P188 Reduces Cell Death and IGF-I Reduces GAG Release Following Single-Impact Loading of Articular Cartilage

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Prior joint injury predisposes an individual to developing post-traumatic osteoarthritis, for which there is presently no disease modifying treatment. In this condition, articular cartilage degenerates due to cell death and matrix breakdown, resulting in tissue with diminished biomechanical function. P188, a nonionic surfactant, and the growth factor IGF-I have been shown to decrease cell death. Additionally, IGF-I is known to have beneficial effects on cartilage matrix. The objective of this study was to determine the efficacy of P188, IGF-I, and their combination following articular cartilage impact injury with two energy levels, 1.1 J ("low") and 2.8 J ("high"), at 24 h and 1 week. Bovine articular cartilage with attached underlying bone was impacted at the low or high level. Impact sites were explanted and examined immediately, or cultured for 24 h or 1 week in serum-free media supplemented with P188 (8 mg/ml), IGF-I (100 ng/ml), or their combination. Gross morphology, cell viability, GAG release to the media, and tissue mechanical properties were assessed. Immediately postimpact, high level impacted tissue had significantly increased gross morphology scores, indicating tissue damage, which were maintained over 1 week. Gross scores following low impact were initially similar to nonimpacted controls, but, at 24 h and 1 week, low impact gross scores significantly increased compared to nonimpacted controls. Additionally, at 24 h, high impact resulted in increased cell death, and both low and high impacts had increased GAG release compared to nonimpacted controls. Furthermore, high impact caused decreased tissue stiffness at 24 h that appeared to worsen over 1 week, evident by the percent decrease from nonimpacted controls increasing from 16% to 26%. No treatment type studied mitigated this loss. The combination did not perform better than either individual treatment; however, following low impact at 1 week, P188 reduced cell death by 75% compared to no treatment and IGF-I decreased GAG release from the tissue by 49%. In conclusion, high impact resulted in immediate tissue changes that worsened over 1 week. Though not causing immediate changes, low impact also resulted in tissue degeneration evident by 24 h. No treatment studied was effective at 24 h, but by 1 week P188 and IGF-I ameliorated established detrimental changes occurring in articular cartilage postimpact. However, further work is needed to optimize treatment strategies to prevent and/or reverse cell death and matrix destruction in a way that maintains tissue mechanical properties, and hence its functionality. [DOI: 10.1115/1.2939368]

Keywords: post-traumatic osteoarthritis, mechanical impact, articular cartilage, P188 treatment, IGF-I treatment, biomechanics, cell death, glycosaminoglycan

Introduction

Osteoarthritis (OA) is the most common form of arthritis, affecting more than 20 million Americans [1,2]. As the population ages, the incidence and economic burden of this disease will increase; however, there is currently no disease modifying treatment. There are several risk factors for OA, with prior joint injury resulting in an increased risk of developing post-traumatic OA [3]. With respect to the articular cartilage in a joint, characteristic features of OA include cell death [4], loss of proteoglycans [5], and softening of the tissue [6]. These three characteristics, in addition to others, have been reproduced in both *in vivo* and *in vitro* models of cartilage impact [7–9].

There has been a wide range of *in vivo* and *in vitro* studies of articular cartilage impact correlating increased cell death with increasing impact energy [10,11], increasing stress or strain [12–14], and the rate of load application [15–18]. Moreover, the various models of cartilage mechanical injury employed in these studies have demonstrated glycosaminoglycan (GAG) loss from the tissue and decreased proteoglycan synthesis [10,14,15,19]. While the majority of explant models use unconfined compression of chondral explants (without underlying bone) following preequilibration, it has been shown that matrix damage and chondrocyte death are increased by these methods compared to injury in the presence of underlying bone without an equilibration period [11,20–22]. Finally, these degenerative changes result in a loss of tissue integrity, represented by decreased stiffness and increased permeability [16,23,24].

While investigations into treatments for established OA have been occurring, treatment of impact injuries is just beginning. One such treatment possibility is Poloxamer 188 (P188). P188 is an ~8.4 kDa triblock copolymer containing both hydrophobic and

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hydrophilic regions. It has been used to protect neurons and skeletal myocytes from necrosis following various types of insult [25,26]. The accepted mechanism of action is selective insertion into damaged cell membranes [26,27], thereby preventing ionic imbalance and loss of important cellular content. Haut and coworkers [28–30] have shown promising results with P188 following injurious unconfined compression of cartilage explants and in an *in vivo* study of rabbit patellofemoral joint trauma. In one study [29], P188 was delivered at 8 mg/ml following a peak load of 25 MPa, and cell viability was examined at 1 h and 24 h. Compared to injured untreated controls, an increased percentage of viable cells in the superficial zone at 1 h, and an increased percentage in all zones at 24 h, was found. In another study [28], though only treating for the first 24 h, percent cell death remained decreased at 4 days and 7 days postinjury compared to no treatment. These studies underscore the utility of P188 in mitigating cell death following articular cartilage injury.

Another potential intervention is insulin-like growth factor-I (IGF-I). Exogenous IGF-I has been shown to enhance chondrogenesis in cartilage defects and promote further IGF-I transcription [31,32]. In cartilage explants, it has been shown that IGF-I increases matrix synthesis, an action further enhanced when combined with dynamic compression. Dynamic compression also resulted in faster IGF-I transport into the tissue [33,34]. Furthermore, it has been shown that IGF-I maintains the physical properties of cartilage explants over 3 weeks in culture [35]. In terms of mechanical injury, D'Lima et al. [36] applied a 500 ms static load of 23 MPa to bovine cartilage explants followed by treatment with 50 ng/ml of IGF-I. A significant decrease in apoptosis was observed in the loaded plus treatment group compared to the loaded untreated group.

Based on work mentioned above, the use of P188 and IGF-I to prevent cell death and of IGF-I to increase matrix synthesis and preserve tissue mechanical properties warrants further examination. Moreover, there are presently no data for the effects of these treatments on GAG loss from the tissue, the tissue's biomechanical properties, or the two agents' effects in combination following impact loading. In this study, we hypothesized that the combination of P188 and IGF-I would reduce cell death postimpact in a synergistic or additive manner. In addition, the combination was expected to maintain matrix integrity, thereby preventing and/or reversing loss of mechanical properties and allowing cartilage to maintain normal function. Hence, the effects of P188, IGF-I, and their combination were examined following articular cartilage injury with two levels of impact loading at 24 h and 1 week.

Materials and Methods

Articular Cartilage Tissue Harvest. A total of 51 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. Beneath the articular cartilage surface, approximately 1.5 cm of bone was left in place to more realistically capture the *in vivo* situation compared to a thin layer of bone. 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 [37]. Briefly, the impact mass was raised to the specified height and dropped onto the impact interface, which connects to a 5 mm diameter, nonporous, flat-ended, rigid, cylindrical impact tip. Two levels of impact were employed. A "low" impact (6 cm drop height with an 18.4 N tup) and a "high" impact (10 cm, 27.8 N tup), delivering 1.1 J and 2.8 J of energy, respectively. Each articular surface, still with subchondral bone attached, was impacted four times—twice at the low level and twice at the high level—in distinct locations that were separated by at least 5 mm. Impact locations were randomized across all groups studied. These levels were chosen based on previous work in our laboratory [37] such that the low level of impact does not cause immediate grossly identifiable surface damage, whereas the high level repeatedly does. Of note, injury to underlying bone resulting from impact was not assessed due to this study's objective of analyzing the effects of the interventions on cartilage tissue alone.

Explant and Culture of Articular Cartilage. Following impact, 5 mm diameter full thickness articular cartilage explants were removed from the subchondral bone using a sterile dermal biopsy punch and a No. 10 scalpel blade. Explants were placed directly into six-well, non-tissue culture treated plastic (TCP) plates for culture. In addition to the four impacted explants, two 5 mm nonimpacted 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 the eight treatment groups. Groups 1–4 were the following: (1) culture media alone, (2) culture media supplemented with 8 mg/ml P188 (Sigma, MO), (3) culture media supplemented with 100 ng/ml IGF-I (Peprotech, NJ), or (4) culture media plus combination of P188 (8 mg/ml) and IGF-I (100 mg/ml). Groups 5–8 were identical to the aforementioned, except they were subjected to ten manual compressions of ~1 MPa at 0.1 Hz (cycle=2 s of load followed by 8 s of recovery) using a custom made stainless-steel nonporous platen every time the culture medium was changed. The motivation for compression was that all prior *in vitro* work with P188 on cartilage explants suggests this compression protocol would be required to see effects of P188 [28,29]. Of note, this protocol is not intended to reflect, or to have the effects, of dynamic compression protocols used to study compression aided delivery of nutrients, growth factors, etc., into articular cartilage.

Explants were cultured in 3 ml of the above formulations, with the culture media defined as serum-free Dulbecco's modified eagle medium (DMEM) with Glutamax™ (Invitrogen, NY) containing 100 units/ml penicillin (Biowhittaker, MD), 100 μg/ml Streptomycin (Biowhittaker), 2.5 μg/ml Fungizone (Biowhittaker), 0.1 mM nonessential amino acids (Invitrogen), and 50 μg/ml ascorbic acid. Explants were cultured for either 24 h or 1 week. For those explants cultured for 1 week, culture medium was replaced every 48 h. Treatment groups had P188, IGF-I, or the combination added fresh with each media change. All culture medium used in this study was collected and stored at −20°C for later use in GAG release quantification. In addition to explants cultured for 24 h and 1 week, another group of explants consisting of nonimpacted controls, low impact, and high impact specimens was examined immediately following injury for gross morphology, cell viability, and biomechanical properties.

Explant Processing. After either 24 h or 1 week, explants were removed from culture and processed for gross morphology, cell viability, and biomechanics testing. Briefly, a 3 mm diameter punch was removed from the center of the 5 mm explant using a dermal biopsy punch. This piece of tissue was placed directly into a 2% solution of India ink (Higgins Violet; Eberhard Faber, Inc., Lewisburg, TN) in PBS and allowed to sit for at least 5 min, after which time no increased staining was observed. The remaining ring of the explant was cut perpendicular to the articular surface to generate several surfaces for confocal viability imaging. These tissue pieces were placed into PBS containing 0.5 μl calcein-AM and 2 μl ethidium homodimer-1 (Live/Dead® assay; Molecular Probes, Eugene, OR) per ml of PBS.

Gross Morphology and Cell Viability. Following India ink staining, images of all explants were collected and randomized before being rated by seven blinded, independent observers. The gross morphology rating scale is shown in Table 1, which is modi-

Table 1 Gross morphology grading scale

Tissue Morphology	
Healthy, no damage	0
Some tissue damage	1
Extensive tissue damage	2
Complete tissue destruction	3
India ink staining	
Homogeneous	0
Some staining heterogeneity	1
Moderate staining heterogeneity	2
Vast staining heterogeneity	3
Surface regularity	
Smooth	0
Small area of surface irregular	1
Moderate area of surface irregular	2
Most of surface irregular	3
Total	9 (max)

fied from a previously used version [37]. Each category was summed to obtain a total score, which was then averaged over the seven raters. Viability images were obtained at 10× magnification using a Zeiss LSM 510 confocal microscope (Carl Zeiss Microscopes, Jena, Germany). Since the sectioning process resulted in some cell death on the sectioned surface, care was taken to image at a tissue depth of ~50 μm. Finally, percent viability was measured over a square area defined by the full thickness of the explant using the "threshold" and "analyze particle" functions of ImageJ (National Institutes of Health, MD). Dead cells were able to be distinguished by number of pixels based on the color the cell stained. Red (indicating a dead cell) was associated with fewer pixels due to ethidium homodimer-1 staining only the nucleus. Conversely, green (indicating a live cell) was associated with more pixels since calcein-AM stains the entire cytoplasm. For calibration, all dead controls, made by incubating cartilage in EtOH for 20 min, were used to determine the upper limit of dead cell size. Particles above this size were counted as alive. Some out-of-plane live cells, having a lower pixel count, were counted as dead. This was corrected for by scaling the counted live cells by 1.16, with the scale factor being determined through analysis of images displaying only live cells. Percent cell death was then calculated as $100\times[1-(\text{live cells}/\text{total cells})]$.

GAG Release to Media. A 1,9-dimethyl-methylene blue (DMMB) colorimetric assay was used to determine the amount of GAG released to the media (Blyscan Sulfated GAG Assay, Accurate Chemical and Scientific Corp., New York). Chondroitin 4-sulfate was used for the standard. The total quantity of GAG released was normalized to tissue volume, which, if in the presence of tissue swelling, represents a lower limit. Tissue volume was calculated knowing the tissue surface area (5 mm diameter) and thickness, as measured upon biomechanical testing (vida infra).

Creep Indentation Biomechanical Testing. Following photography for gross morphology, the 3 mm diam, India ink stained tissue 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 mechanical testing. Prior to testing, samples were thawed for at least 1 h at room temperature in PBS with protease inhibitor solution and then 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 [38]. 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 semianalytic, seminumeric model [39], which needs only the last 30% of the deformation history and no recovery data, was used to determine the linear biphasic properties (aggregate modulus, permeability, and Poisson's ratio) of the tissue from the time-displacement curves. Tissue thickness was measured across the entire 3 mm specimen using digital calipers.

Statistical Analysis. The study consisted of a balanced, full-factorial experimental design. Each time point was conducted and analyzed as an independent experiment because separate controls from distinct pools of animals were used to decrease the effect of interanimal variability. Immediately postimpact, a one-way analysis of variance (ANOVA) was performed with impact level as the predictor variable on gross morphology, cell viability, and biomechanical properties. At both 24 h and 1 week, a three-factor ANOVA (impact level, treatment type, and delivery method) was performed on the gross morphology, GAG release, cell viability, and biomechanical property data. If a factor was found to be significant ($p<0.05$), a Tukey HSD post-hoc test was performed to compare among factor levels. Predominantly, $n=6$ was used for all groups and assays in this study, where a group is a combination of impact, treatment, and delivery method. However, some groups resulted in an $n=5$ due to the sample being of too poor quality for the assay or being identified as an outlier via the method of studentized deleted residuals [40].

Results

Compression Protocol. The compression protocol used was only a significant factor for Poisson's ratios measured at 1 week ($p=0.003$) and 24 h gross morphology scores ($p=0.003$). Compression resulted in increased Poisson's ratios and decreased total gross scores. The compression protocol did not significantly affect GAG loss to the media, the tissue's aggregate modulus or permeability, and, contrary to our expectation, cell viability. Since compression was principally not a significant factor, the remainder of the results is presented in terms of impact level and treatment type.

Gross Morphology. Figures 1(*a*), 2(*a*), and 3(*a*) show the results for gross morphology scoring immediately following impact, 24 h after impact, and 1 week after impact, respectively. Immediately following impact gross morphology showed that high impact resulted in significantly increased scoring, indicative of greater damage, compared to low impact and nonimpacted controls. Low impact and controls had similar gross scores (Fig. 1(*a*)). Figure 1(*d*) shows representative gross morphology pictures. Note the appearance of tissue damage, staining heterogeneity, and surface irregularity resulting from high impact, while low impact specimens appear grossly identical to control specimens.

In contrast, 24 h after impact, gross morphology scoring showed that low impact had significantly increased scores compared to nonimpacted controls. High impact scoring continued to remain significantly increased over both nonimpacted controls and low impact (Fig. 2(*a*)). This same relationship was also observed at 1 week (Fig. 3(*a*)). Gross scores for nonimpacted controls and high impact remained relatively constant over the course of the study, whereas the scores for low impact increased. Treatment type was not a significant factor in gross morphology scoring.

GAG Release to Media. GAG release from explants was significantly affected at 24 h and 1 week by both impact level ($p=0.0004$, 24 h; $p<0.0001$, 1 week) and treatment type ($p=0.02$, 24 h; $p=0.0002$, 1 week). At 24 h, both low and high impacts demonstrated increased GAG release compared to nonimpacted controls. Also, P188 treatment resulted in significantly increased

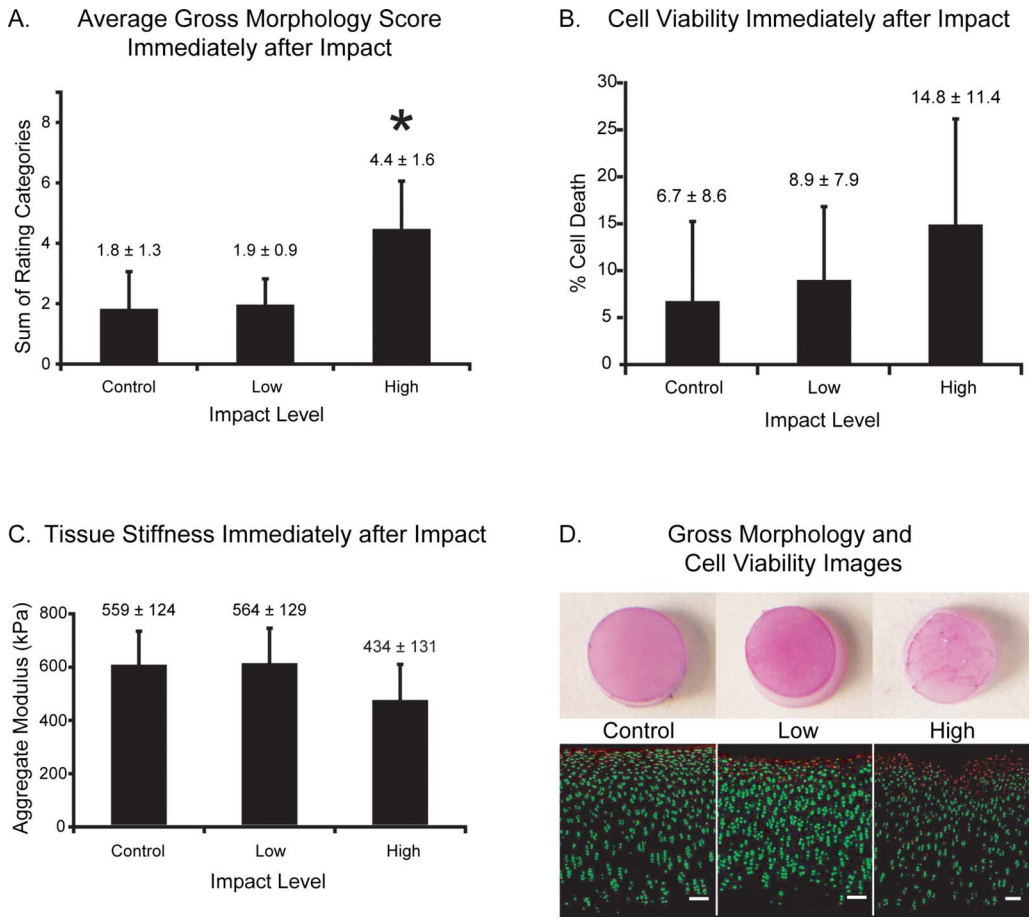

Fig. 1 Response of articular cartilage immediately postinjury. (*a*) Gross morphology scores. * denotes high impact significantly different from nonimpacted control and low impact groups ($p<0.05$). (*b*) Cell viability. (*c*) Tissue stiffness (aggregate modulus). (*d*) Representative gross morphology and cell viability images obtained in this study. Each bar represents mean±SD for $n=5-6$.

GAG loss to the media compared to IGF-I treatment, though neither was significantly different from no treatment or the combination (Fig. 2(*d*)).

Similar to 24 h, at 1 week both low and high impacted specimens had increased GAG loss compared to nonimpacted controls. Further, IGF-I treatment significantly decreased GAG loss compared to all other treatment types. Comparing the low impact, IGF-I treatment group to low impact, no treatment revealed a 49% reduction in GAG release. For both nonimpact controls and high impact, comparing no treatment to IGF-I treatment showed an 18% reduction. In addition, for the groups shown in Figs. 2(*d*) and 3(*d*), the average GAG released in the first 24 h was 34% of the total released over 1 week, with a range from 26% (low impact, no treatment) to 47% (low impact, IGF-I treatment).

Cell Viability. Figure 1(*d*) shows representative confocal images. Immediately after impact, there was little to no cell death in nonimpacted controls, whereas cell death was evident in the superficial zone of low and high impacted specimens. Though not significant, cell death following high impact was over 1.6 times greater than either low impact or nonimpacted controls (Fig. 1(*b*)). However, by 24 h postinjury, impact level significantly affected percent cell death ($p<0.0001$). Post-hoc analysis showed that high impact had significantly more cell death compared to low impact and nonimpacted controls. Treatment type was not significant (Fig. 2(*b*)).

At 1 week, both impact level and treatment type significantly affected cell viability ($p=0.0009$ and 0.03, respectively; Fig. 3(*b*)). Different from 24 h postimpact, post-hoc analysis showed that both low and high impacted specimens had increased cell death compared to nonimpacted controls. Moreover, P188 treatment significantly reduced percent cell death compared to no treatment. This was most evident in the 75% reduction in cell death for low impact, P188 treatment compared to low impact, no treatment. Reductions compared to no treatment were not as dramatic for nonimpacted controls and high impact specimens treated with P188, measuring 66% and 36%, respectively. Treatment with IGF-I or the combination was similar to both the P188 and no treatment groups.

Biomechanical Properties. Figures 1(*c*), 2(*c*), and 3(*c*) show the data for the tissue's aggregate modulus measured by creep indentation immediately postimpact, 24 h after impact, and 1 week after impact, respectively. Immediately following impact, there was a 22% loss of aggregate modulus in high impact specimens from nonimpacted controls, but this change was not significant. However, by 24 h, impact level significantly affected the aggregate modulus ($p=0.02$), with high impacted tissue being significantly softer in compression compared to low impact and nonimpacted controls. Impact level remained significant at 1 week ($p=0.003$); however, while high impact remained significantly different from nonimpacted controls, the aggregate moduli of low impact samples were now indistinguishable from high impact. Treatment type was not a significant factor at 24 h or 1 week.

Tissue permeability and Poisson's ratios are reported in Table 2. Tissue permeability was not significantly affected by impact level or treatment type immediately following impact or at 1 week

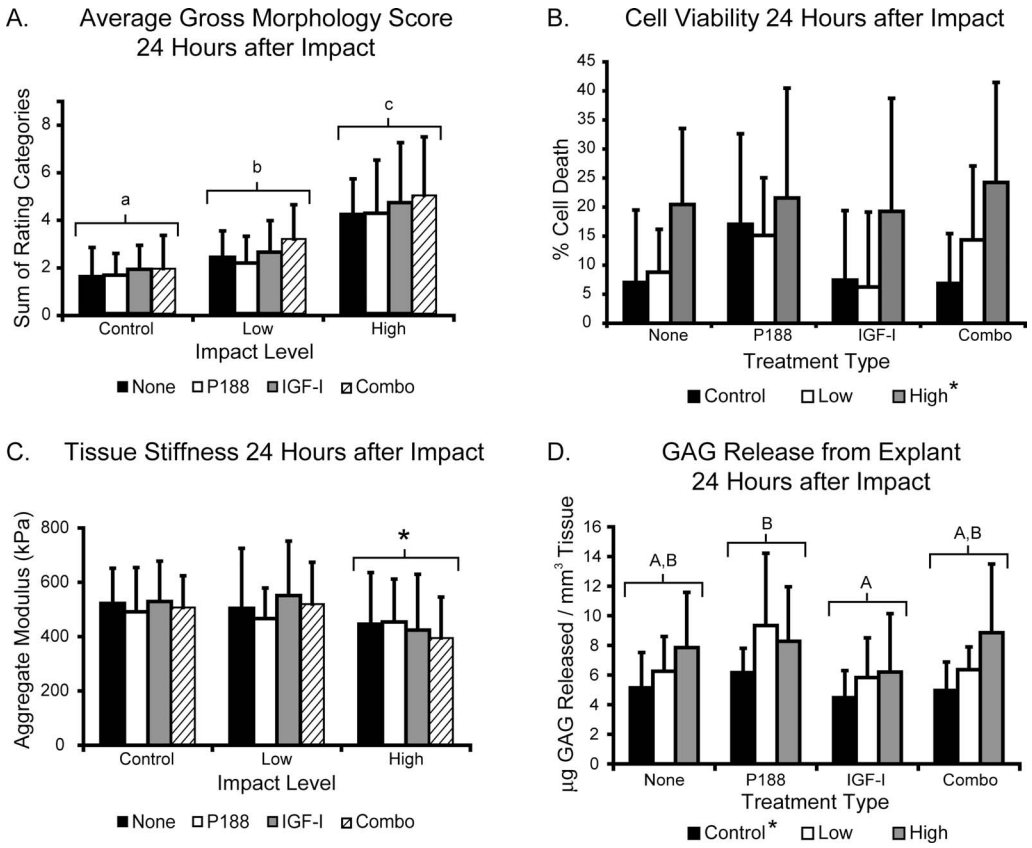

Fig. 2 Response of articular cartilage 24 h postinjury. (*a*) Gross morphology scores. (*b*) Cell viability. (*c*) Tissue stiffness (aggregate modulus). (*d*) GAG release. Within a panel, groups not connected by the same letter are significantly different from one another (lower case reserved for impact level and upper case for treatment type); * denotes significant difference from all other levels of the same factor ($p<0.05$). Each bar represents mean±SD for $n=10-12$.

postimpact. However, at the 24 h time point, treatment type was a significant factor ($p=0.02$), with IGF-I treatment resulting in an increased permeability compared to all other treatment types. Impact level was not a significant factor at 24 h. Poisson's ratios were unaffected by impact level or treatment type at any time point. Finally, it should be noted that the results reported here assume ideal test geometry. While nonimpacted controls and low impact explants fit this geometry well, high impact specimens do not, due to the presence of surface cracks. Thus, the reported values for high impact represent an upper limit for tissue stiffness and lower limit for permeability.

Discussion

The objective of this study was to determine the effects of P188 and IGF-I on gross morphology, cell viability, GAG release, and tissue biphasic material properties following mechanical impact of articular cartilage. At 1 week postinjury, 8 mg/ml P188 treatment reduced percent cell death by 75% and 36% following low (1.1 J) and high (2.8 J) impacts, while 100 ng/ml IGF-I treatment reduced GAG release from the explant by 49% and 18%, respectively. Despite the individual usefulness of P188 and IGF-I, synergy was not observed. The use of these agents in combination resulted in them losing their effects and becoming similar to no treatment. Several possible explanations for this observation are that the concentrations and dosing times used in this study are not yet optimized for use together, simultaneous presence of the two species reduces each other's activities, and P188 may interfere with IGF-I signaling, rendering it ineffective in the presence of P188. Nevertheless, the results of this study show that detrimental changes in cell viability and GAG release occurring after impact can be lessened with targeted bioactive agents.

The gross morphology scale used in this study is a good indicator of articular cartilage damage and captured the effects of impact level and time postinjury. Supporting this claim, the scale correlated with most parameters assayed at both time points; however, low R^2 values (range 0.04–0.20) suggest that there are other important factors. Though the scale was modified, the results of the present study agree with previous work done in our laboratory in that high impact resulted in immediately increased gross scores compared to nonimpacted controls and low impact specimens [37]. Gross morphology has been assessed in other studies of cartilage mechanical injury. Prior work using injurious compression found that increasing load rate from 40 MPa/s to ~900 MPa/s was associated with greater surface fissuring [15], which was reduced when underlying bone was present [20]. In this study, fissuring observed following high impact (2.8 J) was qualitatively less than the images published in the aforementioned studies, consistent with the presence of underlying bone. Other studies have also shown that impact of cartilage removed from subchondral bone results in surface fissuring [41], and Borrelli et al. [42] have shown that cartilage can tolerate injurious loads up to half the joint fracture threshold when underlying bone is present. Further, following injurious compression, increased cell death was noted in the superficial zone [22], as was also found in this study. Another articular cartilage surface damage score has captured the effect of impact, but that score was based on histological evaluation [10]. The scoring method of the current study presents an

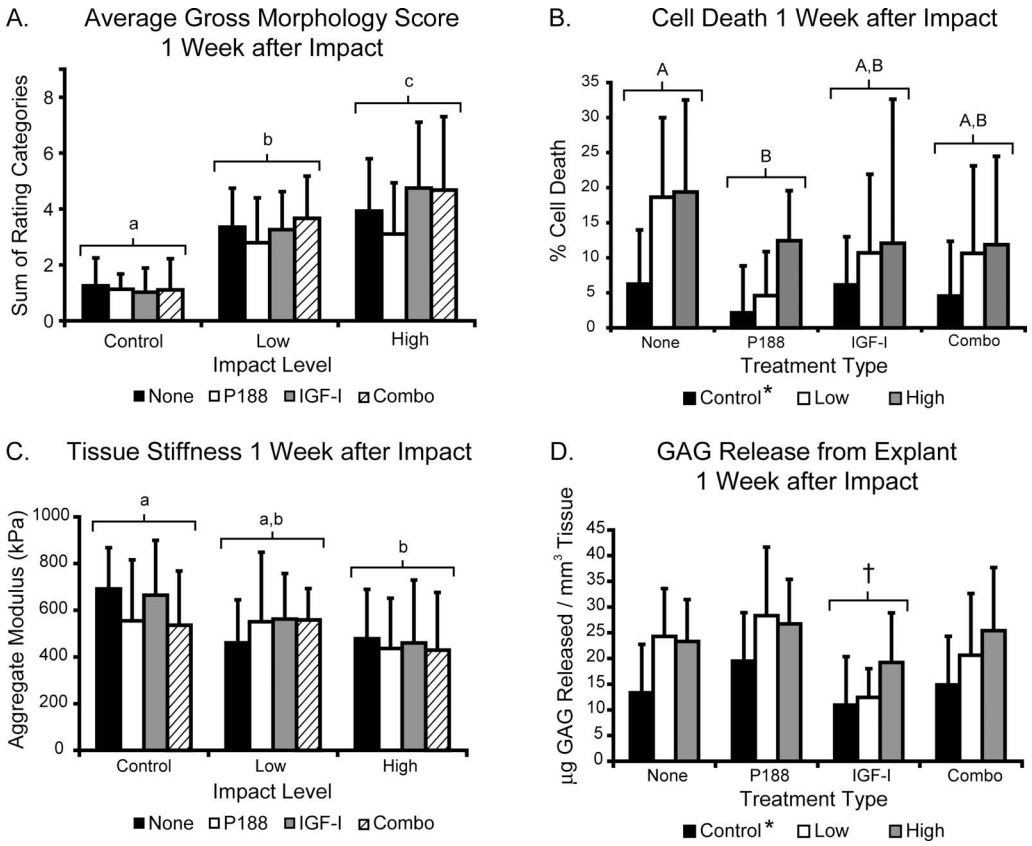

Fig. 3 Response of articular cartilage 1 week postinjury. (*a*) Gross morphology scores. (*b*) Cell viability. (*c*) Tissue stiffness (aggregate modulus). (*d*) GAG release. Within a panel, groups not connected by the same letter are significantly different from one another (lower case reserved for impact level and upper case for treatment type); * and † denote significant difference from all other levels of the same factor ($p<0.05$). Each bar represents mean±SD for $n=10-12$.

improvement, if nothing more than in ease of tissue processing, over the histology-based scale. Further, the scale used in this study was able to capture temporal changes in gross morphology, evident by the increasing scores for low impact (1.1 J), and correlated with a quantitative measure of tissue stiffness.

P188 reduced cell death at 1 week compared to no treatment, but it did not do so at the 24 h time point. This result is not consistent with previous work, which showed that P188 reduced cell death in all zones at 24 h [29]. A possible explanation for the discrepancy is the different loading protocols used, such that the presence of surrounding tissue and underlying bone in the present study provided constraint of the impacted region that protected cells from death more so than in an unconfined compression protocol. Bone likely protects cells from overt necrosis at the moment of injury [42], but the stresses, strains, fluid shear, pore pressure, etc., generated in the tissue during impact may still initiate mechanotransductive pathways leading to apoptosis, perhaps explaining the continued death during culture. Indeed, bone has been shown to spare chondrocytes from death after mechanical loading [20,21], such that ten times the amount of energy is needed to produce similar changes in cartilage when bone is present [11]. In this respect, our results are in good agreement with studies reporting energy as the impact metric [10,11]. For example, a 0.28 J impact would yield 12.24% cell death according to the linear regression in Ref. [11] compared to the 14.84% for the 2.8 J impact seen in the present study immediately postinjury. Notably, compression was not necessary in this study for P188 to be effective, in contrast with two previous studies suggesting it was [28,29]. It is possible that the compression protocol used in this study was different, as the compression frequency was not described in the prior studies. Using that protocol may have furthered the beneficial effects we observed with P188.

Though the current accepted view is that P188 prevents necrosis by repairing damaged cell membranes, data from this study, along with the observation that apoptosis may be occurring at later time points, suggest that P188 may have additional mechanisms of action in chondrocytes. Indeed, work on mechanical trauma to neurons has established P188 reduces apoptosis in that cell type [43]. It has been suggested that cell death by necrosis following impact does not occur past one day [15,28,44], but apoptosis postinjury has been observed to increase with time over seven days in culture [28,45]. In fact, several studies using very different loading protocols have shown that treatment with caspase inhibitors following mechanical injury effectively reduces apoptotic cell death [36,45–47]. In contrast to our results, these studies also found a decrease in GAG release associated with increased cell viability. Moreover, P188 has been previously shown to maintain its efficacy for 1 week, yielding a 46% reduction in DNA fragmentation compared to no treatment when treatment was applied for only the first 24 h [28]. In the current study, there were 75% and 36% reductions in percent cell death at 1 week for low and high impacts, respectively. Compared to the 46% reduction seen in prior work, an explanation for the increased efficacy seen in this study following low impact is that treatment was continuous over the culture period; however, formal proof of this would require comparison of treatment duration following identical injurious loading protocols. On the other hand, less necrosis may lead to decreased apoptosis by preventing the release of apoptosis ini-

Table 2 Tissue permeability and Poisson's ratio as measured by creep indentation. For the immediate time point, values are mean±SD for $n=5-6$. At 24 h and 1 week, values are mean±SD for $n=10-12$. Separate statistical analyses were performed at each time point.

Time point	Treatment type	Impact level	Permeability ($m^4/N\,s \times 10^{-15}$)	Poisson's ratio
Immediate		Control	4.4 ± 1.3	0.035 ± 0.086
		Low	6.0 ± 3.1	0.032 ± 0.070
		High	3.7 ± 2.0	0.074 ± 0.089
24 h after impact	None	Control	2.9 ± 1.8	0.024 ± 0.048
		Low	3.1 ± 2.9	0.055 ± 0.080
		High	3.1 ± 2.1	0.050 ± 0.077
	P188	Control	3.4 ± 1.9	0.026 ± 0.051
		Low	3.5 ± 2.1	0.092 ± 0.095
		High	3.7 ± 2.1	0.092 ± 0.079
	IGF-I[a]	Control	4.6 ± 3.3	0.096 ± 0.095
		Low	4.0 ± 2.3	0.059 ± 0.092
		High	4.8 ± 3.6	0.091 ± 0.091
	Combo	Control	2.4 ± 1.3	0.090 ± 0.115
		Low	4.0 ± 2.3	0.037 ± 0.069
		High	3.6 ± 2.1	0.083 ± 0.094
1 week after impact	None	Control	3.5 ± 2.3	0.046 ± 0.070
		Low	6.3 ± 3.9	0.105 ± 0.081
		High	3.8 ± 2.6	0.120 ± 0.083
	P188	Control	3.0 ± 1.6	0.042 ± 0.057
		Low	4.6 ± 2.6	0.057 ± 0.066
		High	3.7 ± 2.5	0.085 ± 0.074
	IGF-I	Control	3.3 ± 1.9	0.109 ± 0.092
		Low	2.7 ± 2.7	0.066 ± 0.076
		High	4.6 ± 3.3	0.063 ± 0.072
	Combo	Control	3.4 ± 3.5	0.053 ± 0.071
		Low	3.4 ± 2.3	0.090 ± 0.087
		High	3.5 ± 2.7	0.068 ± 0.076

[a]Denotes significant effect ($p=0.02$) of treatment type on permeability at the 24 h time point. Treatment with IGF-I caused a significant increase in permeability compared to no treatment controls and the combination treatment, but was not significantly different than the P188 treatment.

tiators from necrotic cells, thereby preventing further cell death at later time points and explaining the observed efficacy of P188 at 1 week in this model system. Further experimentation is necessary to delineate between these two possibilities.

While a prior study with IGF-I showed it decreased apoptosis postinjury [36], we did not find it effective at preventing cell death, perhaps due to differences in the loading metrics used in our study. Loading regimens in the literature vary with respect to peak stress, time to peak stress, stress rate, and duration [8]. The present study employed impact loading to cartilage with underlying bone attached on a submillisecond time scale [37], while the previous work statically loaded chondral explants to 23 MPa over 500 ms. During impact loading, a higher proportion of the stress is borne by interstitial fluid pressurization [17,48], which may protect chondrocytes when compared to lower loading rates, wherein the solid matrix begins to share the load. With lower loading rates, interaction of cells with the extracellular matrix (ECM) may cause them to respond to the load borne by the solid matrix, triggering cellular death pathways that create a larger pool of dying chondrocytes susceptible to the actions of IGF-I. Work done by Quinn and co-workers [49,50] has shown that cell death following injurious compression is related to macroscopic cracks due to radial strain generated in the superficial zone during loading, and increasing prestrain before loading can alleviate a portion of the cell death. Thus, radial strain is one biomechanical mechanism that may describe the presence of surface fissures and increased cell death immediately following high impact in this study. Additionally, the peak stresses associated with the current study's impact levels are below 8 MPa [37]. It has been suggested that there is a threshold level of peak stress (15–20 MPa) at which cell death begins for low loading rates, 35 MPa/s [14]. Our results demonstrate that this threshold is decreased at higher loading rates, consistent with a previous proposition that peak power delivered during impact may be the important factor [16]. Determining which metrics defining mechanical impact are responsible for the initiation of cell death is an area needing continued research.

Though not effective at preventing cell death, IGF-I reduced GAG release following injury in this model system at 1 week. While IGF-I has been shown to increase GAG synthesis [34], and one would then expect GAG release to increase from the explant, we observed IGF-I to decrease GAG release. Consistent with this observation would be a mechanism by which IGF-I decreases matrix breakdown by inhibiting degradative enzymes, perhaps matrix metalloproteinase (MMPs). DiMicco et al. [51] found that a specific MMP inhibitor (CGS 27023A) was able to decrease GAG loss from articular cartilage following mechanical insult. Furthermore, IGF-I caused an increase in the tissue's permeability at 24 h compared to no treatment, which then recovered by 1 week, a finding consistent with GAG release being decreased at 1 week due to this intervention. This observation exemplifies the importance of evaluating treatment strategies over sufficient time courses in *in vitro* models. Such time course experiments have suggested that there may be optimal "therapeutic windows" [45,51].

While P188 reduced cell death and IGF-I reduced GAG release at 1 week, no treatment in this study was able to prevent and/or reverse the loss of tissue stiffness postimpact. A possible explanation of this observation is that early GAG loss following injury,

which is presumably due to mechanical disruption of the collagen matrix [14,15,51], is enough to cause a decrease in tissue stiffness. One would not then expect tissue stiffness to recover until a repair response has occurred. Decreased tissue stiffness immediately following impact has previously been shown in our system [37], though results of the present study did not reach significance. At 24 h, tissue impacted at the high level (2.8 J) had an average 16% loss in the aggregate modulus, which increased to 26% at 1 week. Due to its avascular, aneural, postmitotic nature, damaged cartilage has a notoriously deficient healing response, with the fibrocartilaginous repair tissue demonstrating inferior mechanical properties [6]. Several *in vivo* studies [23,24,52] impacting the rabbit patello-femoral joint and two *in vitro* studies [16,37] have documented decreased tissue stiffness using various material models at various times postinjury. The requirement of cartilage to function in a demanding mechanical environment necessitates evaluation of the effects of a treatment on its mechanical properties. One *in vivo* study of joint impact showed that treatment with polysulphated GAG was able to prevent the loss tissue stiffness that occurred [53]. Ultimately, it is desirable to have a treatment strategy that prevents tissue degeneration via cell death and matrix breakdown, which also maintains tissue functionality.

In conclusion, the results of this study support the ability of two treatments to mitigate well-known degenerative changes in cartilage following single-impact loading. Based on the time course evaluation, changes occurring following impact injuries at or less than 24 h may not be able to be ameliorated with these interventions, suggesting initial changes are due solely to mechanical trauma and uncoupled from the tissue's biological response. Additional studies must be performed to delineate the magnitude of cell death and matrix damage due to the mechanical insult alone from the subsequent amount resulting from biological responses. Where treatments employed in this experiment only affected the latter, it is desirable to identify agents that can promote a healing response in order to reverse the former changes. Further study with other concentrations and combinations of bioactive agents capable of preventing and/or reversing cell death and matrix breakdown is justified. Additionally, more detailed examination of the mechanisms of action of these agents would be helpful information in the campaign to understand and prevent the development of post-traumatic OA.

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