ARTICLE

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Effects of Doxycycline on Articular Cartilage GAG Release and Mechanical Properties Following Impact

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ABSTRACT: The effects of doxycycline were examined on articular cartilage glycosaminoglycan (GAG) release and biphasic mechanical properties following two levels of impact loading at 1 and 2 weeks post-injury. Further, treatment for two continuous weeks was compared to treatment for only the 1st week of a 2-week culture period. Following impact at two levels, articular cartilage explants were cultured for 1 or 2 weeks with 0, 50, or 100 μM doxycycline. Histology, GAG release to the media, and creep indentation biomechanical properties were examined. The "High" (2.8 J) impact level had gross surface damage, whereas "Low" (1.1 J) impact was indiscernible from non-impacted controls. GAG staining decreased after High impact, but doxycycline did not visibly affect staining. High impact resulted in decreased aggregate moduli at both 1 and 2 weeks and increased permeability at 2 weeks, but tissue mechanical properties were not affected by doxycycline treatment. At 1 week, High impact resulted in more GAG release compared to non-impacted controls. However, following High impact, 100 μM doxycycline reduced cumulative GAG release at 1 and 2 weeks by 30% and 38%, respectively, compared to no treatment. Interestingly, there was no difference in GAG release comparing 2 weeks continuous treatment with 1 week on, 1 week off. These results support the hypothesis that doxycycline can mitigate GAG release from articular cartilage following impact loads. However, doxycycline was unable to prevent the loss of tissue stiffness observed post-impact, presumably due to initial matrix damage resulting solely from mechanical trauma.

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KEYWORDS: post-traumatic osteoarthritis; doxycycline; glycosaminoglycans; biomechanics; articular cartilage

Introduction

Post-traumatic osteoarthritis (PTOA) is a disease state characterized by progressive articular cartilage degeneration, joint pain and dysfunction, and altered cartilage biomechanics (Guilak et al., 2004). While a combination of mechanical, inflammatory, and enzymatic factors are thought to contribute to the sequelae of PTOA (Borrelli and Ricci, 2004; Borrelli et al., 1997; Guilak et al., 2004; Oegema et al., 1993), little is known of the etiology or pathogenesis of PTOA as a clinical disease (Olson and Guilak, 2006). Numerous degenerative changes in articular cartilage occur following traumatic mechanical injury (Borrelli and Ricci, 2004; Ewers et al., 2002; Oegema et al., 1993), including collagen breakdown, reduced proteoglycan synthesis, loss of extracellular matrix (ECM) components, and a decrease in tissue biomechanical integrity, which are also hallmarks of PTOA development (Chen et al., 2001; D'Lima et al., 2001; Huser and Davies, 2006; Lohmander et al., 1999; Silver et al., 2001).

Though difficult to assess articular cartilage injury in vivo, it is possible to observe the effects of articular cartilage damage following impact-induced injuries using ex vivo animal models and explant studies. Such studies have connected mechanical impact to PTOA by a variety of mechanisms that damage both the ECM and chondrocytes (Huser and Davies, 2006; Patwari et al., 2003; Torzilli et al., 1999). Traumatic mechanical injury induces chondrocyte death, disrupts the collagen network, and causes glycosaminoglycan (GAG) release from articular cartilage. One

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study found that human cartilage subjected to a 14 MPa load released GAGs—over two times as much as unloaded samples—during the first 4 days following impact (D'Lima et al., 2001). An in vitro study of the kinetics of GAG release found that a third of the GAG released during the first 24 h occurred during the first 4 h of culture, and remained significantly higher than controls at 24 h (DiMicco et al., 2004). The same study also found a 50–60% reduction in the incorporation of ^{35}S-sulfate and ^{3}H-proline, indicative of decreased GAG and collagen synthesis, in articular cartilage specimens subjected to injurious compression at a strain rate of 1/s. Furthermore, impact level has been found to correlate with tissue damage, demonstrating that there is a direct relationship between the degree of articular cartilage breakdown and the peak stress, stress rate, and energy delivered by the impact (Ewers et al., 2001; Jeffrey et al., 1997; Milentijevic and Torzilli, 2005; Torzilli et al., 1999). One study found progressively greater levels of cell death at 15, 25, and 35 MPa; however, below the threshold stress level of 15 MPa, cells remained viable and the tissue matrix was not damaged. The threshold stress also correlated with proteoglycan biosynthesis, which was found to decrease significantly once the threshold was reached. In addition, Jeffrey et al. (1997) found that greater impact energy, ranging from 0.1 to 1.0 J, corresponded to greater levels of matrix loss over a 2-week culture period. Ultimately, the collective tissue damage leads to detrimental changes in tissue mechanical properties (Kurz et al., 2001).

With better understanding of the mechanisms of osteoarthritis (OA) development, the search to identify and develop successful disease modifying OA drugs to aid in tissue repair and prevent further degeneration in diseased tissue has begun. Much effort has focused on matrix metalloproteinases (MMPs) as potential targets in OA prevention and treatment (Mengshol et al., 2002), though other matrix degrading enzymes, such as aggrecanases and hyaluronidase, may have important roles as well (Glasson et al., 2005; Sandy, 2006; Sugimoto et al., 2004). A study by DiMicco et al. (2004) found that GAG release between 1 and 7 days post-injury was markedly reduced using an MMP inhibitor compared to no treatment. Tetracycline analogs and derivatives have also exhibited success in the inhibition of MMPs by multiple mechanisms in both in vivo and in vitro studies (Arsenis et al., 1992; Golub et al., 1992, 1998; Greenwald et al., 1987, 1992). Furthermore, doxycycline, a member of the tetracycline family and an MMP inhibitor, has been found to reduce collagenolytic activity (Smith et al., 1999; Yu et al., 1991) and levels of MMP-1 and MMP-13 mRNA and protein (Shlopov et al., 1999). Because the development of PTOA typically occurs several years after injury (Krueger et al., 2003), it is difficult to measure the effects of an early intervention. However, a clinical trial by Brandt et al. (2005) found that doxycycline treatment given to patients with unilateral OA for 30 months significantly decreased the rate of joint space narrowing. Also, prophylactic doxycycline has also been shown to effectively reduce the severity of OA in dogs following ACL transection (Yu et al., 1992). These studies underscore the potential for using doxycycline to prevent GAG loss after injury to articular cartilage.

Ameliorating the immediate damage and ensuing degenerative cascade following articular cartilage injury could improve the daily activity of individuals suffering from PTOA. While results from studies involving doxycycline are certainly promising, further examination is needed. Current literature has not established the effects of doxycycline on GAG release or tissue mechanical properties following impact loads, nor has any MMP inhibitor been studied following mechanical trauma for more than 1 week post-injury. In the present study, an experimental system of mechanical injury was utilized to invoke two levels of impact, two concentrations of doxycycline, and two doxycycline treatment regimens in order to investigate its use following impact at 24 h, 1 week, and 2-week time points. It was hypothesized that doxycycline treatment would decrease GAG release by 1 week in a dose-dependent manner. Additionally, the 2-week continuous doxycycline treatment was hypothesized to result in less GAG release and yield greater aggregate moduli in explants compared to treatment for only the 1st week of a 2-week culture period.

Materials and Methods

Articular Cartilage Tissue Harvest

A total of 27 proximal bovine ulnas were obtained from the elbow joints of skeletally mature animals (Animal Technologies, Tyler, TX) within 48 h of slaughter. Under sterile conditions, the proximal ulna was cut parallel to the articular surface using a reciprocating saw (Ryobi, Hiroshima, Japan) with a sterile blade. Approximately 1.5 cm of bone was left in place beneath the articular cartilage surface. The articular surface was then covered with sterile gauze and hydrated with sterile phosphate-buffered saline (PBS). Following tissue harvest, the articular surface, including underlying bone, was placed into a custom designed stainless-steel autoclaved specimen clamp and prepared for impact.

Impact of Articular Cartilage

Cartilage impact was carried out as described previously (Scott and Athanasiou, 2006). Briefly, the impact mass was raised to the specified height and dropped onto the impact interface, which connects to a 5 mm, non-porous, cylindrical impact tip. Two levels of impact were employed: a ''Low'' impact (6 cm drop height with a 1.88 kg mass) and a ''High'' impact (10 cm, 3.43 kg mass), delivering 1.1 and 2.8 J of energy, respectively. Each proximal ulna was impacted four times (two at the High level and two at the

Low level) in distinct locations that were separated by at least 5 mm. Impact location was randomized across all groups studied.

Explant and Culture of Articular Cartilage

Following impact, 5 mm diameter full thickness articular cartilage explants were removed from each ulna using a sterile dermal biopsy punch and a #10 scalpel blade. Explants were placed directly into 6-well plates for culture. In addition to the four impacted areas, two 5 mm non-impacted explants were removed from each joint and used as controls. All cartilage explants (control, Low impact, and High impact) were randomly assigned into one of three treatment groups consisting of either 0, 50, or 100 μM doxycycline supplemented in the media (Sigma, St. Louis, MO). Explants were cultured in 3 mL of serum-free Dulbecco's Modified Eagle's Medium (DMEM) with Glutamax™ (Invitrogen, Carlsbad, CA) containing 100 units/mL Penicillin (Biowhittaker, Walkersville, MD), 100 μg/mL Streptomycin (Biowhittaker), 2.5 mcg/mL Fungizone (Biowhittaker), 0.1 mM non-essential amino acids (Invitrogen), and 50 μg/mL ascorbic acid. Medium was replaced at 24 h, and then every 2–3 days for the remaining time in culture. Collected medium was stored at −20°C for GAG release quantification. Groups assigned to receive treatment had freshly dissolved doxycycline delivered during each media change. As shown in Figure 1, culture duration was either 1 week with continuous treatment, 2 weeks with continuous treatment, or 2 weeks of culture with treatment for the 1st week but not the 2nd. Pilot studies showed 100 μM doxycycline did not cause significant cell death (data not shown).

Histology

Tissue samples were cryoembedded and sectioned at 14 μm. Samples were fixed in 10% phosphate buffered formalin and stained with Safranin O/fast green to examine GAG distribution. Slides were then dehydrated through ascending alcohols before being coverslipped with Permount™ and examined under 10× magnification with a light microscope.

GAG Release to Media

Each sample of collected culture media was removed from −20°C, thawed, and vortexed thoroughly before assaying 50 μL with a 1,9-dimethyl-methylene blue colorimetric assay to detect GAG released to the media (Blyscan Sulfated GAG Assay, Accurate Chemical and Scientific Corp., Westburg, NY). Chondroitin 4-sulfate was used as the standard. Total GAG released into the media was then back calculated and normalized to tissue volume. Tissue volume was calculated knowing the tissue surface area (5 mm diameter circle) and thickness, as measured immediately following biomechanical testing (see below).

Creep Indentation Biomechanical Testing

Following culture, a dermal biopsy punch was used to remove the inner 3 mm diameter portion of the 5 mm diameter explant. These specimens were wrapped in gauze soaked in PBS containing protease inhibitors (10 mM *N*-ethylmaleimide, 5 mM benzamidine, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) and frozen at −20°C until testing. Prior to testing, samples were thawed for at least 1 h at room temperature in the same PBS with protease inhibitor solution and affixed to a flat stainless steel surface with a thin layer of cyanoacrylate glue. A creep indentation apparatus was used to determine the compressive creep and recovery behavior of the cartilage explants (Athanasiou et al., 1995). Testing conditions consisted of a tare load of 0.005 N followed by a test load of 0.02 N applied to the sample through a 0.8 mm diameter, flat-ended, rigid, porous tip. Creep and recovery behavior was recorded using LabView software (National Instruments, Austin, TX). A semi-analytical, semi-numerical model was used to determine the tissue's linear biphasic properties of aggregate modulus, permeability, and Poisson's ratio from the time-displacement curves (Mow et al., 1989). Tissue thickness was measured across the entire 3 mm specimen using digital calipers.

Statistical Analysis

The study was based on a full-factorial experimental design. The JMP IN 5.1 statistical software package (SAS Institute, Cary, NC) was used to perform a two-factor ANOVA (impact level and doxycycline concentration) on the biomechanical properties and amount of GAG released from each explant for the 1- and 2-week continuous treatment groups. This model was also used on media samples collected at 24 h. Another two-factor ANOVA (impact level and treatment regimen) was used to compare the effects of 2-week continuous treatment to 1 week on followed by 1 week off treatment with 50 or 100 μM doxycycline. If significance ($P < 0.05$) was found, a Tukey HSD post-hoc test was performed to compare amongst factor levels. For the GAG release and biomechanics assays, an $n = 6$ was used for each group. For histology, an $n = 2$ was examined for each group. Data are displayed in the figures as mean plus 1 SD.

Results

Histology

Histological staining for GAG with Safranin O/fast green revealed no gross staining differences among the 0, 50, or 100 μM doxycycline concentrations at 1 or 2 weeks

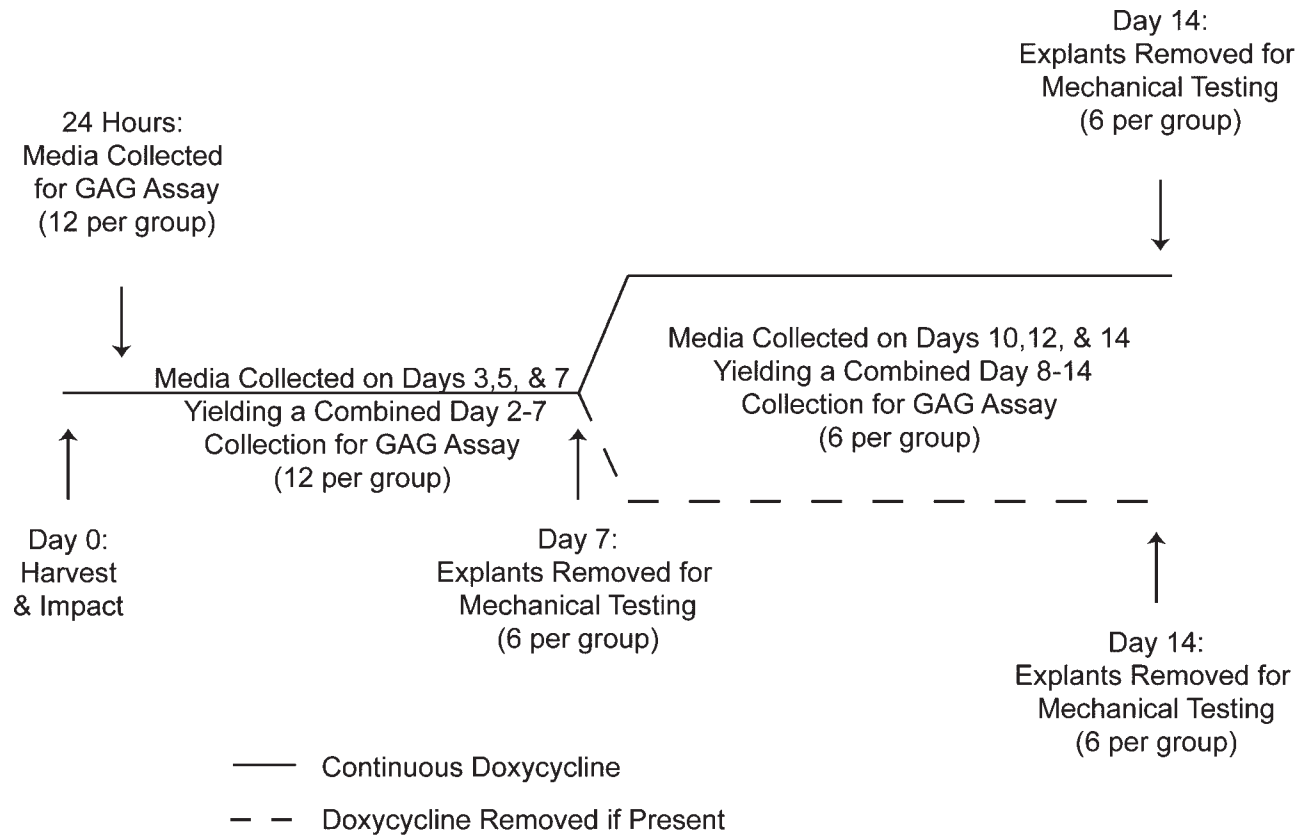

Figure 1. Time line description of experiments. Cartilage was harvested and impacted on day 0 and cultured with 0, 50, or 100 μM doxycycline. Medium was collected and stored separately at 24 h. Medium was also obtained to yield collections corresponding to days 2–7 and days 8–14. At 1 week, six explants per group were removed for mechanical testing while the rest continued to be cultured in the presence (solid line) or absence (dashed line) of doxycycline. After 2 weeks of culture, the remaining explants were removed for mechanical testing.

following injury for all impact levels. Figure 2 shows representative images from the 1 week time point for 0 and 100 μM doxycycline treated control, Low, and High impact specimens. In addition, 2-week samples treated with doxycycline for only 1 week of a 2-week culture period were found to have no identifiable differences compared to samples treated continuously for 2 weeks. Non-impacted controls showed no surface damage, whereas those impacted at the High level had considerable surface damage, including delamination, surface fissures, and gross injury that progressed beyond the superficial layer into the middle-deep zone. Specimens impacted at the Low level showed no gross surface damage. Furthermore, GAG staining appeared noticeably reduced in samples impacted at the High level compared to non-impacted controls, while staining for Low impact appeared similar to controls.

GAG Release

Figure 3A shows GAG release data for 24 h and days 2–7. Impact level was a significant factor 24 h following injury ($P < 0.001$), with High impact resulting in a greater amount of GAG release to media than both Low impact and non-impacted control groups. At 24 h no significant differences were found for GAG release due to doxycycline treatment. For days 2–7, impact level did not significantly affect GAG release ($P = 0.11$); however, doxycycline treatment was a significant factor ($P < 0.001$), with 100 μM doxycycline resulting in decreased GAG release compared to both 0 and 50 μM doxycycline. Cumulative 1 week GAG loss was calculated by adding the 24-hour and 2- to 7-day collections. Both impact level and doxycycline concentration were significant factors ($P = 0.015$ and 0.022, respectively). Post-hoc analysis of factor levels showed High impact caused significantly more GAG to be released to the media compared to non-impacted controls and treatment with 100 μM doxycycline significantly decreased GAG release compared to no treatment. For the control, Low, and High impact levels, treatment with 100 μM doxycycline resulted in 29%, 14%, and 30% reductions in GAG loss, respectively. Notably, of the total GAG released in 1 week, at least 33% was released in the first 24 h for all groups studied.

The amount of GAG released during days 8–14 of culture was not significantly affected by either impact level or doxycycline treatment. However, for 2-week cumulative

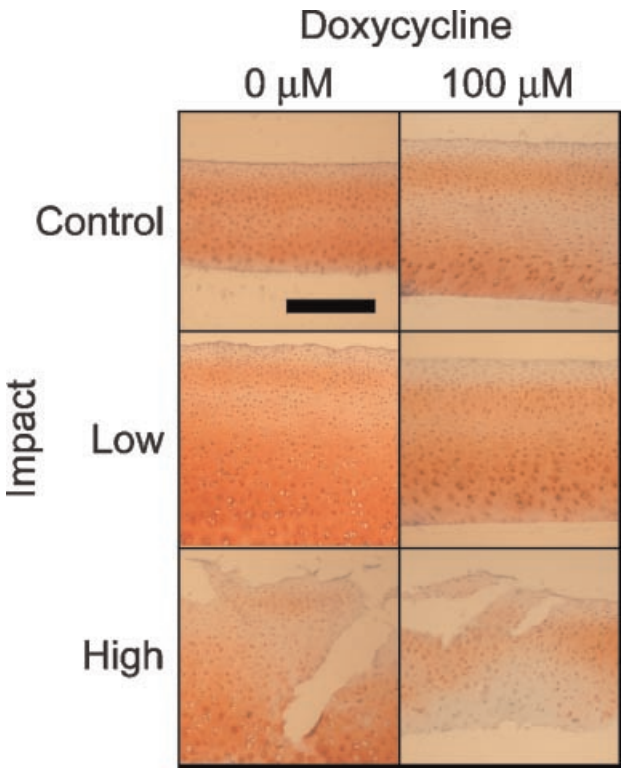

Figure 2. Representative histological images of cartilage explants stained for proteoglycans after 1 week of culture in 0 and 100 μM doxycycline at each impact level. Scale bar = 500 μm. Note the extensive damage caused by High impact and resulting decreased staining. No differences in staining were apparent due to doxycycline treatment. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

GAG release, calculated by adding the 1 week cumulative GAG release with the 8- to 14-day collection, impact level was not a significant factor ($P = 0.32$), but doxycycline concentration was ($P < 0.001$). Post-hoc analysis showed 100 μM doxycycline significantly decreased 2-week cumulative GAG release compared to 0 or 50 μM doxycycline (Fig. 3B). Compared to no treatment, treatment with 100 μM doxycycline of specimens impacted at the control, Low, and High levels resulted in 50%, 49%, and 38% respective decreases in cumulative GAG released over 2 weeks.

To test whether doxycycline's effects are specific to a particular window of time, cartilage explants were treated for only the 1st week of a 2-week culture period and compared to explants treated continuously for 2 weeks. For both 50 and 100 μM doxycycline, there was no significant difference in GAG release between the two treatment regimens (Fig. 3C).

Biomechanical Properties

Biomechanical properties provide indices for the ability of the tissue to function as needed in its mechanical environment. The approach employed in this study, based on the linear biphasic theory (Mow et al., 1980), allows determination of tissue aggregate modulus, permeability, and Poisson's ratio. Figure 4A and B shows the aggregate moduli of all explants that received continuous doxycycline treatment in this study. Data at 1 week and 2 weeks were analyzed separately. Both analyses revealed that impact level was a significant factor ($P = 0.039$ and $P = 0.047$ for 1 and 2 weeks, respectively). Post-hoc analyses showed that High impact resulted in a significant decrease in aggregate modulus at both 1 and 2 weeks compared to non-impacted controls, while the aggregate moduli measured from articular cartilage that was impacted at the Low level were not significantly different from either non-impact control or High impact levels at both 1 and 2 weeks post-injury. At 1 week, the aggregate modulus of the High impact 0, 50, and 100 μM doxycycline treated explants had decreased 32%, 41%, and 36% compared to their respective treated, non-impacted, controls. For the 2-week time point, the same values were 32%, 9%, and 28%. Notably, treatment of Low impact specimens with 100 μM doxycycline resulted in only a 3% decrease in aggregate modulus at 1 week.

In terms of tissue permeability, at 2 weeks post-injury impact level was a significant factor ($P = 0.007$). Post-hoc analysis showed the permeability of articular cartilage following High impact loading was significantly increased (1.5- to 1.8-fold) compared to non-impacted controls (Table I). The permeability of explants subjected to Low impact was similar to both non-impacted controls and the High impact level. Poisson's ratio was not significantly affected by impact level at any time point.

Furthermore, continuous doxycycline treatment had no significant effect on any of the tissue's material properties across all impact levels and time points. Also, looking within the individual impact levels, treatment with doxycycline for the 1st week but not the 2nd did not significantly affect mechanical properties measured at 2 weeks compared to groups that were given 2-week continuous doxycycline treatment.

Discussion

The objective of this study was to determine the effects of doxycycline on GAG release from and mechanical properties of articular cartilage following mechanical impact. Our results indicate that doxycycline mitigates GAG loss from articular cartilage following a single impact injury. In particular, it was demonstrated that treatment with 100 μM doxycycline significantly reduced GAG release from the tissue at both 1 and 2 weeks post-injury. Additionally, doxycycline treatment for only the 1st week of a 2-week culture period was found to provide the same chondroprotective properties as doxycycline delivered continuously for 2 weeks. These results suggest doxycycline merits further study in already established animal models of mechanical

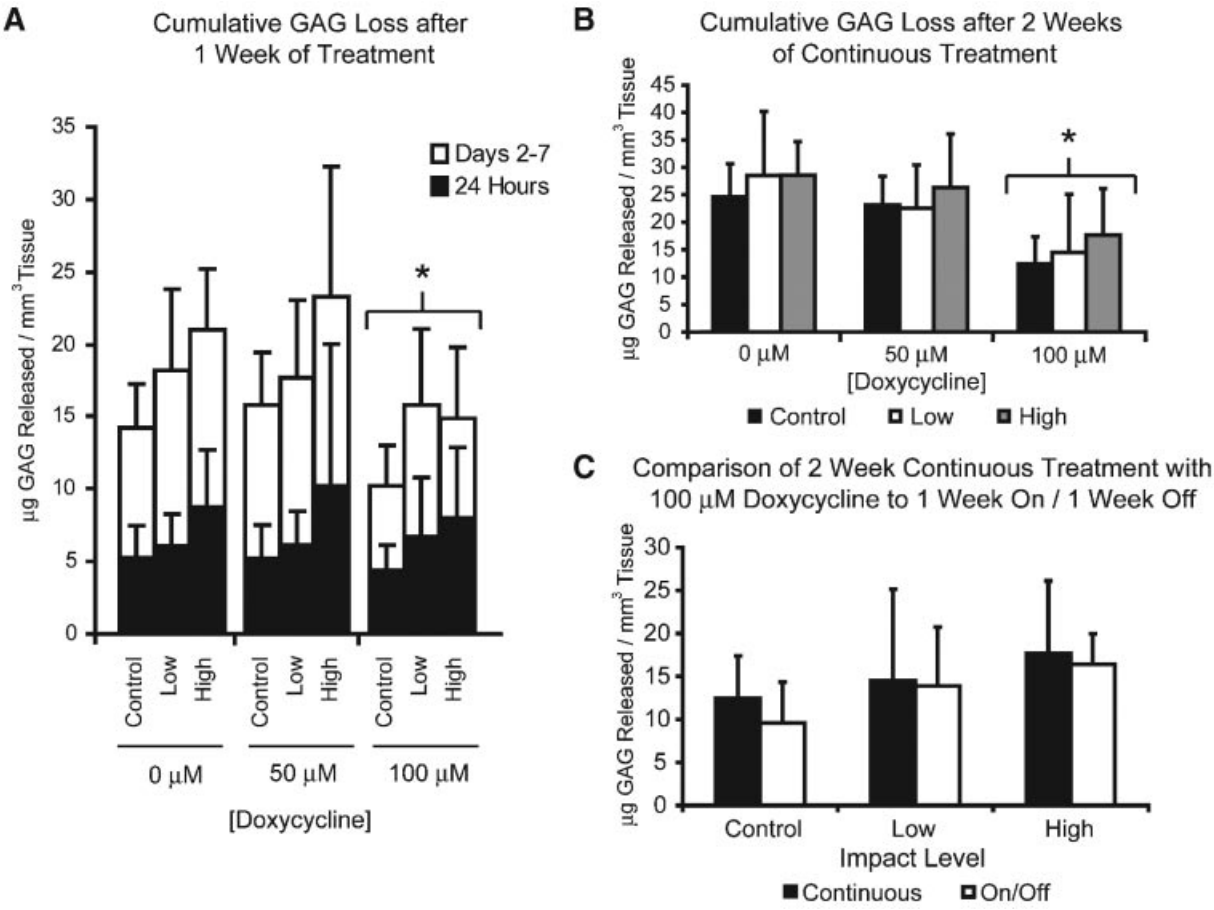

Figure 3. Data from GAG release assay. **A**: Comparison of GAG release for all impact and doxycycline levels at 24 h and 1 week. The top of the white bar represents cumulative 1 week GAG release. While only impact was a significant factor at 24 h ($P < 0.001$), both impact level and doxycycline concentration were significant factors ($P = 0.015$ and 0.022, respectively) for 1 week cumulative GAG release, with High impact resulting in more GAG release compared to non-impacted controls and 100 μM doxycycline resulting in decreased GAG release compared to no treatment ($^*P < 0.05$ with Tukey HSD post-hoc). **B**: Cumulative GAG release after 2 weeks of continuous doxycycline treatment. Treatment with 100 μM doxycycline significantly decreased GAG release compared to 0 and 50 μM groups ($^*P < 0.05$ with Tukey HSD post-hoc). **C**: Comparison of 2 weeks continuous treatment with 1 week of treatment followed by 1 week without at the 100 μM concentration. Treatment regimen did not significantly affect GAG release. The same was seen with 50 μM doxycycline. Each bar represents the mean plus 1 SD of $n = 6$ samples.

injury to articular cartilage (Borrelli et al., 2002; Ewers et al., 2002; Milentijevic et al., 2005; Oegema et al., 1993).

The temporal GAG release profile observed in this study supports a model in which mechanical trauma is responsible for initial GAG loss, while subsequent GAG loss results from enzymatic degradation. In this study, impact loading resulted in GAG release from injured tissue that was significantly greater for the High level of impact (2.8 J) at 24 h compared to both Low impact (1.1 J) and non-impacted controls. These results agree with findings from other groups that show increasing impact energies (0.1–1 J) result in higher levels of GAG release (Huser and Davies, 2006; Jeffrey et al., 1997). Further, studies show that the majority of GAG loss occurs at early time points, typically less than 72 h post-injury (DiMicco et al., 2004; Ewers et al., 2001; Huser and Davies, 2006; Jeffrey et al., 1997). Similarly, in the present study, 33% of the total GAG released over 1 week occurred in the first 24 h. However, doxycycline treatment did not significantly affect immediate GAG release following injury, suggesting that the mechanisms behind initial GAG loss cannot be stopped with an MMP inhibitor alone. Therefore, one possibility is that mechanical damage and loosening of the collagen network is responsible for immediate GAG release following impact injury. DiMicco et al. (2004) demonstrated similar findings, showing that GAG loss during the first 24 h following injury was due to mechanical damage, while GAG loss that occurred in subsequent days could be attributed to MMP activity. Alternatively, GAG release in the first 24 h may also be mediated by other enzymes unaffected by doxycycline, such as hyaluronidases or aggrecanases (Glasson et al., 2005; Sandy, 2006; Sugimoto et al., 2004).

Further, our data suggest that long-term doxycycline treatment following impact injury may not be necessary to result in decreased GAG loss from the tissue, though further in vitro work to identify the minimal treatment time and

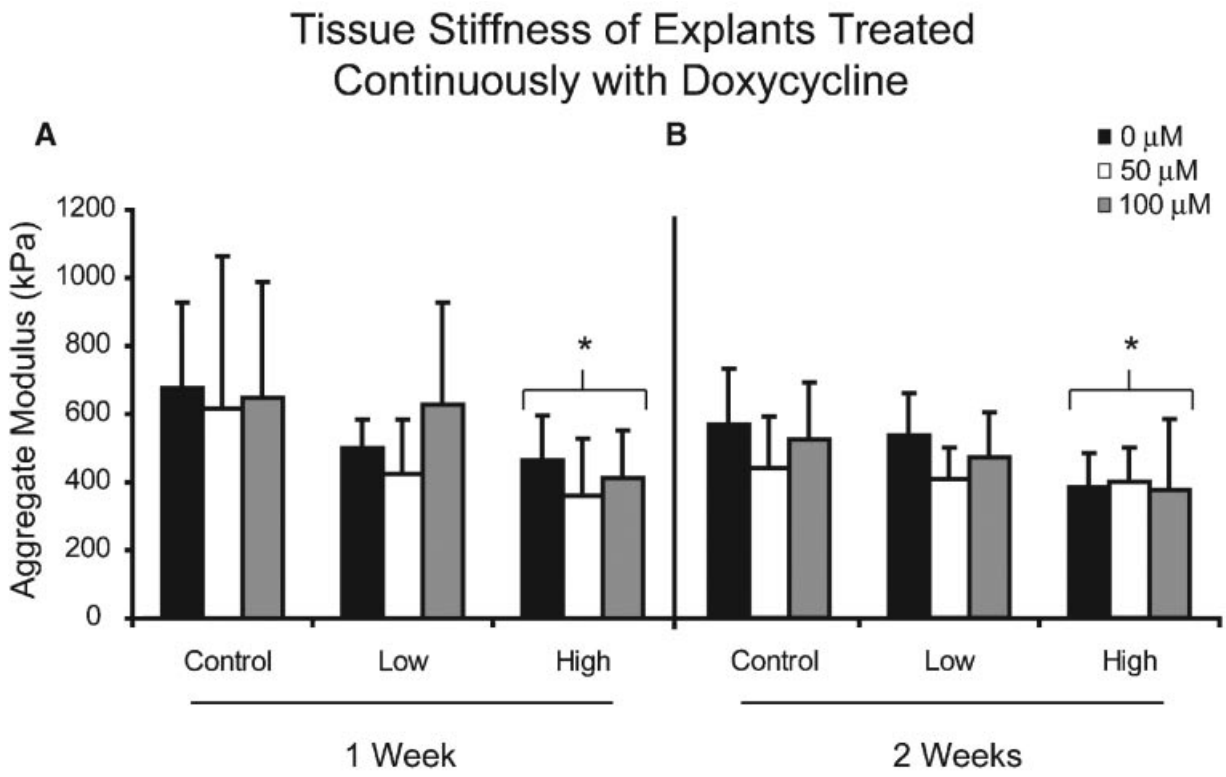

Figure 4. Tissue stiffness. (**A**) Aggregate modulus of explants treated continuously with doxycycline for 1 week of culture. (**B**) Aggregate modulus of explants treated continuously for 2 weeks. Impact level significantly affected the aggregate moduli, with High impact causing a decrease in tissue stiffness at both the 1- and 2-week time points compared to non-impacted controls ($^*P < 0.05$ with Tukey HSD post-hoc). Aggregate moduli following Low impact were similar to both non-impacted controls and High impact at both time points. Doxycycline treatment did not significantly affect aggregate moduli. Each bar represents the mean plus 1 SD of $n = 6$ samples.

Table I. Tissue permeability and Poisson's ratio as measured by creep indentation.

Time point	Impact level	Doxycycline concentration (μM)	Permeability ($m^4/N \cdot s \times 10^{-15}$)	Poisson's ratio
1 Week	Control	0	2.08 ± 1.09	0.123 ± 0.102
		50	4.38 ± 3.82	0.222 ± 0.217
		100	3.85 ± 0.65	0.096 ± 0.108
	Low	0	6.16 ± 3.33	0.070 ± 0.081
		50	4.45 ± 1.75	0.123 ± 0.144
		100	6.21 ± 4.27	0.048 ± 0.065
	High	0	2.52 ± 2.04	0.123 ± 0.107
		50	5.03 ± 2.97	0.057 ± 0.092
		100	4.99 ± 2.49	0.048 ± 0.085
2 Week continuous	Control	0	2.71 ± 0.90	0.048 ± 0.061
		50	3.70 ± 1.29	0.101 ± 0.118
		100	3.95 ± 1.23	0.070 ± 0.115
	Low	0	4.74 ± 2.01	0.039 ± 0.074
		50	3.44 ± 1.84	0.048 ± 0.085
		100	4.41 ± 0.95	0.066 ± 0.077
	High*	0	4.85 ± 1.20	0.131 ± 0.078
		50	5.61 ± 1.88	0.022 ± 0.054
		100	7.26 ± 5.05	0.057 ± 0.073
1 Week on, 1 Week off	Control	50	4.57 ± 1.50	0.022 ± 0.020
		100	4.77 ± 1.74	0.079 ± 0.113
	Low	50	5.31 ± 2.90	0.018 ± 0.032
		100	4.86 ± 1.20	0.005 ± 0.012
	High	50	5.21 ± 1.26	0.066 ± 0.102
		100	7.12 ± 4.48	0.053 ± 0.072

Values given as mean ± 1 SD of $n = 6$ samples.

*Denotes significant effect ($P = 0.007$) of impact level on permeability at the 2-week time point. The "High" level of impact resulted in a significant increase in permeability compared to non-impacted controls.

subsequent in vivo validation are needed. Jeffrey et al. (1997) observed evidence of a repair response through a mechanism in which GAG was synthesized at higher rates by injured tissue compared to control. A higher rate of GAG synthesis is beneficial in a repair response, though it may be hindered by the increase in MMP activity that occurs following injury (Bonassar et al., 1996; DiMicco et al., 2004; Shlopov et al., 1999). Therefore, use of an MMP inhibitor such as doxycycline, coupled with the physiological response of increased GAG synthesis post-injury, may provide an opportunity for tissue recovery, such that the benefits may continue to be seen after treatment is stopped. This is one possible explanation for the results that indicated 2 weeks of continuous doxycycline treatment is equivalent to treatment for only the 1st week of a 2-week culture period. If latent MMP activity and MMP synthesis is only up-regulated following injury for a short period of time, and these enzymes are responsible for a considerable amount of the matrix catabolism, then early treatment with doxycycline may have beneficial outcomes in the long-term. Further supporting this argument, doxycycline treatment has been shown to decrease mRNA and protein levels of MMP-1 and MMP-13 in normal and OA chondrocytes (Shlopov et al., 1999).

While the present study does not specifically address whether the GAG released is due to de novo synthesis or degradation of existing matrix, both literature and our data support a mechanism whereby doxycycline reduces GAG release through inhibition of matrix catabolism post-impact. Following impact there is a decrease in GAG synthesis (Jeffrey et al., 1997; Kurz et al., 2001), which Jeffrey et al. (1997) have shown takes 12 days to recover and increase in impacted cartilage ($\sim$1 J) over uninjured controls. However, increased synthesis is not likely in our system, since, from 8 to 14 days, we observed no differences in GAG release among all groups studied. Further, increased cell death with increasing impact level has been observed both in our system (data not shown) and in previously published work (Huser et al., 2006; Jeffrey et al., 1995). The model that doxycycline prevents tissue degradation is further substantiated by the fact that higher levels of GAG release were still observed despite a greater level of cell death resulting from High impact. With fewer cells to synthesize GAG, decreased GAG release with doxycycline treatment must result from inhibition of matrix degradation, a model supported by literature (DiMicco et al., 2004).

Increased GAG release from the tissue caused by impact was supported histologically. Results showed decreased GAG staining for High impact, especially in areas of surface delamination and fissuring. Though doxycycline was seen to reduce the amount of GAG loss when assayed quantitatively, this was not identifiable by the qualitative nature of histology. Indeed, one study has shown that a histological proteoglycan loss score could not distinguish impacted specimens from controls (Huser and Davies, 2006). Considering that the effects of impact are likely greater than the effects of doxycycline treatment, it is not surprising that a reduction in GAG release due to doxycycline is not observed histologically. However, this contrasts with work done in a rat model of OA that found increased GAG staining in the doxycycline treated group following chemical insult with iodoacetate (Cylwik et al., 2004). The discrepancy is likely due to use of mechanical impact in our study and a different animal model.

Similar to the current study, other investigators have tested the effects of MMP inhibitors on GAG release from articular cartilage. One study utilized iodoacetate to stimulate cartilage damage in rats, and found MMP inhibitors effectively reduced cartilage damage (Janusz et al., 2001). Another study initiated cartilage degeneration by activating latent MMPs with 4-aminophenylmercuric acetate (Bonassar et al., 1996). In that study, two MMP inhibitors were found to inhibit proteoglycan loss, one of them up to 95%. The study also found maintenance of the tissue's streaming potential, electrokinetic coupling coefficient, dynamic stiffness, and equilibrium modulus. In contrast with the present study, though tissue stiffness decreased significantly following impact, we did not observe recovery of tissue biomechanical properties with doxycycline treatment. An explanation for this observation is that the early GAG loss following injury, presumably due to immediate mechanical disruption of the tissue's collagen matrix and, therefore, not preventable by doxycycline, is enough to cause a decrease in tissue stiffness. Though GAG is considered the prominent determinant of tissue stiffness, organization of the collagen matrix has been shown to play a prominent role as well (Kovach and Athanasiou, 1997). Decreased tissue stiffness immediately following impact has previously been shown in our system (Scott and Athanasiou, 2006), and tissue stiffness would not be expected to recover until a repair response has occurred. Further, at 2 weeks a significant increase in tissue permeability in High impacted explants was found. Increased tissue permeability is a hallmark of OA (Hasler et al., 1999), and a 1.3-fold increase has also been reported in a rabbit model of traumatic cartilage injury beginning 7.5 months post-injury, increasing to 2.1-fold by 36 months (Ewers et al., 2002).

While neither 50 nor 100 μM doxycycline may be the optimal concentration, it is noteworthy that 100 μM doxycycline effectively decreased GAG release, while the 50 μM concentration did not. Concentrations used in this study were carefully chosen from values reported previously in literature and pilot studies, though choosing doxycycline concentrations was complicated by using tissue explants, since previous studies used cells or animal models. Shlopov et al. (1999, 2001) utilized concentrations of $\sim$2, 4, and 100 μM when working with OA cells in one study, as well as concentrations of $\sim$20 and 50 μM in another study with healthy chondrocytes. In work to determine the kinetics of doxycycline inhibition of various MMPs, concentrations up to 90 μM were utilized (Smith et al., 1999). Though 100 μM is higher than what is achieved in the tissue following intravenous administration (measured as $\sim$10.5 μg doxycycline per g of cartilage (Legler and Schwemmle, 1975)),

100 μM doxycycline could be delivered via intra-articular injection. Choosing an ideal doxycycline concentration and treatment regimen are just some components of any future clinical application, but we acknowledge that this study has constraints that limit extrapolation to the clinical setting. First, and perhaps most important, is the fact that this study utilized a bovine model. Doxycycline could have other effects in human tissue. Additionally, there are other components of cartilage breakdown that need to be further assessed, such as the effects of doxycycline on collagen release and non-MMP matrix degrading enzymes. Nonetheless, the positive results of this study, namely 100 μM doxycycline decreasing GAG release, indicate future study would be productive.

In conclusion, we found that the mechanical damage of impact loading causes significant tissue disruption, loss of GAG from the ECM, and decreased tissue stiffness. While Low impact did not cause gross surface damage or release as much GAG initially, it became equivalent to the High level of impact at 1 week in terms of tissue stiffness and GAG release. This result indicates that an impact not causing immediate grossly observable damage can result in enough damage at the molecular level to be similar to a High level of impact after 1 week. Though tissue stiffness was not preserved, the data in this study support the hypothesis that doxycycline can mitigate GAG loss following cartilage impact injury. Further, it was interesting that 1 week of doxycycline treatment in a 2-week culture was equivalent to 2 weeks of continuous treatment, suggesting a post-injury treatment regimen may not need to be long. However, further exploration must be undertaken to fully elucidate doxycycline's beneficial effects, and efforts should be made to optimize doxycycline delivery and dosage. Additional study into the use of doxycycline combined with other chondroprotective treatments, such as those that initiate growth and repair mechanisms or specifically target other matrix degrading enzymes, would also be of interest.

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