Comparison of techniques for determination of chondrocyte viability after thermal injury

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Objective—To compare 2 methods of quantitating chondrocyte viability and to determine chondrocyte response to thermal injury over time.

Sample Population—108 stifle joints from 54 adult rats.

Procedures—Cartilage from the distal aspect of the femur was treated ex vivo with radiofrequency energy at a probe setting that would result in immediate partial-thickness chondrocyte death; untreated sections served as controls. Explants were cultured, and cell viability was compared by use of lactate dehydrogenase (LDH) histochemical staining and calcein AM and ethidium homodimer-1 confocal laser microscopy (CLM) cell viability staining. Terminal deoxynucleotidyl transferase–mediated X-dUTP nick end labeling (TUNEL) was used to detect apoptosis. All labeling studies were performed 0, 1, 3, 7, 14, and 21 days after treatment.

Results—In the treated tissues, a greater percentage of viable cells were found with CLM, compared with LDH staining. This result contrasted that of control tissues in which LDH staining indicated a greater percentage of live cells than CLM. The greatest number of TUNEL-positive chondrocytes was present at day 3, declining at later time intervals.

Conclusions and Clinical Relevance—CLM and LDH histochemistry techniques yield different absolute numbers of live and dead cells, resulting in differing percentages of live or dead cells with each technique. These differences may be related to the enzymes responsible for activation in each technique and the susceptibility of these enzymes to thermal injury. Results of TUNEL indicate that apoptosis contributes to chondrocyte death after thermal injury, with a peak signal identified 3 days after insult. (*Am J Vet Res* 2006;67:1280–1285)

Radiofrequency energy is commonly used for thermal modification of joint capsular and ligamentous instability and thermal chondroplasty in human sports medicine.[1–5] Increasing reports exist on its use in veterinary medicine as well.[6] In small animal surgery, RFE is used for meniscectomy, biceps tenotomies, and capsulorrhaphy procedures.[a] In equine surgery, it is used for synovectomy, chondroplasty, and other soft tissue debridement procedures during arthroscopy and tenoscopy.[b]

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Abbreviations

RFE	Radiofrequency energy
Calcein AM	Calcein acetoxymethyl ester
EthD-1	Ethidium homodimer-1
CLM	Confocal laser microscopy
LDH	Lactate dehydrogenase
TdT	Terminal deoxynucleotidyl transferase
TUNEL	TdT-mediated X-dUTP nick end labeling

Radiofrequency energy contours the cartilage surface through the application of heat in the form of electromagnetic energy by use of a generator. Application of RFE can smooth and reshape articular surfaces, anneal chondral fractures, remove delaminated regions, and create a smooth transition between treated and adjacent untreated regions. Results of previous studies[7–9] indicate that treatment of the cartilage with RFE causes discrete regions of chondrocyte death.

Use of the fluorochromes calcein AM to label live cells and EthD-1 to label dead cells is common.[10–13] Calcein AM is an uncharged nonfluorescent substrate that freely diffuses into live cells and is enzymatically converted to the intensely fluorescent calcein by a cytoplasmic esterase. The polyanionic calcein is charged and only retained in live cells, producing green fluorescence on excitation. Ethidium homodimer-1 is excluded by the intact plasma 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. Detection of the presence of these fluorochromes is best accomplished through the use of CLM. Activity of LDH has also been used as an indicator of cell viability.[14–16] Lactate dehydrogenase is an enzyme that catalyzes the reaction of pyruvate and nicotinamide-adenine dinucleotide to form lactate and nicotinamide-adenine dinucleotide. Viable chondrocytes will actively metabolize the substrate and can be identified by the presence of blue formazan granules in their cellular cytoplasm generated by the reaction, whereas devitalized cells are not able to catalyze the reaction and therefore lack the presence of these granules.[14–16]

Two types of cell death exist, necrosis and apoptosis. Necrosis is the death of cells through accidental or toxic insult that results in a passive catabolic process.[11] Apoptosis is an active process producing programmed cell death, a mechanism that regulates cell numbers in tissues and eliminates cells that threaten the survival of an animal.[17–23] Detection of cells undergoing apoptosis can be achieved through the use of modified nucleotides and enzymes to label DNA fragments. Enzymes will bind to 3'-OH termini of broken strands through the use of a

modified nucleotide such as X-dUTP. Terminal deoxynucleotidyl transferase is then incorporated to label the blunt ends of the DNA fragment. This end process is called TUNEL.[19,24–27] The TUNEL method for detection of apoptosis has been widely used.[18,21,25,26,28] The TUNEL method is often used in conjunction with electron microscopy to verify the structural changes of the TUNEL-positive cells, and TUNEL has been found to be an accurate assessment of cells undergoing apoptosis.[11]

Some controversy exists regarding the application of calcein AM and EthD-1 accompanied by CLM to determine chondrocyte viability in cartilage explants,[29] despite its use and presentation in peer-reviewed work.[10,12,13] The purpose of the study reported here was to compare vital cell staining of chondrocytes with 2 accepted methods for determining cell viability in tissues by use of an articular cartilage thermal injury model. In addition, we wished to determine the contribution of apoptosis to the loss of chondrocytes over time. We hypothesized that CLM and LDH techniques would provide similar results and that apoptosis would contribute to chondrocyte loss over the first 7 days of culture.

Materials and Methods

Animals—Fifty-four adult (mean age, 91 days; range, 82 to 103 days) male rats[c] were euthanatized with an overdose of pentobarbital.[d] From these rats, 108 stifle joints were aseptically harvested, and 3 of the available 4 femoral condyles (randomly picked with respect to left or right and medial or lateral condyles) from each rat were collected (osteochondral bone sections).

Treatment protocol—Each bone specimen was sectioned to retain a 5-mm-thick layer of subchondral bone. The subchondral bone was included in each bone specimen to preserve the natural cartilage-bone interface. A third of the bone specimens (tissues from 3 rats for each culture time point) were randomly selected to be in the treatment control group with no trauma. The remaining bone specimens (tissues from 6 rats for each culture time point) were treated with monopolar RFE at a temperature setting designed to uniformly damage the treated tissue.

Treatment with RFE was applied to each condyle immediately after removal from the rat. The treatment was performed by use of a grounding plate and prototype probe attached to a monopolar device[e] set at a temperature of 70°C and a power of 15 W to treat the tissue in a saline (0.9% NaCl) solution bath. On the basis of pilot data, this power setting–probe combination produced thermal injury to the cartilage to a depth of approximately 600 µm with no tissue ablation. After treatment with RFE, all explants were placed into 24-well culture plates and rinsed with serum-free culture media[f] supplemented with gentamicin[g] to remove any particles adhering to the tissue surfaces. Explants were then transferred to fresh 24-well flat bottom microplates and cultured with serum free culture media[f] at 37°C. The media was supplemented with gentamicin (0.05 mg/mL) to prevent contamination during culture. Media was changed with fresh solution every 24 hours. Tissue specimens were subjected to viability and apoptosis analysis at time 0 and at 1, 3, 7, 14, and 21 days after treatment.

Vital cell staining—Viability of the articular cartilage chondrocytes was assessed by use of 2 assay techniques, CLM and LDH histochemistry. For CLM, explants were removed from the culture media and sectioned with a diamond band saw[h] into uniform 1-mm-thick sections under irrigation with sterile saline solution to prevent frictional heating. Sections were then incubated in 1 mL of PBS solution containing 0.6 µL of calcein AM and 10 µL of EthD-1 at room temperature (approx 23°C) for 30 minutes and examined by use of a confocal microscope with a 10× objective lens.[7,11,i] The confocal laser microscope was calibrated by use of a micrometer measured through the objective lens (10×) that was used for this project (20× total magnification [ie, objective plus eyepiece magnification]). The pixel length measured on images was converted to micrometers as previously described,[7,30] and the area of cartilage was determined by use of a computerized imaging program.[j] With the iris settings used, this system provides a depth of focus of 5 µm.

The second method used for cell viability analysis was LDH histochemistry. Specimens were placed in Zamboni fixative for 48 hours after removal from culture media. Specimens were then rinsed in PBS solution and placed in 20% EDTA solution and 5% sucrose solution in 0.1M Tris to decalcify the tissue. After approximately 12 days, specimens were removed from the decalcifying solution and placed in 30% sucrose solution in 0.1M Tris for 24 hours. Complete decalcification was confirmed by use of microradiography. After 24 hours, specimens were sectioned at a thickness of 50 µm by use of a cryostat. Lactate dehydrogenase histochemistry was performed with free-floated osteochondral specimens by use of previously described methods.[16]

Apoptosis analysis—Specimens were again placed in Zamboni fixative for 48 hours and decalcified, as described for LDH histochemistry, and embedded in paraffin for sectioning at a thickness of 5 µm. Sections were then stained by use of a commercial staining kit.[k]

Cell counting and measurement—All sections for CLM were imaged by use of the same laser-microscope system and objective lens as described in the vital cell staining section. All sections for LDH histochemistry and TUNEL were imaged by use of a microscope[l] equipped with a video camera[m] with a 10× objective lens[n] for image capture. This eyepiece and objective lens system provide a depth of focus of 3.06 µm. Within each group, the total number of chondrocytes within a 1-mm-wide area with a full-thickness cartilage depth was counted for each specimen. A 1-mm-wide section was identified that was centered on the margin of the treated region in the treatment group and in a comparable region in the controls. The cartilage thickness was measured in 3 places along the 1-mm-wide area, and the mean of these 3 depths was determined for each specimen. Only cells considered in focus were counted for LDH histochemistry and TUNEL; all fluorescing cells were counted in CLM images.

Statistical analysis—Mean ± SEM values were calculated for live cell numbers, dead cell numbers, and apoptotic cell numbers. Differences between CLM and LDH assay techniques for percentage of live cells and dead cells over all culture time points were determined with a standard ANOVA by use of commercial software.[o] At each culture time point, an ANOVA was used to compare differences between CLM and LDH assay techniques for percentage of live cells and dead cells. A repeated-measures ANOVA was used to determine differences between the total number of TUNEL-positive and TUNEL-negative cells over all culture time points. An ANOVA was used to compare data among all 3 assay techniques for total cell count numbers and mean depth of cartilage for the measured area. Results were considered significant at a value of $P < 0.05$.

Results

RFE treatment—The RFE application produced visible contouring of the articular surface of the cartilage. The settings used did not produce tissue ablation, charring, or caramelization.

Table 1—Mean ± SEM percentage of live cells as indicated by CLM and LDH histochemistry.

	Control tissue		Treated tissue	
Days	**CLM**	**LDH histochemistry**	**CLM**	**LDH histochemistry**
0	72.8 ± 5.5[a]*	99.7 ± 0.3[a]	47.9 ± 11.1[a,b]	59.0 ± 11.2[a]
1	76.8 ± 5.8[a]*	99.4 ± 0.6[a]	45.9 ± 9.1[a,b]	29.4 ± 14.8[b]
3	83.5 ± 5.9[a]	93.0 ± 3.3[a]	70.0 ± 4.8[a]*	30.5 ± 11.1[b]
7	81.7 ± 8.6[a]	96.6 ± 1.3[a]	53.4 ± 5.2[a,b]*	19.1 ± 4.4[b,c]
14	73.2 ± 6.1[a]*	90.2 ± 1.9[a]	44.6 ± 4.9[b]*	7.2 ± 2.2[b,c]
21	32.1 ± 15.8[b]*	83.9 ± 10.7[a]	31.5 ± 9.6[b]*	0.15 ± 0.15[c]

*Significant ($P < 0.05$) difference between CLM and LDH histochemistry at the same culture time point for control or treated tissues.
[a–c]Different letters within a column indicate significant ($P < 0.05$) differences among time points.

Table 2—Mean ± SD percentage of live and dead cells in control versus treated tissues over all culture time points as indicated by CLM and LDH histochemistry.

	Control tissue		Treated tissue	
Assay technique	**Live cells**	**Dead cells**	**Live cells**	**Dead cells**
CLM	70.1 ± 5.2*	29.9 ± 5.2*	48.9 ± 3.6	51.1 ± 3.6
LDH histochemistry	93.8 ± 2.1*	6.2 ± 2.1*	24.2 ± 4.7	75.8 ± 4.7

*Significant ($P < 0.05$) difference in the percentage of live or dead cells between control and treated tissues for an assay technique.

Cell viability analyses—A significantly higher percentage of live cells was seen in the treated tissue with CLM, compared with LDH histochemistry, at days 3, 7, 14, and 21 (**Table 1**). Conversely, for control tissues, LDH histochemistry revealed a significantly higher percentage of live cells than CLM (**Table 2**). A significantly lower percentage of live cells and a significantly higher percentage of dead cells were found for the treatment group over time, compared with the control group for CLM and LDH histochemistry.

Apoptosis—The greatest number of TUNEL-positive chondrocytes was present at day 3, and the numbers declined at later time intervals in the treated tissues (**Figure 1**). The lowest number of TUNEL-positive cells was present on day 0. No significant change was found in the number of TUNEL-negative cells over time. When control groups were compared with the treatment groups, no significant difference was found at any culture time point for the number of TUNEL-negative or TUNEL-positive cells. However, a difference was found in the regional location of the TUNEL-positive cells in the cartilage explants. With RFE treatment, TUNEL-positive cells were seen principally at the margins of the treated region, whereas for the control groups, TUNEL-positive cells were distributed throughout the corresponding region.

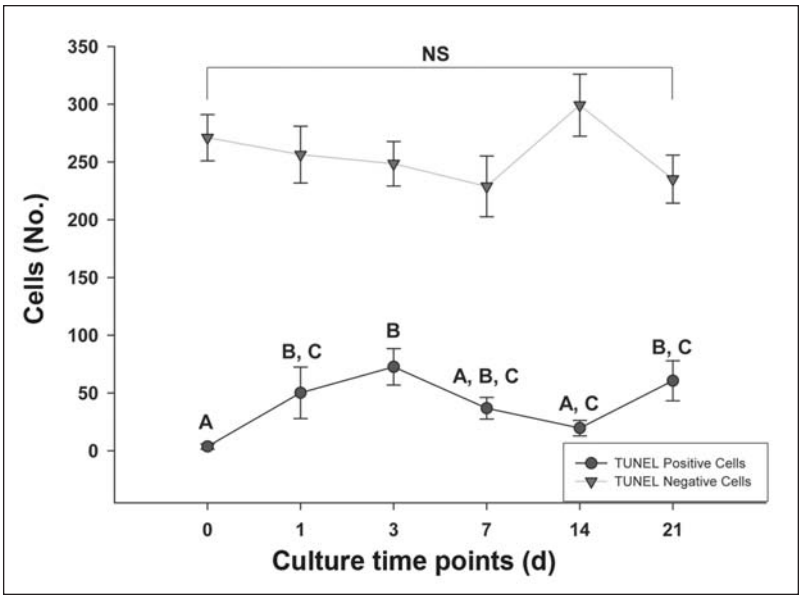

Figure 1—Mean ± SEM number of TUNEL-positive cells and TUNEL-negative cells versus time in culture. No significant change was found in TUNEL-negative cells over time. For TUNEL-positive cells, mean values with different letters (A, B, C) are significantly ($P < 0.05$) different from each other. NS = Nonsignificant change.

Cell counting and measurement—The total number of counted cells for CLM was significantly (P = 0.001) greater than for either LDH histochemistry or TUNEL at all culture time points (**Table 3**). The total numbers of counted cells in tissues by use of TUNEL and LDH histochemistry were significantly (P = 0.019) different from each other only at 1 day. No significant differences were found in total number of cells between the control and treatment groups for CLM. A significant increase was found in the total number of cells for the control tissues, compared with the treated tissues, for LDH histochemistry, whereas for TUNEL, the control group had a significantly lower total number of cells, compared with the treatment group.

Mean cartilage depths for TUNEL and LDH histochemistry were not significantly different from each

Table 3—Mean ± SD total number of cells counted by use of CLM, LDH histochemistry, and TUNEL.

Assay technique	Control tissue	Treated tissue
CLM	800.4 ± 49.1[a]	763.2 ± 19.5[a]
LDH histochemistry	484.4 ± 25.13[b]*	391.4 ± 18.8[b]
TUNEL	259.6 ± 15.4[c]*	297.1 ± 9.6[c]

*Significant ($P < 0.05$) difference in total number of cells between control and treated tissues for an assay technique.
[a–c]Different letters within a column indicate significant ($P < 0.05$) differences among assay techniques.

Table 4—Mean ± SD depth of measured cartilage as determined by use of CLM, LDH histochemistry, and TUNEL.

Assay technique	Control tissues	Treated
CLM (μm)	220 ± 20	220 ± 10
LDH histochemistry (μm)	200 ± 20*	170 ± 10
TUNEL (μm)	140 ± 10	160 ± 10

*Significant ($P < 0.05$) difference in mean depth of measured cartilage between control and treated tissues for an assay technique.

other at any culture time point. Mean cartilage depths for control versus treated tissue with CLM and TUNEL were not significantly different across all culture time points (**Table 4**). However, mean cartilage depth was significantly greater for control tissues, compared with the treated tissues, with LDH histochemistry.

Discussion

The purpose of our study was to compare the results of 2 staining techniques to determine the viability of chondrocytes after thermal injury. In addition, the contribution of apoptosis to the loss of chondrocytes over time was assessed by use of TUNEL. Using LDH histochemistry, we demonstrated that chondrocyte death progressed over time after application of thermal energy to articular cartilage. A greater percentage of live cells were identified with LDH histochemistry and CLM throughout our study in the control group, compared with the RFE treatment group. In treated tissues, CLM revealed a higher total number of cells than LDH histochemistry at each culture time point and a greater percentage of viable chondrocytes. In contrast, LDH histochemistry revealed a greater percent viability in control tissues than CLM. Results of TUNEL indicate that apoptosis contributes to cell loss over time with peak apoptosis staining identified at 3 days after thermal insult.

The reason for a higher percentage of viable cells over all culture time points for the treated tissues by use of CLM versus LDH histochemistry is unknown. It is most likely related to the enzymes responsible for the reactions used by these techniques. It is possible that the ubiquitous esterase responsible for calcein conversion is more resistant to heat inactivation than LDH histochemistry. It is likely that different intracellular enzymes would have different thresholds for injury. It is also possible that green fluorescence associated with the calcein blocks identification of some of the red cells. Fluorescent probes make observation of cells easier because of the intense signal stimulated. Adjacent and overlying cells may mask each other when separate probes are used simultaneously. This could be examined in 2 ways; first, stain adjacent sections separately, one with calcein AM and one with EthD-1, or second, collect and store the red and green channels separately for analysis. The reason for a higher percentage of live cells in the control tissue for LDH histochemistry versus CLM may be related to tissue processing at the completion of incubation. Tissue for CLM was cut on a diamond saw and stained. This saw may produce thermal injury to chondrocytes near the cut surface; similar chondrocyte viability results have been seen in other work in which the same processing and staining techniques were used.[p] Tissues for LDH histochemistry were taken from culture media directly to Zamboni fixation so that no thermal injury associated with processing would occur.

Apoptosis was increased at day 3 after injury. The total number of cells undergoing apoptosis over the area measured did not differ between treated and control tissues. We hypothesized that cells in the treated location already were necrotic, whereas apoptosis occurred at the margins of treated regions. Therefore, in the treatment group, apoptosis is concentrated at the margin of treatment region with some random apoptosis throughout the section. For the control group, apoptosis was randomly distributed throughout the section; therefore, the total number of TUNEL-positive cells is not different between treatment and control groups. This result indicates that the determination of the location of the signal is important, not just the total number of TUNEL-positive cells. The time frame for the increased apoptotic signal and location is consistent with previously reported studies[11,31] evaluating impact injury and laser energy in articular cartilage.

After thermal treatment, chondrocytes may not be killed but only reversibly injured. Use of calcein AM and EthD-1 with CLM was performed in a study by Yetkinler and McCarthy.[29] Results of their study indicated that cell death was overestimated at temperatures < 50°C. At these low temperatures, cells would initially appear devitalized, but then appear viable after recovery from heat shock.[29] However, other studies[7,11,12,32] have supported the validity of the use of CLM and the findings that thermal energy treatment results in irreversible chondrocyte death. In a study performed by Lu et al[33] on sheep cartilage, cell death found at time 0 after a partial-thickness cartilage defect remained until the completion of the study 6 months later. Results of our study support the concept that thermal energy may limit calcein AM fluorescence immediately after heating. Use of CLM revealed that approximately 50% of all cells appeared green on day 0 and day 1 after treatment, which peaked at 70% on day 3, before declining to 53% and less on days 7 to 21. Although not a significant finding, this change indicates that calcein AM fluorescence may be temporarily affected by thermal insult and may rebound for a brief period before necrosis occurs.

Although CLM and LDH histochemistry are common methods for cell viability determination, neither provides an indication of cell metabolic activity relative to the condition of the matrix. Proteoglycan or collagen synthesis within the specimen is potentially a better indicator of whether tissue is metabolically normal.

Future research that would include determination of proteoglycan production by chondrocytes would be especially helpful, as it would provide insight into chondrocyte metabolism after injury. Correlation of proteoglycan synthesis with each vital cell staining technique used in our study would provide more conclusive evidence with regard to which vital staining method best reflects the metabolic state of chondrocytes. In addition, some chondrocytes may react to thermal injury or signaling from adjacent necrotic chondrocytes by the production of cytokines that may influence the cartilage.

It is also important to mention that in our study, we did not perform examination of TUNEL-positive cells with electron microscopy to verify that the cellular structure was truly indicative of apoptotic cells. Unlike previous studies[10,34] in which osteochondral sections have been successfully cultured for ≥ 21 days, substantial chondrocyte death occurs by 21 days in culture. This may be related to the culture media used; however, serum-free media has been used previously to maintain osteochondral sections with extended chondrocyte viability.[10]

In our study, we found that 2 cell viability staining techniques resulted in different absolute numbers of live and dead cells, likely secondary to the different depth of focus for the microscopy systems used. The percentage of viable cells also differed between CLM and LDH histochemistry. This difference may be related to the enzymes responsible for the activation of the stain and their susceptibility to thermal injury. Results of TUNEL indicate that apoptosis contributes to chondrocyte death after thermal injury, with a peak signal at 3 days after insult. On the basis of our findings, direct comparison of cell viability methods may be inappropriate because they may yield differing results. A better understanding of cell and tissue function may result from combining the use of viability stains with determination of other cell function such as proteoglycan, collagen, or cytokine production when evaluating chondrocytes.

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