Cytotoxicity of Local Anesthetics on Human Mesenchymal Stem Cells

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Background: Local anesthetics are frequently delivered intra-articularly to provide perioperative pain control. Previous studies have shown that the commonly used drugs lidocaine, ropivacaine, and bupivacaine can be toxic to human chondrocytes. The present study was conducted to determine whether the toxic effects of local anesthetics on human chondrocytes also extend to human mesenchymal stem cells.

Methods: Human mesenchymal stem cells from three healthy donors were grown in tissue culture and exposed to the following anesthetic treatments for sixty minutes: (1) 1% lidocaine, (2) 2% lidocaine, (3) 0.25% bupivacaine, (4) 0.5% bupivacaine, (5) 0.2% ropivacaine, and (6) 0.5% ropivacaine. The cells were then allowed to recover for twenty-four hours in regular growth media, and viability was measured with use of fluorescent staining for live cells or a luminescence assay for ATP content.

Results: The live cell counts and ATP content were correlated ($r^2 = 0.79$), and 2% lidocaine was found to be significantly more toxic than all doses of bupivacaine and ropivacaine. Treatment with 1% lidocaine resulted in significantly fewer live cells (49%) compared with the control, and the live cell count was also significantly less than that for the other anesthetics. However, the ATP level in the 1% lidocaine group was not significantly lower than those in the other groups. Bupivacaine and ropivacaine did not exhibit significant differences in toxicity compared with the control or with each other.

Conclusions: Ropivacaine and bupivacaine had limited toxicity in human mesenchymal stem cells. However, lidocaine could significantly decrease mesenchymal stem cell viability. Since other studies have shown ropivacaine to be less toxic to chondrocytes than bupivacaine, ropivacaine may be a safer intra-articular anesthetic.

Clinical Relevance: Mesenchymal stem cells likely play a key role in healing following surgical procedures such as microfracture and ligament reconstruction. If local anesthetics are used following joint surgery, selection of an agent with low toxicity toward mesenchymal stem cells, such as ropivacaine, may maximize tissue healing potential.

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isolated human articular chondrocytes and intact articular cartilage.

Although the chondrotoxicity of local anesthetics has been well studied, the effects of these drugs on reparative cells within a joint are unknown. Many orthopaedic procedures, including tendon repair, ligament reconstruction, and surgical marrow stimulation procedures such as microfracture, depend on patient healing responses. Mesenchymal stem cells likely play a key role in these processes. Under appropriate conditions, these cells can form bone, fat, tendon, and cartilage. These regenerative cells are found in multiple tissues, including bone marrow, adipose, and synovium. In addition to the healing resulting from the action of endogenous mesenchymal stem cells, surgical administration of exogenous mesenchymal stem cells may also enhance tissue regeneration.

We hypothesized that the toxicity of local anesthetics on chondrocytes would also extend to mesenchymal stem cells. If so, local anesthetics could both directly injure tissues within a joint and decrease the ability of tissues to heal. In this in vitro study, we evaluated the effects of commonly used doses of lidocaine, ropivacaine, and bupivacaine on the viability of mesenchymal stem cells from three healthy donors. Our goal was to identify treatments that minimized toxicity.

Materials and Methods
Mesenchymal Stem Cell Monolayer Culture

Commercially available bone-marrow-derived human mesenchymal stem cells (Lonza, Walkersville, Maryland) from a twenty-year-old man (Lot 8F354), a thirty-year-old man (Lot 0F4452), and a twenty-two-year-old woman (Lot 0F4825) were plated in monolayer culture in high-glucose DMEM (Invitrogen, Carlsbad, California), supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) and 1% antibiotics/antimycotic solution (Hyclone) on tissue-culture-treated polystyrene at a density of 5000 cells/cm².

The cells were grown at 37°C in a humidified 5% CO₂ incubator until 90% confluent, were trypsinized, and were expanded until the fifth or sixth passage. They were then seeded onto ninety-six-well plates at a density of 2500 cells/well (8000 cells/cm²) and cultured for forty-eight hours, leading to 90% confluence prior to experimental treatment.

Treatment Groups

Mesenchymal stem cell cultures were subdivided into six replicate wells per condition and exposed to one of the following drug dilutions: (1) 2% lidocaine, pH 6 (APP Pharmaceuticals, Schaumburg, Illinois), (2) 1% lidocaine, pH 6, (3) 0.5% bupivacaine, pH 6 (Hospira, Lake Forest, Illinois), (4) 0.25% bupivacaine, pH 6, (5) 0.5% ropivacaine, pH 5 (APP Pharmaceuticals), and (6) 0.2% ropivacaine, pH 5. The higher concentration of each anesthetic was used as provided by the manufacturer. The supplied solutions were preservative-free, with sodium chloride (to adjust osmolality) and sodium hydroxide or hydrochloric acid (to adjust pH) added by the manufacturer. Control groups were treated with 0.9% saline solution (APP Pharmaceuticals), which was also used to prepare the lower concentration of lidocaine and of bupivacaine from the higher concentration. The 0.2% ropivacaine solution was used as provided by the manufacturer or diluted from 0.5% ropivacaine with 0.9% saline solution.

The cell culture media was aspirated, and 200 μL of the appropriate treatment solution was added to each well. Cells were incubated for forty-five minutes at room temperature and then visualized with use of an Axiovert 200M fluorescent microscope (Carl Zeiss Microscopy, Thornwood, New York) with a fluorescein filter. Digital photographs of the center of each well were taken at 5× magnification. The cell viability in each field was quantified by superimposing a grid onto the digital image in Photoshop (Adobe Systems, San Jose, California), and calcein-stained live cells, which exhibit green fluorescence, were counted. The number of dead cells was also quantified in a subset of the experiments by staining with ethidium homodimer-1 and visualization with use of a rhodamine filter. Ethidium-stained dead cells and calcein-stained live cells were counted, and the percentage of live cells for each treatment was calculated.

ATP (Adenosine Triphosphate) Content

The ATP content in treated samples was measured with use of the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, Wisconsin) according to the manufacturer’s instructions. Briefly, the CellTiter-Glo buffer and the CellTiter-Glo substrate were combined to form the CellTiter-Glo reagent. The reagent was added to the culture media in each well in a 1:1 ratio, mixed well, and incubated for ten minutes at room temperature. Luminescence was then measured with use of a Wallac 1420 VICTOR® Multilabel Counter (PerkinElmer, Waltham, Massachusetts).

Statistical Analysis

Live cell counts from the three independent trials, calculated as a percentage of the control, were analyzed together with use of mixed-model analysis of variance (ANOVA), with the specified drug concentration as the fixed factor and trial as a random factor. Live cell count results are reported for each drug concentration as the least-squares mean (the calculated best-fit value across all trials) and the standard error. ATP content results are reported for each of the three individual donors as the mean luminescence (the CellTiter-Glo output) and the standard error. Differences in ATP content among treatment groups were assessed for each donor with use of ANOVA. Results for the three donors were combined with use of mixed-model ANOVA, with the specified drug concentration as the fixed factor and donor as a random factor. ATP content results are reported as the least-squares mean luminescence and the standard error. Because multiple comparisons were made, the significance of differences among treatments was calculated with use of the Tukey honestly significant difference test; a p value of <0.05 was considered significant. Statistical analysis was performed with use of JMP (version 9.0; SAS Institute, Cary, North Carolina).

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Results

Comparison of Cell Viability Measured with the Live Cell Count, Percentage of Live Cells, and ATP Content

In a pilot experiment, mesenchymal stem cells from one of the donors (the twenty-year-old male) were exposed to each of the six anesthetic conditions, and viability was determined with each of three methods: (1) live cell count, (2) percentage of live cells, and (3) ATP content. Six replicates were performed for each of the three evaluation methods for each condition. Linear regression yielded an r² value of 0.84 between the live cell count and the percentage of live cells and an r² value of 0.79 between...
the live cell count and the ATP content. Given the strength of these correlations, we evaluated viability with use of the live cell count and the ATP content for this donor and with use of only the ATP content for the other two donors.

Live Cell Counts
The number of live cells remaining after treatment with local anesthetic was determined with use of mesenchymal stem cells from the twenty-year-old male donor. Treatment with 0.25% and 0.5% bupivacaine in three independent trials reduced the live cell count to 94% and 79%, respectively, of the value in the control group. Treatment with 0.2% and 0.5% ropivacaine reduced the live cell count to 83% and 84%, respectively, of the value in the control group. No difference among these four groups was significant. Treatment with both concentrations of lidocaine, however, resulted in a significant reduction in the live cell count compared with all other groups. Treatment with 1% lidocaine reduced the live cell count to 49% of the value in the control group (p < 0.0001), and 2% lidocaine reduced it to 32% (p < 0.0001) (Figs. 1 and 2).

![Live Cell Counts](image)

**Fig. 1**
The number of live human mesenchymal stem cells present twenty-four hours after a sixty-minute treatment with 0.9% normal saline solution (Control), 1% and 2% lidocaine (Lido), 0.25% and 0.5% bupivacaine (Bup), and 0.2% and 0.5% ropivacaine (Rop). Three separate experiments were performed in each treatment group. The bar heights represent the least-squares mean of the number of live cells relative to the control, and the error bars represent the standard error of the mean. The results for treatments labeled with different letters (e.g., A and B) differed significantly from each other (p < 0.05), whereas treatments labeled with the same letter did not.

![Fluorescent microscopy images](image)

**Fig. 2**
Fluorescent microscopy images of human mesenchymal stem cells stained with calcein AM (acetomethoxy) twenty-four hours after a sixty-minute treatment with 0.9% normal saline solution (Control) (**Fig. 2-A**), 0.5% bupivacaine (**Fig. 2-B**), 0.5% ropivacaine (**Fig. 2-C**), and 1% lidocaine (**Fig. 2-D**) (x5).
ATP Content
As an alternative, independent measure of cell viability, cellular ATP content after local anesthetic treatment was determined with use of mesenchymal stem cells from each of the three donors (the twenty-year-old man, the twenty-two-year-old woman, and the thirty-year-old man). The results for each donor as well as the results of a model combining information from all three donors are shown in Figure 3. In all four cases, 2% lidocaine treatment significantly reduced viability compared with treatment with either ropivacaine or bupivacaine. In the combined model, the ATP content in the 2% lidocaine group was only 49% of that in the 0.2% ropivacaine group. For the twenty-year-old donor, the ATP content in the 1% and 2% lidocaine groups was only 56% and 39%, respectively, of that in the 0.2% ropivacaine group (p < 0.05). However, the difference between the 1% lidocaine group and the other treatment groups was not significant for the other two donors or for the combined model. No significant differences were found between the ropivacaine and bupivacaine groups.

Discussion
The chondrotoxicity of local anesthetics including bupivacaine, ropivacaine, and lidocaine in both human and animal models has been widely reported. However, to our knowledge, the effects of these drugs on human mesenchymal stem cells have not been previously addressed. Given the likely role of these cells in tissue healing following orthopaedic procedures, the toxicity of local anesthetics on these cells may be clinically important. The present study indicated that bupivacaine and ropivacaine at the commonly used 0.5% concentration were significantly less toxic than a concentration of lidocaine with equivalent anesthetic potency. These in vitro results suggest that intra-articular administration of lidocaine may have detrimental effects on mesenchymal stem cell populations.

The relative toxicities of bupivacaine, ropivacaine, and lidocaine on mesenchymal stem cells differed from their toxicities on human chondrocytes. Previous in vitro and in vivo studies have shown that bupivacaine is more toxic to cartilage cells than ropivacaine and lidocaine. Specifically, Piper and Kim directly compared the toxicity of 0.5% ropivacaine with that of 0.5% bupivacaine and found that although ropivacaine...
did induce cell death in human chondrocytes, it was significantly less chondrotoxic than bupivacaine. The observed cytotoxicity differences between chondrocyte and mesenchymal stem cell populations suggest a differential effect of local anesthetics on different cell types.

The inherent differences in physical and chemical properties among these drugs likely contribute to the differences in their relative toxicities on different cell types. Bupivacaine and ropivacaine are highly lipophilic molecules, whereas lidocaine is only slightly lipophilic. Treatment of human leukemia cells with these anesthetics induces different patterns of cell death. Bupivacaine and ropivacaine predominantly cause necrosis, whereas lidocaine induces DNA fragmentation and apoptosis (programmed cell death). Studies of human T-cell lymphoma and neuroblastoma cell lines showed that lidocaine treatment leads to apoptosis but ropivacaine does not. These differences may be due to differential activation of caspasases, which are proteolytic enzymes that play key roles in apoptosis. The three anesthetics may also differentially affect inflammation. Bupivacaine induces nitric oxide synthase-2 activity in rat glial cells and astrocytes but ropivacaine does not. This pathway is normally activated as part of an inflammatory response, suggesting that bupivacaine toxicity may be attributable in part to the production of nitric oxide during ongoing inflammation. In contrast, ropivacaine has anti-inflammatory properties.

The relative potencies of lidocaine, bupivacaine, and ropivacaine were accounted for in our comparisons among the drugs. Bupivacaine and ropivacaine are highly potent anesthetics with long-lasting effects, whereas lidocaine is a moderately potent anesthetic with shorter effects. Specifically, bupivacaine and ropivacaine are approximately four times more potent anesthetics than lidocaine. Therefore, the effects of 0.5% bupivacaine and 0.5% ropivacaine on pain should be similar to that of 2% lidocaine, but with a longer duration of action and less toxicity on mesenchymal stem cells. Although the CellTiter-Glo assay for ATP content was slightly less sensitive to differences among conditions than the live cell count, similar trends were found with both methods of analysis.

One limitation of the present study is the fact that the experiments were performed in vitro. Further experiments are needed to assess the in vivo effects of these drugs on mesenchymal stem cell viability. However, the experimental conditions in the present study (growth of cells in tissue culture to yield a monolayer in the fifth or sixth passage) are commonly used in studies of mesenchymal stem cells, and such cells are defined in part by their ability to adhere, proliferate, and differentiate into mesenchymal tissues under such in vitro conditions. Another limitation is the fact that viability measurements were only performed after sixty minutes of anesthetic treatment. This duration of exposure was chosen on the basis of a previous study of the pharmacokinetics of bupivacaine after intra-articular injection into the knee. The absorption of this anesthetic from the joint into the bloodstream is rapid, with the peak blood concentration being reached within the first hour after injection. We are not aware of similar data for lidocaine or ropivacaine. We did not test for time dependence or for potential early-acting toxicity in the drug treatments. The effect of local anesthetic treatment on the differentiation potential of mesenchymal stem cells is also of interest in future experiments. Although cells treated with bupivacaine and ropivacaine in the present study appeared to be metabolically and morphologically normal, the drugs may subtly affect differentiation patterns. To study these effects, mesenchymal stem cells treated with varying classes and doses of local anesthetic can be assayed for the ability to form bone, cartilage, and fat.

In conclusion, in contrast to the toxicity observed in human and bovine chondrocytes, commonly used concentrations of ropivacaine and bupivacaine appeared to have limited toxicity on human mesenchymal stem cells. In contrast, lidocaine significantly decreased mesenchymal stem cell viability. Although these experiments are limited by their in vitro nature, these data suggest that ropivacaine and bupivacaine may represent better options with respect to mesenchymal stem cell health. Taking into further account the chondrotoxicity of bupivacaine and the chondrolysis associated with postoperative bupivacaine infusions, ropivacaine may be the safest of these three choices for intra-articular analgesia.

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References


