Bupivacaine is an amide local anesthetic with an intermediate onset of action, a high potency because of its lipid solubility, and a long duration of action. When local anesthetics are combined with systemic analgesics, lower doses of systemic analgesics are required and negative adverse effects of the drugs can be minimized. Intra-articular administration of bupivacaine has been used routinely as part of a multimodal perioperative approach to analgesia as arthroscopic day-patient procedures have gained popularity. It is used in dogs as part of a multimodal perioperative approach to analgesia after arthrotomy or arthroscopy.

Currently accepted protocols for intra-articular administration of bupivacaine to dogs involve the use of bupivacaine with methylparaben preservative or bupivacaine in preservative-free solutions at a bupivacaine concentration of 0.5%. Multiple-dose vials of bupivacaine contain the preservative methylparaben to extend shelf life and to limit growth of contaminants that may result from multiple entries into a vial. Intra-articular administration of bupivacaine is used for analgesia despite the lack of a clear understanding of its effects on articular cartilage. Intra-articular use of bupivacaine in dogs has been considered safe on the basis of results of several studies.

Evaluation of chondrocyte death in canine osteochondral explants exposed to a 0.5% solution of bupivacaine

Geoffrey S. Hennig, DVM, MS; Giselle Hosgood, BVSc, PhD; Loretta J. Bubenik-Angapen, DVM, MS; Susanne K. Lauer, Dr med vet; Timothy W. Morgan, DVM, PhD

Objective—To evaluate chondrocyte death in canine articular cartilage exposed in vitro to bupivacaine with and without methylparaben and to compare viability for cartilage with intact or mechanically debrided surfaces.

Sample Population—Both glenohumeral joints from 10 adult canine cadavers.

Procedures—10 osteochondral cores were harvested from each of the 20 humeral heads; synovium and 1 core from each joint were examined to verify joint health, and the other 9 cores were exposed to canine chondrocyte culture medium (CCCM), a 0.5% solution of bupivacaine, or 0.5% solution of bupivacaine with methylparaben for 5, 15, or 30 minutes.

Results—For the superficial zone of surface-intact chondrocytes, bupivacaine with methylparaben caused a significantly higher percentage of chondrocyte death at 5 minutes (47.7%) than did bupivacaine (23.6%) or CCCM (25.4%). Bupivacaine (53.8%) and bupivacaine with methylparaben (62.5%) caused a significantly higher percentage of chondrocyte death at 30 minutes than did CCCM (20.0%). For the superficial zone of chondrocytes with debrided surfaces, bupivacaine with methylparaben caused a significantly higher percentage of chondrocyte death at 30 minutes (59%) than it did at 5 minutes (37.7%). Bupivacaine with methylparaben caused a significantly higher percentage of chondrocyte death at 30 minutes (59.0%) than did CCCM (28.9%). For middle and deep zones of chondrocytes, treatment solution and surface debridement had minimal effects on percentage of chondrocyte death.

Conclusions and Clinical Relevance—Bupivacaine and bupivacaine with methylparaben were cytotoxic to canine articular chondrocytes in vitro. Intra-articular administration of bupivacaine is not recommended for clinical use until additional studies are conducted. (Am J Vet Res 2010;71:875–883)
For in vivo studies in pigs and dogs, structural damage to chondrocytes was not evident for up to 4 to 6 days after intra-articular administration of various concentrations of bupivacaine. In addition, proteoglycan synthesis was inhibited for at least 3 days. In another study, cartilage degeneration (as determined on the basis of histologic evaluation) was not apparent in dogs at 1, 14, or 28 days after intra-articular administration of bupivacaine.

Despite the results of the aforementioned studies, reports of chondrolytic complications following joint surgery in humans have raised concerns that intra-articular administration of bupivacaine may result in chondrodysplasia and a subsequent rapid onset of osteoarthritis. Patients in those reports received intra-articular administration of a constant rate infusion of bupivacaine after surgery via an intra-articular catheter and a pump. After development of joint pain and a decrease in joint function, rapid-onset chondrolysis was diagnosed by use of radiography and arthroscopy.

In vitro exposure of articular cartilage obtained from humans, cattle, dogs, and rabbits to bupivacaine results in chondrocyte death. In a clinical trial conducted to investigate intra-articular administration of 0.5% bupivacaine in rabbits, damage to articular cartilage and synovial inflammation were detected for > 10 days. In vitro exposure of bovine cartilage to 0.5% bupivacaine significantly increased chondrocyte death in 1 study. In that study, chondrocyte death was increased to a lesser extent in intact cartilage (26%), compared with chondrocyte death in cartilage with a mechanically disrupted articular surface (73%). In an in vitro study, a mixture of bupivacaine with methylparaben (as a preservative) and human osteoarthritic synovial fluid resulted in a crystalline precipitate in 2 of 4 samples tested. When cultures of human articular chondrocyte cultures were exposed to various concentrations of bupivacaine for 15 minutes, there were significant effects of time and bupivacaine dose on chondrocyte death.

Because loss of chondrocytes can result in development of chondrosis and osteoarthritis, evidence that intra-articular use of bupivacaine results in chondrocyte death in some species is disconcerting. Although increases in chondrocyte death have been observed in articular cartilage of humans, cattle, dogs, and rabbits to bupivacaine, these results cannot necessarily be directly extrapolated to dogs because of species variations in cartilage composition and physiologic functions. Mechanical properties and thickness of articular cartilage vary among species, so investigation specific to the articular cartilage of dogs is warranted. Canine articular cartilage and synovial tissue cocultures exposed to 0.5% bupivacaine continuously for 2 days resulted in death of almost 100% of the chondrocytes. Furthermore, it remains unknown whether the low pH of bupivacaine or the preservative methylparaben contributes to chondrocyte death in dogs. The purpose of the in vitro study reported here was to measure chondrocyte death in intact articular cartilage of dogs exposed to a 0.5% solution of bupivacaine with and without methylparaben and to compare the effect of these solutions on intact and mechanically debrided articular surfaces. We hypothesized that exposure of canine articular cartilage to bupivacaine would increase chondrocyte death in a time-dependent manner over a period of 5 to 30 minutes and that chondrocyte death would be increased to a lesser extent in surface-intact cartilage cores, compared with chondrocyte death in cartilage cores in which the surface was mechanically debrided.

### Materials and Methods

**Sample population**—Glenohumeral joints were obtained from 10 cadavers of mature dogs that weighed between 14.5 and 27.3 kg (mean, 20.1 kg). Joints were obtained immediately after dogs were euthanized by IV administration of a pentobarbital-phenytoin solution. None of the cadavers had evidence of overt pathological changes in the joints.

**Collection of cartilage samples**—The glenohumeral joints of each dog were assigned by use of a random number table to 1 of 2 preparations of the articular surface prior to tissue collection. The first joint of each dog was assigned according to an odd or even number corresponding to a preparation, with the contralateral joint defaulting to the other preparation, for a total of 10 joints/surface preparation. The articular surface of 10 joints was allowed to remain intact. For the other 10 joints, the surface of the articular cartilage was removed (debrided) by gently rubbing a sterilized abrasive pad on the cartilage surface until there was loss of the typical glistening appearance. Ten cores of articular cartilage and the underlying subchondral bone were aseptically harvested from each glenohumeral joint (20 cores/dog; total of 200 cores). Cores were harvested by cutting the articular surface of each humeral head in a grid-like manner into 6 × 6-mm squares with a sterilized jigsaw. Warm (37°C) PBS solution was flushed over the cartilage surface during cutting to minimize thermal damage. Final dimensions of osteochondral cores were 6 × 6 × 6 mm. Cores were washed with PBS solution and then submerged in CCCM in sterile 60-mm Petri dishes. Dishes were maintained at 37°C in an environment of 95% air and 5% carbon dioxide until further testing was performed, which was conducted < 24 hours after onset of culture.

**Experimental procedures**—One sample of synovial tissue from each joint and 1 randomly selected osteochondral core from each humeral head were fixed in neutral-buffered 10% formalin and prepared for standard histologic examination by use of light microscopy to determine the overall health of the articular cartilage. The formalin-fixed synovial tissue and articular cartilage from each joint were prepared and stained with H&E for microscopic evaluation and verification that the joints were apparently normal. A board-certified veterinary pathologist (TWM) who was unaware of the subsequent treatment of the cores recorded pathological changes and graded the samples for inflammation by use of a scale (Appendix).

One core from each humeral head was assigned to 1 of 3 treatment solutions (CCCM, 0.5% bupivacaine, or 0.5% bupivacaine with methylparaben) and 1 of 3
incubation periods (5, 15, or 30 minutes). Cores were incubated in sterile 60-mm Petri dishes that contained 15 mL of treatment solution maintained at 37°C. The pH of each treatment solution was measured with a digital pH probe and meter at the beginning and end of each incubation period.

Following treatment, 1-mm-thick slices that extended from the articular surface through the subchondral bone were collected from the center of each osteochondral core for analysis of chondrocyte viability. The slices were washed with PBS solution and then stained by incubation with 0.4 µL of calcein AM and 13 µL of ethidium homodimer-1 in 1 mL of PBS solution for 30 minutes. Live cells with intact plasma membranes and active cytoplasm will metabolize calcein and have green fluorescence. Cell membranes of dead, damaged, or dying cells are penetrated by ethidium, and their nuclei will have red fluorescence. After staining, the samples were washed with PBS solution and then mounted on slides prior to evaluation.

Within 1 hour after samples were stained, evaluation at 10X magnification was performed by use of a laser confocal microscope equipped with fluorescein and rhodamine filters. The laser confocal microscope was used to view intact viable tissue by forming serial optical sections through the depth of the specimen. Digital images were acquired of 14 sequential planes of the center field of view from the articular surface to the subchondral bone of each cartilage slice (Figure 1). One of the 14 image planes for each slice was randomly selected for evaluation. The cartilage was divided into 3 equal zones that extended from the surface to the subchondral bone (superficial zone, middle zone, and deep zone), and the live and dead cells were quantified for each zone by use of digital image editing software. For each zone of each image, the number of pixels contained in 10 cells was counted and then used to calculate the mean pixel count for a cell of that zone. This was performed for both the calcein-stained and ethidium-stained cells. The number of cells in each zone was calculated by dividing the total number of green or red pixels in that zone by the respective mean pixel cell count. The percentage of dead chondrocytes ([number of dead cells/number of live cells × number of dead cells] × 100) was determined for the 3 zones of each randomly selected image from each tissue slice.

Statistical methods—Histologic grades for synovial tissue and cartilage and viability of chondrocytes were compared within and between treatment groups by use of a statistical program. All data were categorical and were analyzed by use of Mantel-Haenszel methods, with stratification across time. The frequency of outcomes was compared among treatments. When there was a significant (P < 0.05) difference, ad hoc comparisons were made by use of the Fisher exact test, with type I error maintained at 0.05. The pH of the solution was compared before and after treatment by use of the Mann-Whitney U test (nonparametric), with values of P < 0.05 considered significant. The proportion of dead cells (chondrocyte death) was expressed as a percentage, and square root transformation was performed to provide data with a normal distribution for analysis. The fixed effect of treatments, time, and zones were evaluated by use of a mixed-effect linear model that included the random variance of dogs among treatments. When there were significant fixed effects, pairwise comparisons were performed by use of a Schefte adjustment to maintain type I error at 0.05.

Results
Sample population—All 10 dogs were considered free of overt orthopedic disease during physical examination performed immediately before they were euthanatized, and no gross lesions were evident on the articular cartilage. Histologic examination of the synovium and articular cartilage revealed that 19 of 20 joints were histologically normal. One joint had a focal area with grade 1 inflammation of the synovium; however, the articular cartilage of that joint had no histologic evidence of disease.

Cartilage with an intact surface—The percentage of chondrocyte death in the superficial zone was significantly higher for all treatments at each time, compared with the percentage of chondrocyte death in the middle and deep zones (Table 1). In the superficial zone, bupivacaine with methylparaben caused a significantly higher percentage of chondrocyte death at 5 minutes (47.7%) than did bupivacaine alone (23.6%) or CCCM (25.4%); however, by 30 minutes, bupivacaine with methylparaben and bupivacaine alone caused a similar percentage of chondrocyte death (62.5% and 53.8%, respectively), whereas CCCM caused a significantly lower percentage of chondrocyte death (20.0%). In the middle and deep zones, percentage of chondrocyte death did not change over time for bupivacaine, bupivacaine with methylparaben, or CCCM. In the middle zone, bupivacaine with methylparaben caused a significantly higher percentage of chondrocyte death at 30 minutes (27.6%), compared with the results for CCCM (7.6%). In the deep zone, bupivacaine with methylparaben caused a significantly higher percentage of chondrocyte death at 30 minutes (14.1%), compared with the results for bupivacaine alone (6.4%).

Cartilage with the surface removed—Percentage of chondrocyte death in the superficial zone was significantly higher for all treatments at each time, compared with the percentage of chondrocyte death in the middle and deep zones (Table 1). In the superficial zone, bupivacaine with methylparaben caused a significantly higher percentage of chondrocyte death at 30 minutes (59.0%) than at 5 minutes (37.7%), bupivacaine with methylparaben caused a significantly higher percentage of chondrocyte death at 30 minutes (59.0%) than did CCCM (28.9%), and the percentage of chondrocyte death did not change over time for bupivacaine alone or CCCM. In the middle and deep zones, percentage of chondrocyte death did not increase over time or on the basis of treatment with bupivacaine alone, bupivacaine with methylparaben, or CCCM.

Comparison between cartilage with an intact surface or from which the surface was removed—For the superficial zone, percentage of chondrocyte death was significantly higher at 5 minutes in cartilage from which the surface was removed and that was treated
with bupivacaine (48.2%) or CCCM (42.7%) than in cartilage with the surface intact that was treated with bupivacaine (23.6%) or CCCM (25.4%). In addition, the percentage of chondrocyte death in the superficial zone was significantly higher at 15 minutes in cartilage from which the surface was removed and that was treated with CCCM (43.3%) than in cartilage with an intact surface that was treated with CCCM (25.3%). In the middle zone, percentage of chondrocyte death was significantly higher at 5 minutes in the cartilage from which the surface was removed and that was treated with CCCM (19.3%) than in cartilage with an intact surface that was treated with CCCM (25.3%).

Figure 1—Confocal microscopic digital images of representative samples of canine cartilage with an intact surface (A, C, and E) or from which the surface was removed (B, D, and F) and incubated for 30 minutes with CCCM (A and B), a 0.5% solution of bupivacaine (C and D), or a 0.5% solution of bupivacaine with methylparaben (E and F). Chondrocytes were stained with calcein AM (green) and ethidium homodimer-1 (red). Notice the 3 zones (superficial, middle, and deep) indicated in panel C. White bar in panel F = 200 μm.
Table 1—Mean ± SD percentage of chondrocyte death for various zones of canine cartilage with an intact surface or from which the surface was removed and that was incubated for 5, 15, or 30 minutes with a 0.5% solution of bupivacaine, a 0.5% solution of bupivacaine with methylparaben, or CCCM.

<table>
<thead>
<tr>
<th>Zone</th>
<th>Surface</th>
<th>Treatment</th>
<th>5 minutes</th>
<th>15 minutes</th>
<th>30 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>Superficial</td>
<td>Intact</td>
<td>Bupivacaine</td>
<td>23.8 ± 14.07</td>
<td>49.9 ± 21.53</td>
<td>53.8 ± 14.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCCM</td>
<td>47.7 ± 16.54</td>
<td>37.6 ± 23.00</td>
<td>62.5 ± 19.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bupivacaine with methylparaben</td>
<td>26.4 ± 18.42</td>
<td>25.3 ± 18.95</td>
<td>20.0 ± 23.29</td>
</tr>
<tr>
<td></td>
<td>Removed</td>
<td>Bupivacaine</td>
<td>48.2 ± 27.06</td>
<td>38.8 ± 28.29</td>
<td>47.2 ± 19.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCCM</td>
<td>37.7 ± 20.72</td>
<td>46.6 ± 29.59</td>
<td>50.0 ± 19.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bupivacaine with methylparaben</td>
<td>42.7 ± 25.94</td>
<td>43.3 ± 23.09</td>
<td>26.9 ± 19.76</td>
</tr>
<tr>
<td>Middle</td>
<td>Intact</td>
<td>Bupivacaine</td>
<td>8.8 ± 6.84</td>
<td>24.0 ± 24.42</td>
<td>18.0 ± 15.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCCM</td>
<td>16.7 ± 20.68</td>
<td>28.8 ± 20.62</td>
<td>27.6 ± 18.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bupivacaine with methylparaben</td>
<td>8.4 ± 9.35</td>
<td>11.9 ± 10.68</td>
<td>7.6 ± 8.10</td>
</tr>
<tr>
<td></td>
<td>Removed</td>
<td>Bupivacaine</td>
<td>9.2 ± 16.13</td>
<td>12.1 ± 11.45</td>
<td>19.8 ± 18.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCCM</td>
<td>18.6 ± 19.28</td>
<td>17.1 ± 18.75</td>
<td>29.5 ± 19.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bupivacaine with methylparaben</td>
<td>19.3 ± 16.67</td>
<td>13.3 ± 16.92</td>
<td>14.8 ± 11.30</td>
</tr>
<tr>
<td>Deep</td>
<td>Intact</td>
<td>Bupivacaine</td>
<td>5.0 ± 4.64</td>
<td>10.7 ± 19.92</td>
<td>6.4 ± 12.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCCM</td>
<td>13.0 ± 24.60</td>
<td>12.2 ± 16.98</td>
<td>14.1 ± 11.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bupivacaine with methylparaben</td>
<td>5.2 ± 6.42</td>
<td>10.38 ± 8.04</td>
<td>7.2 ± 7.06</td>
</tr>
<tr>
<td></td>
<td>Removed</td>
<td>Bupivacaine</td>
<td>7.8 ± 12.70</td>
<td>15.5 ± 14.29</td>
<td>17.0 ± 13.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCCM</td>
<td>12.2 ± 16.49</td>
<td>10.5 ± 7.29</td>
<td>16.1 ± 17.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bupivacaine with methylparaben</td>
<td>8.9 ± 9.03</td>
<td>5.9 ± 5.60</td>
<td>11.0 ± 17.53</td>
</tr>
</tbody>
</table>

*Within a time point within a zone, the value differs significantly (P < 0.05) from the value for the same treatment in the cartilage from which the surface was removed.

a,Within a row, values with different superscript letters differ significantly (P < 0.05). a,Within a column, values with different superscript letters differ significantly (P < 0.05).

Each time point/surface/treatment involved 10 osteochondral cores, and each sample was analyzed 3 times (once for each zone).

Table 2—Mean ± SD pH of a 0.5% solution of bupivacaine, a 0.5% solution of bupivacaine with methylparaben, and CCCM before and after incubation for 5, 15, or 30 minutes with canine cartilage with an intact surface or cartilage from which the surface was removed.

<table>
<thead>
<tr>
<th>Cartilage surface</th>
<th>Treatment</th>
<th>5 minutes</th>
<th>15 minutes</th>
<th>30 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>Intact</td>
<td>Bupivacaine</td>
<td>5.8 ± 0.42</td>
<td>6.3 ± 0.26**</td>
<td>5.8 ± 0.42</td>
</tr>
<tr>
<td></td>
<td>Bupivacaine with methylparaben</td>
<td>5.6 ± 0.22</td>
<td>6.1 ± 0.17**</td>
<td>5.6 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>CCCM</td>
<td>7.5 ± 0.26</td>
<td>7.8 ± 0.28</td>
<td>7.5 ± 0.42</td>
</tr>
<tr>
<td>Removed</td>
<td>Bupivacaine</td>
<td>5.8 ± 0.36</td>
<td>6.3 ± 0.24**</td>
<td>5.8 ± 0.36</td>
</tr>
<tr>
<td></td>
<td>Bupivacaine with methylparaben</td>
<td>5.6 ± 0.23</td>
<td>6.1 ± 0.16**</td>
<td>5.6 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>CCCM</td>
<td>7.6 ± 0.28</td>
<td>7.7 ± 0.28</td>
<td>7.6 ± 0.28</td>
</tr>
</tbody>
</table>

*Within a time point within a treatment, pH is significantly (P < 0.05) higher than before incubation.

a,Within a column, values with different superscript letters differ significantly (P < 0.05).

Each time point/surface/treatment involved 10 osteochondral cores, and each sample was analyzed twice (once before and once after).

The surface that was treated with CCCM (8.4%). In the deep zone, percentage of chondrocyte death was significantly higher at 30 minutes in cartilage from which the surface was removed and that was treated with bupivacaine (17.0%) than in cartilage with an intact surface treated with bupivacaine (6.4%).

In the osteochondral cores for both the cartilage with an intact surface and the cartilage from which the surface was removed, pH significantly increased over all time periods for all treatments, except for CCCM at 5 minutes (Table 2). The pH was significantly lower at all times for bupivacaine (5.81 and 6.52 for the surface-intact and surface-removed cartilage, respectively) and bupivacaine with methylparaben (5.36 and 6.36 for the surface-intact and surface-removed cartilage, respectively) than for CCCM (7.51 and 7.85 for the surface-intact and surface-removed cartilage, respectively). The pH for bupivacaine with methylparaben at 30 minutes (6.38 and 6.35 for the surface-intact and surface-removed cartilage, respectively) was significantly lower than for bupivacaine at 5 minutes (6.34 and 6.32 for the surface-intact and surface-removed cartilage, respectively) and 15 minutes (6.45 and 6.48 for the surface-intact and surface-removed cartilage, respectively) but was similar (6.52 and 6.51 for the surface-intact and surface-removed cartilage, respectively) at 30 minutes. Comparison of the surface-intact and surface-removed groups revealed that surface integrity did not influence pH.

**Discussion**

In the study reported here, in vitro exposure with a 0.5% solution of bupivacaine with and without methylparaben for 5 to 30 minutes was cytotoxic to canine articular cartilage. At every time point, a significantly higher percentage of chondrocyte death was detected in the superficial zones of cores from both surface-intact and surface-removed cartilage, compared with results for the middle and deep zones. These results suggest that an intact articular surface is susceptible to chondrocyte death when exposed to 0.5% bupivacaine and that debriding the surface may in-
crease chondrocyte death but not consistently among treatments or over time. These findings have potential clinical relevance because many dogs undergoing joint-related surgical procedures have concurrent chondrosis. Pathological changes in a joint characterized by loss of the surface continuity of the articular cartilage is evident with conditions such as osteochondritis dissecans, cranial cruciate ligament rupture, articular fractures, and patellar luxations. These pathological changes result in subsequent chondrosis and osteoarthritic human cartilage exposed to bupivacaine in vitro resulted in greater chondrocyte death, compared with results for cartilage with an intact surface.

Concerns regarding safety for the intra-articular use of bupivacaine are supported by the mounting evidence that exposing cartilage of cattle, humans, and rabbits to bupivacaine increases chondrocyte death. Coculture of canine articular cartilage and synovial tissue exposed to 0.5% bupivacaine for 2 days resulted in death of almost 100% of chondrocytes. This finding of time-dependent chondrocyte death is important because intra-articular catheters are placed in joints of humans after joint-related surgeries and are used to deliver a constant flow of local anesthetics during approximately the subsequent 2 days. A common protocol for intra-articular administration of bupivacaine in dogs is a single injection of a 0.5% solution of bupivacaine before or after surgery (0.5 mL/kg but not to exceed a total dose of 2.0 mg/kg). Peak plasma concentrations of bupivacaine were achieved at 11 minutes after a single intra-articular injection of 0.5% bupivacaine in a stifle joint of a dog, but the concentrations of bupivacaine within the joints of dogs after a single injection remain unknown. The study reported here was designed to examine a single-injection scenario, and we found that exposing articular cartilage to bupivacaine with methylparaben for as little as 3 minutes or to bupivacaine or bupivacaine with methylparaben for 15 minutes resulted in increased chondrocyte death in the superficial zone of articular cartilage, compared with results for the control (CCCM) treatment. On the basis of these findings, even a single intra-articular injection of 0.5% bupivacaine has the potential to cause chondrocyte death, which suggests that intra-articular administration of bupivacaine should be avoided until further in vivo studies are performed.

Methylparaben is the preservative most commonly used in local anesthetics. Multiple-dose vials of bupivacaine contain this preservative to maximize shelf life and to minimize growth of potential contaminants following multiple entries into a vial. Multiple-dose vials may be an attractive option for clinicians from the perspective of cost-efficiency; however, the potential for bacterial contamination and allergic reactions exists. For this reason, contents of multiple-dose vials are not recommended for use in spinal or epidural anesthesia. When synovial fluid from humans with osteoarthritis was mixed with a 0.5% solution of bupivacaine with methylparaben, a crystalline precipitate formed in 2 of 4 samples. The precipitate was not characterized further, and it was suggested that preservative-free bupivacaine should be used for intra-articular injection. We are not aware of any other reports of studies conducted to evaluate the effects of intra-articular injection of bupivacaine containing methylparaben on cartilage. In the present study, osteochondral cores exposed to bupivacaine with methylparaben did not consistently have a greater percentage of chondrocyte death, compared with results for cores exposed to bupivacaine alone, which suggested that methylparaben may minimally contribute to increases in chondrocyte death.

On the basis of the results of our study, loss of surface integrity was not consistently associated with greater chondrocyte death in cartilage incubated with bupivacaine or bupivacaine with methylparaben. However, in some cartilage incubated with CCCM, cores from cartilage from which the surface was removed had a greater percentage of chondrocyte death than did the cores from cartilage with an intact surface. This suggests that our method for articular surface debridement may have been excessive and disrupted so many chondrocytes that death was imminent regardless of the treatment solution used. Also, mechanical debridement may not have caused uniform disruption of the articular surface among cores. Histologic assessment of our surface debridement method revealed disruption of the superficial layers of the articular surface. The intent of the disruption was to mimic surface disruption that may result from chondropathy. In vivo studies of induced osteoarthritis in dogs have been reported; however, to the authors' knowledge, an in vitro osteochondral surface disruption protocol has not been established. Researchers removed the superficial 1 mm of cartilage with a sharp scalpel from bovine osteochondral explants for studies of bupivacaine-associated chondrocyte death. The thickness of cartilage in the humeral head of dogs is considerably thinner than that of the stifle joint of cattle; thus, this method could not be duplicated with consistency in the present study.

In the study reported here, we evaluated the pH of the treatment solutions to determine whether differences in pH could explain differences in chondrocyte death among the treatments. The pH increased significantly for all treatments during the treatment period (except for CCCM at 5 minutes), which suggests that ongoing metabolism offers some buffering effects. Both bupivacaine and bupivacaine with methylparaben had a consistently and significantly lower pH than did CCCM. Although a causal relationship has not been established, the lower pH of both bupivacaine solutions may have played a role in the death of canine chondrocytes. Synovial fluid is an ultrafiltrate of blood and has a pH of 7.4, whereas disease states such as osteoarthritis can decrease the synovial fluid pH to 7.2. The pH of bupivacaine solutions used in the present study ranged from 5.6 to 5.8. In another study, investigators treated bovine osteochondral explants with saline (0.9% NaCl) solution at pH of 7.4, 7.0, and 5.0. Chondrocyte death was not increased at pH 5.0 and 7.0, compared with chondrocyte death for the control group of pH 7.4; this suggested that a pH as low as 5.0 had little or no role in increased chondrocyte death. Analysis of results of the study reported here did not reveal whether the low pH of either bupivacaine solution contributed to increased...
chondrocyte death. The effect of pH on canine cartilage remains unknown. Furthermore, the relationship between pH and bupivacaine-associated death of canine chondrocytes also remains unknown. It is possible that a low pH could accentuate bupivacaine-associated death of canine chondrocytes.

Because of the in vitro evidence that bupivacaine causes chondrocyte death in bovine and human articular cartilage, the effects of other local anesthetics on chondrocytes have been evaluated. Lidocaine and ropivacaine can both cause increases in chondrocyte death in vitro, compared with results for saline (control) solution. Similar to bupivacaine, lidocaine and ropivacaine caused greater chondrocyte death in cell cultures than in intact tissues. These local anesthetics are effective as intra-articular analgesics, and they reportedly have fewer adverse systemic effects than does bupivacaine, especially with regard to the heart and brain. Lidocaine and ropivacaine can cause chondrocyte death, but not to the same degree as for bupivacaine, which suggests that an inherent property of bupivacaine is responsible for chondrocyte death. Mepivacaine also can cause increased cell death in explants of equine articular cartilage exposed to lipopolysaccharide. Other studies have revealed that chondrocyte cultures are much more sensitive to the effects of local anesthetics than is intact cartilage from which the surface has been debrided. This suggests that an intact articular surface may have a protective effect and also that the cartilage matrix may have an integral role in chondrocyte protection.

The mechanisms responsible for bupivacaine-associated death of canine chondrocytes are currently unknown. There is a relationship between exposure to local anesthetics and cartilage inflammation. An in vivo study in rabbits revealed an increase in inflammation of articular cartilage by 24 hours after intra-articular administration of a single dose of 0.3% bupivacaine. Bupivacaine causes inflammation through potentiating the activity of nitric oxide synthase-2 activity in rat glial cells and astrocytes. Activity of nitric oxide synthase-2 can be induced in physiologically normal cells through immunostimulation, and bupivacaine can exacerbate ongoing inflammation through the production of nitric oxide. These findings support the fact that bupivacaine stimulates inflammation in cartilage as well as in nervous tissue. Exposing cocultures of canine cartilage and synovium treated with interleukin-1 to 0.5% bupivacaine decreased the production of nitric oxide and prostaglandin E, in study; however, that decrease in inflammatory markers was attributed to a decrease in production subsequent to bupivacaine-associated chondrocyte death. Lidocaine can selectively upregulate proinflammatory proteins and downregulate anti-inflammatory and some proresolution peptides and proteins, thereby inhibiting resolution of ongoing inflammation. It is possible that bupivacaine shares this characteristic for inhibition of inflammation with lidocaine.

Bupivacaine can induce apoptosis. Bupivacaine caused time- and dose-dependent apoptosis in Schwann cell cultures by stimulating the production of reactive oxygen species in 1 study. Blocking the reactive oxygen species with antioxidants resulted in a significant inhibition of bupivacaine-induced apoptosis. Apoptosis of human articular chondrocytes has also been observed and is proposed to have at least a partial role in chondrolysis following exposure to 0.25% bupivacaine. Staining chondrocytes with calcein AM and ethidium homodimer-1 allows only the assessment of cell viability and does not provide information regarding pathophysiologic processes. Therefore, for the study reported here, it cannot be determined whether apoptosis contributed to chondrocyte death following exposure to bupivacaine.

Bupivacaine can disrupt mitochondrial homeostatic mechanisms and therefore contributes to bupivacaine-associated chondrocyte death. Local anesthetics (such as bupivacaine) that have high lipid solubility can reach the mitochondria and disrupt their transmembrane potential and subsequently cause apoptosis. Bupivacaine causes mitochondrial depolarization and opening of permeability transition pores, which plays a key role in many forms of cell death. The extracellular matrix of articular cartilage may provide protection to chondrocytes exposed to bupivacaine. Alginate bead cultures of bovine chondrocytes and intact bovine cartilage cores exposed to 0.5% bupivacaine for 30 minutes resulted in death of 99% of the chondrocytes, compared with death of 42% of the chondrocytes in the intact cartilage. Alterations in water, collagen, and glycosaminoglycan content within the extracellular matrix were investigated by exposing cocultures of canine cartilage and synovium to 0.5% bupivacaine. Results of that study did not reveal significant variations in water, collagen, or glycosaminoglycan content in the synovium following exposure to bupivacaine. Because of a lack of alterations in the extracellular matrix following exposure to bupivacaine, chondrocyte death is most likely the result of direct effects of bupivacaine on chondrocytes.

The study reported here has several limitations. The in vitro design did not account for the physiologic processes and homeostasis of healthy canine joints. Attempts were made to minimize this through the use of intact tissues and maintaining all samples in environments that were as ideal as possible. Intact osteochondral explants were used in vitro to mimic the in vivo intra-articular environment; however, lack of the synovium may have contributed to chondrocyte death. The method of cartilage disruption used in this study has not been described elsewhere and does not reflect the typical pathological progression of naturally developing disease. This method was potentially too aggressive and, in fact, may have contributed to increased cell death in the samples, which was supported by the finding of increased percentages of chondrocyte death in the control samples. Therefore, our study did not clearly evaluate the effect of cartilage disruption, but the results do suggest a potential protective effect for articular cartilage with an intact surface.

Intra-articular administration of bupivacaine has been used clinically as an effective perioperative analgesic; however, concern has been raised regarding its safety. The in vitro design of the present study did not account for the dynamics of physiologic processes
in normal canine joints. Further in vitro and in vivo studies will be necessary to evaluate the effects of time, dose, single injection versus continuous infusion, healthy versus diseased cartilage, and pH in bupivacaine-associated chondrolysis. Until additional studies are conducted, intra-articular administration of a 0.5% solution of bupivacaine with or without methylparaben should be avoided in dogs.

References


Appendix
Grading system used for histologic assessment of canine cartilage and synovium.24,25

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No inflammation</td>
</tr>
<tr>
<td>1</td>
<td>Minimal inflammation; minimal congestion and edema</td>
</tr>
<tr>
<td>2</td>
<td>Mild inflammation; increase in thickness of the cell lining and a few inflammatory cells (neutrophils)</td>
</tr>
<tr>
<td>3</td>
<td>Moderate inflammation; increase in thickness of the cell lining, hyperplasia of synoviocytes, and increase in the number of inflammatory cells (neutrophils and macrophages)</td>
</tr>
<tr>
<td>4</td>
<td>Severe inflammation; increase in thickness of the cell lining, hyperplasia of synoviocytes, marked numbers of inflammatory cells (neutrophils and macrophages), and exudation of fibrin</td>
</tr>
</tbody>
</table>