

# Comparison of Ropivacaine and Bupivacaine Toxicity in Human Articular Chondrocytes

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**Background:** It has been shown that bupivacaine, the most commonly used local anesthetic for postoperative intra-articular use, is cytotoxic to bovine articular chondrocytes in vitro. Ropivacaine is as effective as bupivacaine for intra-articular analgesia and has less systemic toxicity. We compared the in vitro viability of human articular chondrocytes after exposure to bupivacaine, ropivacaine, and saline solution control.

**Methods:** Macroscopically normal human articular cartilage was harvested from the femoral head or tibial plateau of five patients. Full-thickness cartilage explants and cultured chondrocytes isolated from these patients were treated with 0.9% normal saline solution, 0.5% ropivacaine, or 0.5% bupivacaine for thirty minutes. Twenty-four hours after treatment, chondrocyte viability was measured with use of the LIVE/DEAD Viability/Cytotoxicity Kit for cartilage explants and with use of the CellTiter-Glo Luminescent Cell Viability Assay for cultured chondrocytes.

**Results:** Chondrocyte viability in cartilage explants was significantly greater after treatment with ropivacaine as compared with bupivacaine ( $94.4\% \pm 9.0\%$  compared with  $78\% \pm 12.6\%$ ;  $p = 0.0004$ ). There was no difference in viability after treatment with ropivacaine as compared with saline solution ( $94.4\% \pm 9.0\%$  compared with  $95.8\% \pm 5.7\%$ ;  $p = 0.6$ ). The viability of cultured chondrocytes was significantly greater after treatment with ropivacaine as compared with bupivacaine ( $63.9\% \pm 19\%$  as compared with  $37.4\% \pm 12\%$  of the value in the saline solution group;  $p < 0.0001$ ).

**Conclusions:** In vitro, 0.5% ropivacaine is significantly less toxic than 0.5% bupivacaine in both intact human articular cartilage and chondrocyte culture.

**Clinical Relevance:** Although bupivacaine is the most commonly used local anesthetic for intra-articular analgesia, the demonstrated toxicity to human articular chondrocytes is cause for concern. The present study demonstrated that ropivacaine is less chondrotoxic than bupivacaine and, therefore, may be safer for intra-articular analgesia.

Intra-articular injection of local anesthetics is routinely used to enhance postoperative analgesia following arthroscopic surgery. This technique has been shown to provide effective, reliable, and safe postoperative pain relief. Currently, 0.5% bupivacaine is the best-studied and most commonly used agent for this purpose<sup>1</sup>. However, several recent studies have demonstrated that even a brief exposure to bupivacaine may result in chondrotoxicity. Chu et al. found that 0.5% bupivacaine is cytotoxic to bovine articular chondrocytes in vitro<sup>2</sup>. Bovine cartilage explants and chondrocytes

cultured in vitro underwent apoptosis as early as one hour after a fifteen-minute exposure to 0.5% bupivacaine. While an intact native matrix provided some protection from cytotoxicity, chondrocytes grown in alginate bead culture experienced 99% cell death after bupivacaine treatment as compared with 20% cell death after saline solution treatment. Gomoll et al. showed that a forty-eight-hour continuous infusion of 0.25% bupivacaine into the glenohumeral joints of rabbits resulted in significantly increased chondrolysis when compared with infusions of saline solution<sup>3</sup>. Dogan et al. observed an increase in

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inflammation in the articular cartilage of rabbit knee joints when the injection of 0.5% bupivacaine was compared with the injection of normal saline solution<sup>4</sup>.

The loss of viable chondrocytes and the cellular changes observed in these animal studies are consistent with the characteristics of early osteoarthritis<sup>5</sup>; however, the long-term effect of bupivacaine exposure on articular health is unknown. The short and long-term viability of human articular chondrocytes after exposure to bupivacaine has not been well studied, and evidence of immediate chondrotoxic effects, even *in vitro*, would be clinically pertinent because of the frequency of intra-articular injection of bupivacaine. An alternative, less chondrotoxic local anesthetic could then be considered for intra-articular analgesia.

A promising alternative to bupivacaine for intra-articular pain relief is ropivacaine. Ropivacaine is a long-acting aminoamide member of the pipecoloxylidide group of local anesthetics that differs from bupivacaine only by the replacement of the butyl group on the piperidine nitrogen atom of the molecule with a propyl group. However, this variation makes ropivacaine less lipid-soluble than bupivacaine is, reducing its ability to diffuse into the heart and brain<sup>6</sup>. As a result, ropivacaine is known to have fewer systemic toxicities than bupivacaine, allowing for higher doses to be used in all sites<sup>7,8</sup>.

Ropivacaine also has been shown to be as effective or more effective than bupivacaine for providing postoperative analgesia following knee arthroscopy<sup>9-11</sup>. The effects of ropivacaine on the viability of human articular chondrocytes has not been reported; therefore, its safety profile and effectiveness warrant further investigation.

Given the reports that bupivacaine is chondrotoxic to animal articular chondrocytes and the lack of data on human tissue, we chose to investigate the viability of human articular chondrocytes after exposure to 0.5% bupivacaine, 0.5% ropivacaine, and 0.9% normal saline solution. The purpose of the present study was to test the hypotheses that short-term exposure to 0.5% bupivacaine is toxic to human articular chondrocytes *in vitro* and that 0.5% ropivacaine is a less toxic alternative for intra-articular injection.

## Materials and Methods

### Cartilage Explants

Macroscopically normal articular cartilage was harvested from the femoral head of three patients who were undergoing hemiarthroplasty of the hip for the treatment of an acute femoral neck fracture and from the tibial plateau of two patients who were undergoing total knee replacement as limb salvage for the treatment of a distal femoral tumor. Human tissue collection protocols were reviewed and approved by the institutional review board at the University of California at San Francisco. The articular cartilage was carefully screened for any gross evidence of preexisting damage or degeneration such as softening, fibrillation, or fissuring. Cartilage was isolated and processed under sterile conditions. A 4-mm-diameter dermatological biopsy punch was used to remove fifteen full-

thickness cartilage explants from each specimen. Cartilage explants were placed into individual wells of a ninety-six-well tissue-culture plate (BD Biosciences, San Jose, California), submerged in culture media (high glucose Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, 1% penicillin/streptomycin, 1% Fungizone) and incubated at 37°C with 5% CO<sub>2</sub> immediately following isolation. Explants were used for experimental treatment within forty-eight hours after harvesting.

### Chondrocyte Monolayer Culture

After the cartilage explants were harvested, the remainder of the articular cartilage was removed from bone with a sterile scalpel and was digested in sterile 0.2% hyaluronidase (Sigma-Aldrich, St. Louis, Missouri) for twenty minutes at 37°C followed by sterile 0.1% collagenase (Sigma-Aldrich) for four to six hours at 37°C. Following digestion, chondrocytes were plated in monolayer culture in fresh media into a 75-cm<sup>3</sup> flask at a density of 10<sup>4</sup> cells/cm. Chondrocytes were allowed to recover from digestion for at least one week prior to experimental treatment and were used for experimentation within one to two weeks after harvesting to prevent dedifferentiation. Twenty-four hours before experimental treatment, culture specimens were visualized under phase microscopy to verify a cell morphology consistent with differentiated chondrocytes, and the cells were then replated into a ninety-six-well Falcon plate at a density of 1000 cells per well. Only first-passage chondrocytes were used.

Cultured chondrocytes were maintained in high-glucose Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% Fungizone and were kept in an incubator at 37°C with 5% CO<sub>2</sub>. The medium was changed every three or four days.

### Experimental Groups

Cartilage explants and chondrocyte cultures were each subdivided into three treatment groups. Each group was exposed to one of the following: (1) 0.5% bupivacaine (Hospira, Lake Forest, Illinois), (2) 0.5% ropivacaine (AstraZeneca, Wilmington, Delaware), (3) 0.9% normal saline solution (Baxter, Deerfield, Illinois). All samples were treated according to the same protocol. Specifically, culture medium was aspirated; 200 µL of 0.9% normal saline solution, 0.5% bupivacaine, or 0.5% ropivacaine was added to each well; samples were incubated in 5% CO<sub>2</sub> at 37°C for thirty minutes; and the treatment solution was aspirated and fresh culture medium was added. Samples were returned to the incubator, and chondrocyte viability was measured twenty-four hours later.

We assessed chondrocyte viability in cartilage explants from three patients (with the explants being obtained from the tibial plateau in two patients and from the femoral head in one patient) and in cultured chondrocytes from five patients (including the three patients just mentioned as well as two additional patients from whom the specimens were obtained from the femoral head). Each independent experiment comparing ropivacaine and bupivacaine treatment was performed

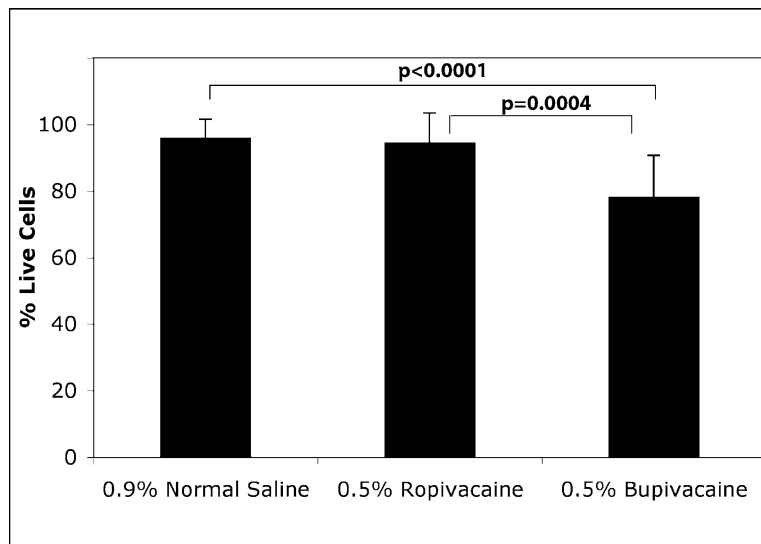


Fig. 1

Bar graph showing the percentage of live chondrocytes within cartilage explants twenty-four hours after a thirty-minute treatment with 0.9% normal saline solution, 0.5% ropivacaine, or 0.5% bupivacaine. Live and dead cells were quantified with use of the LIVE/DEAD Cell Viability/Cytotoxicity Kit. P values were determined with use of the Student t test.

with use of cartilage obtained from a single donor to avoid the potential confounding influence of cartilage source.

### Assessment of Viability

#### LIVE/DEAD Cell Viability/Cytotoxicity Assay in Cartilage Explants

Twenty-four hours after treatment, cartilage explants were sectioned orthogonally to the articular surface at 100  $\mu$ m per section. Three sections were obtained from each explant. Chondrocyte viability was assessed with use of the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, Oregon) following the kit protocol. Briefly, sections were incubated in 60  $\mu$ L of 1- $\mu$ M calcein AM/1  $\mu$ M ethidium homodimer-1 solution at room temperature in the dark for thirty minutes. Sections were then viewed with a fluorescent microscope, and digital photographs were made at five times magnification with use of

both a fluorescein and a Texas Red filter. The photographs included an area extending from the articular surface to 1 mm below the articular surface. Live and dead cells in an area measuring 1 mm  $\times$  1 mm  $\times$  100  $\mu$ m were quantified with use of a cell-counting software program previously developed and validated in our laboratory (Adobe Photoshop, ImageJ).

#### CellTiter-Glo Luminescent Cell Viability Assay in Cultured Chondrocytes

Twenty-four hours after treatment, culture medium was aspirated from the wells, chondrocytes were washed once with 1X phosphate-buffered saline solution, and 50  $\mu$ L of CellTiter-Glo Reagent (Promega, Madison, Wisconsin) was added to each well. The cells were incubated at room temperature in the dark for ten minutes. Next, 40  $\mu$ L of chondrocyte lysate from each well was transferred to an empty well in an opaque-walled

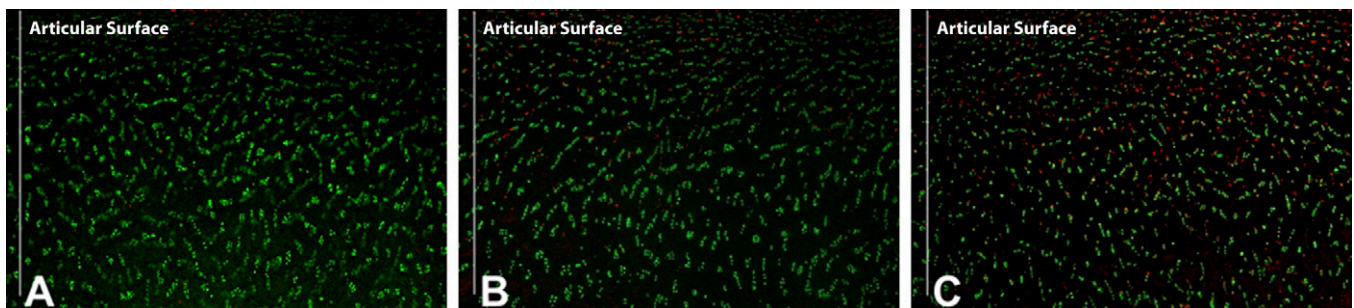


Fig. 2

Fluorescence microscopy images of human articular cartilage explants stained with 1- $\mu$ M calcein AM and 1- $\mu$ M ethidium homodimer-1 twenty-four hours after a thirty-minute treatment with (A) 0.9% normal saline solution, (B) 0.5% ropivacaine, or (C) 0.5% bupivacaine. Live cells fluoresce green, and dead cells fluoresce red (magnification,  $\times 5$ ; white calibration bar = 1 mm).

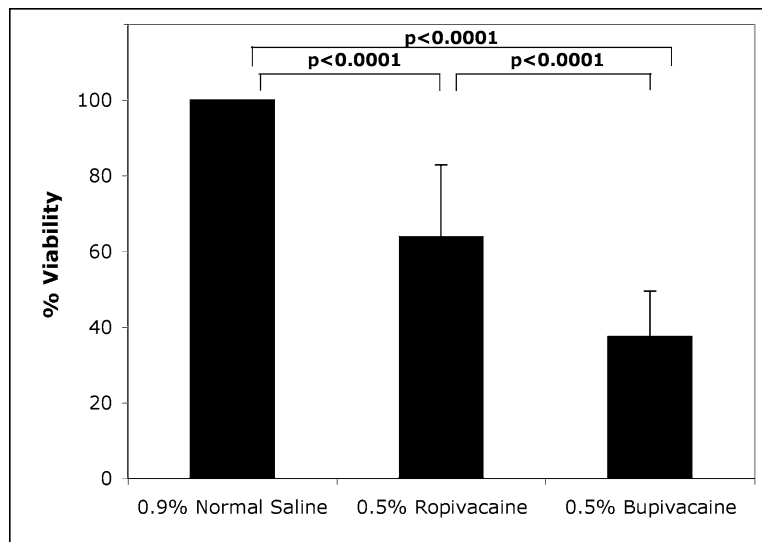


Fig. 3

Bar graph illustrating chondrocyte viability in monolayer tissue culture twenty-four hours after a thirty-minute treatment with 0.9% normal saline solution, 0.5% ropivacaine, or 0.5% bupivacaine. Cell viability is defined as the mean luminescence of treated cells normalized to the mean luminescence of saline control-treated cells, with luminescence being quantified with use of the CellTiter-Glo Luminescent Cell Viability Assay. P values were determined with use of the Student t test.

ninety-six-well plate, and luminescence was measured. Luminescence output data were standardized by dividing the measured luminescence of treated cells by the luminescence of cells treated with saline control for each sample.

### Statistical Methods

For statistical comparison of chondrocyte viability, the data were analyzed with the two-tailed Student t test, with the level of significance defined as  $p < 0.05$ .

## Results

### Cartilage Explants

Chondrocyte viability (as determined with use of cartilage explants from three patients, with five samples per explant per treatment group) was significantly greater after treatment with ropivacaine than it was after treatment with bupivacaine ( $94.4\% \pm 9.0\%$  compared with  $78\% \pm 12.6\%$ ;  $p = 0.0004$ ). There was no difference in viability after treatment with ropivacaine as compared with saline solution ( $94.4\% \pm 9.0\%$  compared with  $95.8\% \pm 5.7\%$ ;  $p = 0.6$ ) (Fig. 1).

In saline solution and ropivacaine-treated explants, predominantly live cells were present from the articular surface throughout the depth of the explant (1 mm). However, in bupivacaine-treated explants, predominantly dead cells were present from the articular surface to a depth of 0.5 mm (Fig. 2).

### Cultured Chondrocytes

The viability of cultured chondrocytes (as determined with use of chondrocyte cultures from five patients, with five replicates per treatment group) was significantly greater after treatment

with ropivacaine than it was after treatment with bupivacaine ( $63.9\% \pm 19\%$  as compared with  $37.4\% \pm 12\%$  of the value in the saline solution group;  $p < 0.0001$ ) (Fig. 3). While the viability of cultured chondrocytes treated with ropivacaine was lower than saline solution-treated controls, ropivacaine was significantly less chondrotoxic than bupivacaine.

## Discussion

The results of the present study demonstrate that a thirty-minute exposure to 0.5% bupivacaine is cytotoxic to human articular chondrocytes, both in human articular cartilage explants and in chondrocyte culture. In intact cartilage, treatment with 0.5% ropivacaine showed no toxicity as compared with treatment with normal saline solution. While 0.5% ropivacaine was toxic to cultured chondrocytes, it was significantly less chondrotoxic than 0.5% bupivacaine was. These data suggest that 0.5% bupivacaine may have unintended detrimental effects when used for intra-articular analgesia. Our results also show that 0.5% ropivacaine is a less chondrotoxic local anesthetic than bupivacaine is, especially for intact cartilage; therefore, ropivacaine may be a safer alternative to bupivacaine for intra-articular analgesia.

While the chondrotoxicity of bupivacaine has been established in several animal models<sup>2-4</sup>, there has been little previous research regarding the effects of bupivacaine or ropivacaine on the viability of human articular chondrocytes. Jaureguito et al.<sup>12</sup> measured proteoglycan synthesis on the basis of the uptake of radiolabeled sulfate in human osteoarthritic cartilage that had been treated with a combination of morphine and 0.25% bupivacaine and found that, while synthesis

was transiently decreased in the saline solution and morphine groups, it was slightly increased in the groups with added bupivacaine. However, that study involved the use of cartilage from patients with severe osteoarthritis and did not test the effects of bupivacaine alone, making it difficult to apply the results to healthy human articular cartilage. In contrast, Nole et al.<sup>13</sup> reported that treatment of porcine and canine cartilage explants with a mixture of saline solution and bupivacaine acutely inhibited proteoglycan synthesis more than treatment with saline solution alone did. While that study involved the use of animal rather than human articular cartilage, the tissue was free of disease and therefore may better reflect the response of healthy human articular cartilage to bupivacaine treatment. To our knowledge, the present study is the first to directly quantify and compare the effects of bupivacaine and ropivacaine on the viability of healthy human articular chondrocytes. Our data confirm that the chondrotoxicity from bupivacaine exposure seen in animal models is applicable to human articular chondrocytes and show that ropivacaine is less chondrotoxic.

The mechanism of chondrotoxicity induced by local anesthetics is not well understood. Feinstein et al.<sup>14</sup> found that bupivacaine potentiates nitric oxide synthase-2 activity in rat glial cells and astrocytes while ropivacaine does not. Nitric oxide synthase-2 is normally absent from cells, but its expression and activity is induced on immunostimulation of cells, suggesting that bupivacaine is more likely than ropivacaine to exacerbate an ongoing inflammatory process through the production of nitric oxide<sup>14</sup>. Ropivacaine has been shown to have anti-inflammatory properties; Martinsson et al. reported that ropivacaine reduced the release of selected eicosanoids from activated human granulocytes in a dose-dependent manner<sup>15</sup>. Although these properties of bupivacaine and ropivacaine have not been tested in human articular chondrocytes, they may play a role in the differing effects that the two anesthetics have on chondrocyte viability and warrant further investigation.

Like Chu et al.<sup>2</sup>, we noted a difference in the toxicity of both bupivacaine and ropivacaine in the presence and absence of an intact extracellular matrix. While bupivacaine was significantly chondrotoxic in both environments, an intact extracellular matrix appeared to be protective against the toxicity that ropivacaine had on cultured chondrocytes. The treatment

of cartilage explants in vitro represents the in vivo environment during intra-articular injection of local anesthetics because articular cartilage will have an intact extracellular matrix and chondrocytes will not be directly exposed to anesthetic agents, as they are in tissue culture. In vivo studies of the effects of both local anesthetics on articular cartilage will be useful for further characterizing the chondrotoxicity seen in vitro.

Our results were highly reproducible, with little variation between experiments; however, the limitations of the present study were that it was done in vitro, that it involved the use of a single treatment duration and a single concentration of both bupivacaine and ropivacaine, and that it assessed chondrocyte viability at a single time point after treatment. Future experiments might compare chondrocyte viability after longer durations of exposure and at later time points after exposure and might produce a dose-response curve to the anesthetic agents. In vivo studies on the effects of intra-articular injection of ropivacaine and bupivacaine are also important not only to assess chondrocyte viability after exposure to bupivacaine and ropivacaine but also to further compare their pharmacokinetics.

In conclusion, we have shown that, in vitro, 0.5% bupivacaine is significantly toxic to human articular chondrocytes both in intact cartilage and in cultured chondrocytes after only a thirty-minute exposure. We also have shown that 0.5% ropivacaine is significantly less chondrotoxic than 0.5% bupivacaine is in both of these settings. Our results suggest that intra-articular injection of 0.5% bupivacaine may be detrimental to articular chondrocytes, although long-term clinical effects need to be evaluated. Because ropivacaine is significantly less chondrotoxic than bupivacaine is, it may prove to be a safer alternative for intra-articular analgesia. ■

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