Long-Term Effects of Bupivacaine on Cartilage in a Rabbit Shoulder Model

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Background: Previous investigations have reported on the chondrotoxicity of bupivacaine in short-term in vivo and in vitro models. This study was designed to provide additional information on the long-term effects of bupivacaine infusion on articular cartilage in an established rabbit shoulder model.

Hypothesis: Infusion of bupivacaine into the rabbit shoulder will have long-term deleterious effects on articular cartilage.

Study Design: Controlled laboratory study.

Methods: Thirty-six rabbits were randomized into 3 groups and were infused over 48 hours with saline (S), bupivacaine alone (B), or bupivacaine with epinephrine (B+E) into the glenohumeral joint. Animals were sacrificed after 3 months, and tissue samples were analyzed with live/dead cell assay, proteoglycan (PG) synthesis and content assays, and conventional histological evaluation.

Results: No macroscopic or radiographic changes were detected in the infused shoulders. Sulfate uptake of infused shoulders relative to controls was elevated to 112% ± 39% (S), 166% ± 67% (B), and 210% ± 127% (B+E). Statistical analysis of PG content demonstrated significantly increased levels in bupivacaine groups compared with saline. There were no significant differences among groups in cell count, percentage of living cells, or histological grade.

Conclusions: No permanent impairment of cartilage function was detected 3 months after intra-articular infusion of bupivacaine. Cartilage metabolism was increased, indicating a possible reparative response. This suggests that, at least in the model used, articular cartilage has the ability to recover from the chondrotoxic effects of bupivacaine infusion. Before extrapolating these results to human cartilage, other factors including underlying cartilage injury or disease, decreased chondrocyte density, and increased bupivacaine dosing need to be taken into account.

Clinical Relevance: Bupivacaine toxicity has recently been implicated in the development of chondrolysis after arthroscopic shoulder procedures, but these findings suggest that additional noxious stimuli might be required before permanent damage ensues.

Keywords: shoulder arthroscopy; articular cartilage; bupivacaine toxicity; pain pump

Complications from the use of pain pumps in orthopaedic surgery have lately received considerable interest. The potential for cartilage damage due to toxicity of the commonly used local anesthetic bupivacaine is particularly concerning and was the focus of 3 recent studies. Using an in vitro model of chondrotoxicity, Chu et al[3] investigated the effects of bupivacaine exposure, both in cell culture and in osteochondral explants. Our group recently published the results of an in vivo study in a rabbit shoulder model that closely replicated the clinical scenario by infusing bupivacaine through a catheter into the glenohumeral joint over 48 hours.[7] Both studies demonstrated significant toxicity with chondrocyte death and decreased cartilage metabolism, but these studies were not designed to detect long-term sequelae of such cellular responses. A third study by Nole et al,[12] although only looking at a single injection of 0.5% bupivacaine into the knees of 6-week-old pigs, found there was no difference in the radiolabeled sulfate uptake in this group compared with a control group 3 days after treatment. The present investigation uses our

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previous experimental setup of intra-articular bupivacaine infusion into the rabbit shoulder, but included long-term, 3-month follow-up to assess whether the chondrotoxicity observed earlier was only transient or would lead to overt, permanent changes in the articular cartilage.

MATERIALS AND METHODS

Study Animals

This study was conducted in accordance with established animal care protocols and was approved by our institutional animal review board. New Zealand White rabbits were used for all procedures, following a previously established model.[7] The rabbits were young adults with confirmed closure of growth plates, which was thought to be similar to the clinical situation where the target population consists of mainly adolescents and young adults presenting for instability surgery.

Thirty rabbits were divided into 3 different groups: group S, normal saline solution (control) with a pH of 5.9 (Baxter Healthcare, Deerfield, Illinois); group B, bupivacaine hydrochloride (0.25%) with a pH of 5.7 (Abbott Laboratories, Abbott Park, Illinois); and group B+E, bupivacaine hydrochloride (0.25%) with epinephrine (1:200 000), with a pH of 4.0 (Hospira Inc, Lake Forest, Illinois). All catheters were introduced surgically into the rabbits' left shoulders; the contralateral sides remained unoperated. All rabbits were randomly assigned to groups, and the surgeons were blinded to the infusate until statistical analysis was performed.

Surgical Procedure

Induction of each rabbit was conducted through an intramuscular injection of ketamine (40 mg/kg), acepromazine (1 mg/kg), and xylazine (5 mg/kg). The animals were then intubated and ventilated with isoflurane in oxygen. Preoperatively, intramuscular buprenorphine (0.01-0.03 mg/kg) was given for pain control. This was also used postoperatively every 10 to 12 hours or as needed for pain. Cefazolin (22 mg/kg) was injected subcutaneously to achieve antibiotic prophylaxis at the time of surgery and daily, thereafter, for 48 hours.

The animals were prepared and draped in a sterile fashion, and the glenohumeral joint capsule was exposed through a 2-cm incision over the dorsolateral aspect of the left shoulder. After capsulotomy, a soft polyurethane catheter was then introduced into the joint space under direct visualization. The free end of the catheter was advanced 5 mm into the joint, and the incisions in the capsule and rotator cuff musculature were closed over the catheter. To maintain the catheter's location, suture flanges attached to the catheter were secured with 3-0 Vicryl (Ethicon, Somerville, New Jersey) to the soft tissues of the proximal humerus. To externalize the catheter, its free end was tunneled medially in a subcutaneous fashion where it exited and was sutured to the skin. Skin closure was performed in a subcutaneous and subcuticular fashion. The catheter was then connected to an infusion disk, which was placed into a jacket that protected the incision and disk.

Postoperative Considerations

The infusion disk was set to run for 48 hours at a constant flow rate of 210 μL/h. This rate was calculated according to proportional weight because no data exist regarding the human-equivalent dosing of intra-articular bupivacaine in the rabbit. Assuming a flow rate of 4.16 mL/h in an average human weighing 70 kg, the proportional infusion rate for a 3.5-kg rabbit was calculated as 0.21 mL/h.

Postoperatively, the animals were placed in individual cages where they were allowed unrestricted ambulation, food, and water. At 48 hours, the animals returned to the operating room where the incisions were reopened under general anesthesia to confirm that the catheter tips had remained intra-articularly. The infusion catheters were then removed and the incisions closed as described above. All animals were closely monitored for any complications during the entire postoperative period. Three months after the original operation, the animals were sedated with intravenous acepromazine (1 mg/kg) and ketamine (25 mg/kg) and then euthanized through an overdose of pentobarbital.

Gross/Radiographical Analyses

Immediately postmortem, anteroposterior radiographs of the glenohumeral joints were obtained to assess any degenerative changes. The shoulder joints were then explored in a manner similar to the index surgical procedure. Gross changes in the surrounding glenohumeral soft tissue and synovial fluid were noted, and digital images of the articular surface were attained. After sterile arthrotomy, culture swabs were used to obtain aerobic, anaerobic, and fungal cultures from the synovial fluid and joint.

Histopathological Analyses

Proteoglycan Synthesis as Detected by Sulfate Uptake. Sulfate uptake is a standard measure of cartilage proteoglycan (PG) metabolism,[11] which provides an understanding of cartilage anabolism. Full-thickness sections of cartilage were shaved from the anterior and posterior portions of the humeral head, leaving a single strip of cartilage through the midsection of the humeral head. To analyze PG synthesis, cartilage explants were incubated overnight with $Na^{35}SO_4$ (10 μCi/mL) in 3 mL of a 1:1 ratio mixture of Dulbecco's Modified Eagle/Ham's F-12 medium containing 10% fetal bovine serum (FBS) with gentamycin (50 μg/mL) in sterile 12-well plates at 37°C under an atmosphere of 95% air and 5% CO_2. The labeling media were saved, and the tissue explants were rinsed with unlabeled media and weighed. The cartilage was placed in 2 mL of 4 M guanidine hydrochloride (pH 7.0) and 100 mM EDTA solution overnight at 4°C on a shaker. The solution was then dialyzed (1-kD cutoff) against 10 mM EDTA at 4°C overnight to remove free nucleotides and guanidine hydrochloride.

This volume was subsequently measured, and 200 μL thereof was placed into a scintillation vial along with 2.5 mL of ScintiSafe fluid (Fischer Sci, Hanover Park, Illinois). The resultant counts per minute (CPMs) were then corrected to the total volume of the dialyzed solution (in mL) and normalized to the DNA content of each explant. The level of PG synthesis was calculated as a percentage of the CPMs detected in the infused shoulder relative to the control shoulder for each group.

Proteoglycan Content. Dimethylmethylene blue (DMMB) assay can quantitatively determine the amount of PG/glycosaminoglycan (GAG) present in the cartilage or released into the media. To determine PG content, the dialyzed solution used to determine sulfate uptake was prepared in a serial dilution using 0.05 M sodium acetate and 0.05% TWEEN-20 buffer (pH 6.8). Two hundred microliters of 1,9-DMMB chloride was added to each sample to allow for colorimetric analysis, which was performed in triplicate on a Wallac 1420 Multilabel Counter (Perkin Elmer, Waltham, Massachusetts).[2,5,6] The data for each sample were averaged and then normalized to its respective DNA content and analyzed as a percentage of the infused shoulder relative to the control shoulder for each group.

DNA Content. DNA content was determined using Hoechst 33258 dye (Polysciences, Warrington, Pennsylvania). Each dialyzed sample was combined with 100 μL of 1 μg/mL Hoechst dye for fluorometric analysis. Emissions were measured using a Wallac 1420 Multilabel Counter (Perkin Elmer) and used individually for normalization.[9]

Confocal Live/Dead Cell Assay. From the remaining central strip of cartilage, 2-mm sections were removed including the subchondral bone. These were incubated in sterile phosphate buffered saline with 4 μmol/L calcein ac toxymethyl ester (Molecular Probes, Eugene, Oregon) and 8 μmol/L ethidium homodimer (Molecular Probes) for 1 hour to label live and dead cells, respectively.[8] Live and dead cells were viewed and analyzed with confocal laser microscopy (488-nm excitation) (Fluoview IX70 confocal laser microscope; Olympus, Melville, New York). Live and dead cells were manually counted by 4 independent, blinded observers and then normalized to the surveyed surface area, yielding cells per unit area. The total number of cells present was analyzed along with the percentage of living cells in each shoulder for each group.

Histological Evaluation. The remaining tissue (humeral head with remaining cartilage) was decalcified in aqueous solution of formic acid (22%)/sodium citrate (10%) and fixed in 10% neutral buffered formalin. The synovial tissue was also fixed in this same buffer, and both were processed for paraffin embedding. The tissue was sectioned at 4 to 6 μm, placed on glass slides coated with Vectabond (Vector Laboratories, Burlingame, California), and stained with hematoxylin and eosin for routine histological analysis. The osteochondral sections from the humeral head were also stained with safranin O and fast green.[13] Both the cartilage and synovium sections were assessed with a modified Mankin grading scale for 5 aspects of histopathological changes.[14] Each section of the grading scale was added for a total histological grade for each specimen. This analysis was performed by 2 independent blinded observers.

Statistical Analysis

One-way analysis of variance (ANOVA) was used when variables were normally distributed. Significant results were followed by a Tukey multiple comparisons post hoc test. Similarly, the nonnormally distributed variables were analyzed with a Bonferroni-Kruskal-Wallis test and further evaluated with a Bonferroni-adjusted 2-sided Mann-Whitney test for pairwise comparisons. All analyses were performed with a significance level set at $P < .05$.

RESULTS

One animal from group S was excluded from the study due to premature displacement of the infusion catheter during the first 48 hours after surgery. This left 11 animals in group S and 12 animals each in groups B and B+E for a total of 35 specimens.

Gross/Radiographical Analysis

Radiographs did not show evidence of narrowing of the glenohumeral joint space or osteophyte formation for any of the treatment groups. During dissection, there was no presence of injected tissue or soft tissue overgrowth in any of the animals. Culture swabs of the synovial fluid at the time of harvest did not grow any organisms.

PG Synthesis

The PG synthesis of infused shoulders relative to their contralateral controls was 112% ± 39% for group S, 166% ± 67% for group B, and 210% ± 127% for group B+E. The levels seen in groups B and B+E were significantly higher than that of group S ($P < .05$); however, groups B and B+E were not significantly different from each other (Figure 1).

PG Content

Statistical analysis of PGs indicated significant differences when analyzing the infused shoulders relative to the corresponding controls across all groups (ANOVA, $P = .02$). Further subanalysis demonstrated significantly higher levels of PGs detected in groups B and B+E compared with group S (Tukey post hoc, $P < .01$ and $P < .05$, respectively) (Figure 2). No significant difference was noted between groups B and B+E.

Live/Dead Cell Assay

The absolute number of cells (live and dead) per area unit of cartilage was not statistically different between groups (ANOVA, $P = .09$). Using the same information but calculating the ratio of live to dead cells, one again found no difference between groups (ANOVA, $P = .34$) (Figure 3).

Histological Evaluation

Histological analysis of samples revealed no significant differences in the histological scores of cartilage and synovial

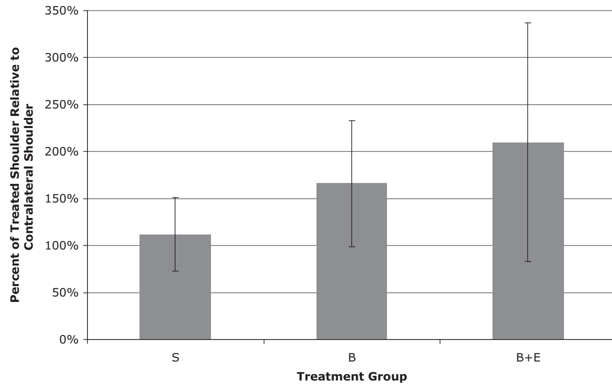

Figure 1. Sulfate uptake expressed as ratio between infused and control shoulders. S, saline; B, bupivacaine; B+E, bupivacaine and epinephrine.

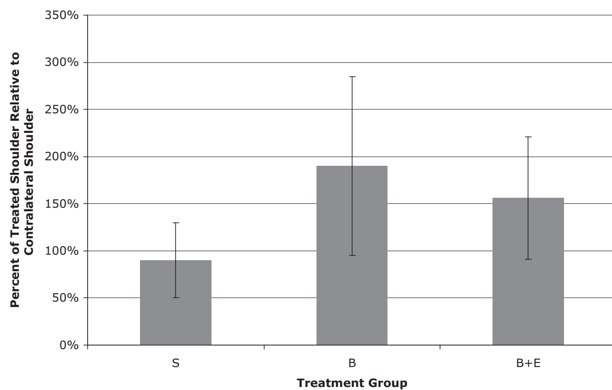

Figure 2. Proteoglycan content expressed as ratio between infused and control shoulders. S, saline; B, bupivacaine; B+E, bupivacaine and epinephrine.

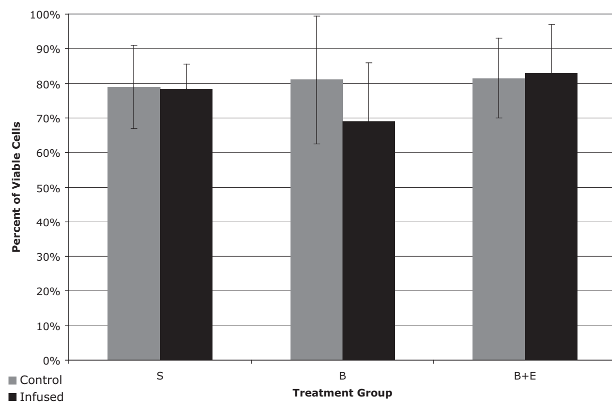

Figure 3. Results of live/dead-cell analysis showing the ratio of live versus dead cells in infused and control shoulders for each group. S, saline; B, bupivacaine; B+E, bupivacaine and epinephrine.

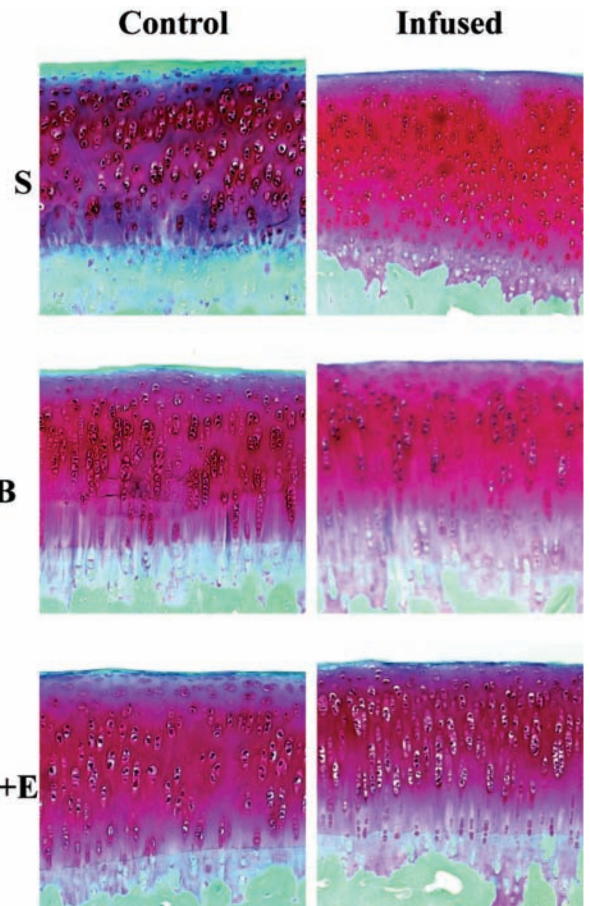

Figure 4. Safranin O staining of the control and infused shoulders is depicted within each treatment group. S, saline; B, bupivacaine; B+E, bupivacaine and epinephrine.

samples stained with hematoxylin and eosin and safranin O between groups (ANOVA, $P = .39$) (Figure 4 and Table 1).

DISCUSSION

This in vivo study of intra-articular bupivacaine infusion in a rabbit shoulder model did not demonstrate any permanent impairment of cartilage function at 3 months. The literature includes studies using in vitro and short-term in vivo models yielding mixed results regarding the deleterious effects of bupivacaine on chondrocytes. Chu et al[3] demonstrated significant chondrotoxic effects in an in vitro model using bovine chondrocyte cultures as well as osteochondral explants, with up to 90% cell death in culture experiments and 42% cell death in samples with intact cartilage. Our group demonstrated similar results using an in vivo rabbit model[7]; here, the glenohumeral joints were surgically catheterized, and the cartilage was exposed to a continuous bupivacaine infusion, resulting in 20% to 30% cell death and a 50% reduction in cell metabolic activity after 1 week.

Our current study did not demonstrate any permanent impairment of cartilage function at 3 months in a rabbit

TABLE 1
Results of Histological Analysis[a]

	Group S		Group B		Group B+E	
	Control	Infused	Control	Infused	Control	Infused
Articular surface, mean (SD)	1.0 (0.0)	1.4 (1.3)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)
Safranin O, mean (SD)	2.4 (0.8)	2.4 (1.1)	1.9 (0.4)	2.4 (0.9)	2.2 (0.8)	1.9 (0.6)
Clone formation, mean (SD)	1.2 (0.4)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	1.0 (0.2)	1.0 (0.0)
Cellularity, mean (SD)	2.0 (0.8)	1.9 (1.2)	1.9 (0.3)	2.0 (0.5)	1.9 (0.7)	1.8 (0.6)
Total, mean (SD)	6.5 (1.9)	6.8 (3.4)	5.8 (0.7)	6.4 (1.3)	6.1 (1.4)	5.7 (1.1)

[a]S, saline; B, bupivacaine; B+E, bupivacaine and epinephrine; SD, standard deviation.

model. Conversely, we found an increase in chondrocyte anabolic activity, as evidenced by an elevated sulfate uptake and higher PG content in cartilage exposed to bupivacaine when compared with contralateral controls, or with shoulders treated with saline infusion. These apparently conflicting results suggest that for the experimental model used, cartilage function was only transiently impaired by exposure to bupivacaine. Cartilage metabolism after bupivacaine exposure was significantly decreased at 1 week as presented in our initial study; however, by 3 months it not only had recovered but was higher than in shoulders that were either treated with saline or not infused at all. A possible explanation of our results is that the increased PG synthesis seen at 3 months might indicate a reparative response to the earlier noxious stimulus, analogous to the reactions observed with injection of chymopapain into the rabbit knee.[14] This study demonstrated that lower levels of chymopapain transiently decreased PG content by 50% during the first week, which later recovered without permanent joint damage. Only the highest concentration of chymopapain resulted in progressive degenerative changes. This suggests a dose-dependent mechanism that should be evaluated in future experiments.

Although 20% to 30% of chondrocytes appeared nonviable 1 week after infusion with bupivacaine, the current study found no significant differences in cell viability at 3 months when comparing saline infusion with bupivacaine infusion. There was also no difference in the number of cells present across groups. Therefore, corroborating this finding with a lack of empty lacunae or cloning on microscopy leads one to believe that the chondrocytes had repopulated the empty lacunae. There has been considerable controversy about the ability of human adult chondrocytes to divide and produce matrix after noxious stimulation. Archer et al[1] demonstrated that chondrocytes have the ability to repopulate when placed adjacent to devitalized cartilage. Lu et al[10] have demonstrated robust outgrowth of chondrocytes and production of a hyaline-like matrix after mechanical trauma through mincing of cartilage. This suggests that even adult human chondrocytes have the ability to divide and exert anabolic effects even after considerable trauma.[10] Therefore, some of the cells could have undergone a sublethal dosing of bupivacaine from which they were able to survive, increase their metabolic rate, and repopulate the tissue.

Another important point to take into consideration is the limitation of using a rabbit model. In articular cartilage samples taken from the medial femoral condyle, chondrocyte density measures 12.2% in the rabbit and only 1.7% in humans. Each human chondrocyte therefore has to maintain an approximately 8 to 10 times larger area of surrounding matrix.[4] It is possible that the higher chondrocyte density allows the rabbit to more readily recover from articular damage than humans, possibly through cell division. Furthermore, it is possible that rabbit cartilage is simply more resilient to noxious stimuli than human cartilage given a higher chondrocyte density of rabbit cartilage. This model is also treating pristine cartilage, where some authors have suggested that previous damage to the cartilage surface can increase its susceptibility to toxic effects.[3] The equivalency dosing of bupivacaine in the rabbit is not well established and was based on weight. Because in vitro studies using cell and tissue culture can neither provide long-term term follow-up nor demonstrate macroscopic degenerative changes, further animal studies should be conducted, using larger species with potentially less regenerative capabilities than the rabbit. Last, there is a question as to the potential influence of the different pH levels of the infusate solutions. In our initial study using the same experimental setup,[7] albeit with a shorter follow-up of only 1 week, we found much larger differences in chondrocyte function between groups treated with infusions of similar pH, such as saline (pH 5.9) versus bupivacaine (pH 5.7), than between groups treated with infusions that had similar biochemical structures but different pH, such as bupivacaine (pH 5.7) versus bupivacaine with epinephrine (pH 4.0). This led us to believe that the effects seen were a function of "biochemical trauma," rather than a confounding effect of the different pH levels.

CONCLUSION

In conclusion, 3 months after intra-articular infusion of bupivacaine in a rabbit shoulder model, no permanent impairment of cartilage function could be detected. Cartilage metabolism was increased, possibly beyond that of normal chondrocyte physiology, as a reparative response to the damage reported at 1 week after infusion. This suggests that, at least in the model used, articular cartilage has the ability to recover from the

chondrotoxic effects of bupivacaine infusion. If these findings can be extrapolated to human cartilage, surpassing a yet to be determined threshold concentration, or other factors besides bupivacaine exposure, would likely be required before permanent cartilage damage such as chondrolysis ensues. Additional experiments will be necessary before the phenomenon of bupivacaine toxicity is fully understood.

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