

In Vitro Exposure to 0.5% Bupivacaine Is Cytotoxic to Bovine Articular Chondrocytes

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Purpose: Intra-articular use of 0.5% bupivacaine is common in arthroscopic surgery. This study was conducted to test the hypotheses that (1) 0.5% bupivacaine is toxic to articular chondrocytes, and (2) the intact articular surface protects chondrocytes from the effects of short-term exposure to 0.5% bupivacaine. **Methods:** Freshly isolated bovine articular chondrocytes were prepared into alginate bead cultures and were treated with 0.5% bupivacaine solution or 0.9% saline for 15, 30 or 60 minutes, washed, and returned to growth media. Chondrocytes were recovered from alginate 1 hour, 1 day, and 1 week after bupivacaine exposure; they were fluorescently labeled to identify apoptotic and dead cells and were analyzed by flow cytometry. Twelve osteochondral cores were harvested from bovine knees. The superficial 1 mm of cartilage was removed from 6 cores (top-off). Intact and top-off cores were submerged in 0.9% saline or 0.5% bupivacaine solution for 30 minutes and then maintained in chondrocyte growth media for 24 hours. Live-cell/dead-cell fluorescent imaging was assessed using confocal microscopy. **Results:** Greater than 99% chondrocyte death/apoptosis was observed in all bupivacaine-exposed alginate bead cultures compared with 20% cell death in saline-treated controls ($P < .05$). Osteochondral cores with intact surfaces treated with 0.5% bupivacaine showed 42% dead chondrocytes. When the articular surface was removed, 0.5% bupivacaine resulted in increased cell death, with 75% dead chondrocytes ($P < .05$). **Conclusions:** Results show that 0.5% bupivacaine solution is cytotoxic to bovine articular chondrocytes and articular cartilage in vitro after only 15 to 30 minutes' exposure. The intact bovine articular surface has some chondroprotective effects. **Clinical Relevance:** Because healthy chondrocytes are important for maintenance of the cartilage matrix, chondrocyte loss may contribute to cartilage degeneration. This study shows a cytotoxic effect of 0.5% bupivacaine solution on bovine articular chondrocytes in vitro. Although these results cannot be directly extrapolated to the clinical setting, the data suggest that caution should be exercised in the intra-articular use of 0.5% bupivacaine. **Key Words:** Articular cartilage—Cytotoxicity—Bupivacaine—Knee.

Intra-articular use of bupivacaine is common in arthroscopic surgery. Bupivacaine is important as a local and intra-articular anesthetic agent. Surgeons may also inject bupivacaine solution with epinephrine into the intra-articular space to reduce intraoperative bleeding. Postprocedural injections of bupivacaine are frequently given as well. Recently, continuous intra-

articular infusions of bupivacaine have been used for analgesia after shoulder surgery.

Despite the common use of intra-articular bupivacaine, its effects on articular cartilage are not well understood. Evidence suggests that bupivacaine may be harmful to articular cartilage and chondrocytes. Intra-articular injection of 0.5% bupivacaine into rabbit knee joints caused increased inflammation of synovium and cartilage within 24 hours.¹ Reports of chondrolysis following continuous infusion of bupivacaine into the shoulder raise additional concerns that bupivacaine may have negative effects on articular cartilage (Freddie H. Fu, M.D., personal communication, July 2005).

Given that chondrocyte loss has been implicated in the development of chondrosis and osteoarthritis,^{2,3}

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additional research into the effects of bupivacaine on articular chondrocytes has been needed. This study was undertaken to assess the effects of 0.5% bupivacaine on the viability of bovine articular chondrocytes in vitro and was designed to test the hypotheses that (1) 0.5% bupivacaine is toxic to articular chondrocytes, and (2) an intact articular surface protects chondrocytes from the effects of short-term exposure to 0.5% bupivacaine.

METHODS

Survival of articular chondrocytes after brief exposures to 0.5% bupivacaine was assessed with the use of flow cytometry of chondrocytes recovered from 3-dimensional alginate cultures of freshly isolated bovine articular chondrocytes. Fluorescent viability staining and confocal microscopy of fresh bovine articular cartilage were used to study the effects of cartilage integrity on chondrocyte viability after exposure to 0.5% bupivacaine.⁴ Methods used to detect dead cells have been previously validated in our laboratory with the use of HCl to kill chondrocytes prior to fluorescent viability staining and confocal microscopy, and with the use of 70% ethanol to kill chondrocytes prior to flow cytometry (unpublished data).

Alginate Bead Cultures

Bovine knees obtained within 4 hours of slaughter were dissected to expose the articular cartilage. Articular chondrocytes were isolated from the cartilage through enzymatic digestion and were encapsulated in alginate beads at a density of 4×10^6 cells per mL as described by Masuda et al.⁵ For 1 week before bupivacaine exposure, beads were kept in a tissue culture incubator at 37°C/5% CO₂ in chondrocyte growth medium that consisted of Dulbecco's Modified Eagle's Medium/Medium F12 (Invitrogen, Grand Island, NY) (1:1) plus 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen).

Experimental Groups

After 1 week of culture, alginate beads were segregated into 6 groups of 60 beads each. Experimental groups were immersed in 1 mL of 0.5% bupivacaine HCl (Abbott Laboratories, Abbott Park, IL), and control groups were placed in 1 mL of sterile 0.9% saline (Invitrogen, Grand Island, NY) for 15 minutes, 30 minutes, or 60 minutes. Beads were then washed and reincubated in chondrocyte growth media. Cell viability was assayed at 3 time points: 1 hour, 24 hours, and

1 week. For each assay time point, 20 beads were removed from each group and assayed by flow cytometry and histology, as described later. This entire experiment was performed 3 times with the use of freshly isolated preparations of bovine articular chondrocytes from 3 different animals.

Flow Cytometry

At designated times, 10 beads from each treatment group were removed for labeling with the use of the Vybrant Apoptosis Assay Kit #3 (Molecular Probes, Eugene, OR). Alginate beads were transferred to Eppendorf tubes (Eppendorf International, Hamburg, Germany) containing 1 mL of cold 55 mM sodium citrate to dissolve the alginate and release the chondrocytes. The Eppendorf tubes were refrigerated at 4°C until the beads had fully dissolved and chondrocytes were collected by centrifugation. Cells were resuspended in 1 mL of phosphate-buffered saline. Tubes were centrifuged, and cells were washed once more and then resuspended in 400 μ L of 1X Annexin Binding Buffer. To 100 μ L of each suspension, 5 μ L of Alexa Flour 488 Annexin V and 1 μ L of propidium iodide (PI) were added to stain the cells. Cells were incubated at room temperature for 15 minutes; then, 400 μ L of 1X Annexin Binding Buffer was added. Samples were analyzed by flow cytometry to identify Annexin V- and PI-positive cells. Live cells were not labeled with either stain, apoptotic cells had only Annexin V staining, and necrotic cells were stained with both Annexin V and PI (Fig 1).

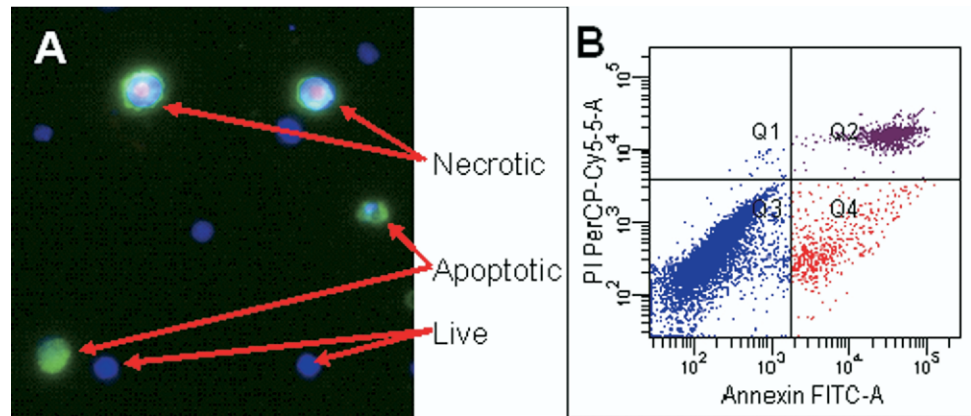
Histology

Eight beads were removed from each group and were placed in 3 mL of 10% buffered formalin acetate with 105 mM CaCl₂ for 1 hour. These beads were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

Treatment of Bovine Cartilage With Bupivacaine

An 8-mm Mitek coring device (DePuy Mitek, Norwood, MA) was used to cut 18 osteochondral cores from the trochlear groove; cores were randomly assigned to 6 groups of 3 cores each. Three groups (9 cores) were left intact, and in the other 3 groups (9 cores), the top 1 mm of cartilage was removed with a sharp scalpel (Fig 2). The 3 groups of intact cores and the 3 groups with the surface removed were submerged in 0.9% saline, 0.25% bupivacaine, or 0.5% bupivacaine for 30 minutes; they were then washed and returned to chondrocyte growth medium

FIGURE 1. Fluorescent staining and sorting of chondrocytes. (A) Fluorescent image of stained chondrocytes with propidium iodide (PI) staining appearing as red and Annexin V as green. Nuclei of live cells are shown here stained blue with DAPI (4',6-diamidino-2-phenylindole) for demonstration purposes; however, this dye was not used in flow cytometry. (B) Example of flow cytometry results showing the intensity of PI fluorescence (ordinate) plotted against the intensity of Annexin V fluorescence (abscissa) for a batch of control chondrocytes.



for 24 hours. Live and dead cells were imaged when orthogonal sections approximately 0.5 mm thick were cut from the middle of the cores and stained with 5 μ M 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes, Eugene, OR) and 1.5 μ M propidium iodide. Stained sections were washed with PBS and imaged by confocal microscopy.

Confocal Microscopy

Three pieces of cartilage were prepared for each treatment group. Fluorescently stained cartilage pieces were placed on the stage of an inverted confocal microscope (Olympus IX81-DSU; Olympus America, Melville, NY). With the use of a 40 \times objective, confocal stacks were obtained from 3 separate areas of each section, which were located 250 to 500 μ m from the cartilage surface. Each stack represented a volume that was 250 μ m \times 250 μ m \times 25 μ m thick. Red and green cells were counted in each image series with the use of image analysis software (Metamorph Imaging System; Molecular Devices, Sunnyvale, CA).

Statistical Analysis

For statistical comparison of saline and bupivacaine treatments, data from 3 separate experiments were analyzed by analysis of variance (ANOVA) and by Bonferroni's *t* test with significance set at a *P* value less than .05.

RESULTS

Alginate Bead Cultures and Flow Cytometry

After 1 week in culture, control alginate bead cultures contained an average of 69% viable cells, with

20% dead and 6.9% apoptotic cells. One hour after exposure of alginate bead chondrocyte cultures to 0.5% bupivacaine, fewer than 1% of cells were viable, compared with 69% in saline-treated controls (Fig 3). No difference was observed in viability between exposure times of 15 minutes, 30 minutes, and 60 minutes (Fig 4). One week after exposure of alginate bead cultures to 0.5% bupivacaine, empty lacunae were seen in H&E-stained sections (Fig 5).

Cores With Intact Articular Surfaces: Saline-treated intact cores had predominantly live cells near the articular surface (Fig 6A). Following exposure to 0.5% bupivacaine for 30 minutes, dead cells were observed extending from the articular surface to depths ranging from 80 to 200 microns (Fig 6B). In intact cores, the percentages of live cells per 250 μ m \times 250 μ m \times 25 μ m section was 74% \pm 11% for saline-treated controls and 58% \pm 11% for 0.5% bupivacaine-treated cores. This difference was significant (*P* < .05).

Cores Following Removal of the Top 1 mm of Cartilage: In saline-treated control cores, viability staining with CMFDA (live cell) and PI (dead cell)

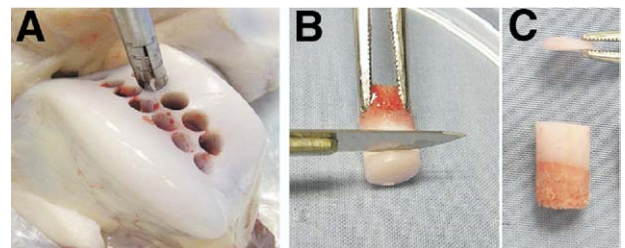


FIGURE 2. Preparation of osteochondral cores from bovine knees. (A) An 8-mm coring device is used to cut osteochondral cores from the trochlear groove. (B) A scalpel is used to remove the superficial layer of cartilage. (C) Resultant osteochondral core with its top cut off.

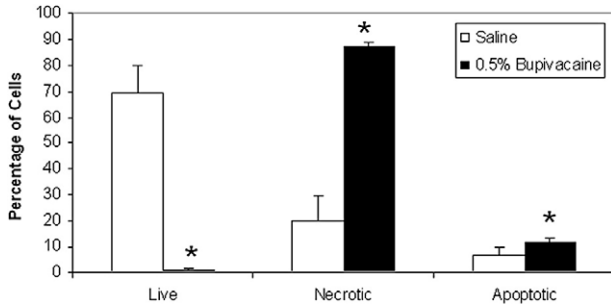


FIGURE 3. Results of flow cytometry of alginate bead cultures treated with 0.5% bupivacaine. Combined results 1 hour after treatment for 15, 30, and 60 minutes. * $P < .001$ compared with saline-treated controls.

and fluorescent microscopy showed that saline-treated controls contained predominantly live cells near the freshly cut surfaces (Fig 6C). However, treatment of these cores with 0.5% bupivacaine resulted in a zone of dead cells that extended to a depth of 0.5 to 1 mm from the exposed surface (Fig 6D). These qualitative observations were confirmed with quantitative analysis of confocal sections of treated cartilage pieces. In cores with the surface removed, the percentage of live cells was $76\% \pm 9\%$ for saline-treated controls. Exposure of cores with the surface removed to 0.5% bupivacaine for 30 minutes decreased ($P < .05$) viable chondrocytes to $25\% \pm 14\%$ (Fig 7).

DISCUSSION

Results show that 0.5% bupivacaine is cytotoxic to bovine articular chondrocytes in alginate culture and

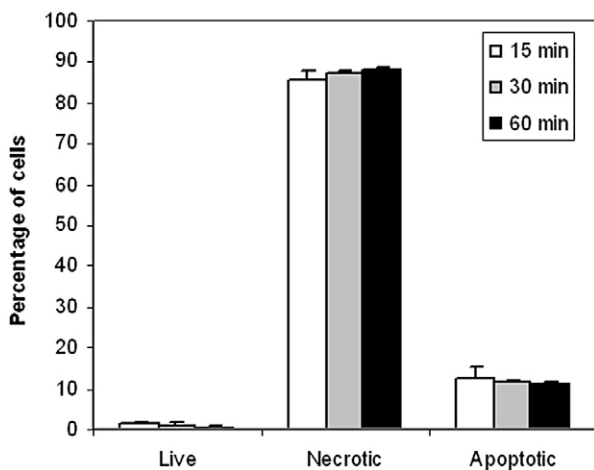


FIGURE 4. Percentages of live, necrotic, and apoptotic cells treated with 0.5% bupivacaine for 15, 30, and 60 minutes. No difference in viability was seen with these 3 exposure times.

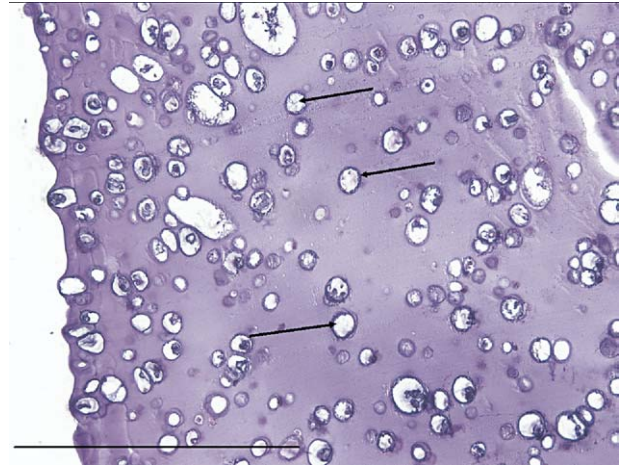


FIGURE 5. H&E stain of alginate bead culture of bovine chondrocytes, 1 week after exposure to 0.5% bupivacaine. Arrows point to empty lacunae. 200 \times magnification. Calibration bar = 50 microns.

in osteochondral cores after only 15 to 30 minutes' exposure in vitro. Although the data suggest that the intact articular surface in bovine articular cartilage provides some protection from the cytotoxic effects of bupivacaine, the articular cartilage of patients undergoing arthroscopic surgery is frequently fissured and fibrillated.⁶ The deeper layers of cartilage in these areas of chondrosis would not be protected by an articular surface, and the chondrocytes in these areas would therefore be more exposed to agents injected

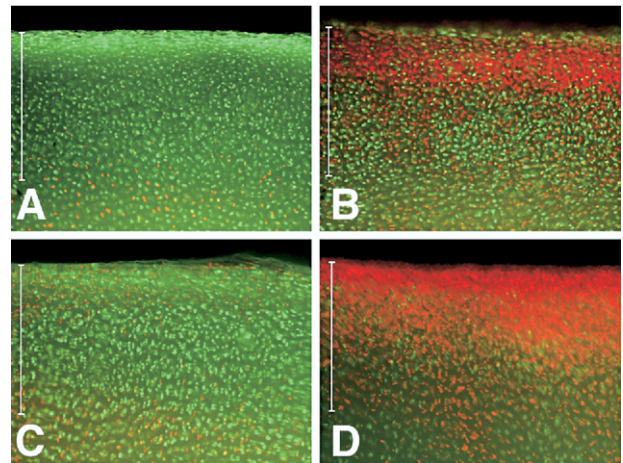


FIGURE 6. Sections of bovine cartilage stained with 5-chloromethylfluorescein diacetate (CMFDA) and propidium iodide (PI). (A, B) Intact surfaces at the top. (C, D) Top surface removed. Specimens were treated for 30 minutes with saline (A, C) or 0.5% bupivacaine (B, D). 400 \times magnification wide field images are shown. Calibration bars = 0.5 mm.

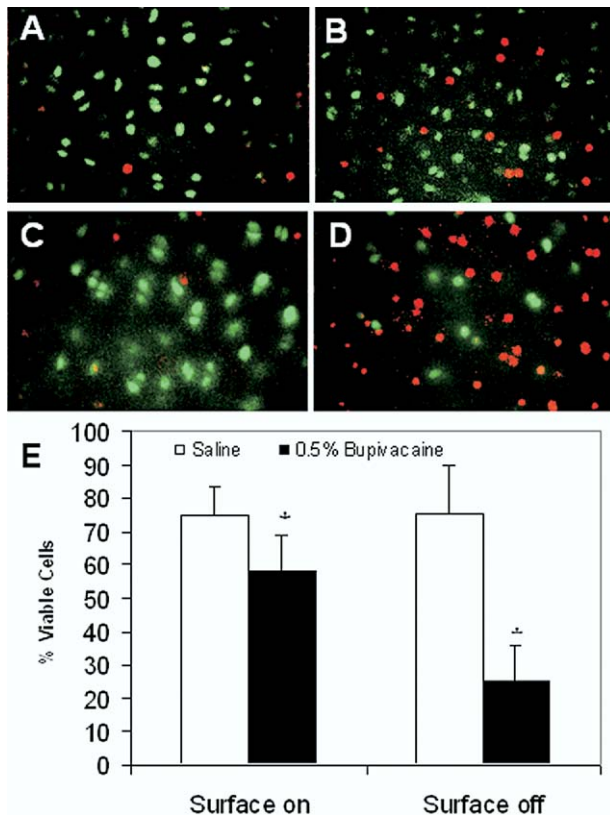


FIGURE 7. Cytotoxicity of bupivacaine in bovine cartilage cores. (A, B) Confocal images of cartilage with intact surfaces treated for 30 minutes with saline or 0.5% bupivacaine, respectively. (C, D) Confocal images of cartilage with surfaces removed treated for 30 minutes with saline or 0.5% bupivacaine, respectively. (E) Percentage of live cells in 9 volumetric reconstructions for each condition. * $P < .05$ as determined by analysis of variance (ANOVA) and Bonferroni's t test.

into the joint during arthroscopy. Although the effects of bupivacaine on human cartilage may differ from the results obtained *in vitro* with bovine tissue, these results suggest that caution is needed in the intra-articular use of 0.5% bupivacaine solution.

Few studies to date have reported on the effects of bupivacaine on articular cartilage. In an *in vivo* rabbit study undertaken to assess intra-articular bupivacaine and neostigmine, the authors concluded that both agents caused histopathologic change, with 0.5% bupivacaine causing greater articular cartilage inflammation at day 10. It is notable that increased inflammation of synovium and cartilage was seen within 24 hours.¹ Jaureguito et al.⁷ reported on the effects of 0.25% bupivacaine in concert with morphine on human articular cartilage from osteoarthritic knees without apparent adverse effects to articular cartilage

histology or proteoglycan metabolism. Although proteoglycan synthesis transiently decreased in the morphine and saline groups, addition of 0.25% bupivacaine resulted in a slight increase in proteoglycan synthetic activity. This study differs from ours in that a lower concentration of bupivacaine was used. Histology and radiolabeling studies also do not directly assess chondrocyte viability, which was the focus of our study. In a large number of experiments in which similarly obtained human cartilage from elderly osteoarthritic knees was used, we have found a high degree of variability in proteoglycan synthetic activity, which may confound results (unpublished data).

To reduce tissue variability for quantitative analysis, we used bovine articular cartilage from freshly slaughtered young animals. We first treated bovine articular chondrocytes suspended in alginate with 0.5% bupivacaine, a dosage commonly used clinically, and then analyzed the recovered cells by flow cytometry. The use of alginate to encapsulate chondrocytes within a 3-dimensional matrix maintains the cartilage phenotype.⁵ Alginate culture therefore permitted treatment of chondrocytes in physiologic suspension culture followed by ready recovery of a large number of cells for quantitative flow cytometry. This method provided the means for more precise quantification of live, dead, and apoptotic cells following bupivacaine treatment than could be obtained with fresh tissues.

Although alginate beads allow articular chondrocytes to maintain a spherical morphology that is similar to their native shape, alginate is fairly homogeneous and does not fully approximate the exquisite matrix structure of articular cartilage. Articular cartilage is anisotropic, with a distinct surface architecture that is completely different from that of the matrix structure in the deeper zones of the tissue.² As such, alginate may permit more complete penetration of bupivacaine than is possible with native articular cartilage.

Our results with intact bovine articular cartilage, however, further support a toxic effect of 0.5% bupivacaine on articular chondrocytes. Increased chondrocyte cell death was observed in the superficial regions of both intact articular cartilage and cartilage with the articular surface removed following 30 minutes' exposure to 0.5% bupivacaine. The depth of cell death was less for intact articular cartilage suggesting that the articular surface may provide a partial barrier to bupivacaine. Observations for greater chondrocyte viability in intact bovine articular cartilage over that of chondrocytes suspended in alginate further suggest

that the bovine cartilage matrix may provide some protection from bupivacaine.

It is important to note that this study was performed with the use of bovine tissues and cells, and the results may thus be different from those observed in humans. Intra-articular bupivacaine has a long clinical history, with no apparent detrimental effects. Demonstration of cytotoxic effects on bovine chondrocytes *in vitro* should not be interpreted to mean that 0.5% bupivacaine has harmful effects clinically. Clinical study would be required to determine clinical effects. However, clinical assessment tools are limited, and currently, no diagnostic technologies are available for clinical use in assessing *in situ* chondrocyte viability.⁸ Clinical assessment through available methods of patient outcomes, magnetic resonance imaging, and radiographic examination may not detect negative effects of chondrocyte cytotoxicity until cartilage degeneration or even osteoarthritis has occurred. At these late endpoints, it would be difficult to determine what caused the degeneration.

Careful delineation of the dose- and time-dependent effects of bupivacaine on human chondrocytes and human articular cartilage is critically important for proper risk-benefit analysis regarding intra-articular use of bupivacaine. Bupivacaine is important as a local anesthetic agent for arthroscopic surgery because its anesthetic effects persist despite subsequent inflow of saline. This property is advantageous in permitting arthroscopic procedures to be done with the patient under local anesthesia, thereby reducing the need for general or regional anesthesia.

Clinical studies on the pain relief efficacy of bupivacaine compared with other analgesic agents have yielded mixed results. At least 1 report shows that 0.25% bupivacaine is no more effective than saline for pain management.⁹ Several reports indicate that bupivacaine is more effective when used in combination with epinephrine,¹⁰ tenoxicam,¹¹ and morphine¹² than when used alone. Other reports show that 0.5% bupivacaine is less effective than intra-articular ketorolac¹³ or morphine¹⁴ in alleviating postoperative pain.

Most of these studies combined different types of arthroscopic surgery; this complicates interpretation because the type of surgery may affect the efficacy of bupivacaine. Marchal et al.¹⁵ showed that following surgeries with minimal inflammation such as diagnostic arthroscopy and partial meniscectomy, 0.25% bupivacaine with epinephrine was more effective than morphine in controlling pain. However, in more invasive arthroscopic procedures such as anterior cruciate ligament reconstruction, lateral release, patellar shav-

ing, and plica removal, 5 mg of morphine was more effective.

Efficacy questions concerning bupivacaine must also be weighed against the potential for toxicity. Bupivacaine is known to cause toxicity to the nervous and cardiovascular systems through its effects on plasma membrane sodium channels. Bupivacaine is considered to have higher levels of cardiotoxicity than other local anesthetic agents.^{16,17} Studies measuring peak serum bupivacaine levels after intra-articular injection suggest that bupivacaine is absorbed from the joint over several hours, with peak serum levels seen within the first hour.^{18,19} Wasudev et al.²⁰ showed that in procedures involving resection or division of the synovium, higher levels of bupivacaine were measured in serum following intra-articular injection; they advised a reduction in the concentration used to avoid systemic toxicity. Results of this study additionally raise the possibility of direct adverse effects of 0.5% bupivacaine on articular chondrocytes.

With the use of flow cytometry and confocal microscopy, this study showed significant articular chondrocyte cell death following brief *in vitro* exposures to 0.5% bupivacaine solution. An intact articular surface and the cartilage matrix provided partial protection from bupivacaine. However, disruption of the articular surface exposed chondrocytes to the toxic effects of bupivacaine, and increased cell death was seen. Because arthroscopic procedures are performed on injured joints with a high prevalence of chondrosis, the potential for chondrocyte bupivacaine toxicity may be amplified clinically.

Although study methods permitted reproducible quantitative analysis, limitations were that this was an *in vitro* study that used bovine cartilage, and there was no dose-response curve. Important next studies would be undertaken to determine the dose and time effects of bupivacaine on human chondrocytes and human articular cartilage within clinically relevant ranges. *In vivo* studies on the intra-articular pharmacokinetics of bupivacaine following single injection and continuous infusion would enhance understanding of the intra-articular effects of bupivacaine injection. The relative safety of other anesthetic agents, such as lidocaine, and of the combined effects of bupivacaine and epinephrine should also be studied. Because basic science studies suggest that loss of healthy chondrocytes contributes to cartilage degeneration,³ the *in vitro* bovine chondrocyte data as presented here support the need for comprehensive additional studies and suggest that caution should be exercised in the intra-articular use of undiluted 0.5% bupivacaine.

CONCLUSIONS

Results show that 0.5% bupivacaine solution is cytotoxic to bovine articular chondrocytes in alginate culture and bovine articular cartilage in vitro after only 15 to 30 minutes' exposure. Data additionally show that the intact bovine articular surface provides some degree of protection from the toxic effects of bupivacaine. Because the articular cartilage of patients undergoing arthroscopy is frequently fissured or fibrillated, results suggest that caution should be exercised in the intra-articular use of 0.5% bupivacaine solution.

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