Effect of Bipolar Radiofrequency Energy on Human Articular Cartilage: Comparison of Confocal Laser Microscopy and Light Microscopy

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Purpose: To evaluate chondrocyte viability using confocal laser microscopy (CLM) following exposure to bipolar radiofrequency energy (bRFE) and to contrast CLM with standard light microscopy (LM) techniques. **Type of Study:** In vitro analysis using chondromalacic human cartilage. **Methods:** Twelve fresh chondral specimens were treated with the ArthroCare 2000 bRFE system (ArthroCare, Sunnyvale, CA) coupled with 1 of 2 types of probes and at 3 energy delivery settings (S2, S4, S6). A sham-operated group was treated with no energy delivered. Specimens were analyzed for chondrocyte viability and chondral morphology with CLM using fluorescent vital cell staining and with LM using H&E and safranin-O staining. **Results:** LM with H&E staining showed smoothing of fine fronds of fibrillated cartilage; thickened fronds were minimally modified. Chondrocyte nuclei were present and not morphologically different than nuclei within sham-operated and adjacent untreated regions. LM with safranin-O staining showed a clear demarcation between treated and untreated regions. CLM, however, showed chondrocyte death: the depth and width of chondrocyte death increased with increasing bRFE settings. **Conclusions:** CLM showed that bRFE delivered through the probes investigated created significant chondrocyte death. These changes were not apparent using LM techniques. **Key Words:** Articular cartilage—Chondromalacia—Cartilage defect—Radiofrequency—Knee joint.

Radiofrequency energy (RFE) has gained popularity for the treatment of partial-thickness cartilage defects and chondromalacic cartilage in clinical orthopaedic surgery. Several studies have attempted to characterize the effects of RFE in in vitro and in vivo settings.[1-4] Using in vitro and in vivo studies, Kaplan et al.[2] and Turner et al.[3] reported that RFE delivered through bipolar devices appeared to be safe for use on chondromalacic articular cartilage with better histologic outcomes than traditional methods such as mechanical shaving. However, Lu et al.[1,4] reported that both monopolar (mRFE) and bipolar (bRFE) RFE caused immediate chondrocyte death when RFE was used to treat partial-thickness defects and chondromalacic cartilage. We hypothesize that the reason for these contradictory results is the different analytic methods used.

Confocal laser microscopy (CLM) has gained widespread use over the past 10 years, especially in the field of fluorescent microscopy. CLM uses a laser source to stimulate the fluorescent-labeled sample and projects the resulting image onto a monitor through computer-controlled software. CLM with vital cell

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staining is recognized as an accurate and sensitive method of determining cell viability.[1,5-13] This study describes the use of CLM in conjunction with vital cell staining (Live/Dead Viability/Cytotoxicity Kit [L-3224], Molecular Probes, Eugene, OR). This technique uses calcein-AM (acetoxymethylester) to label live cells and ethidium homodimer-1 (EthD-1) for dead cells. Calcein-AM is an uncharged, nonfluorescent substrate that freely diffuses into live cells and is enzymatically converted to the intensely fluorescent calcein by an esterase. The polyanionic calcein is charged and only retained in live cells, producing green fluorescence on excitation (ex/em ≈495 nm/ ≈515 nm). EthD-1 has low cell-membrane permeability and is excluded by the intact membrane of live cells. However, EthD-1 readily enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding nucleic acids, producing a strong red fluorescence in nonviable cells.[5,13,14] Light microscopy (LM) techniques have and continue to be used to evaluate tissue architecture and tissue type. The viability of cells using LM is inferred from the appearance of the cell cytoplasm, organelles, and the nucleus. Fixation, dehydration, embedding, and staining techniques each affect the cellular appearance within the tissue. The staining combination of hematoxylin and eosin (H&E) provides a method for polychromatic staining of the nucleus (hematoxylin) and cytoplasm (eosin).[15,16] Safranin-O is an orange metachromatic dye that readily shows cartilage architecture and relative proteoglycan content.[17-19] With proteoglycan loss, the staining intensity is reduced.

In the studies by Kaplan et al.[2] and Turner et al.,[3] LM techniques were used to determine chondrocyte viability. In the studies by Lu et al.,[1,4] CLM and fluorescent vital cell staining were used to differentiate live and dead chondrocytes.

The purpose of this study was to evaluate chondrocyte viability in vitro with CLM following thermal chondroplasty using bRFE. We have reproduced the study by Kaplan et al.,[2] but used CLM (in addition to LM) to determine the extent of chondrocyte viability. We hypothesized that CLM would show significant chondrocyte death that was not apparent using standard LM techniques.

METHODS

All procedures were approved by the Institute Review Board and Human Subjects Committee at participating universities. Twelve fresh femoral condyles or patellae from 12 patients undergoing total or partial knee arthroplasty were used for this study. Chondromalacia was graded using a modified Outerbridge system[20,21]: grade 1, softened cartilage surface; grade 2, softened cartilage with fine fibrillations; grade 3, fibrillated surface with pitting to subchondral bone; and grade 4, fibrillation of cartilage and exposed subchondral bone. Each specimen was placed on a custom-designed jig with an inflow pump maintaining a constant flow (120 mL/min) of saline at 37°C. Under arthroscopic visualization, an ArthroCare 2000 bRFE system coupled with a 3-mm, 90° ArthroWand or CoVac 50° angle probe (ArthroCare, Sunnyvale, CA) was used to deliver RFE over a 10-mm linear pass, noncontact mode (1 mm above cartilage surface) at 1 of 3 settings: S2 (133 to 147 kHz), S4 (160 to 179 kHz), or S6 (190 to 210 kHz). For each setting/probe combination, 6 independent samples from separate patients were used (total, 36 treatments). Another sham-operated group of 6 specimens served as a control. The total treatment time for each pass was 3 seconds.

After RFE treatment, each treated area was processed for analysis by LM, using H&E[15,16] and safranin-O[17-19] staining and CLM.[1,5,13,14] A diamond-wafering blade (Isomet 2000 Precision Saw; Buehler, Lake Bluff, IL) was used to cut osteochondral sections 2.5-mm thick for LM and 1.5-mm thick for CLM. Phosphate-buffered saline (PBS) was used for irrigation to avoid thermal injury.

The 2.5-mm sections were fixed in 10% neutral buffered formalin, decalcified, and embedded in paraffin. Sections 5.0-μm thick were cut and stained with H&E[15,16] to show general chondrocyte morphology, and with safranin-O[17-19] to stain proteoglycan within the extracellular matrix. All sections were coded to prevent identification of the probe type or setting used. The sections were examined and the chondrocyte appearance and safranin-O staining were assessed.

Vital cell staining was performed using EthD-1 and calcein-AM in conjunction with CLM. The 1.5-mm sections were stained by incubation in 1.0 mL of PBS containing 0.4 μL calcein-AM per 13 μL EthD-1 (Live/Dead Viability/Cytotoxicity Kit [L-3224]) for 30 minutes at room temperature. The 1.5-mm osteochondral section was placed on a glass slide and moistened with several drops of PBS. A confocal laser microscope (MRC-1000; Bio-Rad, Hemel Hempstead/Cambridge, England) equipped with an argon laser and necessary filter systems (fluorescein and rhodamine) was used with a triple labeling technique. In this technique, the signals emitted from the double-

stained specimens can be distinguished because of their different absorption and emission spectra.[5,13,14] These images are displayed on a monitor in an RGB (red, green and blue) mode. All cartilage samples were examined blindly.

The depth and width of chondrocyte death were determined for each RFE-treated region in the CLM image. Again, all images were coded to prevent identification of the probe and setting used. The confocal laser microscope was calibrated using a micrometer measured through the objective lens (2×) used for this project (20× total magnification; objective plus eyepiece magnification). The pixel length measured on images was converted to micrometers as previously described.[1] The depth and width of chondrocyte death were determined in each confocal image of the osteochondral section with Adobe PhotoShop (Version 5.5; Adobe Systems, San Jose, CA).

Mean depth and width of chondrocyte death for each probe/setting combination and subject age were compared among groups using analysis of variance (ANOVA, SAS version 7.1; SAS Institute, Cary, NC). Factors included in the analysis were patient number, age, grade, bRFE probe type, and bRFE probe setting. ANOVA was used to identify differences between the 2 probe types and probe setting for the depth and width of chondrocyte death. When differences among settings were shown by ANOVA, paired *t* tests were used to identify differences among settings within each probe type. Cartilage grades and patient gender were compared using Wilcoxon signed-rank tests; $P < .05$ was considered significant, P values between .05 and .08 indicated a trend.

RESULTS

There were no significant differences in the age, gender, or chondromalacia grade among treatment groups (mean age, 62 ± 7 years; 6 males and 6 females per group; mean grade, 2.3; $P > .05$). All specimens were either grade 2 (softened fibrillated cartilage) or grade 3 (softened, fibrillated cartilage with pitting to subchondral bone).

Macroscopically, the bRFE-treated cartilage changed color from white to light yellow and caramelized with some areas becoming concave. H&E staining showed a rough and fibrillated surface in the sham-operated cartilage surface (Fig 1A), whereas the bRFE-treated cartilage surface was smoother (Fig 1 B-D). Chondrocyte nuclei within the treated regions were present and not different in appearance from chondrocytes within sham-operated cartilage and adjacent untreated cartilage at all RFE settings and for both ArthroWand and CoVac 50° probes (Fig 1). Using higher magnification, the chondrocytes in the treated area appeared to have no alterations in nuclear and surrounding lacunar structure (Fig 2). Safranin-O staining was weaker (lighter orange in color) within the superficial layer of treated cartilage with a clear demarcation visualized between treated and untreated regions (Fig 3).

CLM showed that the ArthroWand and CoVac 50° probes created immediate chondrocyte death, the demarcation of thermal injury was clearly present and, in some specimens, thermal injury reached to subchondral bone (Figs 4 and 5). The depth and width of chondrocyte death after the application of bRFE are documented in Table 1. In the ArthroWand group, the depth of chondrocyte death at S6 was significantly

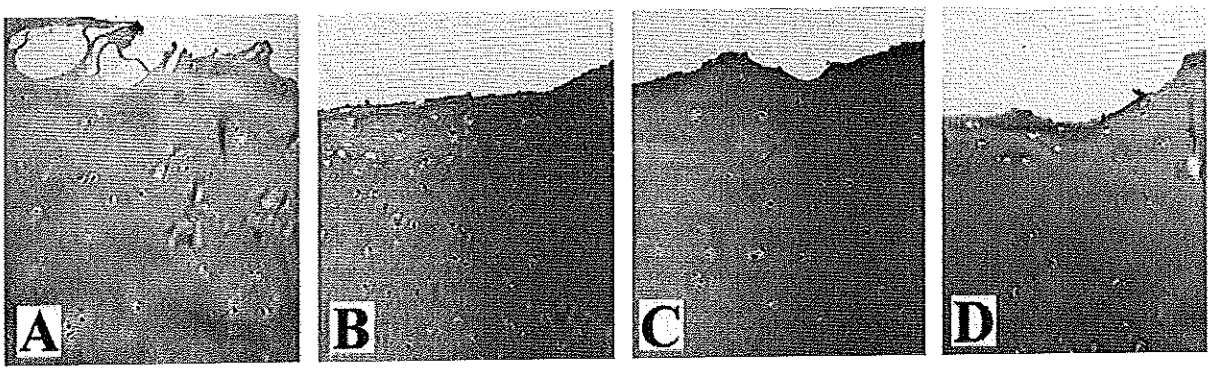

FIGURE 1. (A) Sham-operated control cartilage with fibrillated and rough surface. (B) Cartilage treated by ArthroWand probe at the setting 2. (C) Cartilage treated by ArthroWand probe at the setting 4. (D) Cartilage treated by ArthroWand probe at the setting 6. The chondrocytes and their nuclei are visible and are not different between control tissue and cartilage treated with bRFE at settings 2, 4, and 6 (H&E, original magnification ×100).

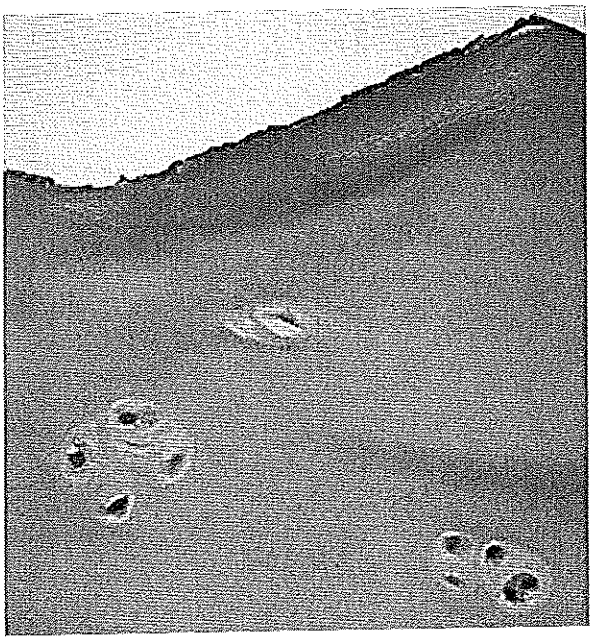

FIGURE 2. Cartilage treated by ArthroWand probe at setting 4. The nuclei of chondrocytes in the bRFE-treated region were present and appeared no different than adjacent untreated cartilage (H&E, original magnification ×400).

deeper than at S4 ($P < .05$), and the depth of chondrocyte death at S4 was significantly deeper than that at S2 ($P < .05$). The width of chondrocyte death increased as the setting was increased, but this increase was not significant ($P = .3$). In the CoVac 50° group, depth ($P = .3$) and width ($P = .2$) of chondrocyte death increased as the setting was increased, but this increase was not significant. Penetration to subchondral bone occurred in 2 of 18 ArthroWand samples and 3 of 18 CoVac 50° samples. There was a trend for the CoVac 50° probe to kill chondrocytes to a greater depth than the ArthroWand probe ($P = .08$).

DISCUSSION

The purpose of this project was to evaluate chondrocyte viability using CLM following exposure to bRFE and to compare CLM with standard LM techniques. While thermal chondroplasty performed with bRFE can contour the articular surface of chondromalacic cartilage, it resulted in immediate chondrocyte death and a reduction in proteoglycan staining. For the limited time (≈3 seconds) and single linear pass used in this study, the fine fronds of fibrillated cartilage were smoothed, but thickened fronds were minimally modified. CLM showed that the ArthroCare CoVac 50° probe tends to cause greater depth and width of chondrocyte death than the ArthroWand probe. With an increase in the setting (S2, S4, S6), the depth of thermal penetration in the ArthroWand group increased significantly. The results of this study contradict reports by Kaplan et al.[2] and Turner et al.[3] that concluded bRFE could modify the articular cartilage surface without deleterious effects on matrix architecture and chondrocyte viability.

FIGURE 3. Cartilage treated by ArthroWand probe at setting 4. The bRFE-treated cartilage is stained blue with a clear demarcation of the treated region (arrow heads). Adjacent untreated cartilage appeared red. Arrows indicate folding artifacts (safranin-O, original magnification ×20).

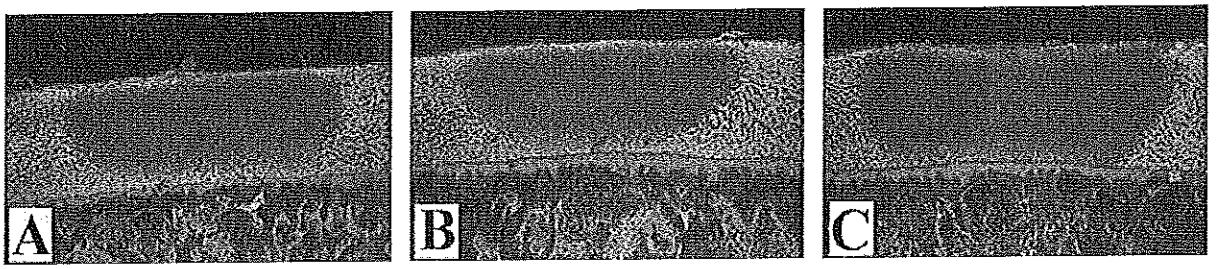

FIGURE 4. Confocal images indicated thermal damage with clear demarcation in the ArthroWand group (top, cartilage surface; bottom, subchondral bone; green dots, live chondrocytes; red region, dead chondrocytes). (A) Setting 2, (B) setting 4, (C) setting 6 (original magnification ×20).

We propose that LM cannot accurately assess chondrocyte viability in the acute phase following injury. Based on previous unpublished work from our laboratory, chondrocyte death occurs around 50°C, similar to findings with osteoblasts.[22] These findings are supported by work performed by others investigating heat shock proteins in chondrocyte culture.[23] Previous reports indicate that temperatures of 100°C to 160°C are generated by the bRFE probe (ArthroWand, settings 2, 4, and 6) used in this study.[2] In addition, temperatures greater than 70°C are reached at least 2,000 μm below the articular surface of articular cartilage during application of bRFE with the CoVac 50° probe and ArthroCare 2000 bRFE system (unpublished data). Immediately following thermal chondroplasty, even though chondrocyte necrosis with disruption of the plasma membrane occurs, the nuclei remain intact and do not appear morphologically abnormal with LM techniques. Based on the results of this study, LM is insensitive in identifying chondrocyte viability immediately following thermal injury. Future work could investigate microstructural changes using alternative techniques such as high-pressure cryopreservation for evaluation of cartilage architecture.[13,24-26]

CLM and LM were used in this study to analyze the bRFE-treated osteochondral sections and to compare these techniques for determining cell viability. In the histologic slides, normal chondrocyte morphology was clearly identifiable and nuclei were visible in the bRFE-treated regions; however, cartilage matrix staining was reduced with clear demarcation of reduced staining within the treated regions. CLM of these sections showed chondrocyte death in the bRFE-treated regions with red staining and a clear demarcation of thermal injury. Some investigators have questioned whether chondrocytes are actually dead after thermal treatment or whether their function has been temporarily impaired.[27] There are 2 bodies of work that support the fact that thermal treatment results in irreversible chondrocyte death (unpublished data).[1] In an in vivo ovine study investigating treatment of a partial-thickness articular cartilage defect with RFE, chondrocyte death was identifiable at time 0 and persisted until the termination of the project 6 months after surgery.[1] In no case did the investigators observe return of cell viability in areas where previous cell death had been established. In fact, a greater depth of chondrocyte death was identified in samples analyzed 14 days after treatment, compared with those evaluated immediately after RFE treatment. This indicates that chondrocyte death may result both from immediate thermal necrosis and, following treatment, from

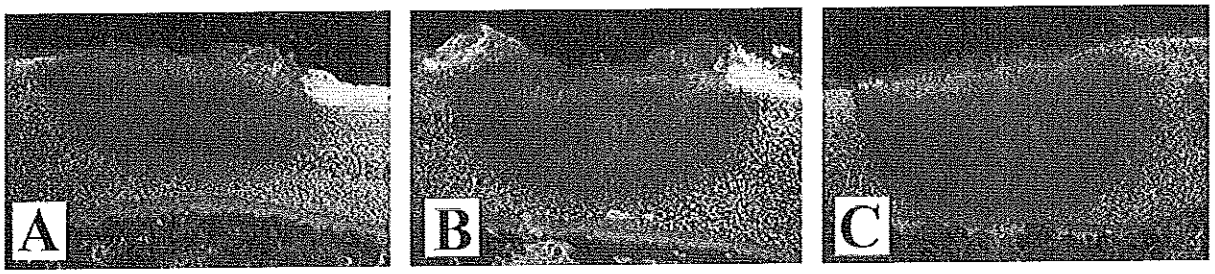

FIGURE 5. Confocal images indicated thermal damage with clear demarcation in the CoVac 50° group. (A) Setting 2, (B) setting 4, (C) setting 6 (original magnification ×20).

TABLE 1. *Depth and Width of Death Chondrocyte Following Application of bRFE*

Probe	RFE Generator Setting S2	S4	S6
Depth of chondrocyte death (μm)			
ArthroWand	1,512[a] ± 324	1,886[b] ± 312	2,269[c] ± 233
CoVac 50°†	1,929 ± 612	2,193 ± 439	2,445 ± 532
Width of chondrocyte death (μm)			
ArthroWand†	4,188 ± 846	4,944 ± 469	5,029 ± 571
CoVac 50°†	4,563 ± 800	5,077 ± 634	5,314 ± 476

NOTE. Values represent mean ± SD of the maximum depth of chondrocyte death measured from 6 independent samples for each probe/setting combination. Different letters within a row represent significant differences for an individual probe at different settings. Significance set at $P < .05$.

† No significant difference among settings.

continued necrosis or induction of apoptosis. Apoptosis is defined as cell deletion by fragmentation into cell membrane–bound particles, and has been documented by numerous investigators in chondrocytes.[28-36] Further support of irreversible chondrocyte death is derived from in vitro temperature measurements during the application of bRFE to osteochondral sections. Temperatures exceeding 100°C have been recorded 200 μm below the articular surface, and mean temperatures exceeding 70°C have been recorded 2,000 μm below the surface with the bipolar device used in the study reported here (unpublished data).

Our results contradict the findings of Kaplan et al.,[2] who stated that bRFE appeared to be safe for performing thermal chondroplasty at settings 2, 4, and 6 with the ArthroCare 2000 and the ArthroWand based on LM analysis. We believe LM to be an insensitive technique for evaluating cell viability. Using ArthroCare's currently recommended chondroplasty probe, the CoVac 50°, CLM showed that the depth of chondrocyte death was 1,900 to 2,500 μm, with a trend for the CoVac 50° to kill chondrocytes to a greater depth than ArthroWand. In some instances, thermal injury extended to the level of subchondral bone. The results of this study show that bRFE caused deeper chondrocyte death in articular cartilage than described for the holmium:YAG laser (500 to 1,000 μm),[37] and was consistent with the results of a previous in vitro study using bovine osteochondral sections.[4] We believe that these depths of chondrocyte death are significantly deeper than the depths of chondrocyte loss expected with mechanical debridement. With minimal mechanical shaving, the surgeon can expect to remove 200 to 300 μm of cartilage and have chondrocyte death extend 100 to 200 μm from the margins of this debridement. The cause of this extended chondrocyte death is not well described but has occurred following fracture, incision, and debridement of cartilage, and is most likely related to chondrocyte matrix interactions. The total chondrocyte loss following mechanical debridement, therefore, is approximately 500 μm. This death is far less than the depths found with the limited bRFE treatment used in this study and far below the 95% confidence intervals for all treatments presented in Table 1.

Another important result of this study relates to the treatment time investigated. We found that 3 seconds of bRFE treatment of chondromalacic cartilage allowed smoothing of fine fronds of fibrillated cartilage, but that thickened fronds were minimally modified. Fine fronds refer to the disruption of the articular cartilage surface that results in fronds less than 1 mm in diameter uniformly distributed over an area of the articular surface. The thicker fronds (>1 mm) are cartilage flaps that are not easily smoothed with a single pass of the bRFE probe. Clinicians might attempt to smooth thickened fronds by using longer treatment times or increased power settings. This more aggressive treatment could result in deeper thermal injury and potentially could create thermal injury to underlying subchondral bone.

We believe that in vivo clinical application of bRFE could result in full-thickness cartilage death as well as death to subchondral bone. Based on the results of this study, we conclude that (1) bRFE delivered through the probes investigated creates significant chondrocyte death in chondromalacic human articular cartilage in vitro, posing a great danger for creating full-thickness chondrocyte death and subchondral bone necrosis clinically, and (2) CLM is a sensitive technique for revealing this finding and LM is not.

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