

Apoptosis and Mitochondrial Dysfunction in Human Chondrocytes Following Exposure to Lidocaine, Bupivacaine, and Ropivacaine

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Background: Several mechanisms have been proposed to explain toxicity of local anesthetics to chondrocytes, including the blockade of potassium channels and mitochondrial injury. The purposes of this investigation were to study the effects of lidocaine, bupivacaine, and ropivacaine on human chondrocyte viability and mitochondrial function in vitro and to characterize the type of cell death elicited following exposure.

Methods: Primary chondrocyte cultures from patients with osteoarthritis undergoing knee replacement were treated with saline solution and the following concentrations of local anesthetics: 2%, 1%, and 0.5% lidocaine, 0.5% and 0.25% bupivacaine, and 0.5% and 0.2% ropivacaine for one hour. Cell viability and apoptosis were measured by flow cytometry at twenty-four hours and 120 hours after treatment. Nuclear staining and caspase 3 and 9 cleavage assays (Western blot) were used to further establish the induction of apoptosis. Mitochondrial dysfunction was evaluated by the accumulation of mitochondrial DNA damage (quantitative Southern blot), changes in adenosine triphosphate production (bioluminescence kit), and mitochondrial protein levels (Western blot analysis).

Results: Exposure of primary human chondrocytes to a 2% concentration of lidocaine caused massive necrosis of chondrocytes after twenty-four hours, 1% lidocaine and 0.5% bupivacaine caused a detectable, but not significant, decrease in viability after twenty-four hours, while 0.5% lidocaine, 0.25% bupivacaine, and both concentrations of ropivacaine (0.5% and 0.2%) did not affect chondrocyte viability. Flow cytometry analysis of chondrocytes 120 hours after drug treatment revealed a significant decrease in viability ($p < 0.05$) with a concomitant increase in the number of apoptotic cells at all concentrations of lidocaine, bupivacaine, and ropivacaine analyzed, except 0.2% ropivacaine. Apoptosis was verified by observation of condensed and fragmented nuclei and a decrease in procaspase 3 and 9 levels. Local anesthetics induced mitochondrial DNA damage and a decrease in adenosine triphosphate and mitochondrial protein levels.

Conclusions: Lidocaine, bupivacaine, and ropivacaine cause delayed mitochondrial dysfunction and apoptosis in cultured human chondrocytes.

Clinical Relevance: Local anesthetics cause deleterious effects on human chondrocytes in vitro. The results of the present study establish a basis for the further investigation of local anesthetic toxicity in an in vivo system.

Drugs that block voltage-gated sodium channels have been a mainstay of pain medicine since the introduction of cocaine more than 120 years ago. At the same time, these local anesthetics are known to have both beneficial and adverse effects on a variety of cellular activities, such as wound-healing, thrombosis, and inflammatory responses, and to cause cellular toxicity.

Intra-articular injection of local anesthetics is widely used to control pain following arthroscopic surgery. Currently, bupivacaine is the best studied and most commonly used agent during this type of surgical procedure¹. However, chondrotoxicity has been demonstrated after treatment with bupivacaine^{2,3} as well as other anesthetics⁴⁻⁶. Moreover, there are a number of reports concerning local anesthetic toxicity on

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other cell types, including the induction of necrosis and apoptosis⁷⁻⁹. Previous studies have been related to the immediate or short-term effects of bupivacaine or lidocaine. The long-term effects of bupivacaine, lidocaine, or ropivacaine on human chondrocytes are not known.

The exact mechanisms of local anesthetic toxicity have not been fully elucidated. It appears that the mechanism of toxicity is probably unrelated to the primary action of all local anesthetics, the blockade of the voltage-gated sodium channels^{10,11}.

Recent investigations have shown that local anesthetics have an influence on potassium and calcium channels¹²⁻¹⁶.

Several studies have suggested that local anesthetics may affect mitochondrial energetics, and mitochondrial insults can induce either apoptosis or necrosis, with less severe injuries leading to apoptosis, a form of programmed cell death¹⁷⁻¹⁹. It is well established that various toxic stimuli can unbalance the electron transport chain and enhance endogenous free radical production in mitochondria²⁰. Reactive oxygen species pro-

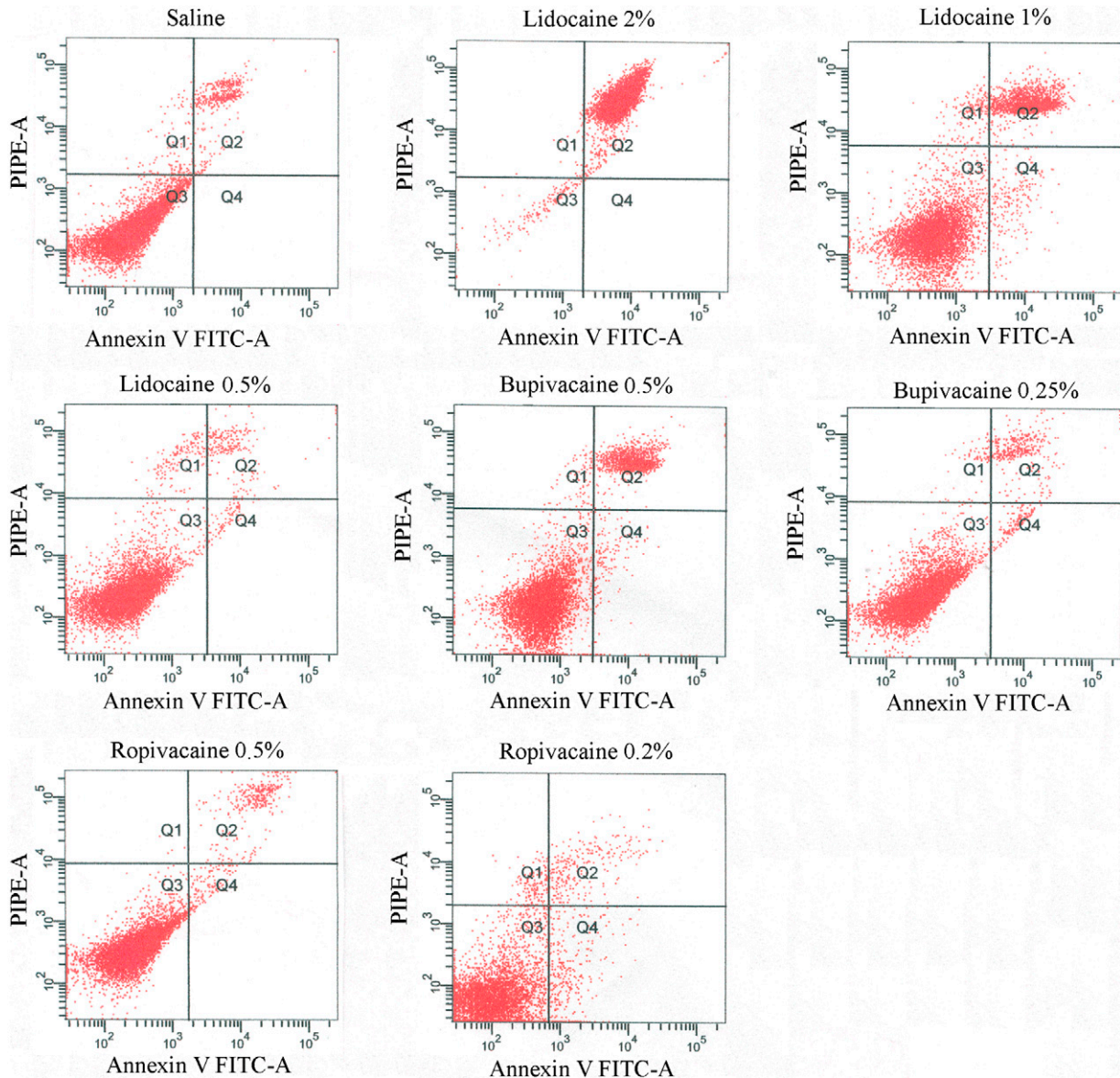


Fig. 1

Scatterplots of flow cytometry determinations of the numbers of necrotic, apoptotic, and viable cells twenty-four hours after a one-hour exposure to the following (from left to right, from top to bottom): saline solution, 2% lidocaine, 1% lidocaine, 0.5% lidocaine, 0.5% bupivacaine, 0.25% bupivacaine, 0.5% ropivacaine, and 0.2% ropivacaine. Propidium iodide fluorescence (PIPE-A; ordinate) was plotted against annexin-V fluorescence (FITC-A; abscissa). Quadrant 3 (Q3) shows live cells, quadrants 1 and 2 (Q1 and Q2) show necrotic cells, and quadrant 4 (Q4) shows apoptotic cells. Note the presence of necrotic cells and the absence of apoptotic cells at twenty-four hours after exposure to local anesthetics.

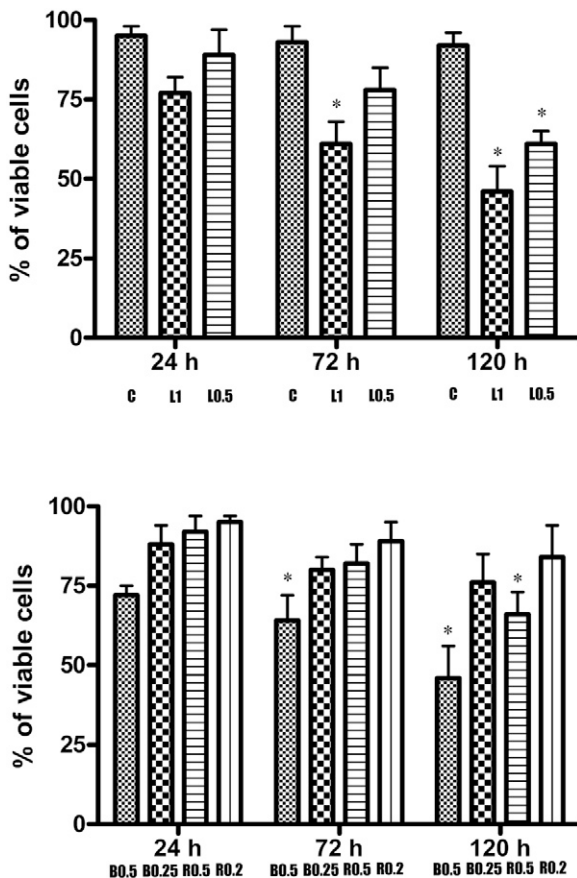


Fig. 2
Viability of human chondrocytes as determined by flow cytometry analysis at twenty-four hours, seventy-two hours, and 120 hours after exposure to saline solution (C), 1% and 0.5% lidocaine (L) (top panel); 0.5% and 0.25% bupivacaine (B), and 0.5% and 0.2% ropivacaine (R) (bottom panel). The results were obtained from a minimum of seven independent experiments. The shaded bars represent the mean percentage of viable cells, and the I-bars represent the standard error of the mean. An asterisk indicates a significant difference ($p < 0.05$) between local anesthetic-treated and nontreated chondrocytes.

duction induces damage to lipids, proteins, and nucleic acids in mitochondria. Reactive oxygen species-induced mitochondrial DNA (mtDNA) damage and mutations lead to the synthesis of functionally impaired respiratory chain subunits, causing respiratory chain dysfunction and augmented reactive oxygen species production^{21,22}. This vicious cycle has been proposed to cause an exponential increase in mtDNA damage and mutations over time, resulting in functional failure and cell death²³.

We hypothesized that the accumulation of mtDNA damage, mediated by blockade of potassium channels, is a key factor contributing to the development of chondrocyte toxicity induced by local anesthetics.

The purposes of this study were to compare the effects of lidocaine, bupivacaine, and ropivacaine on human chondrocyte mitochondrial function and viability in vitro and to char-

acterize the type of cell death elicited following exposure to local anesthetics.

Materials and Methods

Cartilage Specimens and Chondrocyte Cultures

We used cartilage (from both femoral condyles and tibial plateaus) obtained from patients with osteoarthritis who were an average (and standard deviation) of 53 ± 16 years old and undergoing total knee replacement. The cartilage was removed by cuts through the cancellous bone during preparation for a total knee arthroplasty. The total time from cartilage harvest to culture initiation varied from one to three hours. After specimen inspection, all cartilage was removed for culture initiation except for that from surrounding areas devoid of cartilage, which likely contained only dead cells.

Primary chondrocyte cultures were generated by overnight digestion of minced cartilage samples with 5 mg/mL of collagenase B (Roche, Indianapolis, Indiana) in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, Utah) and antibiotics. Cells were plated and used for experiments after reaching confluence (seven to ten days). In order to preserve chondrocyte phenotype, primary chondrocyte cultures were never passaged. Confluent cultures were routinely checked for the presence of collagen II by Western blot analysis with anticollagen-II antibody to ensure that the chondrocytes studied had a normal phenotype (data not shown).

The chondrocyte cultures generated for this study represent a mixed population of less and more-damaged cartilage cells, but this likely reflects what is present in osteoarthritic cartilage. Thus, the results obtained from this study show the overall effect of local anesthetics on osteoarthritic cartilage. Likely the effect of these drugs on chondrocytes from advanced lesions would be more severe than on less-damaged cells. However, it is not technically possible to separate out sufficient numbers of chondrocytes with high or low levels of damage for independent study. Therefore, a different approach was used, with each experiment carried out within cultures generated from a single specimen, including all necessary controls. Because there was no dramatic variation between experiments, we believe we had uniform preparations of cultures, which responded similarly to the introduced drugs.

Normally, about twenty to twenty-five confluent 100-mm dishes were obtained from culture preparations, and after drug exposure each dish provided a yield of about 20 to 30 μ g of DNA, which was sufficient to do three or four Southern blot analyses. For protein isolation, smaller dishes were used, and ATP (adenosine triphosphate) assays required only 10,000 cells. For example, with use of a single 100-mm dish, cells were counted after trypsinization; 10,000 cells were lysed to perform an ATP assay, about 100,000 cells were used for flow cytometric analysis, and the rest of the cells were used for the isolation of DNA, RNA, or proteins. As mentioned above, the amount of DNA was sufficient to do three or four Southern blot analyses, while the total protein amount obtained by this procedure was

sufficient to perform ten to twelve Western blot analyses. To perform DAPI staining (4',6-diamidino-2-phenylindole, Hoechst 33342; Invitrogen, Carlsbad, California), cells were seeded in six-well or twelve-well plates.

Drug Preparation and Exposure

After reaching confluence, primary human articular chondrocyte cultures were exposed for one hour to the following

concentrations of local anesthetics: 2%, 1%, and 0.5% lidocaine (Hospira, Lake Forest, Illinois); 0.5% and 0.25% bupivacaine (Hospira); and 0.5% and 0.2% ropivacaine (Astra Zeneca, Wilmington, Delaware). All drugs used were preservative-free and contained only local anesthetics dissolved in saline solution. Control cultures were exposed to saline solution (Hospira) under the same conditions. After sixty minutes, cells were immediately lysed or placed in

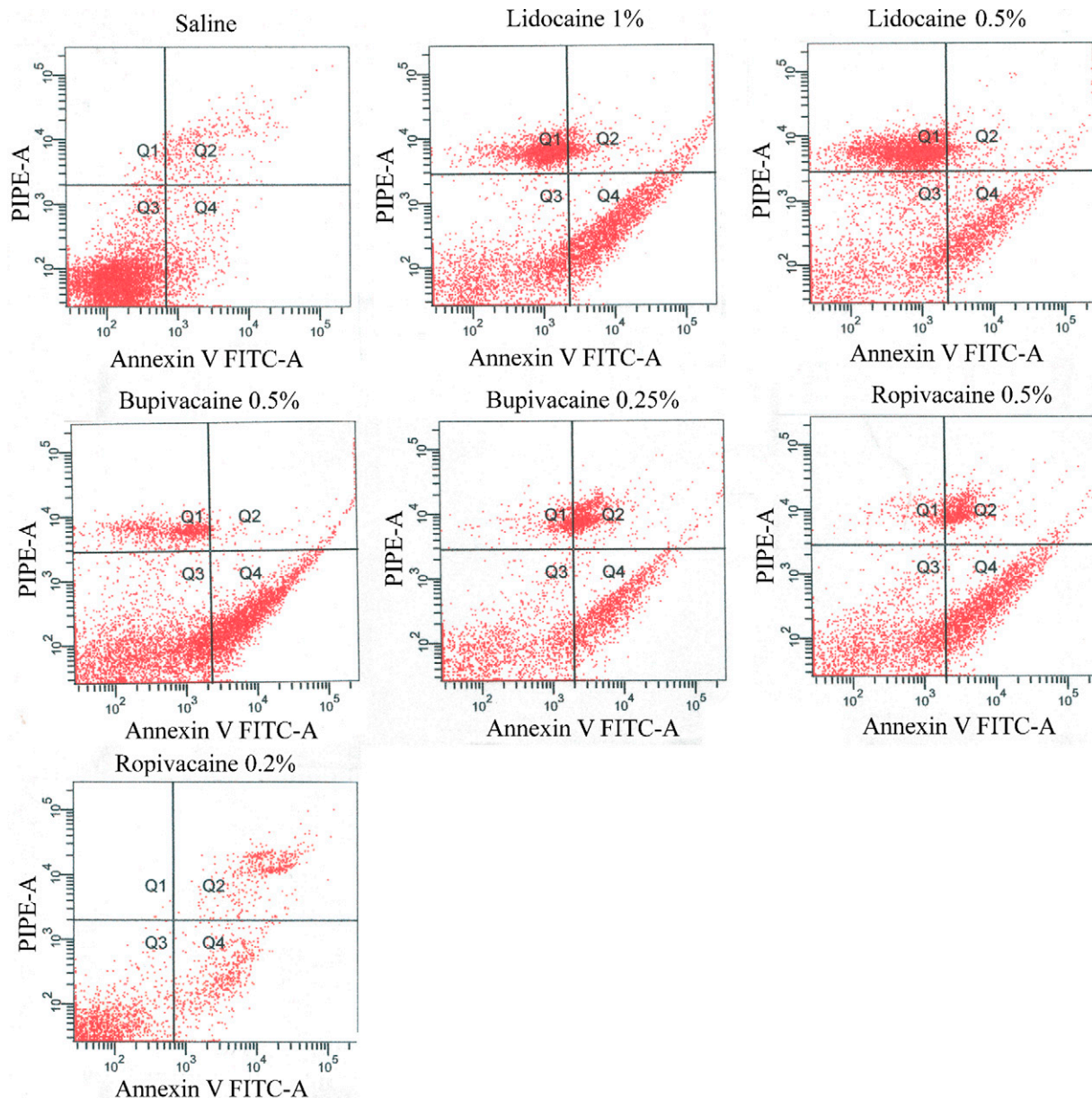


Fig. 3

Scatterplots of flow cytometry determinations of the numbers of necrotic, apoptotic, and viable cells 120 hours after a one-hour exposure to the following (from left to right, from top to bottom): saline solution, 1% lidocaine, 0.5% lidocaine, 0.5% bupivacaine, 0.25% bupivacaine, 0.5% ropivacaine, and 0.2% ropivacaine. Propidium iodide fluorescence (PIPE-A; ordinate) was plotted against annexin-V fluorescence (FITC-A; abscissa). Quadrant 3 (Q3) shows live cells, quadrants 1 and 2 (Q1 and Q2) show necrotic cells, and quadrant 4 (Q4) shows apoptotic cells. Note the increase of dead cells with the prevalence of apoptosis at 120 hours after exposure to local anesthetics.

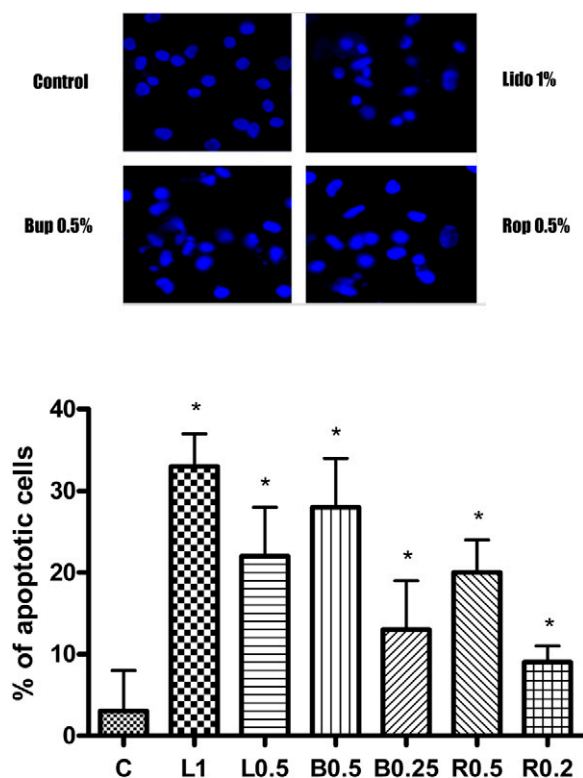


Fig. 4
Apoptosis in human chondrocytes at 120 hours following a single one-hour exposure to local anesthetics. The top panel represents the DAPI staining showing the condensation and fragmentation of chondrocyte nuclei following the induction of apoptosis as observed in a fluorescent microscope ($\times 40$); the bottom panel shows the calculation of the percentage of apoptotic cells according to flow cytometry experiments. The results were obtained from a minimum of six independent experiments. The shaded bars represent the mean percentage of apoptotic cells, and the I-bars represent the standard error of the mean. An asterisk indicates a significant difference ($p < 0.05$) between local anesthetic-treated and nontreated chondrocytes. C = control, L or Lido = lidocaine, R or Rop = ropivacaine, and B or Bup = bupivacaine.

normal culture medium to allow time for recovery for twenty-four, seventy-two, and 120 hours.

To investigate whether blockade of sodium channels was involved in mitotoxicity and cytotoxicity of local anesthetics, we exposed some cultures to tetrodotoxin (Sigma, St. Louis, Missouri). Tetrodotoxin is another selective blocker of sodium channels that is widely used to study the role of these channels in normal physiology and disease²⁴⁻²⁶. Tetrodotoxin was diluted in water, and chondrocytes were treated with this agent to a final concentration of 1 or 20 μM for one hour alone or in combination with lidocaine. Some cultures were exposed to local anesthetics in Earle's Balanced Salts formulation media (EBSS; HyClone), where 60 mM of sodium chloride was replaced with 60 mM of potassium chloride. Following exposure or recovery, cells were collected and used for the evaluation of

mtDNA repair and damage, ATP synthesis, the induction of apoptosis, or change in concentration of specific mitochondrial proteins.

Flow Cytometry

The ApoScreen Annexin V apoptosis kit (SouthernBiotech, Birmingham, Alabama), which employs fluorescein-labeled annexin V (Annexin V-FITC) in concert with propidium iodide to evaluate subpopulations of cells undergoing apoptosis, was used for the current investigation²⁷. At designated time points following recovery from local anesthetic exposure, chondrocytes were trypsinized and collected by centrifugation. To ensure that all cells were harvested, cell culture media from each dish was combined with the resulting cell suspension from the same dish following trypsinization. Cells were washed twice in cold phosphate-buffered saline solution and labeled with V-FITC and propidium iodide for thirty minutes according to the manufacturer's suggestions. Samples were analyzed by a FACSDiva flow cytometry machine (Becton Dickinson, Franklin Lakes, New Jersey) to identify apoptotic (V-FITC-labeled), necrotic (propidium iodide-labeled), and viable cells.

Programmed Cell Death Evaluation

To further evaluate the involvement of apoptosis in chondrocyte death following lidocaine, bupivacaine, or ropivacaine exposure, primary human chondrocyte cultures were exposed to predetermined concentrations of these drugs for sixty minutes. After treatment, the normal growth media was replenished, and, twenty-four, seventy-two, or 120 hours later, the appearance of apoptosis was evaluated by the observation of condensed and fragmented nuclei following DAPI staining.

To analyze whether caspase activation was involved in the initiation of apoptosis following exposure to local anesthetics, Western blot analysis with use of antibodies against caspase 3 and caspase 9 was employed. Anti-actin antibody was used to ensure equal loading of protein samples.

For total cellular protein isolation, cells were lysed in cell lysing buffer (Cell Signaling Technology, Danvers, Massachusetts) and processed according to the manufacturer's suggestions. Chondrocyte suspensions were briefly sonicated on ice, were centrifuged once more at 5000 g to pellet any remaining debris, and the supernatant protein was used for Western blot assays. The protein concentration was determined with use of the Bio-Rad protein dye micro-assay (Bio-Rad, Hercules, California), according to the manufacturer's recommendation.

Mitochondrial DNA Damage Assay

Following sixty minutes of exposure to 2%, 1%, and 0.5% lidocaine; 0.5% and 0.25% bupivacaine; and 0.5% and 0.2% ropivacaine, DNA was extracted from primary chondrocyte cultures that were lysed in buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.5% sodium dodecyl sulfate, and 300 $\mu\text{g/mL}$ proteinase K (Roche) overnight. DNA was isolated by standard phenol-chloroform extraction, precipitated with cold ethanol, and digested overnight with

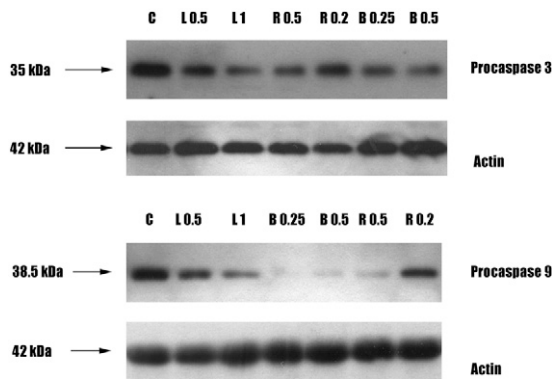


Fig. 5

Western blot analysis of procaspase 3 and 9 levels in human chondrocytes at 120 hours following exposure to 0.5% and 1% lidocaine (L0.5 and L1), 0.5% and 0.2% ropivacaine (R0.5 and R0.2), and 0.5% and 0.25% bupivacaine (B0.5 and B0.25). Note the diminished levels of procaspase 3 and 9 after higher drug concentrations. Anti-actin antibody was used to ensure equal loading. C = control.

BamHI. Prior to loading on an alkaline agarose gel for Southern blot analysis, each sample containing 5 μ g of total DNA was incubated with 0.1 N of NaOH to reveal single-strand breaks. After gel electrophoresis under alkaline conditions, DNA was transferred by means of vacuum transfer to a nylon membrane. Membranes were hybridized with a polymerase chain reaction-generated radioactive specific probe representing a part of the cytochrome-c oxidase subunit-III human mitochondrial gene. BamHI was selected because hu-

man mtDNA has a single restriction site for this enzyme so that, on digestion, it linearizes the mtDNA, and hybridization with the human mitochondrial gene-specific probe to cytochrome-c oxidase subunit III recognizes the restriction band of 16,569 bp, corresponding to the whole mitochondrial genome²⁸. Autoradiographs were scanned for hybridization band intensity. DNA damage was evaluated as the number of DNA breaks per 16.6-kb fragment. Break frequency was determined with use of the Poisson expression ($s = -\ln P_0$, where s is the number of breaks per fragment and P_0 is the fraction of fragments free of breaks).

ATP Bioluminescence Assay

Human primary chondrocyte cultures were exposed to different doses of local anesthetics (the same as for apoptosis and mtDNA damage studies) for sixty minutes and then were replenished with normal media; three hours later, the ATP levels in cells were evaluated with use of an ATP bioluminescence assay kit (Roche). This technique is well established and uses the ATP dependency of the light-emitting luciferase-catalyzed oxidation of luciferin for the measurement of extremely low concentrations of ATP.

Western Blot Analysis

To analyze changes in mitochondrial proteins, Western blot analysis was employed. For total cellular protein isolation, cells were lysed in cell lysing buffer (Cell Signaling Technology) and processed according to the manufacturer's suggestions. This analysis was used to evaluate levels of subunit III of cytochrome-c oxidase. This subunit is one of thirteen proteins encoded by mitochondria, and accumulation of mitochondrial dysfunction

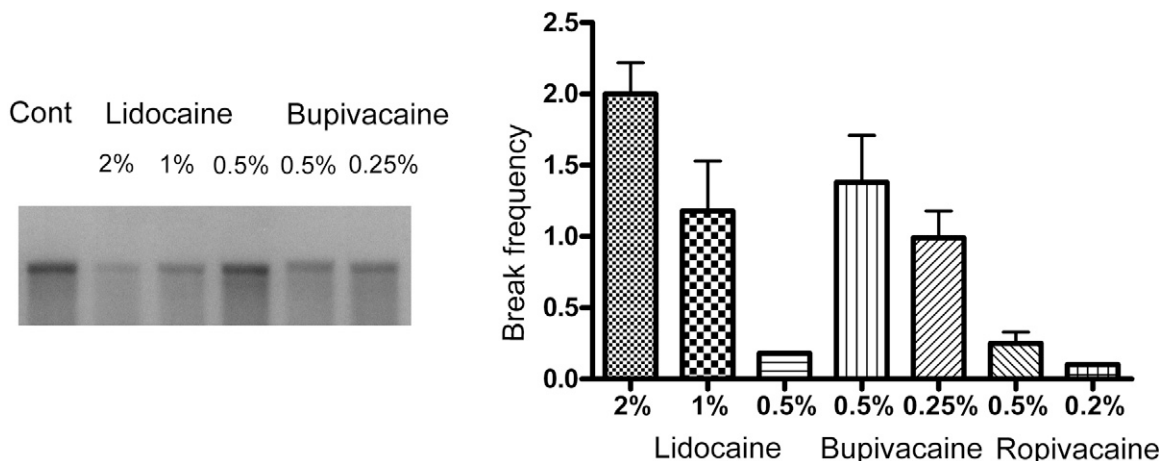


Fig. 6

Mitochondrial DNA damage induced by local anesthetics in human chondrocytes. The left panel is a representative autoradiograph from Southern blot analyses of mtDNA from osteoarthritic chondrocytes incubated for one hour with various doses of lidocaine and bupivacaine. Human chondrocytes were lysed for DNA extraction following overnight digestion with collagenase B. The decreased intensity of the hybridization bands indicates mtDNA damage. The right panel displays quantitation of the mtDNA damage following the use of all three anesthetics. The results were obtained from a minimum of eight independent experiments. The shaded bars represent the mean break frequency, and the I-bars indicate the standard error of the mean. Each independent experiment was performed with use of cultures obtained from an individual cartilage specimen. Cont = control.

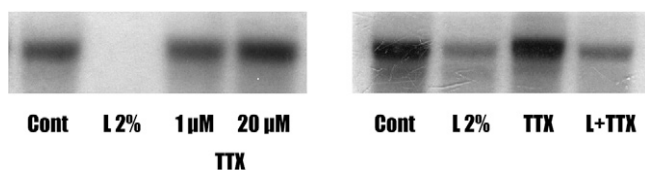


Fig. 7

Effect of tetrodotoxin (TTX) on human chondrocyte mtDNA integrity. Representative autoradiographs of quantitative Southern blot analysis on DNA from cells exposed to tetrodotoxin and 2% lidocaine (L) are displayed. Note the absence of damage in mtDNA from cultures treated with tetrodotoxin alone. Cont = control.

tion should change the levels of this protein. The antibody to this protein was obtained from MitoSciences (Eugene, Oregon). Anti-actin antibody was used to ensure equal loading of total protein fractions.

Statistical Analysis

All of the data from similar experiments on single cultures obtained from separate patients were averaged to give an average value relating to each analysis performed. Statistical analyses were performed with use of the Student t test or one or two-way analysis of variance (GraphPad Prism; GraphPad Software, La Jolla, California), where appropriate. A difference was considered significant when $p < 0.05$. The Bonferroni post hoc test was used to determine the source of observed differences.

Source of Funding

There was no external funding source to support the present study.

Results

Twenty-four hours after exposure of chondrocyte cultures to local anesthetics, chondrotoxicity was observed with 2% lidocaine, with an almost complete loss of viable cells due to massive necrosis (Figs. 1 and 2). Exposure of primary human chondrocytes to 1% lidocaine and 0.5% bupivacaine for one hour caused a detectable, but not significant, decrease in viability after twenty-four hours. Lower doses of 0.5% lidocaine and 0.25% bupivacaine, as well as both concentrations of ropivacaine (0.5% and 0.2%), did not affect chondrocyte viability compared with saline solution controls (Figs. 1 and 2). The decrease in viability at all of the concentrations of local anesthetics used was primarily due to necrosis; there was no significant increase in the number of apoptotic cells twenty-four hours after exposure. However, flow cytometry analysis of chondrocytes 120 hours after drug treatment revealed a significant decrease in viability ($p < 0.05$), with a concomitant increase in predominantly apoptotic cells at all concentrations of lidocaine, bupivacaine, and ropivacaine analyzed, except 0.2% ropivacaine (Figs. 2 and 3). We did not include 2% lidocaine in the 120-hour study because >90% of the chondrocytes were dead by twenty-four hours after treatment. These results also were confirmed by the increased number of condensed and fragmented apoptotic nuclei observed following DAPI staining (Fig. 4). To further establish the

induction of apoptosis and the possible involvement of mitochondrial damage in its initiation, caspase 3 and 9 activation-cleavage assays were performed. The increase in caspase-3 cleavage showed that apoptosis occurred in chondrocytes exposed to local anesthetics, and caspase-9 cleavage showed that apoptosis observed in the chondrocytes, following local anesthetic exposure, involved mitochondrial dysfunction (Fig. 5).

To evaluate whether local anesthetic exposure causes mitochondrial dysfunction in human chondrocytes in vitro, mitochondrial DNA damage and changes in ATP and mitochondrial protein levels were investigated. As can be seen in Figure 6, local anesthetics caused damage to mtDNA in human chondrocytes following one hour of exposure. Tetrodotoxin is known to be a sodium channel blocker of a different chemical nature, which has been extensively used by many researchers to study mechanisms of pain and its blockade²⁴⁻²⁶. While lidocaine, bupivacaine, and ropivacaine belong to the amino amide group of anesthetics and block sodium channels by interaction with their intracellular part, tetrodotoxin (anhydrotetrodotoxin 4-epitetrodotoxin, tetrodonic acid) binds to the extracellular side of sodium channels. To explore whether the chondrocyte toxicity is the result of sodium channel blockade, we studied the toxic effects of tetrodotoxin on human chondrocytes. We treated chondrocyte cultures with 1 and 20 μ M of tetrodotoxin for one hour, isolated DNA, and subjected it to quantitative Southern blot analysis. A tetrodotoxin concentration of 1 μ M is commonly used and has been proven to be sufficient to block sodium channels in different cell types²⁹⁻³¹. In order to ensure channel blockade, a much higher concentration of tetrodotoxin was also used. Also, we exposed cells to

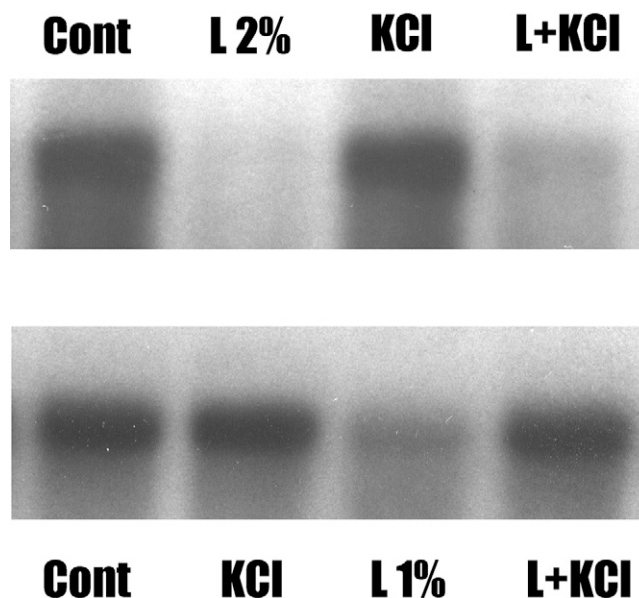


Fig. 8

Effect of high potassium (KCl) on mtDNA damage in human chondrocytes following local anesthetic exposure. Note the diminished mtDNA damage when high potassium was added during exposure to local anesthetics. Cont = control, and L = lidocaine.

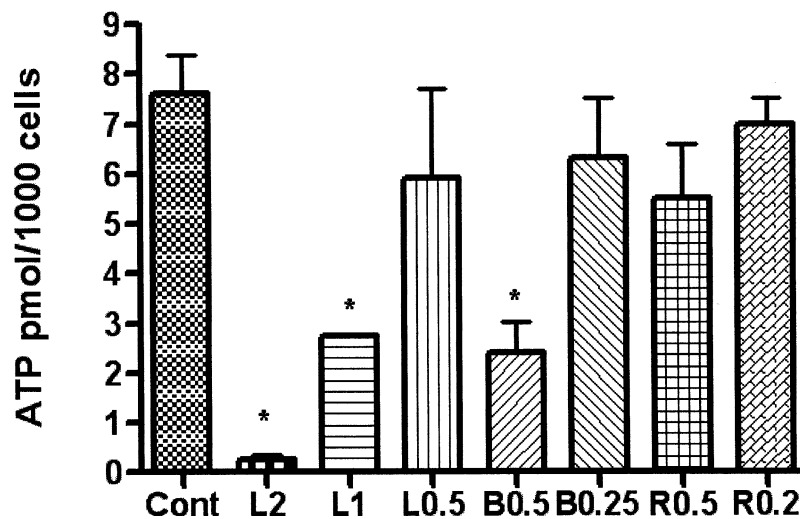


Fig. 9
ATP levels (in pmol/1000 cells) in human chondrocytes following exposure to 0.5%, 1%, and 2% lidocaine (L), 0.5% and 0.25% bupivacaine (B), and 0.5% and 0.2% ropivacaine (R). An asterisk indicates a significant difference ($p < 0.05$) between local anesthetic-treated and nontreated chondrocytes. The shaded bars represent the mean, and the I-bars indicate the standard error of the mean. Cont = control.

a combination of tetrodotoxin and lidocaine for the same duration. We hypothesized that, if mtDNA damage is due to blockade of sodium channels, then tetrodotoxin and lidocaine should have additive effects on chondrocyte mtDNA integrity. Figure 7 displays our findings, which demonstrate that tetrodotoxin alone, even in high concentration, did not have an effect on chondrocyte mtDNA integrity. Additionally, there was no additive effect of tetrodotoxin on mtDNA damage induced by lidocaine.

It has been widely appreciated that high potassium concentrations in the media can cause the depolarization of cell membranes and collapse of the ionic gradients and thereby disable proper function of potassium channels³². Because local anesthetics can efficiently block potassium and calcium channels, we hypothesized that, in the presence of high potassium, the channel blockade can be overcome and mtDNA damage can be ameliorated. To test this hypothesis, chondrocytes were incubated in a modified EBSS media, in which 60 mM of sodium chloride was replaced with 60 mM of potassium chloride (to avoid osmotic swelling from high salt concentration); in EBSS with 2% or 1% lidocaine; and in modified EBSS with 2% or 1% lidocaine. Figure 8 displays the results of quantitative Southern blot analysis in cells treated as described above. As can be seen in this figure, there was attenuation of mtDNA damage following treatment with 2% or 1% lidocaine when high potassium levels were present.

Because production of energy is the main function of mitochondria, we evaluated whether ATP levels are affected following local anesthetic exposure. Human primary chondrocytes were exposed for sixty minutes to the previously used concentrations of local anesthetics for viability and mtDNA damage studies; were replenished with normal media; and,

three hours later, were evaluated with use of an ATP bioluminescence assay kit (Roche) to determine the ATP levels. As can be seen in Figure 9, lidocaine and bupivacaine at higher concentrations significantly decreased ATP levels ($p < 0.05$), while ropivacaine minimally affected ATP content.

We also investigated whether mitochondrial protein levels were affected by local anesthetic exposure. Figure 10 displays a Western blot analysis showing the levels of the mitochondrially encoded subunit III of cytochrome-c oxidase twenty-four hours following exposure to different doses of lidocaine. Anti-actin antibody was used to ensure equal loading of protein samples. As can be seen in this figure, after exposure of human chondrocytes to 2% lidocaine, we could not detect any cytochrome-c oxidase subunit III, while 1% and 0.5% lidocaine diminished the level of this protein compared with the control.

Discussion

The novel finding of this study is that exposure of human chondrocytes to lidocaine, bupivacaine, or ropivacaine

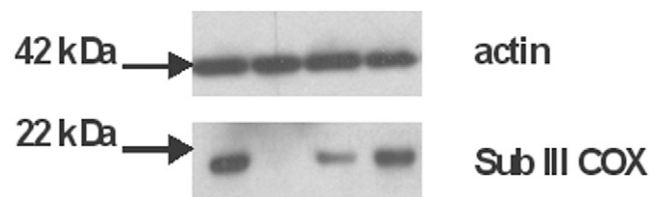


Fig. 10
Western blot analysis of cytochrome-c oxidase subunit III (Sub III COX) levels in human chondrocytes following exposure to saline solution and to 2%, 1%, and 0.5% lidocaine (from left to right).

in vitro causes a decrease in cellular viability and an increase in the induction of apoptosis after the drugs have been removed. Our results suggest that a mitochondrial pathway is involved in this induction of apoptosis. Also, we demonstrate that the chondrotoxicity of local anesthetics is associated with mitochondrial dysfunction resulting from damage to the mitochondrial genome. This leads to a decrease in energy production and, ultimately, to cell death.

Multiple studies have described the detrimental effects of local anesthetics on chondrocyte viability. For example, Chu et al. evaluated the effects of administering 0.5% bupivacaine to bovine chondrocytes in vitro. They reported that >99% of the chondrocytes were killed in all bupivacaine-exposed cultures². Gomoll et al. similarly showed histopathologic and metabolic changes with continuous infusion of 0.25% bupivacaine with and without epinephrine in rabbit shoulders³. Karpie and Chu also reported chondrotoxic effects of 1% and 2% lidocaine on bovine chondrocytes⁶. Although the use of static and alginate bead cultures, bovine chondrocytes, or rabbit shoulders prevent a direct comparison with human disease, these studies clearly establish the chondrotoxicity of bupivacaine and lidocaine. Recently, chondrocyte toxicity was reported by Piper and Kim⁴ after exposure of human cartilage explants and chondrocytes in primary culture to bupivacaine and ropivacaine. Those investigators demonstrated that, similar to our findings, 0.5% ropivacaine was not toxic to human chondrocytes twenty-four hours after a thirty-minute exposure, while the same concentration of bupivacaine significantly decreased chondrocyte viability. However, these investigators did not evaluate at a time point longer than twenty-four hours after exposure and could have missed the toxic effects of bupivacaine and lidocaine that we observed.

The complete understanding of the exact mechanisms involved in local anesthetic toxicity to cartilage cells remains to be fully elucidated. While some data related to the toxicity of these drugs in other cell types have been reported, little is known about the effects of local anesthetics on chondrocytes. As mentioned above, several investigations have suggested that local anesthetics may affect mitochondrial energetics, and mitochondrial insults can induce either apoptosis or necrosis¹⁷⁻¹⁹. Mitochondrial injury is likely to be important in the toxicity of local anesthetics, but this needs to be carefully investigated because of the multiple cross talk and feedback loops that are involved in the different mitochondrial death pathways. Also, it should be noted that we did not perform studies on normal chondrocytes obtained from healthy donors. Therefore, it is likely that osteoarthritic chondrocytes already are stressed and may not be able to recover as well as normal cells. In support of this notion is our recent study, which demonstrates that mitochondrial dysfunction was present in osteoarthritic chondrocytes³³. It is conceivable that similar mechanisms are involved in the cell damage and death caused by the pathogenesis of osteoarthritis and local anesthetics, and these effects are additive.

Recent studies have shown that local anesthetics have an effect on mitochondrial function in different cell types: bupivacaine at a concentration of ≤ 1.5 mM uncouples isolated heart

muscle mitochondria, while higher concentrations inhibit respiration¹⁹. Other support for mitochondrial involvement in local anesthetic toxicity has been provided by studies of the effects of lidocaine on neuronal cell lines. It has been shown that, in ND7 cells obtained from the rat dorsal root ganglion, lidocaine-induced apoptosis correlated with mitochondrial membrane depolarization, cytochrome-c release from mitochondria into the cytosol, and activation of caspases⁸. Interestingly, equimolar Tris buffer and equipotent tetrodotoxin controls were not toxic, indicating that neither osmotic nor sodium-blockade effects explain lidocaine neurotoxicity. Our data support this notion. We did not observe mtDNA damage after exposure to tetrodotoxin.

Damage to mtDNA appears to be important in mitochondrial pathways of apoptosis. It has been shown that the loss of mitochondrial membrane potential, which leads to swelling of mitochondria and release of proapoptotic factors from mitochondria, is linked to the presence of mtDNA damage³⁴. Also, it has been reported that oxidative damage to mtDNA is significantly higher in apoptotic cells than in controls^{35,36}. In addition, the differential susceptibility of glial cell types to oxidative stress and apoptosis correlates with increased oxidative mtDNA damage³⁷. Finally, a number of studies have shown that targeting DNA repair enzymes into mitochondria not only can enhance the repair of mtDNA lesions but also can increase the viability of a variety of cell types and protect against the induction of apoptosis^{38,39}.

We believe that a particularly novel aspect of our investigation is that potassium modulates some of the toxic effects of local anesthetics. However, this finding requires further investigation. In support of this notion are the findings that potassium channel blockers affect the proliferation and apoptotic behavior of human osteoarthritic chondrocytes⁴⁰. Also, it has been shown that bupivacaine is a very potent blocker of potassium channels, and lidocaine also has the ability to block potassium channels, although to a lesser extent than bupivacaine^{15,41}. Potassium channel openers recently have been studied and used as important tools to explore the role of potassium channels in cell function. Many of them are used as potent drugs to prevent pathologic changes in various organs^{42,43}. Moreover, in a recent work presented by Sun et al., a direct connection between mitochondrial injury and potassium channels has been demonstrated⁴⁴. They found that the K_{ATP} channel opener diazoxide prevented the dramatic early accumulation of reactive oxygen species in the mitochondria of ischemic spinal cord neurons by amelioration of oxidative mitochondrial DNA damage and deletions, thereby arresting apoptosis. We believe that similar mechanisms may be involved in chondrocytes.

We understand that our studies only correlate mtDNA damage, mitochondrial dysfunction, and cell death. In future studies, we plan to transfect our cells with DNA repair enzymes and definitely establish whether enhanced mtDNA repair, which leads to a decreased steady state of mtDNA damage, can reduce the toxicity caused by local anesthetics and, thus, enhance the viability of chondrocytes following exposure to these drugs. ■

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