



Amide-Type Local Anesthetics and Human Mesenchymal Stem Cells: Clinical Implications for Stem Cell Therapy

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ABSTRACT

In the realm of regenerative medicine, human mesenchymal stem cells (hMSCs) are gaining attention as a cell source for the repair and regeneration of tissues spanning an array of medical disciplines. In orthopedics, hMSCs are often delivered in a site-specific manner at the area of interest and may require the concurrent application of local anesthetics (LAs). To address the implications of using hMSCs in combination with anesthetics for intra-articular applications, we investigated the effect that clinically relevant doses of amide-type LAs have on the viability of bone marrow-derived hMSCs and began to characterize the mechanism of LA-induced hMSC death. In our study, culture-expanded hMSCs from three donors were exposed to the amide-type LAs ropivacaine, lidocaine, bupivacaine, and mepivacaine. To replicate the physiological dilution of LAs once injected into the synovial capsule, each anesthetic was reduced to 12.5%, 25%, and 50% of the stock solution and incubated with each hMSC line for 40 minutes, 120 minutes, 360 minutes, and 24 hours. At each time point, cell viability assays were performed. We found that extended treatment with LAs for 24 hours had a significant impact on both hMSC viability and adhesion. In addition, hMSC treatment with three of the four anesthetics resulted in cell death via apoptosis following brief exposures. Ultimately, we concluded that amide-type LAs induce hMSC apoptosis in a time- and dose-dependent manner that may threaten clinical outcomes, following a similar trend that has been established between these particular anesthetics and articular chondrocytes both *in vitro* and *in vivo*. *STEM CELLS TRANSLATIONAL MEDICINE* 2014;3:365–374

INTRODUCTION

Increasing interest in cell-based therapies as a method for treating tissue defects and disease has turned the focus of medical investigators and professionals to multilineage progenitor cells, which are capable of repairing several tissues that exist within the same niche [1]. Currently, many emerging tissue-repair concepts revolve around the use of allogeneic and autologous-derived human mesenchymal stem cells (hMSCs) for treatment via surgical implantation [2–5] or local injection [6–8]. The common interest in hMSCs derives from their innate (multipotent) capacity to differentiate into cartilage, bone, and adipose tissue as well as more specialized neurons, tendons, ligaments, and muscle [9].

Therapeutic applications for hMSCs are largely reliant on site-specific delivery and signaling from the respective niche microenvironment to cue differentiation [10, 11], allowing the clinical use of hMSCs to span multiple medical fields including cardiovascular repair, diabetes, stroke, multiple sclerosis, and orthopedic diseases, with additional applications developing on a regular basis [12]. Despite the ongoing characterization of these cells

from a functional perspective, few have addressed the influence that adjuvants to the respective therapy might have on hMSC integrity. Among the more commonly used adjuvant compounds in orthopedic practices are anesthetics, which are used to minimize patient sensitivity and discomfort [13, 14] and have been well characterized in the native joint niche with respect to cartilage.

Preceding the entry of hMSCs into the clinical realm, chondrocytes were a popular cell source for the treatment of orthopedic injuries [15], which are often characterized by eroding cartilage and chondrocyte death. Due to their suspected link to chondrolysis, various common local anesthetics (LAs) have been studied extensively in combination with chondrocytes [16–23]. Although many groups have demonstrated LA-induced chondrocyte toxicity and the coinciding upregulation of cartilage-degradation factors [16], little work has been performed with LAs with regard to hMSCs, which hold promise for repairing various tissues of orthopedic interest including cartilage [9]. In orthopedic injuries, the delivery of hMSCs is often required intra-articularly and at regions surrounding damaged ligament or tendon (e.g., anterior cruciate

ligament), where it is common to introduce LAs before, during, and after a procedure [13, 14]. Because of this practice, it is of critical importance to gain a working knowledge of the risks associated with the use of LAs in combination with hMSCs and to recognize the implications it may have for clinical outcomes.

Previous work performed by Lucchinetti et al. with reference to mesenchymal stem cells (MSCs) and LAs demonstrated that ropivacaine limited murine MSC proliferation and influenced the cells' sensitivity to environmental cues but did not induce cell death at the doses administered [24]. More recently, Rahnama et al. illustrated that 1-hour exposure of hMSCs to stock concentrations of amide-type LAs significantly affected cell viability 23 hours following exposure and concluded that bupivacaine is the least threatening to hMSC viability (of those examined) [25]. In this paper, we investigate the impact of various LAs on hMSC viability, adhesion, and mechanisms of LA-induced cell death *in vitro*. We expose bone marrow-derived hMSCs from three separate donors to the aminoamide-type LAs: lidocaine, bupivacaine, ropivacaine, and mepivacaine. Each hMSC line was exposed to the four LAs at various dilutions from the stock solution (SS) over time courses ranging from 40 minutes to 24 hours as a method of replicating the operational concentrations and potential resident times of the anesthetics when delivered into an average knee joint capsule containing 0.5–4.0 ml of synovial fluid [26]. In orthopedic practice, it is common that the volume of anesthetics delivered intra-articularly can range from half of the total synovial capsule's capacity to as little as one-eighth. Hence, we investigated concentrations of each anesthetic ranging from one-half to one-eighth of their SSs to replicate doses that are often used in orthopedic procedures. Throughout this study, we demonstrate that therapeutic concentrations of certain LAs can result in a delayed adherence phenotype over extended time courses and induce apoptosis within as little time as 40 minutes after exposure.

MATERIALS AND METHODS

hMSC Isolation and Culture

Primary hMSCs were culture expanded from bone marrow of three consenting patients/donors, as described previously [27]: a 27-year-old male, a 45-year-old male, and a 56-year-old female with osteoarthritis diagnosis in multiple joints. hMSCs were cultured in minimum essential medium- α (α MEM) (Gibco, Grand Island, NY, <http://www.invitrogen.com>; catalog no. 12571) with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, <http://www.atlasbio.com>; catalog no. F-0500-A). For all experimental set-ups, cultures were established from cryo-containment in passage 1 and used between passage 2 and passage 3; total days in culture (*ex vivo*) did not exceed 18 days. When prepared for experimentation, hMSCs were removed using TrypLE (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>; catalog no. 12563029) and seeded in 24-well plates (BD Biosciences, San Diego, CA, <http://www.bdbiosciences.com>; catalog no. 353047) at a density of 20,000 cells per well with media containing the respective LA or saline component and incubated at 37°C and 5% carbon dioxide until analyzed.

Validation of hMSC Lines

Characterization of hMSCs to validate our process was done in one patient by *in vitro* functional differentiation and four-color flow cytometry, consistent with the requirements established by the International Society for Cellular Therapy [28]. The cells were blocked

with 1% BSA in phosphate-buffered saline (PBS) and then stained with directly conjugated fluorescent antibodies: CD44-FITC, CD105-PE, CD73-PerCP-Cy5.5, CD90-APC and CD34-FITC (BD Biosciences; catalog nos. 560977, 560839, 561260, 561971, and 555821) and analyzed via the BD Accuri C6 flow cytometer and associated software. Cells were >99% CD44⁺/CD73⁺/CD90⁺/CD105⁺ and CD34⁻ (>10,000 cells counted in gated area). The differentiation capacity of the hMSCs was evaluated using the Human MSC Functional Identification Kit (R&D Systems Inc., Minneapolis, MN, <http://www.rndsystems.com>; catalog no. SC006). The cells were incubated in differentiation media for 4 weeks and then evaluated for adipocyte, chondrocyte, and osteocyte differentiation according to the manufacturer's instructions.

LA Preparation for Cell Culture

hMSCs were exposed to the LAs diluted to 50%, 25%, and 12.5% of the SSs in α MEM with 10% FBS immediately prior to cell exposure. Purchased SSs were 1% lidocaine (1 mg/ml; Hospira Inc., Lake Forest, IL, <http://www.hospira.com>; NDC catalog no. 0409-4276-02), 0.5% ropivacaine (0.5mg/ml; APP Pharmaceuticals, Lake Zurich, IL, <http://www.fresenius-kabi.us/>; NDC catalog no. 63323-286-30), 2% mepivacaine (2 mg/ml; Hospira; NDC catalog no. 0409-2047-50), and 0.5% bupivacaine (0.5 mg/ml; Hospira; NDC catalog no. 0409-1163-01). Dulbecco's PBS (Gibco; catalog no. 14190) was used as a saline control and occupied culture medium component at the same respective volume/volume as LAs tested. The pH of all LAs at each dilution in complete media was measured (Hanna Instruments, Smithfield, RI, <http://www.hannainst.com>; catalog no. HI 32) and compared with complete media alone (α MEM plus 10% FBS). There were no major changes in pH as a consequence of adding LAs at any concentration to total media; therefore, it is not considered to be a confounding factor in the study (supplemental online Table 1).

24-Hour LA Exposure Live/Dead Assay

hMSCs from three separate patients (described in the hMSC Isolation and Culture section) were analyzed concurrently while exposed to the diluted LAs for 24 hours. After the exposure, the media was collected and the wells trypsinized to determine viability of both the adherent and nonadherent cells. A live/dead assay was performed using CellTrace calcein red-orange AM at 2 μ m (Molecular Probes, Eugene, OR, <http://probes.invitrogen.com>; catalog no. C34851) to detect live cells and the nucleic acid stain SYTOX green-1 AM (SYTOX; Molecular Probes; catalog no. S-34860) diluted 1:2,000 from stock to detect dead cells. Each was added according to the manufacturer's instructions, and cells were analyzed using flow cytometry. Gates were established by running an unlabeled/single-labeled CellTrace and SYTOX in untreated samples. Live cells were positive in FL3 only (calcein red-orange AM⁺), whereas dead cells were positive in FL1 (SYTOX green⁺) or FL1 plus FL3 (SYTOX green⁺/calcein red-orange AM⁺), with color compensation applied. For analysis, 5,000 cell counts were obtained in the gated area per run per patient hMSC line as a measure of consistency across all samples. Additional counts were collected thereafter until the sample was limited by the volume available; the distribution of fluorescence did not differ between these primary and secondary collection subsets. Sample volume limitations were the result of the formation of dense crystals by some anesthetics that settled in the cuvette. This marked a threshold for collection volume because analyzing these particles added significant background to the data set within the gated area. Live versus dead counts were compared with determined

viable percentage in each condition based on gating respective to the unlabeled controls. Data are presented as a mean percentage and the associated standard error of the mean and represent the merged data from the three hMSC lines per condition ($n = 3$).

Cells collected from the medium fraction of culture were analyzed independently of the adhered population via flow cytometry using the live/dead staining technique described. This provided a method for determining “percentage adhered.” Cell counts (live and dead) from adhered and unadhered cell populations were compared with determined percentage adhered. Data are presented as a mean percentage and the associated SEM and represent the merged data from the three hMSC lines per condition ($n = 3$).

40-, 120-, and 360-Minute Apoptotic Assay

hMSCs from three separate patients (described in the hMSC Isolation and Culture section) were analyzed concurrently following exposure to the listed dilutions of LAs for 40, 120, and 360 minutes. Adherent cells and cells in suspension were pooled, centrifuged and washed two times in PBS. Apoptotic cells were labeled with annexin-V-conjugated APC antibody (BD Biosciences; catalog no. 550475) and dead cells with SYTOX Green. Annexin-V antibody and SYTOX were added according to the manufacturer’s instructions and analyzed via flow cytometry in FL1 and FL4. Apoptotic and necrotic controls were established prior to investigation using camptothecin at $6 \mu\text{M}$ (Sigma-Aldrich, St. Louis, MO, <http://www.sigmaaldrich.com>; catalog no. C9911) and hydrogen peroxide at $400 \mu\text{M}$ (Sigma-Aldrich; catalog no. H1009), respectively, to verify the assay gating technique (supplemental online Fig. 1). It is noteworthy to state that at these concentrations of hydrogen peroxide, a degree of apoptosis is to be expected [29]. Single-labeled and unlabeled control samples were used to establish gating criteria as shown in supplemental online Figure 1; viability $>80\%$ was a minimum criteria for annexin-V/SYTOX-stained controls (supplemental online Fig. 2A). For analysis, 5,000 cells were counted in the gated area per run per patient hMSC line as a measure of consistency across all samples. Additional events were collected beyond 5,000 counts until limited by sample volume; these additional counts showed no difference in marker distribution when compared with the initial 5,000 cells counted (supplemental online Fig. 2). Sample volume limitations were the result of the formation of dense crystals by some anesthetics that settled in the cuvette. This marked a threshold for collection volume because analyzing these particles added significant background to the data set within the gated area. Data are presented as a mean percentage and the associated SEM and represent the merged data from the three hMSC lines per condition ($n = 3$).

Adherent hMSC Toxicity Assay

In order to assess the effects of the delayed adherence on cell viability, hMSCs were allowed to adhere until 80% confluent and then exposed to the 50% diluted LAs for 60 minutes. The medium was removed, and the wells were lightly washed with PBS. The live/dead staining protocol was performed with CellTrace calcein red-orange AM (live) and SYTOX green (dead), and images were captured in monochrome via the AMG EVOS fl inverted microscope (Life Technologies, Rockville, MD, <http://www.lifetech.com>) using the RFP Light Cube (531 nm excitation, 593 nm emission) and GFP Light Cube (470 nm excitation, 525 nm emission), respectively.

Quantification of hMSC metabolism during exposure to LAs was determined by the use of the PrestoBlue cell viability reagent

(Molecular Probes; catalog no. A-13261) according to the manufacturer’s instructions. The 96-well plates were seeded with 5,000 hMSCs per condition, and each LA was added to a respective well at 50% SS, as described previously. Once treated with LAs, wells were excited with a 544-nm beam, and emission was collected at 590 nm using a Fluoroskan Ascent FL plate reader (Thermo Fisher Scientific, Waltham, MA, <http://www.thermoscientific.com>). Cell-free blanks were prepared with media and each anesthetic at 50% SS to normalize each treatment against the respective background. Relative fluorescent units (RFUs) were obtained from treatments and blanks at “time zero” and 60 minutes. Treatments were normalized to their respective blanks. Normalized RFUs from time zero were subtracted from the 60-minute values to calculate actual RFUs resulting from cellular metabolism. Data are presented as mean RFUs and the associated SEMs ($n = 2$).

Endoplasmic Reticulum and Intracellular Calcium Labeling

Cells were allowed to adhere until 80% confluent, with the media discarded and the wells washed with PBS. ER-Tracker Blue-White DPX (Molecular Probes; catalog no. E12353) at $0.75 \mu\text{M}$ was added according to the manufacturer’s instructions. Images are shown in red for contrast. The wells were washed with PBS, and $5 \mu\text{M}$ Calcium Green-1, AM (Molecular Probes; catalog no. C3012) was added according to the manufacturer’s instructions. The wells were washed with PBS and visualized with fluorescent microscopy using the AMG EVOS fl microscope. Monochrome images were captured for the Calcium Green-1, AM, with the GFP Light Cube (470 nm excitation, 525 nm emission; shown as green), and the ER-Tracker Blue-White DPX was captured using the DAPI Light Cube (357 nm excitation, 447 nm emission; shown as red for contrast). Cells were then exposed to 90% SS LA to exaggerate the effects noted at lower doses; images were captured at 0, 5, 10, and 20 minutes after exposure.

Statistical Analysis

Analysis was performed in LA exposures for 24-hour experiments as well as 40-, 120-, and 360-minute experiments ($n = 3$). For each treatment (e.g., exposure to bupivacaine at 50% SS for 40 minutes), raw data from each of the three independent hMSC lines were used as the input for a one-way analysis of variance. Following output, all p values were generated by Tukey’s post-test. Results were considered to be significant when $p < .05$. In metabolic assays, experiments were run in duplicate ($n = 2$) and significance was determined by one-way analysis of variance and Tukey’s post-test where the resulting p values $< .05$ were considered to be significant.

RESULTS

24-Hour Exposure to Diluted LAs Reduces hMSC Viability and Adhesion

hMSCs were seeded with each of the respective LAs diluted in culture media to 50%, 25%, and 12.5% SS (Table 1) for 24 hours. We found that the percentage of cell viability when treated with ropivacaine, lidocaine, and mepivacaine at 12.5% SS did not differ significantly from the saline control ($p > .05$) (Fig. 1A). Bupivacaine treatment alone at this concentration resulted in significantly lower cell viability compared with the control ($p < .01$) (Fig. 1A). In addition, hMSC viability when treated with bupivacaine at this concentration was significantly lower when compared with all other LAs examined ($p < .05$; not depicted). Percent viability of hMSCs

Table 1. Dilution of stock LAs to working solutions

LA	Stock concentrations		Experimental concentrations					
	100% of stock (%)	mg/ml	50.0% of stock (%)	mg/ml	25.0% of stock (%)	mg/ml	12.5% of stock (%)	mg/ml
Lidocaine	1.00	10	0.500	5	0.250	2.5	0.1250	1.25
Ropivacaine	0.50	5	0.250	2.5	0.125	1.25	0.0625	0.625
Bupivacaine	0.50	5	0.250	2.5	0.125	1.25	0.0625	0.625
Mepivacaine	2.00	20	1.000	10	0.500	5	0.2500	2.5

Stock concentrations of LAs purchased are shown as the percentage of LA in solution and milligrams per milliliter. Experimental concentrations are shown as the final percentage of LA in solution and milligrams per milliliter once stock LA was diluted into culture media.

Abbreviation: LA, local anesthetic.

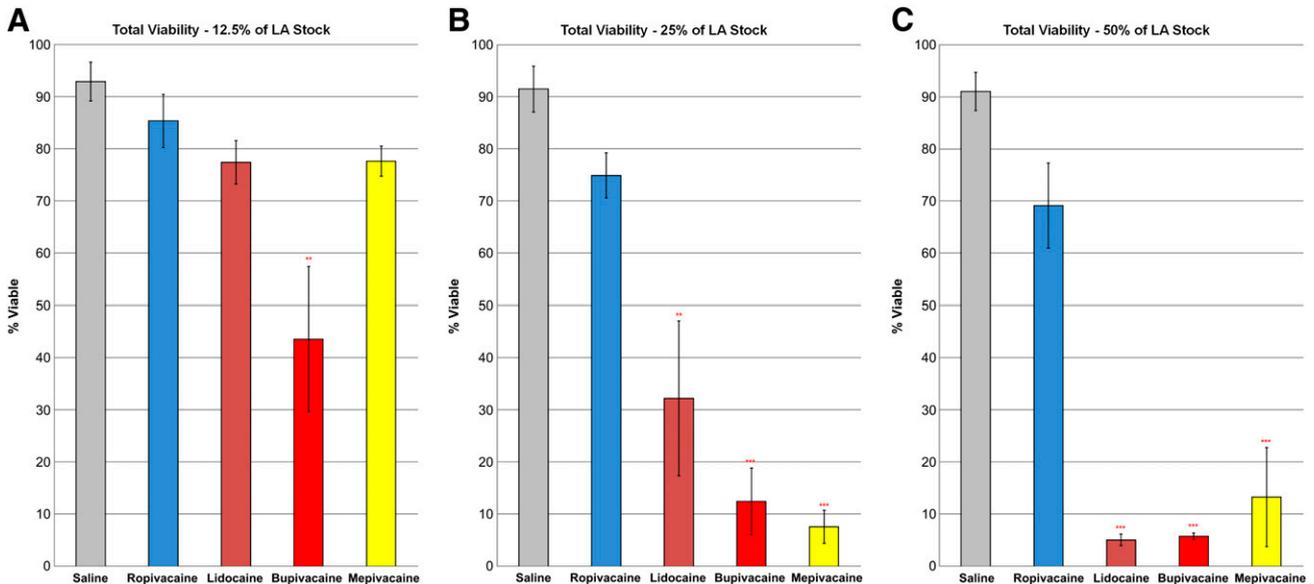


Figure 1. Percentage of viable human mesenchymal stem cells following 24-hour treatment with LAs. Human mesenchymal stem cells were treated with LAs at 12.5% (A), 25% (B), and 50% (C) of the stock solution. Cells were stained with calcein red-orange AM and SYTOX green and then analyzed via flow cytometry. All data are represented as a mean with the associated SEM ($n = 3$). Levels of significance compared with control are depicted as follows: *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviation: LA, local anesthetic.

exposed to 25% and 50% SS of mepivacaine, bupivacaine, and lidocaine was statistically lower than control ($p < .001$) (Fig. 1B, 1C); however, ropivacaine did not differ from the saline control in terms of hMSC viability at these levels ($p > .05$) (Fig. 1B, 1C). Furthermore, hMSC viability following ropivacaine treatment at these levels was significantly higher than that of hMSCs treated with the corresponding levels of mepivacaine, bupivacaine, and lidocaine ($p < .001$; not depicted).

Interestingly, hMSC adhesion was significantly reduced when exposed to 12.5% SS of bupivacaine ($p < .001$) and mepivacaine ($p < .01$) for 24 hours (Fig. 2A). Treatment with higher concentrations (25% and 50% SS) of lidocaine, bupivacaine, and mepivacaine resulted in significantly less cell adhesion compared with the control ($p < .001$) (Fig. 2B, 2C). Likewise, the percentage of adhered hMSCs treated with ropivacaine at these higher levels differed from the saline control as well ($p < .01$) but also was characterized by a significantly higher percentage of adhered cells when compared with the other three LAs ($p < .001$; not depicted).

Short-Term LA Exposure Initiates an Apoptotic Cascade in hMSCs

To investigate the mechanism of hMSC death following LA administration, we exposed each of the three cell lines to the equivalent

concentrations of the aminoamide-type LAs described above for 40, 120, and 360 minutes and then labeled cells with the apoptotic marker annexin-V and the nuclear stain SYTOX. Annexin-V staining is dependent on flippase activity to translocate phosphatidylserine from the cytoplasmic membrane interface to the extracellular leaflet [30]. To accurately detect apoptotic populations, gates were established based on unlabeled cell populations, and annexin-V/SYTOX staining specificity was verified by the use of camptothecin and hydrogen peroxide, respectively (supplemental online Fig. 1). From these controls, we identified a “live” population (annexin-V⁻/SYTOX⁻), an “early apoptotic” population (annexin-V⁺/SYTOX⁻ only), a “late apoptotic” population (annexin-V⁺/SYTOX⁺), and a “necrotic” population (annexin-V⁻/SYTOX⁺ only). In this paper, early and late apoptotic populations are combined and are referred to as “apoptotic.”

At 40 minutes, apoptosis was only evident following hMSC treatment with bupivacaine at 50% SS, marked by the significant increase in annexin-V⁺ cells coupled with the significant reduction in live cells compared with the saline controls ($p < .001$), whereas lidocaine, ropivacaine, and mepivacaine remained normal respective to the controls ($p > .05$) (Fig. 3C). Increasing LA exposure to 120 minutes resulted in the significant increase of annexin-V⁺ cells over the respective control with bupivacaine treatment at the lower dose of 25% SS ($p < .01$) and the corresponding

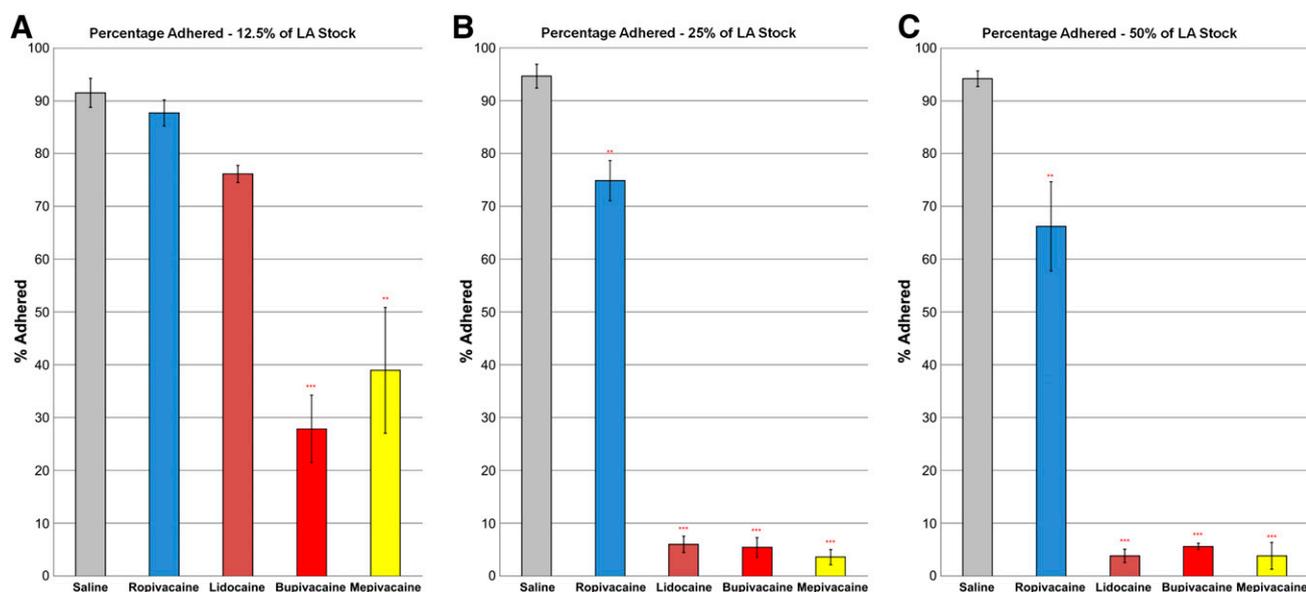


Figure 2. Percentage of adhered human mesenchymal stem cells following 24-hour treatment with LAs. Human mesenchymal stem cells were treated with LAs at 12.5% (A), 25% (B), and 50% (C) of the stock solution. Cells in suspension were analyzed independently of those adhered to determine percentage adhered. All data are represented as a mean with the associated SEM ($n = 3$). Levels of significance compared with control are depicted as follows: *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviation: LA, local anesthetic.

decrease in live cells ($p < .001$) (Fig. 4B). When treated with lidocaine, bupivacaine, and mepivacaine for 120 minutes at 50% SS, the increase of annexin- V^+ cells and decreased live cells reached significance respective to the control ($p < .001$) (Fig. 4C). hMSCs treated with mepivacaine at 50% SS also demonstrated a necrotic phenotype (annexin- V^- /SYTOX $^+$) elevated over control ($p < .01$) (Fig. 4C).

hMSCs treated for 360 minutes with the lowest levels of bupivacaine (12.5% SS) led to an increase in annexin- V^+ cells along with the expected decrease in the live population (compared with the saline control) ($p < .01$) (Fig. 5A). Increasing LA concentrations to 25% SS resulted in significantly reduced live cell counts and increased annexin- V^+ counts over the control when exposed to lidocaine ($p < .01$), bupivacaine ($p < .001$), and mepivacaine ($p < .001$) (Fig. 5B). Ropivacaine-treated cells did not differ from the control in any terms. Increasing exposure to 50% SS resulted in greater statistical separation from the control in the case of lidocaine ($p < .001$), whereas bupivacaine and mepivacaine remained consistent with data from 25% SS. In contrast, ropivacaine maintained $>70\%$ viability with no difference versus the controls ($p > .05$) (Fig. 5C), complementing the 24-hour viability data.

LA Exposure Induces Death in Adherent hMSCs

Adherent hMSCs were treated with LAs at 50% SS for 60 minutes to demonstrate that the onset of LA-induced cell death was independent of the delayed adhesion phenotype. When stained concurrently with calcein and SYTOX, it is qualitatively evident that bupivacaine and mepivacaine induce rapid cell death within an hour at the concentrations used, demonstrated by (positive) nuclear staining with SYTOX. Alternatively, lidocaine and ropivacaine exposure showed a low level of cells with stained nuclei (Fig. 6). In addition, calcein staining of ropivacaine-treated hMSCs was comparable to the saline control, characterized by cells saturated with signal, which is indicative of high cellular metabolism. In contrast, calcein signal in cells treated with lidocaine, bupivacaine, and mepivacaine was weak (comparatively). Furthermore, mepivacaine and

bupivacaine treatments were characterized by extensive cell detachment, evident by the disruption of the adherent-type morphology in the phase contrast and fluorescent images. Lidocaine treatment also resulted in hMSC detachment but to a much lesser degree, whereas ropivacaine exposure had no noticeable influence on cell adherence. These qualitative assessments were verified by measuring metabolic activity following 60-minute exposure to each LA at 50% SS using the PrestoBlue reagent and measuring RFUs. Bupivacaine was the only LA treatment that differed significantly from the saline control ($p < .05$) (Fig. 6B).

To determine whether hMSC death in response to specific LAs is connected to calcium dysregulation, we labeled the endoplasmic reticulum (ER), a major calcium storage organelle, and intracellular calcium in adhered hMSCs, much of which colocalized with the ER (and nucleus). The influence of each LA on hMSCs was examined at 50% SS over a 50-minute time course (data not shown). Consistent with trends identified in our previous experiments, bupivacaine appeared to have the most prominent effect on hMSCs of the LAs investigated with respect to intracellular calcium fluorescence, whereas ropivacaine treatment resulted in the least. To exaggerate the response observed at 50% SS, hMSCs were exposed to a lower dilution of ropivacaine and bupivacaine over a shortened time course. A brief 10-minute exposure to bupivacaine (90% SS) resulted in the rapid decrease of intracellular calcium in regions colocalized with the ER. By 20 minutes, we observed the near complete evacuation of intracellular calcium localized with the ER under these conditions (supplemental online Fig. 3A). Concurrently, the morphology of the ER in bupivacaine-treated cells became disrupted, taking on a fragmented appearance (supplemental online Fig. 3B). In contrast, exposure to ropivacaine (90% SS) did not result in an observable loss of intracellular calcium, and calcium signal localized with the ER (and nucleus) maintained fluorescent intensity over the time course examined (supplemental online Fig. 3A). In addition, the structure of the ER remained unaltered over the course of exposure (supplemental online Fig. 3B).

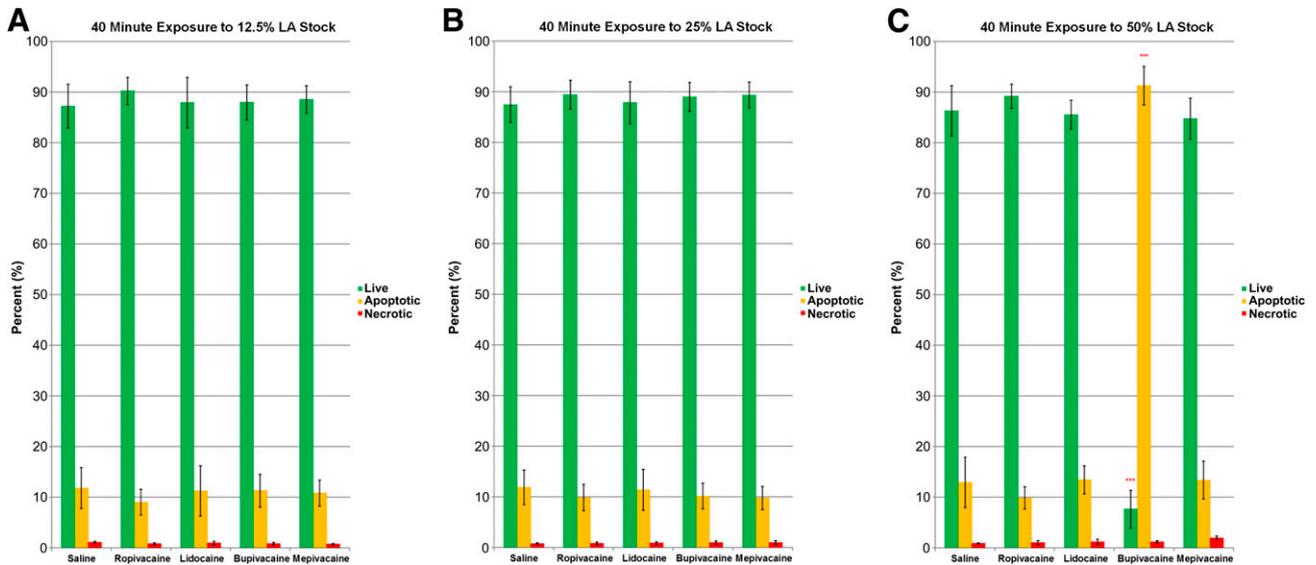


Figure 3. Apoptosis of human mesenchymal stem cells following 40-minute exposure to LAs. Human mesenchymal stem cells were treated with LAs at 12.5% (A), 25% (B), and 50% (C) of the stock solution. Unstained cells are “live”; annexin-V⁺ and annexin-V⁺/SYTOX⁺ cells are apoptotic; SYTOX⁺ cells are necrotic. All data are represented as a mean with the associated SEM ($n = 3$). Levels of significance compared with control are depicted as follows: *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviation: LA, local anesthetic.

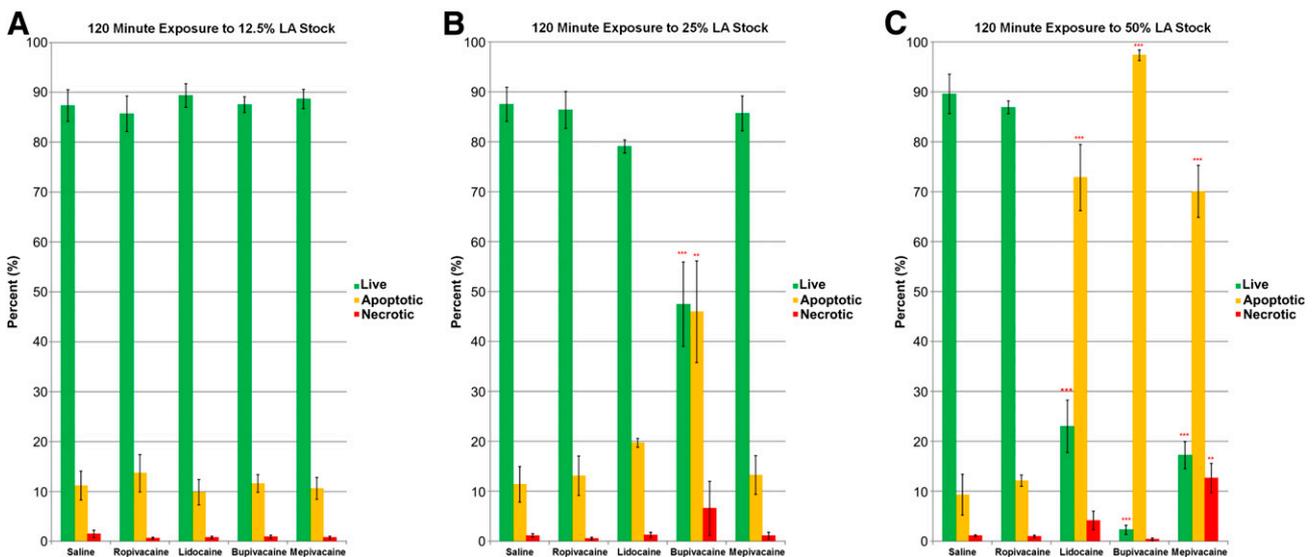


Figure 4. Apoptosis of human mesenchymal stem cells following 120-minute exposure to LAs. Human mesenchymal stem cells were treated with LAs at 12.5% (A), 25% (B), and 50% (C) of the stock solution. Unstained cells are “live”; annexin-V⁺ and annexin-V⁺/SYTOX⁺ cells are apoptotic; SYTOX⁺ cells are necrotic. All data are represented as a mean with the associated standard error of the mean ($n = 3$). Levels of significance compared with control are depicted as follows: *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviation: LA, local anesthetic.

DISCUSSION

Allogeneic and autologously sourced hMSCs have gained significant attention in the past several years as a potential method for the repair and regeneration of orthopedic tissues [9]. Among the first attempts to investigate the role for hMSCs in the orthopedic field was Steadman et al. via microfracture [31]. This approach (in part) is aimed to liberate hMSCs from long-bone marrow to allow for restructuring of chondral defects and fill the chondral void with the respective tissue. More recently, bone marrow aspirate concentrate has been used as an hMSC source for cartilage repair [32, 33].

Often, such procedures warrant intra-articular application of LAs to minimize patient discomfort. We demonstrated that relevant orthopedic doses intended to mimic clinical dilutions of stock LAs in the synovial capsule (or subcutaneously) have an adverse effect on hMSCs that may negatively affect clinical outcomes. Likewise, extensive studies that investigated chondrocyte viability and biological effectiveness in response to LA exposure are consistent with our findings, which are among the first to characterize the impact of LAs on hMSCs related to current clinical applications.

In the studies conducted, we investigated the impact of four amide-type LAs on three separate hMSCs lines as a method to

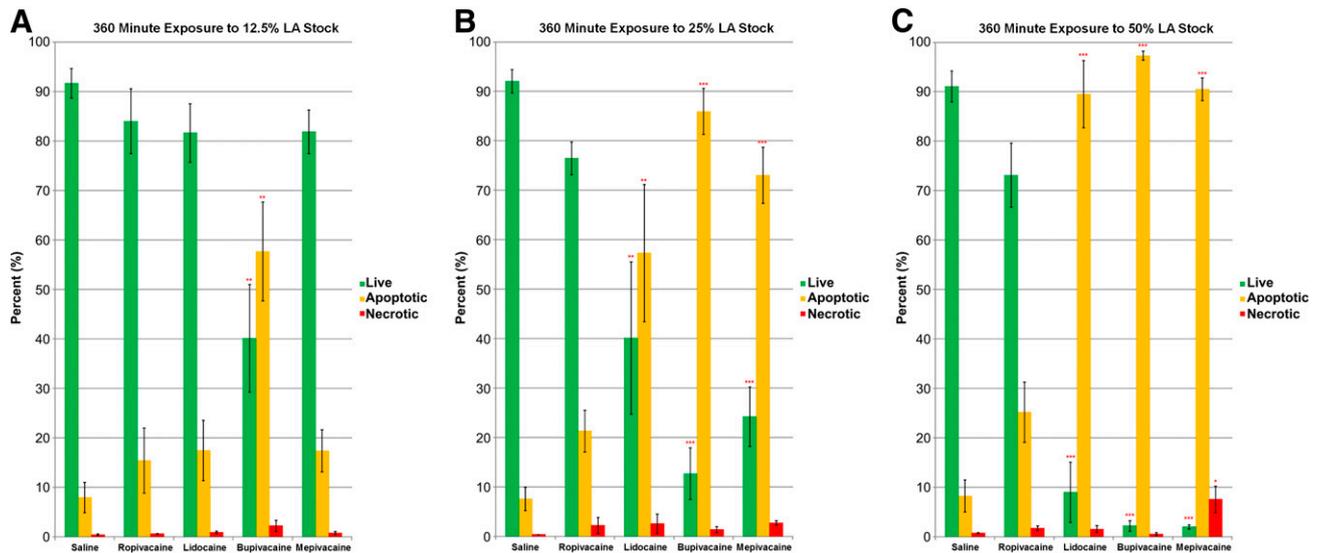


Figure 5. Apoptosis of human mesenchymal stem cells following 360-minute exposure to LAs. Human mesenchymal stem cells were treated with LAs at 12.5% (A), 25% (B), and 50% (C) of the stock solution. Unstained cells are “live”; annexin- V^+ and annexin- V^+ /SYTOX $^+$ cells are apoptotic; SYTOX $^+$ cells are necrotic. All data are represented as a mean with the associated SEM ($n = 3$). Levels of significance compared with control are depicted as follows: *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviation: LA, local anesthetic.

demonstrate the consistency of the hMSC response to LAs regardless of the donor background. The LAs examined included ropivacaine, lidocaine, mepivacaine, and bupivacaine, diluted to 50%, 25%, and 12.5% of their stock. To establish a baseline for cell survival, hMSCs were treated with the respective diluted stock LAs for 24 hours. Adherent cells and those in suspension were collected, stained with calcein red-orange and SYTOX and analyzed by flow cytometry. At the lowest dose of LAs administered, bupivacaine treatment alone resulted in a significant reduction of viable cells respective to the control. At higher levels (25% and 50% SS), hMSC exposure to three of the four LAs led to the significant loss of viability against the saline control. Treatment with ropivacaine was the lone exception, for which hMSC viability was comparable to the control at all doses.

To evaluate the influence of LAs on hMSC adhesion, cells remaining in suspension after 24 hours were compared with the number of adherent cells for each treatment. Surprisingly, we found that bupivacaine and mepivacaine inhibited a significant portion of hMSCs from adhering at all concentrations respective to the saline control, whereas lidocaine differed only at 25% and 50% SS. Compared with the control, ropivacaine treatment also inhibited hMSC adhesion at these levels; however, it is clinically relevant to note that the percentage of adhered cells in ropivacaine treatments at 25% and 50% SS was significantly elevated over the other three LAs, despite contrast with the control groups.

In order to determine whether short-term hMSC exposure to LAs resulted in cell death via apoptosis or necrosis, we labeled cells treated with LAs for 40, 120, and 360 minutes for the apoptotic marker annexin-V and the nuclear stain SYTOX. In this study, we classified apoptotic cells as annexin- V^+ /SYTOX $^-$ (early apoptotic) and annexin- V^+ /SYTOX $^+$ (late apoptotic), whereas annexin- V^- /SYTOX $^+$ were necrotic (annexin- V^- /SYTOX $^-$ are live cells). At the highest concentration of bupivacaine administered, we observed significant apoptosis following 40 minutes of exposure. Similarly, 120 minutes of bupivacaine exposure at 25% SS led to increasing apoptotic counts. In contrast, 120-minute exposure to 50% SS of bupivacaine, lidocaine, and mepivacaine resulted in the vast

majority of the cell population undergoing apoptosis, with the exception being ropivacaine treatment. At 360 minutes, the lowest dose of bupivacaine resulted in an apoptotic population that drastically exceeded that of the live population. At this time point, both the 25% and 50% SS of lidocaine, bupivacaine, and mepivacaine were characterized by >50% of the population in an apoptotic state. Interestingly, ropivacaine treatment at all doses and time points investigated did not result in an apoptotic population that differed from the saline controls. Cumulatively, these findings suggest that the initiation of apoptosis by amide-type LAs, specifically bupivacaine, lidocaine, and mepivacaine, is a time- and dose-dependent process. Consequently, lower doses require longer time courses to initiate an apoptotic cascade, whereas the high doses lead to the rapid onset. This suggests that the large, nonviable hMSC population at 24 hours, when treated with bupivacaine, mepivacaine, and lidocaine, was attributable to an LA-induced apoptotic cascade during early exposure.

To address the ability of LAs to induce apoptosis in adhered cells and rule out delayed adherence as a major contributor, hMSCs were adhered to the culturing well prior to LA administration. Using the higher concentration of each LA (50% SS), we observed extensive cell death in hMSCs treated with bupivacaine, mepivacaine, and lidocaine within 1 hour; however, cells treated with ropivacaine maintained a large number of viable cells by comparison. Furthermore, adherent hMSCs exposed to mepivacaine and bupivacaine—and, to a lesser extent, lidocaine—showed extensive detachment phenotypes, whereas ropivacaine-treated cells remained attached. Thus, we identified a cell-death phenotype that develops concurrently with a detachment phenotype in three of the four LAs. This serves as additional evidence to support the concept that reduced hMSC viability during 24-hour LA treatments was not the consequence of delayed adherence, suggesting that the two phenotypes develop independently.

The rapid induction of cell death via the apoptotic pathway as the consequence of failed calcium regulation is a well-studied process [34, 35], including apoptosis as the result of coordinated release of intracellular calcium stores [36]. In addition, cell

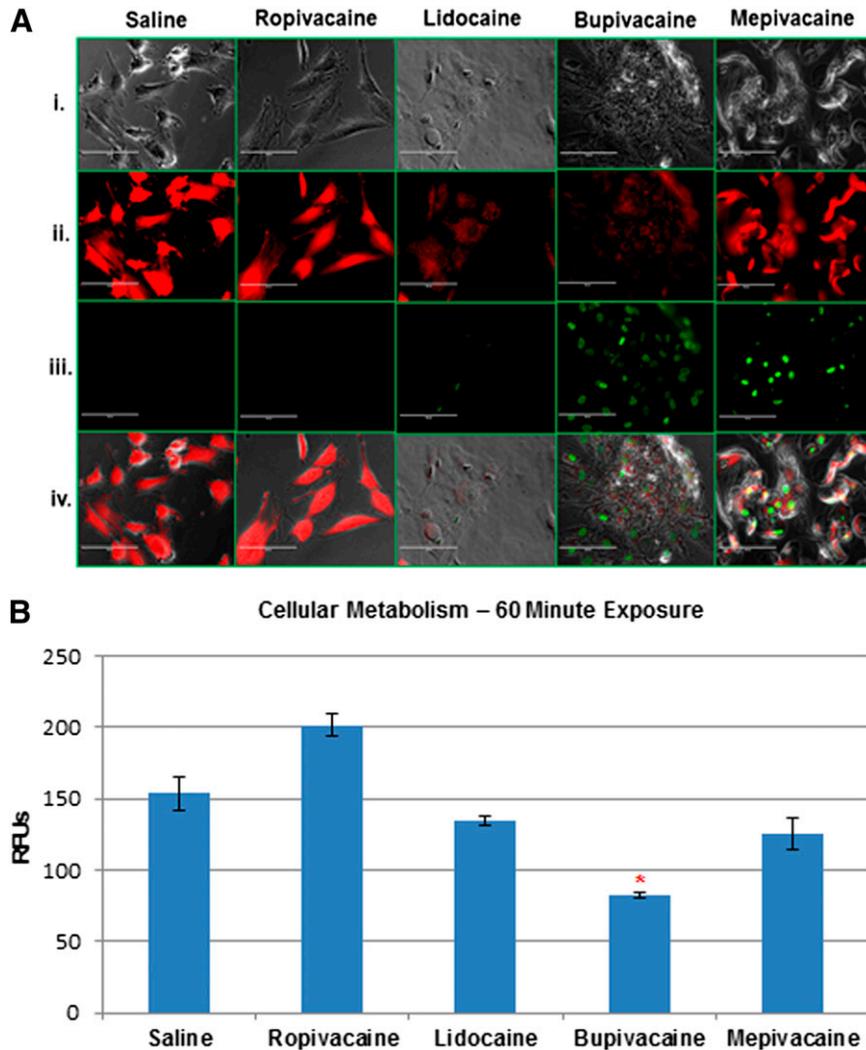


Figure 6. Local anesthetics (LAs) induce cell death in adherent human mesenchymal stem cells. Following 60-minute exposure to LAs at 50% of the stock solution, cells were visualized ($\times 1,172$ magnification). **(A):** Morphological changes by phase-contrast (i) and viability using calcein red-orange (ii), SYTOX green (iii), and merged channels (iv). Scale bars = 100 μm . **(B):** Quantification of cellular metabolism via PrestoBlue is represented as RFUs following 60-minute exposure to LAs at 50% of the stock solution. All data are represented as a mean with the associated SEM ($n = 2$). Level of significance compared with control is depicted as follows: *, $p < .05$. Abbreviation: RFU, relative fluorescent unit.

adhesion is a process that is mediated (in part) by intracellular calcium and is largely dependent on the activity of intracellular calcium stores for regulation [37–39]. Thus, we postulated that intracellular calcium stores are depleted during LA exposure and that specific LA types are more effective in activating this cascade; the consequences include hMSC apoptosis and abrogated adhesion.

In the interest of determining whether calcium played a role in LA-mediated hMSC death, we labeled the ER of adherent hMSCs concurrently with an intracellular calcium dye and exaggerated the effect of the LAs by adding them at 90% SS. Bupivacaine and ropivacaine were chosen based on our earlier data regarding apoptotic/cell death phenotypes following exposure, where bupivacaine was the most robust in inducing the phenotypes and ropivacaine the least. Immediately prior to LA administration, the labeled cells were visualized by fluorescent microscopy to identify the ER structure and intracellular calcium deposits. We found that punctate calcium stores were colocalized with the ER as well as the nucleus. The addition of bupivacaine at 90% SS resulted in

calcium depletion within 10 minutes, marked by the loss of fluorescent intensity. At 20 minutes, most calcium appeared to have been evacuated from intracellular calcium stores localized with the ER, along with a noticeable loss of cytoplasmic calcium. Furthermore, the ER structure became distorted and resembled a fragmented-type morphology, a phenotype that has been previously linked to calcium-induced apoptosis [40]. These observations suggest a pivotal role for calcium regulation in the initiation of the apoptotic cascade during LA exposure and potentially explain the interruption of hMSC adhesion, consistent with previous work in other cell types [37–39]. It is possible that bupivacaine toxicity results from interaction with the membrane protein sodium potassium ATPase [41], which may trigger ER calcium depletion via interaction with the inositol triphosphate receptor [42, 43]. Alternatively, hMSCs treated with ropivacaine did not follow this trend. Calcium stores remained intact, and the ER did not undergo an observable morphological change; however, there does appear to be the formation of calcium-rich membrane-based vesicles that extend into the

extracellular environment. We question whether this may be an hMSC-specific response to ropivacaine, characterized by the mobilization of ropivacaine-bound plasma membrane proteins, providing a potential mechanism for hMSC resistance to this specific LA.

Although performed in murine MSCs, our findings complement the results reported by Lucchinetti et al. [24], and we find that ropivacaine does not induce a significant degree of cell death at clinically relevant doses. Contrary to the recent findings of Rahnama et al. [25], our work identified diluted doses of bupivacaine to be highly toxic to hMSCs within a 60-minute time course. Furthermore, we submit that ropivacaine is the ideal LA for use with hMSC-based therapies (when required). We suspect that experimental design plays a role in this discrepancy because initial exposure of hMSCs to LAs by Rahnama et al. was for a period of 1 hour before being washed and the cells allowed to expand [25]. It is possible that the surviving fraction was able to propagate over the ensuing 23 hours before measures were taken. In our study, we find that the majority of adhered cells die within 60 minutes of bupivacaine exposure (50% SS), as demonstrated by positive nuclear staining in this hMSC population. Complementing these data is our 40-minute time point, at which hMSCs exposed to 50% SS bupivacaine resulted in >90% of the cell population in apoptosis.

Across the spectrum of our study, we found that our results are most comparable to those performed with articular chondrocytes [44]. Previously, short-term exposure to bupivacaine at high doses (equivalent and higher than used in our study) for as little as 15 minutes was shown to result in significant human chondrocyte death compared with the control [17]. Similarly, a comparative study with human articular chondrocytes exposed to ropivacaine (stock 0.5%) and bupivacaine (stock 0.5%) for 30 minutes showed significantly greater viability in the ropivacaine-treated cells compared with bupivacaine [22]; complementing results were derived from articular bovine cartilage exposed to bupivacaine in vitro and in vivo [17, 22, 45]. Our results support the previous claim that lidocaine is less toxic than bupivacaine [20] but with respect to hMSCs. Although mepivacaine appears to be the least characterized in prior work, others suggest it is less toxic than bupivacaine and lidocaine in equine chondrocytes [21], and this is partially supported by our findings in hMSCs.

CONCLUSION

We have demonstrated that several common amide-type LAs are capable of inducing apoptosis in hMSCs in a dose- and time-dependent manner, possibly by means of intracellular calcium store depletion and dysregulation of calcium signaling. Furthermore, LAs influence the ability for hMSCs to adhere in vitro, suggesting an important role for delivery vehicles (e.g., fibrin glue) to successfully place and maintain hMSCs at the sites of interest when used in conjunction. The major exception was ropivacaine, which, at the doses used in our study, did not induce significant apoptosis and was not subject to the scale of failed adherence observed with bupivacaine, lidocaine, and mepivacaine. In light of our findings, it is of critical importance that medical providers exercise extreme caution in the selection of the amide-type LA used and the dose administered during hMSC therapies to avoid compromising the integrity and potency of the cell-based therapy.

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AUTHOR CONTRIBUTIONS

R.C.D.: experimental design, data collection, data analysis and interpretation, assembly of data, manuscript writing; N.F.L.: experiment execution, data collection, manuscript revision; P.D.R. and C.J.C.: conception, administrative support, manuscript revision.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

R.C.D. has compensated employment. N.F.L. and P.D.R. compensated employment with Regenerative Sciences. C.J.C. is the compensated CEO of Regenerative Sciences, is an uncompensated intellectual property rights/patent holder with Regenerative Science and Biorestorative Therapies, is a compensated consultant for Biorestorative Therapies, and has stock options in Regenerative Sciences, Neostem, and Bioregenerative Therapies.

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