EXPERIMENTAL STUDY

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Cytotoxicity of local anesthetics to rats' articular cartilage: an experimental study

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Objective: The aim of this study was to evaluate the effects of both in vivo and in vitro bupivacaine, levobupivacaine and tramadol on articular cartilage and chondrocytes in experimental rat models.

Methods: Thirty mature Sprague Dawley rats weighing 230-300 g were randomized into 3 groups. Bupivacaine (Group 1), levobupivacaine (Group 2) and tramadol (Group 3) were injected into the right knee joints and a physiological 0.9% saline into the left. From each group, 5 rats were executed 48 hours following drug administration after 5 and 10 days. The specimens were fixed, decalcified and stained with hematoxylin & eosin and toluidine blue. All samples were histopathologically evaluated according to the recommendation of ICRS' osteoarthritis and cartilage histopathology grading and staging system. Articular cartilage cells of the rats were cultured and seeded into cell culture flasks. Cartilage cell seeded samples (104 cells/ml) were incubated in three different anesthetic agents (0.5%); bupivacaine, levobupivacaine, and tramadol, respectively. CellTiter 96® Non-Radioactive Cell Proliferation (MTS) assay was used to determine the cell density on the samples.

Results: Statistically significant higher OARSI grades and OA stage and scores were detected when comparing the group injected with levobupivacaine and executed after 10 days with the levobupivacaine injected group killed after 48 hours ($p<0.01$ [$p=0.008$]). Although, statistical analysis could not be done due to insufficient number of samples in the in vitro part of the experiment, it can be concluded that tramadol is cytotoxic to rat chondrocyte in vitro after 30 min of exposure. Additionally, cell numbers in both the bupivacaine- and levobupivacaine-treated wells showed decrease throughout 15, 30 and 60 minute exposures.

Conclusion: Although chondrotoxicity of bupivacaine was less harmful than levobupivacaine and tramadol, these findings suggest that local anesthetics may negatively affect articular cartilage and chondrocytes.

Key words: Articular cartilage; bupivacaine; chondrocyte; levobupivacaine; tramadol.

Intra-articular use of anesthetic agents is common for postoperative pain relief following arthroscopic knee surgery. Different combinations for intra-articular analgesic injections with different efficiency have been reported.[1-7] Tramadol is an analgesic drug with a local anesthetic effect that is not opioid receptor related. It has been shown that 50 mg of intra-articular tramadol provides analgesia equivalent to 5 mg of intra-articular mor-

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phine.[8] Bupivacaine is a local anesthetic that has been used alone or in combination with other drugs.[9] Levobupivacaine has recently been introduced to daily clinical practice, rapidly replacing other conventional local anesthetics.[10]

The off-label use of intra-articular injection of local anesthetics to provide a comfortable postoperative period following outpatient surgery have been reported. However, the side effects of these drugs on articular cartilage have been mostly neglected by clinicians, despite reports on their negative effects.

In this study, we aimed to evaluate and compare the effects of bupivacaine, levobupivacaine and tramadol on articular cartilage and chondrocytes in both in vivo and in vitro experimental rat models.

Materials and methods

Approval for this study was obtained from the Istanbul University Animal Research Committee, Turkey, in accordance with established animal care protocols and institutional guidelines. The study was performed in two parts, both in vivo and in vitro.

Thirty mature Sprague Dawley rats weighing 230 to 300 g were randomized into 3 groups and anesthetized using 6-8 mg/kg intramuscular ketamine. Bupivacaine (Group 1) (0.25% ml), (Marcaine Spinal Heavy 0.5%; AstraZeneca, Istanbul, Turkey); levobupivacaine (Group 2) (0.25% ml), (Chirocaine® 0.5%; Nycomed Pharma AS, Elverum, Norway) and tramadol (Group 3) (0.25% ml), (Contramal 100 mg; Grünenthal GmbH, Aachen, Germany) were injected into the right knee joints and 0.25 ml of physiological 0.9% saline into the left, under aseptic conditions. Animals were then allowed to awaken and were returned to their cages without any restrictions on ambulation. Five rats from each group were then killed by a lethal injection of phenobarbital, 48 hours and 10 days following drug administration. The knee joints were resected.

Specimens were fixed in a neutral saline-buffered formalin fixative (10% formalin fixative; Bio-Optica Milano SpA, Milan, Italy). After fixation, decalcification was performed for 6 days in Shandon TBD-2 tissue decalcification solution (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature. Following decalcification, tissues were rinsed in tap water and routine tissue processing was performed in a Shandon Excelsior tissue processor (Thermo Fisher Scientific, Waltham, MA, USA). Sections of 0.4 µm thickness were prepared from the paraffin embedded tissues. The slides were stained with hematoxylin and eosin (H&E) and toluidine blue (Toluidine blue, ready-to-use kit; Bio-Optica Milano SpA, Milan, Italy).

All slides were examined by the same pathologist, who was blinded to the injectate used in each joint. Samples were evaluated histopathologically for the presence of inflammation and osteoarthritis grade (OARSI grades 0-6), osteoarthritis stage (OA stages 0-4) and osteoarthritis scoring (OA scoring: [OARSI grade] x [OA stage]) according to the recommendation of the International Cartilage Repair Society's osteoarthritis and cartilage histopathology grading and staging system.[11] The severity of articular cartilage changes was divided into six grades and four major stages. The four major stages according to this system were: (Stage 0) No osteoarthritis activity seen, (Stage 1) Less than 10% of articular surface affected, (Stage 2) 10-25% of articular surface affected, (Stage 3) 25-50% of articular surface affected, and (Stage 4) More than 50% articular surface affected.

In the in vitro experiment, articular cartilage cells were obtained from the knees of 6 to 8 week-old Sprague Dawley rats and cultured on T-25 flasks in RPMI-1640 with 10% FCS, 100 units/ml penicillin and streptomycin. The cells were incubated at 37°C in a CO_2 incubator.

When the monolayers reached confluency, the cells were enzymatically lifted from the flask using a 625 µg/ml solution of trypsin-EDTA solution. The cells were concentrated by centrifugation at 2000 rpm for 5 minutes and re-suspended in 3 ml of RPMI-1640 (10% FCS, 100 units/ml penicillin and streptomycin). Aliquots of 25 µl of cell suspension containing 104 cells were seeded onto each well of a 24-well plate. The samples were left undisturbed in an incubator for 2 hours to allow cells to attach to the surface of the samples. Then, 1 ml of growth medium was added to each well and incubated for 1 day at 37 °C in a CO_2 incubator.

For three time points (15, 30 and 60 minutes), the cells were incubated in three different anesthetic agents (0.5%); bupivacaine, levobupivacaine, and tramadol, respectively. The cells were then washed one time with PBS and returned to chondrocyte growth media.

After anesthetic agent treatment, cartilage cells (104 cells/well) were incubated for 24 hours in the CO_2 incubator at 37 °C. CellTiter 96® Non-Radioactive Cell Proliferation (MTS) assay was used to determine cell density. A MTS/PMS reagent (100 µL) was added to each well of the 24-well plate and incubated for 135 minutes at 37 °C in a CO_2 incubator. Absorbance was determined at 490 nm using an Elisa Plate Reader

(ELx800 Absorbance Microplate Reader; BioTek US, Winooski, VT, USA). Even though experiments were performed three times, statistical analysis could not be performed due to the insufficient number of samples.

The histopathologic results were analyzed by SPSS for Windows v10.0 (SPSS Inc., Chicago, IL, USA), using Mann-Whitney U and Wilcoxon rank tests with significance level set at $p<0.05$.

Results

Histopathologic findings according to OARSI grading and scoring system are summarized in Tables 1, 2 and 3. In the group injected with tramadol, cartilage hypertrophy and active chronic inflammation with abscess formation were detected in one of the joints after 48 hours (Fig. 1). No additional pathologic finding was observed and no statistically significant difference was found between the tramadol-injected group and the control group after 48 hours ($p>0.05$). Ten days after

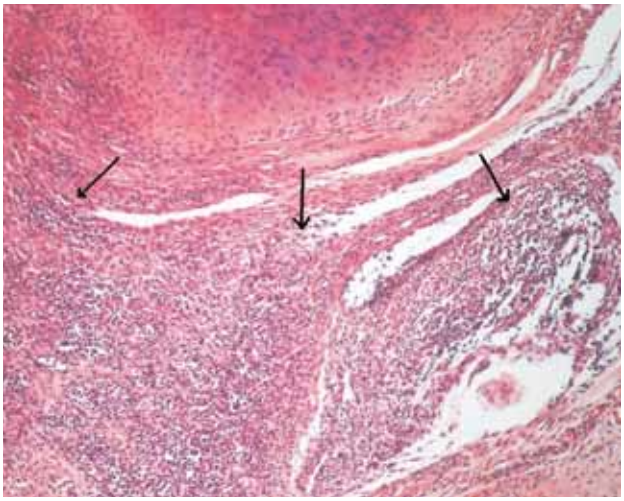

Fig. 1. Right knee of tramadol-injected rat number 2. Cartilage hypertrophy and active chronic inflammation with abscess formation after 48 hours in the injection site was detected. Chronic inflammation area could be seen under arrows (H-E x100). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

Table 1. Histopathologic findings of the specimens of tramadol-injected group.

Time and group		Specimen number	OARS1 grade	Grade advanced note	OA stage*	OA score	Mean OARS1 grade/SD	Mean OA stage*/SD	Mean OA score/SD
48 hours after injection	Tramadol-injected group	1	0	Normal	0	0	0.20/0.45	0.80/1.79	0.80/1.79
		2	1	Cartilage hypertrophy and active chronic inflammation with abscess formation	4	4			
		3	0	Normal	0	0			
		4	0	Normal	0	0			
		5	0	Normal	0	0			
	Tramadol control group	1	0	Normal	0	0	0/0	0/0	0/0
		2	0	Normal	0	0			
		3	0	Normal	0	0			
		4	0	Normal	0	0			
		5	0	Normal	0	0			
10 days after injection	Tramadol-injected group	1	1	Surface is intact, cellular proliferation is found	1	1	1.20/ 1.30	0.60/0.55	1.20/1.30
		2	3	Focal vertical fissures and cartilage hypertrophy	1	3			
		3	2	Focal discontinuity on cartilage matrix at superficial zone	1	2			
		4	0	Normal	0	0			
		5	0	Normal	0	0			
	Tramadol control group	1	0	Normal	0	0	0.60/ 1.34	0.20/0.45	0.60/1.34
		2	0	Normal	0	0			
		3	3	Focal vertical fissures	1	3			
		4	0	Normal	0	0			
		5	0	Normal	0	0			

*0: No osteoarthritic activity seen; 1: Less than 10% of articular surface is affected; 2: 10-25% of articular surface is affected; 3: 25-50% of articular surface is affected 4: More than 50% articular surface is affected; SD: standard deviation

tramadol injection, one specimen showed cellular proliferation, one focal vertical fissures and cartilage hypertrophy and one focal discontinuity on the cartilage matrix at the superficial zone. One of the cases in the control group showed focal vertical fissures on 10% of the articular surface. These histopathologic findings were not found statistically significant when compared to the OARSI grade, OA stage and OA score of the tramadol-injected group with the control group 10 days after injection (p>0.05). No statistically significant difference was found in the OARSI grade, OA stage and OA score of tramadol-injected group after 48 hours when compared to the tramadol-injected group after 10 days as well (p>0.05). No pathologic finding was observed both in the bupivacaine- and levobupivacaine-injected and control groups after 48 hours (Fig. 2). Superficial abrasion was detected in 3 of the specimens 10 days following injection of bupivacaine (Fig. 3), but these findings were not statistically significant (p>0.05). There was no statistically significant difference in aspects of OARSI grade, OA stage and OA score between control groups and groups injected with bupivacaine and executed at 48 hours and 10 days, respectively (p>0.05, p>0.05). There was no statistically significant difference in OARSI grade, OA stage and OA score between the control group and the levobupivacaine-injected group after 48 hours (p>0.05). Superficial abrasion and/or superficial fissures were detected in 4 of the specimens taken 10 days after injection of levobupivacaine (Fig. 4). There was no statistically significant difference in OARSI grade, OA stage and OA score between the control group and the levobupivacaine-injected group after 10 days (p>0.05). However, statistically significant higher OARSI grades, OA stages and OA scores were detected when comparing the levobupivacaine-injected group after 10 days and 48 hours (p<0.01 [p=0.008]). No specimens were determined as Grade 5 or 6 which involve the subchondral bone.

Cell growth was measured by MTS assay for rat chondrocytes cultured on TCP after treatment with different anesthetic agents: bupivacaine, levobupivacaine, and tramadol after a 15, 30, 60 min incubation period, respectively. MTS results show that 0.5% tramadol is cytotoxic to rat chondrocyte in vitro after 30

Table 2. Histopathologic findings of the specimens of bupivacaine-injected group.

Time and group		Specimen number	OARS1 grade	Grade advanced note	OA stage*	OA score	Mean OARS1 grade/SD	Mean OA stage*/SD	Mean OA score/SD
48 hours after injection	Bupivacaine-injected group	1	0	Normal	0	0	0/0	0/0	0/0
		2	0	Normal	0	0			
		3	0	Normal	0	0			
		4	0	Normal	0	0			
		5	0	Normal	0	0			
	Bupivacaine control group	1	0	Normal	0	0	0/0	0/0	0/0
		2	0	Normal	0	0			
		3	0	Normal	0	0			
		4	0	Normal	0	0			
		5	0	Normal	0	0			
10 days after injection	Bupivacaine-injected group	1	0	Normal			0.60/0.55	1.20/1.30	1.20/1.30
		2	1	Focal superficial fibrillation (abrasion)	1	1			
		3	0	Normal	0	0			
		4	1	Superficial fibrillation (abrasion)	3	3			
		5	1	Superficial fibrillation (abrasion)	2	2			
	Bupivacaine control group	1	0	Normal	0	0	0.20/0.45	0.20/0.45	0.20/0.45
		2	0	Normal	0	0			
		3	1	Focal superficial abrasion	1	1			
		4	0	Normal	0	0			
		5	0	Normal	0	0			

*0: No osteoarthritic activity seen; 1: Less than 10% of articular surface is affected; 2: 10-25% of articular surface is affected; 3: 25-50% of articular surface is affected 4: More than 50% articular surface is affected; SD: standard deviation

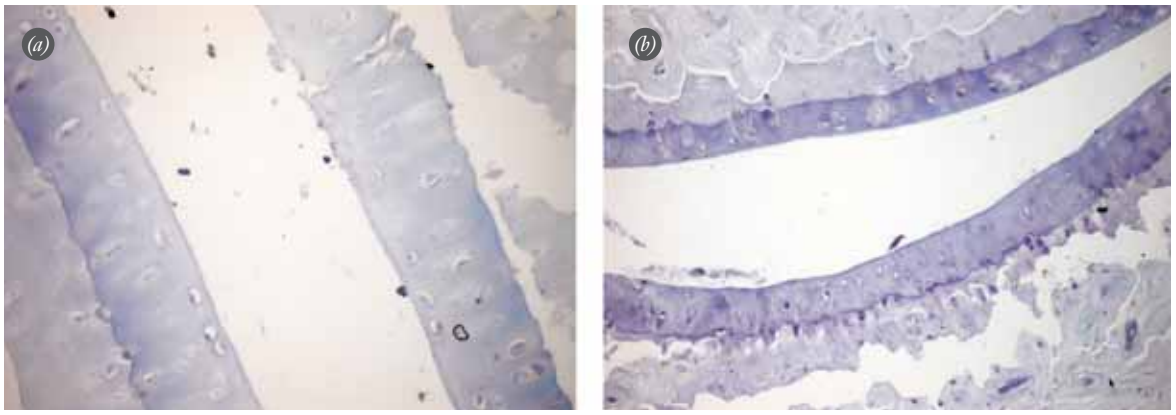

Fig. 2. **(a)** Left knee of bupivacaine-injected rat number 1 (toluidine blue x200). **(b)** Right knee of bupivacaine-injected rat number 1. A view of the injection site after 48 hours showing no pathologic finding when compared with the control side (left knee) of the same rat (toluidine blue x100). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

min of exposure (Fig. 5). Additionally, the cell number in both the bupivacaine- and levobupivacaine-treated wells showed a decrease throughout 15, 30 and 60 min exposures.

Discussion

There are few studies about the musculoskeletal side effects of bupivacaine, especially on joint cartilage. To the authors' knowledge, no one has yet examined and

Table 3. Histopathologic findings of the specimens of levobupivacaine-injected group.

Time and group		Specimen number	OARSI grade	Grade advanced note	OA stage*	OA score	Mean OARS1 grade/SD	Mean OA stage*/SD	Mean OA score/SD
48 hours after injection	Levobupivacaine-injected group	1	0	Normal	0	0	0/0	0/0	0/0
		2	0	Normal	0	0			
		3	0	Normal	0	0			
		4	0	Normal	0	0			
		5	0	Normal	0	0			
	Levobupivacaine control group	1	0	Normal	0	0	0/0	0/0	0/0
		2	0	Normal	0	0			
		3	0	Normal	0	0			
		4	0	Normal	0	0			
		5	0	Normal	0	0			
10 days after injection	Levobupivacaine-injected group	1	3	Vertical fissures	1	3	1.40/0.89	1.20/0.45	1.60/0.89
		2	1	Focal superficial fibrillation (abrasion)	1	1			
		3	1	Focal superficial fibrillation (abrasion)	1	1			
		4	1	Superficial fibrillation (abrasion)	1	1			
		5	1	Superficial fissures and superficial fibrillation (abrasion)	2	2			
	Levobupivacaine control group	1	0	Normal	0	0	1.20/1.64	0.40/0.55	0.55/1.64
		2	0	Normal	0	0			
		3	1	Focal superficial abrasion	1	1			
		4	3	Vertical fissures	1	3			
		5	3	Vertical fissures	1	3			

*0: No osteoarthritic activity seen; 1: Less than 10% of articular surface is affected; 2: 10-25% of articular surface is affected; 3: 25-50% of articular surface is affected 4: More than 50% articular surface is affected; SD: standard deviation

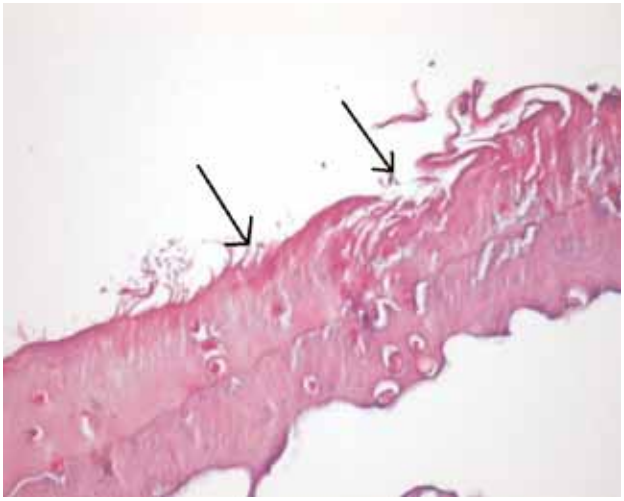

Fig. 3. Right knee of bupivacaine-injected rat number 5. Superficial abrasion on the articular surface 10 days after bupivacaine injection was detected. This finding was graded as OARSI Grade 1. Arrows point out to superficial abrasion (H-E x100). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

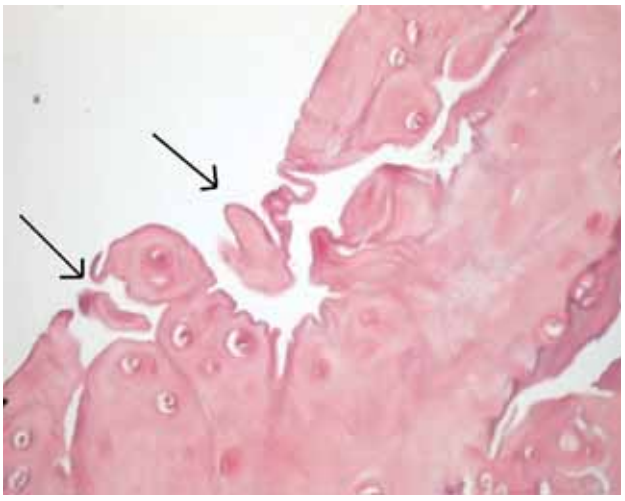

Fig. 4. Right knee of levobupivacaine-injected rat number 5. Superficial fissures on the articular surface 10 days after levobupivacaine injection were detected. This finding was graded as OARSI Grade 1. Arrows point out to superficial fissures (H-E x200). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

reported the effects of levobupivacaine and tramadol on articular cartilage. Additionally, no report has been published comparing the effects of the mentioned three drugs, both in vivo and in vitro.

Dogan et al.[12] conducted an in vivo rabbit study with intra-articular bupivacaine and neostigmine. They concluded that both agents caused histopathologic changes, such as inflammatory cell infiltration, hypertrophy, and hyperplasia of the synovial membrane, with 0.5% bupivacaine causing greater articular cartilage inflammation at Day 10. Although Gomoll et al. demonstrated significant histopathologic and functional changes in articular cartilage after infusion of bupivacaine with or without epinephrine in rabbit shoulders,[13] the same author reported no permanent impairment of cartilage function at 3 months after intra-articular infusion of bupivacaine.[14] In our study, major cartilage destruction was histopathologically observed with tramadol use. However, the only statistically significant higher OARSI grade, OA stage and OA scores were detected with levobupivacaine when comparing the 10 day and 48 hour groups ($p<0.01$ [$p=0.008$]).

Chu et al. showed that 0.5% bupivacaine has a cytotoxic effect on bovine articular chondrocytes in vitro.[15] Although there was no statistical analysis, our in vitro study also supported these findings. The number of both bupivacaine- and levobupivacaine-treated cells showed decreases throughout 15, 30, and 60 minutes exposures. Between the two, bupivacaine-treated cells showed less decrease in cell growth than those treated with levobupivacaine. Tramadol, on the other hand, destroyed all of the cells after 30 minutes of exposure. This study, including both in vivo and in vitro components, confirms prior in vivo or in vitro investigations on the chondrotoxic effects of bupivacaine.

A limitation of our study is that because it was performed on animal models, our results may be different from those observed in humans as it has yet to be determined if human cartilage is equally susceptible. On the other hand, Hansen at al. reported chondrolysis in shoulders after the use of intra-articular pain pump catheters eluting bupivacaine with epinephrine

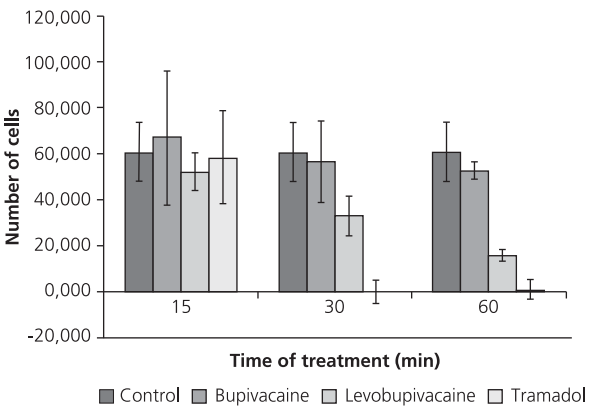

Fig. 5. Cartilage cell growth by MTS assay upon exposure of three different anesthetic agents: bupivacaine, levobupivacaine, and tramadol.

following arthroscopy.[16] Their clinical findings also support our results. However, our study showed that among the other two anesthetic agents (tramadol and levobupivacaine), bupivacaine gave better results with respect to cell growth, in vitro and histopathological effects, in vivo.

Recently, Baker et al. demonstrated a reduced toxic effect on the articular chondrocyte after the addition of magnesium to a local anesthetic.[17] This report supports additional care for less invasive local anesthetic solutions following arthroscopy.

In a previous randomized, study we compared the effect of intra-articular and pericapsular injections with different local anesthetic combinations.[1] Although the absorption of the local anesthetics into the joint has not been clearly determined, pericapsular incisional injections might be taken into consideration to diminish the harmful effects of the drugs on articular cartilage.

Another limitation of our study is the lack of a demonstration and identification of the absorption of anesthetics into joint tissues (i.e. articular cartilage). Future experiments on the absorption of anesthetics after pericapsular or intra-articular injections into articular tissues (e.g. via recovery/visualization of labeled compound in situ, etc.) would be a significant and valuable contribution to the literature.

In conclusion, three local anesthetic agents commonly used after arthroscopy for pain control have devastating effects on articular cartilage, especially in in vitro experimental models; tramadol being the worst of the three. Although the chondrotoxicity of bupivacaine was less harmful than that of levobupivacaine and tramadol, these findings suggest that all three local anesthetics negatively affect articular cartilage and chondrocytes. Given that chondrocyte loss has been implicated in the development of chondrosis and osteoarthritis,[18] orthopaedic surgeons should be careful in their choice of intra-articular drug injections pain control after arthroscopic procedures.

Conflicts of Interest: No conflicts declared.

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