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Effects of Caffeine on Intervertebral Disc Cell Viability in a Whole Organ Culture Model

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Abstract

Study Design: Controlled laboratory study.

Objective: To investigate the impact of exposure to physiologically relevant caffeine concentrations on intervertebral disc (IVD) cell viability and extracellular matrix composition (ECM) in a whole organ culture model as potential contributing mechanisms in development and progression of IVD disorders in humans. Primary outcome measures were IVD viable cell density (VCD) and ECM composition.

Methods: A total of 190 IVD whole organ explants from tails of 16 skeletally mature rats—consisting of cranial body half, endplate, IVD, endplate, and caudal body half—were harvested. IVD explants were randomly assigned to 1 of 2 groups: uninjured (n = 90) or injured (20G needle disc puncture/aspiration method, n = 100). Explants from each group were randomly assigned to 1 of 3 treatment groups: low caffeine (LCAF: 5 mg/L), moderate caffeine (MCAF: 10 mg/L), and high caffeine (HCAF: 15 mg/L) concentrations.

Results: Cell viability was significantly higher in the low-caffeine group compared with the high-caffeine group at day 7 ($P = .037$) and in the low-caffeine group compared with the medium- and high-caffeine groups at day 21 ($P \leq .004$). Analysis of ECM showed that all uninjured and control groups had significantly higher ($P < .05$) glycosaminoglycan concentrations compared with all injured groups. Furthermore, we observed a temporal, downward trend in proteoglycan to collagen ratio for the caffeine groups.

Conclusions: Caffeine intake may be a risk factor for IVD degeneration, especially in conjunction with disc injury. Mechanisms for caffeine associated disc degeneration may involve cell and ECM, and further studies should elucidate mechanistic pathways and potential benefits for caffeine restriction.

Keywords

intervertebral disc, caffeine, nicotine, disc degeneration, degenerative disc, cell viability, whole organ culture

Introduction

Intervertebral disc (IVD) disorders are prevalent and often associated with pain and dysfunction.[1,2] In the United States alone, direct and indirect medical costs of these conditions are estimated to be between $50 billion and $100 billion each year, highlighting the importance of pathophysiologic understanding and interventional development.[3-7] Although the precise mechanisms driving disc degeneration have not been fully characterized, a number of suspected contributors have been described, including patient age, genetics, nutrition, metabolism, environmental factors, mechanical stress, injury, and comorbidities.[6-9] Importantly, nutritional and environmental factors may contribute to the pathologic processes and represent modifiable risk factors, in contrast to other factors such as age, genetics, or injury.

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Among the environmental factors associated with IVD disorders, nicotine use has long been considered a potential contributor in IVD degeneration and more recently, its relationship with back pain and disc disease have been more elegantly described and understood.[2,10-23] Recently, our laboratory used a novel whole organ culture model to assess the effects of nicotine exposure, as well as its metabolite cotinine, on IVD health and cell viability, and found significantly decreased cell viability and glycosaminoglycan (GAG) content in IVDs exposed to nicotine or cotinine for as little as 7 days. Importantly, these detrimental changes were profoundly more rapid and severe in injured discs.[2]

Although structurally different and with unique receptors and mechanisms of actions, caffeine and nicotine have a number of shared physiologic effects, pathways, and end-results.[24-26] While caffeine has not been directly implicated as a risk factor for IVD degeneration, studies have reported an association between caffeine intake and back pain.[27,28] Historically, moderate caffeine consumption has not been associated with proven long-term adverse health effects. However, consumption patterns and caffeine concentrations in beverages and supplements have continued to rise over the past 2 decades such that dependence syndromes have been well described.[29-33] Importantly, while only 15.5% of US citizens are current smokers, up to 89% of US citizens consume at least one caffeinated beverage daily.[27,34,35] Given the known detrimental effects of nicotine on IVDs and shared physiologic effects of caffeine and nicotine in conjunction with the growing prevalence and amount of caffeine consumption, it is germane to characterize the effects of caffeine on IVD health at the cell and tissue levels in order to understand the potential clinical ramifications with regard to IVD degeneration, pain, and disability. While controlled laboratory studies cannot fully mimic cell and matrix responses that occur in patients, the whole organ rat tail IVD model provides an ethical and valid method for providing initial data for subsequent translational studies. Therefore, the purpose of this study was to investigate the effects of caffeine on IVD cell viability and extracellular matrix (ECM) composition using our validated whole organ IVD culture model.[7] The study was designed to test the hypothesis that caffeine exposure will be associated with significant dose-dependent loss of IVD cell viability and ECM composition, which will be further exacerbated by injury, when compared with controls.

Methods

Tissue Harvest and Culture

Experimental design methods are based on previous work.[2,7] With institutional animal care and use committee (IACUC) approval, tails were harvested from skeletally mature Sprague-Dawley rats (n = 16) euthanized for reasons unrelated to this study. Soft tissues were aseptically dissected from the caudal vertebrae, and whole organ explants consisting of cranial vertebral body half, cartilaginous endplate, IVD, cartilaginous endplate, and the caudal vertebral body half were harvested. Whole organ explants were then randomly assigned to either uninjured (n = 90) or injured (n = 100) groups. Uninjured discs (n = 22) were assessed immediately (time 0) as controls. For the injured group, the posterolateral annulus fibrosus (AF) of each explant was penetrated with a 20G needle to the approximated center of the nucleus pulposus (NP) based on needle penetration depth and tactile feedback. Aspiration of a portion of the NP was then performed using a 1-mL syringe with the plunger pulled back to 0.5-mL. Uninjured discs received no insult. Explants from each group were further randomly assigned to 1 of 3 treatment groups (n = 24/treatment uninjured and n = 32/treatment injured): low caffeine (LCAF) (5 mg/L), moderate caffeine (MCAF) (10 mg/L), and high caffeine (HCAF) (15 mg/L). Caffeine concentrations were determined based on serum levels reported for the average US consumer with normal serum concentrations of coffee drinkers being 2 to 10 mg/L and abuse defined as levels >15 mg/L.[36] IVDs were cultured in a rotating wall vessel (RWV) bioreactor (Synthecon, Inc) at 50 rpm in Dulbeccos's modified Eagle's medium supplemented with sodium pyruvate, L-glutamine, ascorbic acid, Minimum Essential Medium non-essential (MEM N-E) amino acid solution, insulin-transferrin-selenium acid (ITS), and penicillin-streptomyocin. Media were refreshed every 7 days and IVDs were harvested on day 7 (n = 6 uninjured and 8 injured from each of the 3 groups [low, medium, and high caffeine]), day 14 (again, n = 6 uninjured and 8 injured from each of the 3 groups), and day 21 (n = 12 uninjured and 16 injured from each of the 3 groups) and compared against the 22 control discs harvested at day 0. This accounts for 18 uninjured and 24 injured discs harvested at day 7 and again at day 14, and for 36 uninjured and 48 injured discs harvested at day 21, compared against 22 time-0 control discs, totaling 190 discs.

Assessments for Cell Viability and Extracellular Matrix Composition

A microscopic fluorescent cell viability stain assay (Invitrogen) was used to determine the viability of the IVD days 0, 7, 14, and 21 of culture.[37] For this assay, each whole organ explant was bisected through its center to expose the AF and NP in cross-section. The explant halves were placed in solution containing the live-cell stain calcein AM and the dead-cell stain ethidium homodimer and incubated at 37 °C for 30 minutes. After incubation, explant halves were washed in phosphate buffered saline (PBS) for 5 minutes at 25 °C, and then images of both halves of each IVD AF and NP were captured at 4× using a fluorescent microscope (Olympus BX51) with attached digital camera (F-View II 12 Bit B&W CCD). Using Microsuite software (Olympus), separate images for green-staining live cells and red-staining dead cells were obtained. The viable cell density (VCD) of the AF for each IVD was determined by counting the number of green-staining viable cells in AF using an in-house cell counting program. Minimum threshold level of pixel intensity was set to ensure background staining was eliminated. Difference in pixel intensity was used

to delineate cell boundaries to verify accurate cell counting. The computer program counted total number of green-staining cells for each image. The VCD of the NP was not determined because the cells of the NP are too clumped to identify discrete cells and there was variability in NP content between injured and uninjured samples. The area of the respective tissue section counted was measured using MicroSuite (Olympus), and the VCD was determined by dividing the number of green-staining viable cells by the area of the tissue (number of viable cells/area of tissue in mm^2). After cell viability assessments were completed, tissue sections were processed for biochemical evaluations of ECM composition.

Assessments for Extracellular Matrix Composition

AF and NP tissues were dissected from IVD bone with a scalpel blade and weighed to determine wet weight. The tissues were then digested in papain buffer (300 μg/mL dithioreitol; 300 μg/mL papain, 20 mM sodium phosphate pH 6.8; 1 mM ethylenediaminetetraacetic acid [EDTA]) overnight at 65 °C. Digested tissues were tested for total IVD proteoglycan (PG) content using the dimethylmethylene blue (DMMB) assay and total IVD collagen content was determined using α-chymotrypsin and hydroxyprolene (HP) assays, as previously described.[38,39] Briefly, to determine proteoglycan content, a portion of the tissue digest was added the DMMB reagent, and then level of absorbance was measured at 525 nm and compared with a chondroitin sulfate standard curve to determine concentrations of sulfated proteoglycans in the digest. For determination of collagen content, a portion of the tissue digest was mixed with 4 N NaOH and the samples were hydrolyzed by autoclaving at 120 °C for 20 minutes. After autoclaving, samples were mixed with chloramine-T and incubated for 25 minutes at room temperature. Next, Ehrlich's reagent was added to the samples and incubated for 30 minutes at 65 °C for color development. After incubation, absorbance level was determined at 550 nm and compared with a hydroxyproline standard to determine concentrations of collagen. In order to assess relative effects on ECM composition, the PG-to-HP ratio was calculated by dividing the PG content measured in the tissue to the HP content measured in the tissue.

Statistical Analysis

Statistical analysis was performed using SigmaPlot (Systat Software, Inc) using 1-way analysis of variance with the Bonferroni method to test for statistically significant differences among groups with significance set at $P < .05$.

Results

Cell Viability

Day 0 control IVDs had significantly ($P < .05$) higher VCD when compared with most injured and uninjured MCAF and HCAF groups at days 14 and 21 (Figures 1 and 2). The injured LCAF group had significantly ($P < .05$) higher VCD at day 7 than at days 14 and 21. Injured MCAF VCD at day 7 was also significantly ($P < .05$) higher than at days 14 and 21. For the HCAF groups, VCD was significantly ($P < .05$) higher in uninjured IVDs than in injured IVDs at days 7 and 14 (Figure 2). Taken together, this data indicates that AF puncture with NP aspiration injury had detrimental effects on IVD cell viability through 21 days in explant culture. In addition, significant caffeine dose-dependent loss of IVD viable cell density was noted, which magnified and expedited the detrimental effects of injury when compared with healthy (time 0) controls.

Biochemical Extracellular Matrix Analyses

Proteoglycan concentrations were significantly ($P < .05$) higher in all control and uninjured groups compared to all concentration-matched injured groups (Figure 3). At day 21, the injured LCAF group demonstrated significantly ($P < .05$) greater PG content than injured MCAF and HCAF groups. Also at day 21, all injured groups had significantly ($P < .05$) lower PG-to-HP ratios compared with the control group (Figure 4). All injured groups, with the exception of HCAF at day 14, had significantly ($P < .05$) lower PG-to-HP ratios compared with all corresponding uninjured groups. Taken together, this data indicates that AF puncture with NP aspiration injury had detrimental effects on IVD proteoglycan content through 21 days in explant culture. In addition, significant caffeine dose-dependent loss of IVD proteoglycan was noted, which magnified and expedited the detrimental effects of injury when compared with healthy (time 0) controls. Based on PG-to-HP ratio data, IVD collagen was relatively spared from the effects of injury and caffeine.

Discussion

To the authors' knowledge, this is the first study to characterize the effects of caffeine on injured and uninjured IVDs using a whole organ explant model. The results of the present study suggest that caffeine exposure was associated with significant dose-dependent loss of IVD cell viability and ECM composition, which magnified and expedited the detrimental effects of injury, compared to controls. There was a negative, progressive effect on IVD health in culture with increasing concentrations of caffeine. Moderate and high caffeine concentrations were associated with detrimental effects on uninjured IVDs, suggesting that more excessive caffeine consumption may negatively affect even healthy IVDs. Higher levels of caffeine were associated with more rapid and more severe effects, and this dose-dependent relationship was magnified in injured IVDs.

The model used in the present study has been validated to represent changes observed in symptomatic IVD degeneration in humans, specifically with regard to cell viability, biochemical, and biomechanical changes.[7] Cadaveric studies have confirmed inflammatory, apoptotic, and PG degradation mechanisms of disease in patients with IVD degeneration.[40-46] Loss of cell viability is a hallmark and other established criteria of IVD degeneration include (1) increased type I collagen

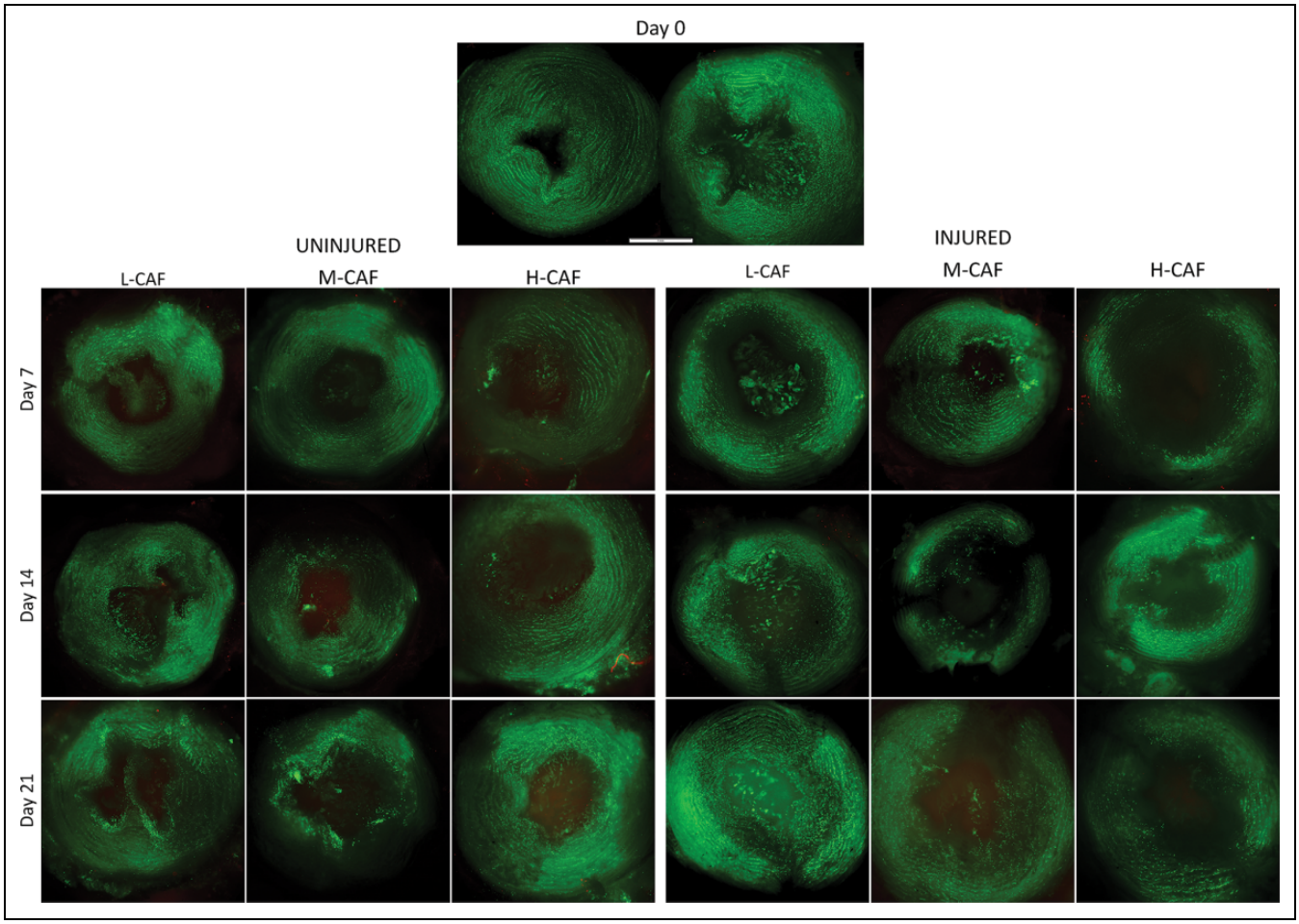

Figure 1. Intervertebral disc (IVD) explant viability. Representative 4× cell viability photomicrographs for injured and uninjured IVD explants on days 0, 7, 14, and 21 of culture for the low-caffeine group (LCAF), medium-caffeine group (MCAF), and high-caffeine group (H-CAF). Green staining indicates viable cells in the tissue (scale bar = 1 mm).

production within the NP; (2) decreased PG, specifically aggrecan, production; (3) upregulation of matrix-degrading enzymes; (4) elevated levels of inflammatory cytokines; and (5) presence of neural growth promoters.[46-48] The most significant biochemical transformation during disc degeneration is ECM loss through decreased production of proteoglycans and type II collagen within the NP.[46] Loss of normal ECM composition results in direct and immediate negative effects on the disc's material properties, and the resultant mechanical overloading further drives loss of cell viability and ECM homeostasis.[46,49-52] This cycle leads to the biomechanical changes that present as clinical manifestations of stiffness, pain, and dysfunction due to IVD protrusion, extrusion, and/or failure.[46,53-55]

The causes of disc degeneration appear to be multifactorial and include genetic, mechanical, and environmental contributions.[46,47,56,57] Cigarette smoking, more specifically nicotine, is a modifiable environmental factor that is strongly associated with back pain, failed spinal fusions, and revision spine surgery.[10-12,15-23] While this relationship is well established, the underlying mechanisms are incompletely understood, are multifactorial, and are likely accompanied by other contributors (eg, toxins, free radicals, carbon monoxide).[58] Nicotine appears to have indirect effects on IVD health through blood flow and nutrient restriction, while recent data suggest that nicotine and its breakdown product, cotinine, may have direct effects on the health and homeostasis of IVD cells and ECM.[5,11,16,59-61] Data from our laboratory using the model described in the present study provided evidence in support of these direct effects by demonstrating dose-dependent negative effects on IVD cell viability and ECM composition (decreased GAG content and altered PG:HP ratios) with more rapid and severe changes in injured discs, and this mirrors the findings associated with caffeine exposure in the present study.[2]

Caffeine is widely consumed throughout the world with reports of average US consumption rates between 85% and 89% of the population and average daily intake between 165 and 305 mg.[27,35,62] Serum caffeine concentrations for the average US coffee drinker range between 2 and 10 mg/L with abuse of caffeine defined as levels >15 mg/L.[36] The relationship of caffeine with IVD health is not well established and while some studies report correlations between caffeine intake

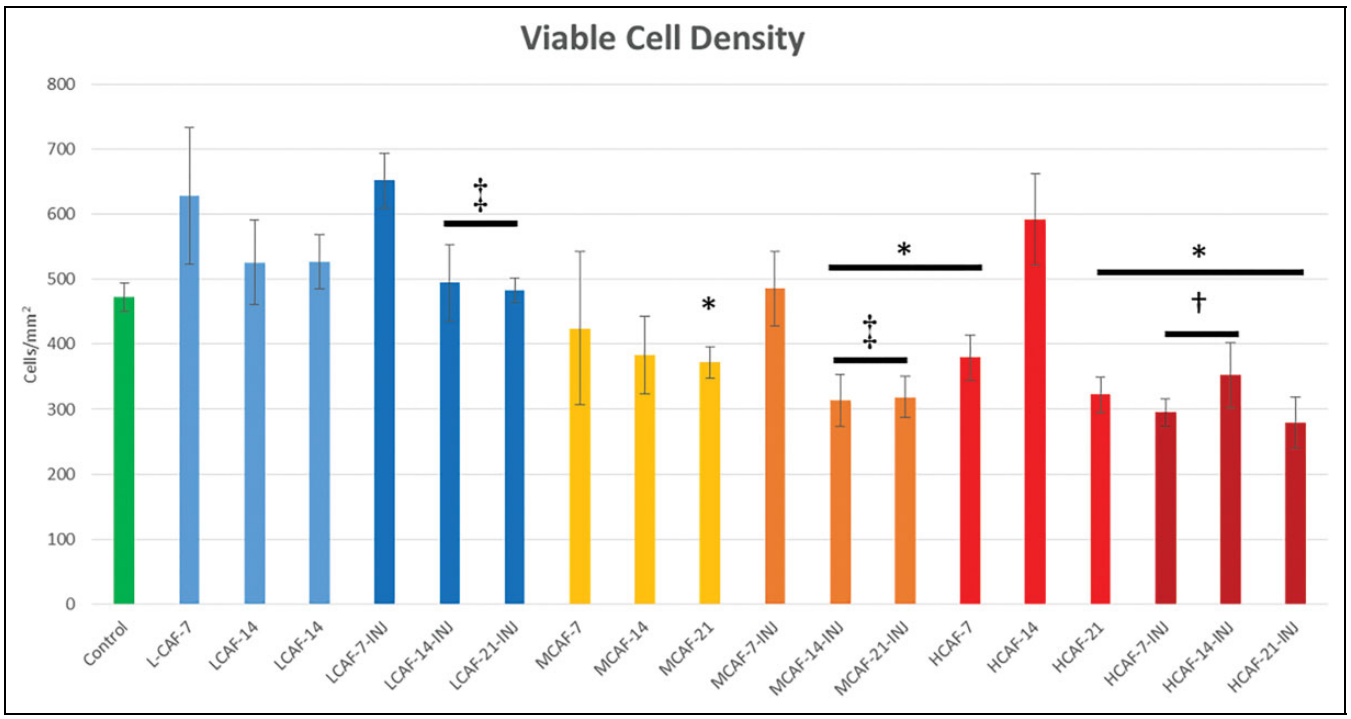

Figure 2. Mean viable cell density (VCD) for all groups at all time points. (*) Significantly ($P < .05$) lower than day 0 control; (†) significantly ($P < .05$) lower than corresponding uninjured group; (‡) significantly ($P < .05$) lower than corresponding day 7 group.

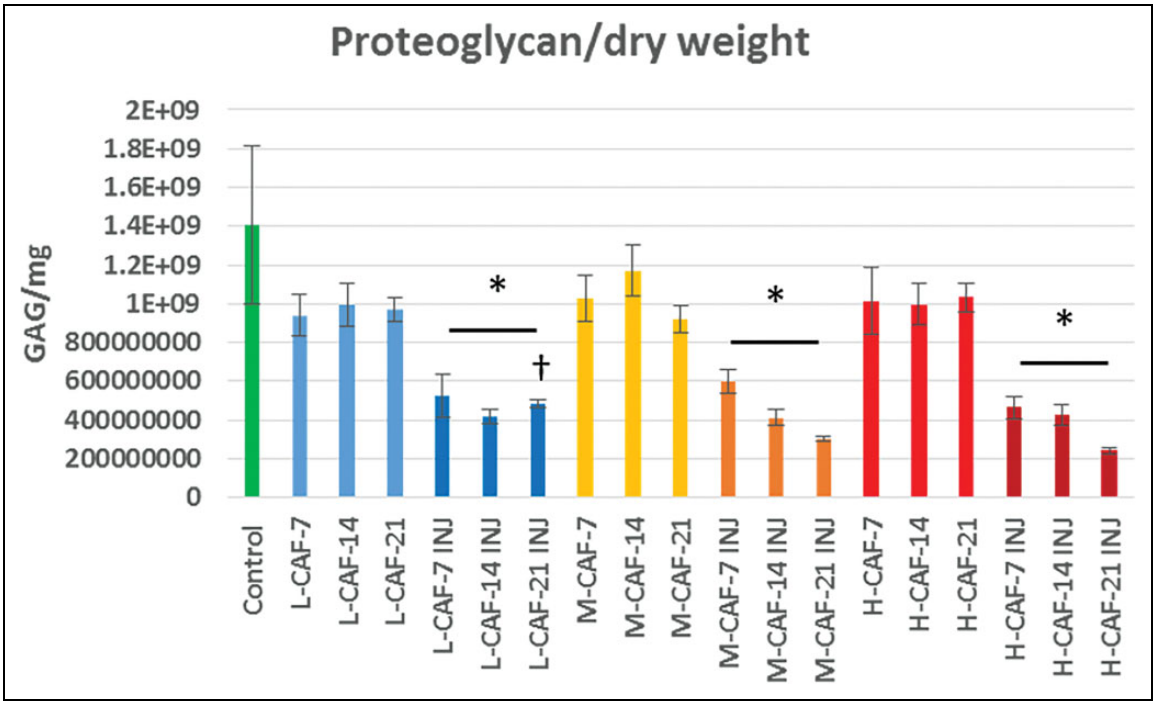

Figure 3. Mean proteoglycan content standardized to the dry weight of the tissue after days 7, 14, and 21. (*) Significantly ($P < .05$) less than the uninjured counterparts and the controls; (†) significantly ($P < .05$) greater than other injured day 21 groups.

and back pain, others do not.[27,28,63] Even in those studies that support a correlation, controversy exists with regard to the cause-and-effect nature of the relationship.[27] This controversy centers on the fact that caffeine serves to potentiate the effects of analgesics such that patients consuming higher levels of caffeine may be consciously or subconsciously self-medicating, which may confound the results of these studies.[27,64] This potential confounder highlights the need for controlled laboratory studies, such as the present study, in order to delineate and differentiate direct and indirect effects,

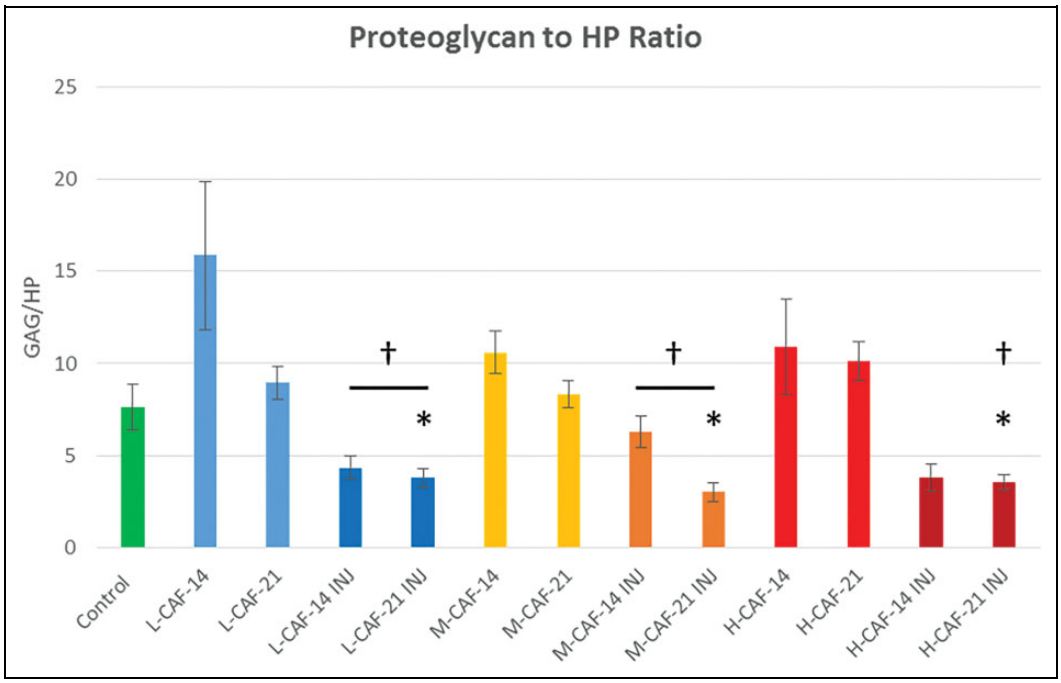

Figure 4. Mean ratio of tissue proteoglycan to hydroxyprolene (HP) content for the day 0 control and at days 14 and 21 of culture. (*) Significantly ($P < .05$) lower than day 0 control; (†) significantly ($P < .05$) lower than corresponding uninjured groups.

relationships, and mechanisms for caffeine in IVD health and disease. Interestingly, caffeine shares several physiologic effects with nicotine despite acting through different receptors and pathways. Nicotine primarily exerts its effects by binding to nicotinic receptors where it acts as an agonist, potentiating the effects of acetylcholine. Caffeine acts by antagonizing adenosine receptors, preventing adenosine-driven neuronal suppression and drowsiness.[25] Additionally, suppression of adenosine receptor activation is stimulatory at medullary centers of respiration, vagal, and vasomotor control, yielding vasoconstriction, increased heart rate, and respiration.[26] Caffeine also stimulates acetylcholine release and activates ryanodine receptors that mediate calcium release from the sarcoplasmic reticulum and endoplasmic reticulum of cells, potentiating muscle contraction.[24] While neither of these mechanisms of action explain caffeine's seemingly direct, toxic effects on IVD health noted in the present study, they do provide potential pathways to investigate, which are the focus of ongoing studies in our laboratory.

Although the factors involved in the mechanisms of caffeine's effects on IVD cell and ECM are not yet elucidated, it is clear from the present study that caffeine has the potential to contribute to IVD degeneration beyond a simple association with back pain. Based on previous studies, potential mechanistic factors for caffeine-related effects on cells and ECM production include mineralization and alkaline phosphatase activity, as well as decreased cartilage-specific matrix gene expression via the adenosine type 1 receptor pathway.[65] In addition, caffeine has been associated with increased apoptosis, chondrocyte phenotype alterations, and ECM perturbations in offspring exposed in utero.[66-69] As such, these factors and pathways deserve further attention for elucidating caffeine-related disease mechanisms for IVD degeneration.

There are limitations to the present study that must be considered when interpreting and applying the results. The experimental design was based on the use of rodent tissues in culture for 21 days. While this design does not allow for direct translation to the clinical scenario, this model has demonstrated reproducible biomechanical and biologic changes reflective of the pathophysiologic processes known to occur in symptomatic IVD degeneration while allowing for control of important variables.[7,70,71] Similarly, media concentrations of caffeine were based on reported serum levels in humans, which may not accurately reflect the concentrations to which IVDs are actually exposed. Therefore, it is possible that even the lowest caffeine concentration used in culture may be supraphysiologic, requiring further investigation using an in vivo model. The model also included only time 0 controls in order to maximize numbers for treatment groups. While it would have been ideal to have comprehensive time-matched controls, previous studies have demonstrated maintenance of cell viability over the duration of culture used in the present study, and time 0 controls represent the most accurate and stringent comparison to healthy cells and matrix.[2] In addition, media pH was not assessed. While caffeine is a base that could raise media pH, media color changes indicative of pH changes were not noted in any group at any time point.

The methods for inciting injury and subsequent loading must be considered experimental conditions. However, changes reflective of those observed in degenerative IVDs (ie, cell death, loss of ECM integrity and architecture, decreased disc height, water content, PG content, and increased

stiffness) have been successfully reproduced using needle puncture to simulate disc injury.[2,65-69,72] RWV bioreactors recreate the physiologic microgravity environment inducing sheer stresses on cultured tissues, generating laminar fluid-flow that reduces diffusional limitations, producing more efficient nutrient-waste exchange.[7,73-76] Furthermore, this flow serves as a cell signaling and communication pathway.[7,73,74] Using this model, we previously reported successful culture of whole-organ rat-tail IVDs such that they retained cell viability, tissue composition, and architecture, as well as material properties in both the AF and NP using a RWV bioreactor.[7] It should be reiterated that the injury used in the present study is associated with decreased cell viability and ECM changes.[72] Thus, the study was designed to compare the effects of caffeine on injured and noninjured discs over time. While the experimental design and its limitations require further study toward translational applicability, previous data in conjunction with the results of the present study suggest that the detrimental effects on IVD cells and ECM associated with caffeine exposure are valid and relevant.

Conclusions

In this whole organ culture model, caffeine exposure was associated with marked loss of IVD cell viability and altered ECM composition in a dose-dependent manner, which was magnified by needle puncture and aspiration injury. These negative effects seen in conjunction with caffeine exposure are similar to those reported for symptomatic IVD degeneration in patients. As such, caffeine intake may be a risk factor for IVD disorders, especially if the disc is already injured or degenerative with potentially detrimental effects on both cells and ECM. Further studies are warranted to confirm these results in vivo, elucidate mechanisms of disease, and investigate potential benefits of education regarding caffeine restriction or avoidance for prevention and treatment of patients with or at risk for IVD injury and degeneration.

Authors' Note

This research was presented as a poster at the 2017 Orthopaedic Research Society's Annual Meeting, March 19-22, in San Diego, CA.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Dr Stoker reports personal fees from Arthrex, Inc, personal fees from Musculoskeletal Transplant Foundation, outside the submitted work. Dr Choma reports other from AO Spine North America, personal fees from Gentis, Inc, other from North American Spine Society, other from Scoliosis Research Society, outside the submitted work. Dr Cook reports grants and personal fees from Arthrex, Inc, personal fees from AthleteIQ, grants from ConforMIS, personal fees from CONMED Linvatec, grants from Coulter Foundation, grants from DePuy Synthes, grants and personal fees from Eli Lilly, other from *Journal of Knee Surgery*, grants from Merial, other from Midwest Transplant Network, grants, personal fees, and other from Musculoskeletal Transplant Foundation, grants from National Institutes of Health (NIAMS and NICHD), grants from Purina, grants from Sites Medical, personal fees and other from Thieme, grants from TissueGen Inc, personal fees from Trupanion, grants from the US Department of Defense, grants from Zimmer-Biomet, outside the submitted work.

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