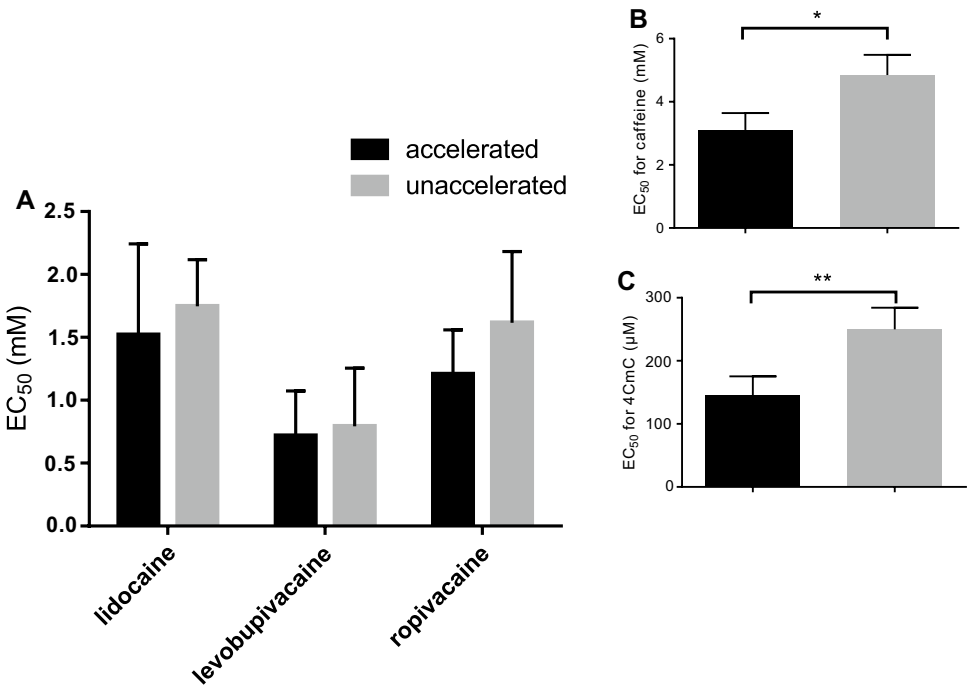

Fig. 5 50% effective concentration (EC_{50}) for local anesthetics (**a**), caffeine (**b**), and 4-chloro-m-cresol (4-CmC) (**c**) in accelerated and unaccelerated groups. There were no differences in EC_{50} for local anesthetics between accelerated and unaccelerated groups. EC_{50} for both caffeine and 4-CmC was significantly higher in unaccelerated group than accelerated group. $*p < 0.0001$, $**p < 0.0001$

Characteristics of myotubes

The diagnosis of MH takes into account several factors: clinical findings; genetic testing; muscle biopsy; and functional testing, specifically the caffeine–halothane contracture test in North America and the in vitro contracture test in Europe [28, 29]. In Japan, MH susceptibility is diagnosed based on the fact that CICR rates vary by Ca^{2+} concentrations. After muscles are chemically stripped with saponin, the CICR test can specifically detect SR abnormalities, eliminating other mechanisms related to the onset of MH, such as excitation–contraction coupling or Ca^{2+} homeostasis mediated by other Ca^{2+} transporters. The caffeine–halothane contracture test and the in vitro contracture test are based on the contracture of intact muscle fibers in the presence of halothane or caffeine as the RYR1 activator. Compared to the CICR test, which indicates only abnormalities in the SR, the caffeine–halothane contracture test /the in vitro contracture test can detect various abnormalities such as those of DHPR in the skeletal muscle. The results of the CICR rate test and the caffeine–halothane contracture test /the in vitro contracture test are not necessarily identical [13]; therefore, whether the CICR rate test can accurately assess MH predisposition remains a matter of debate. Therefore, we performed additional functional analysis using myotubes. Functional tests using myotubes can evaluate overall Ca^{2+} homeostasis in muscle cells, and the results were shown to correlate with those of the in vitro contracture test [30]. Our previous study demonstrated a good correlation between the CICR test and the response to RYR1 activators in human cultured myotubes [14]. Susceptibility to RYR1 agonists (caffeine and 4-CmC) was confirmed in myotubes, and all cells in the accelerated CICR group in this study had low RYR1 agonist EC_{50} values. This indicates that all cells in the accelerated CICR group in this study were predisposed to MH.

Limitations

The limitations of this study include its small sample size. As MH is a rare disease, few individuals underwent muscle biopsy during the study period. Various gene mutations have been reported in MH, and the muscle cells used in this study are not representative of all cases of MH. Also, some cells that demonstrated CICR acceleration was derived from individuals without apparent MH. Furthermore, whether the CICR rate test can accurately assess MH predisposition remains a matter of debate. Susceptibility to RYR1 agonists (caffeine and 4-CmC) was confirmed in myotubes, and all cells in the accelerated CICR group in this study had low RYR1 agonist EC_{50} values.

We used local anesthetics at lower concentrations than those used clinically. This is because the myotubes entered apoptosis when loaded with local anesthetics above the concentrations used in this study. These concentrations were considered to be the upper limits for conducting experiments with myotubes. Despite these limitations, this study showed that cells from individuals with or without MH predisposition exhibited no significant difference in fluctuations of intracellular Ca^{2+} dynamics caused by local anesthetics.

As MH is rare and difficult to investigate clinically, we conducted this experiment using skeletal muscle specimens from MH-susceptible individuals. We conclude that the myotoxicity of local anesthetics is equivalent in individuals with and without predisposition to MH.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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