ANTI-APOPTOTIC TREATMENTS PREVENT CARTILAGE DEGRADATION AFTER ACUTE TRAUMA TO HUMAN ANKLE CARTILAGE

Cecilia Pascual Garrido¹, Arnavaz A. Hakimiyan¹, Lev Rappoport¹, Theodore R. Oegema¹,², Markus A. Wimmer¹,², and Susan Chubinskaya¹,²,³

¹Department of Biochemistry, Rush University Medical Center, Chicago, IL, 60612
²Department of Orthopedic Surgery, Rush University Medical Center, Chicago, IL, 60612
³Section of Rheumatology, Rush University Medical Center, Chicago, IL, 60612

Abstract

Objectives—To investigate the effect of anti-apoptotic agents on cartilage degradation after a single impact to ankle cartilage.

Design—Ten human normal tali were impacted with the impulse of 1 Ns generating peak forces in the range of 600 N using a 4mm diameter indenter. Eight mm cartilage plugs contained the 4mm diameter impacted core and a 4mm adjacent ring were removed and cultured with or without P188 surfactant (8mg/ml), caspase-3 (10μM), or caspase-9 (2μM) inhibitors for 48hrs. Results were assessed in the superficial and middle-deep layers immediately after injury at day 0 and at 2, 7 and 14 days after injury by live/dead cell and Tunel assays and by histology with Safranin-O/fast green staining.

Results—A single impact to human articular cartilage ex vivo resulted in cell death, cartilage degeneration, and radial progression of apoptosis to the areas immediately adjacent to the impact. The P188 was more effective in preventing cell death than the inhibitors of caspases. It reduced cell death by more than 2-fold (P<0.05) in the core and by about 30% in the ring in comparison with the impacted untreated control at all time points. P188 also prevented radial expansion of apoptosis in the ring region especially in the first 7 days post impaction (7.5% Tunel-positive cells vs. 46% in the untreated control; p<0.01). Inhibitors of caspase-3 or 9 were effective in reducing cell death in the impacted core only at early time points, but were ineffective in doing so in the ring. Mankin score was significantly improved in the P188 and caspase-3 treated groups.

Conclusions—Early intervention with the P188 and caspase-3 inhibitor may have therapeutic potential in the treatment of cartilage defects immediately after injury.
**Keywords**
cell death; apoptosis; cartilage degradation; impact; acute trauma; ankle cartilage

**Introduction**
Recent studies have shown that acute cartilage injury induces cell death that may contribute to the subsequent development of post-traumatic osteoarthritis (OA)\(^1,\)\(^2\), the treatment of which remains one of the main challenges faced by orthopedists. Poor intrinsic regenerative capacity of hyaline cartilage as well as inconsistent results with existing treatments stimulated efforts to better understand cellular responses to acute trauma in order to develop novel strategies that may protect cartilage from degradation and prevent or delay the development of secondary OA.

Normal cartilage homeostasis and structural integrity depend on chondrocytes, which account for only 5% of the total cartilage volume\(^3\). Reduced cellularity (attributed either to necrosis or apoptosis) has been shown to predispose matrix degeneration and may be associated with the etiopathogenesis of post-traumatic OA\(^4,\)\(^5\). Markedly increased level of chondrocyte apoptosis and release of proteoglycans have been reported in cartilage explants subjected to mechanical injury which resulted in progressive cartilage degradation\(^1,\)\(^5,\)\(^6\). Activation of caspases-3 and -9 was suggested as one of the main mechanisms leading to chondrocyte death by apoptosis after mechanical trauma\(^7,\)\(^8\). Therapy aimed at inhibiting apoptosis with synthetic caspase inhibitors has been proposed as a potential strategy to impede the development of post-traumatic OA\(^9\).

The amphipathic nonionic surfactant P188 has been shown to significantly prevent cell death by sealing the plasma membrane and arresting the leakage of intracellular materials and influx of calcium ions from the damaged cells. It has also been suggested that P188 should be present at the time or immediately after the injury in order to prevent cell damage\(^10\)–\(^12\).

For the current study we selected an open joint *ex vivo* model of acute injury with force characteristics that induce damage primarily to the superficial layer of cartilage. This model has been previously developed and applied by us and others *in vitro* and *in vivo*\(^13,\)\(^14\); however, it was not tested *ex vivo* on human cartilage in the intact joint when cartilage was still attached to the subchondral bone.

Our overall hypothesis is that post-traumatic OA is caused by initial death and apoptosis of chondrocytes adjacent to, and within, the injury site and the failure of surviving cells to mount a robust reparative response. The aim of the current study was to investigate if anti-apoptotic agents (a membrane stabilizing surfactant P188 and caspase-3 and -9 inhibitors) applied immediately after a single impact to human ankle cartilage will promote cell survival and reduce the development of post-traumatic cartilage degeneration.

**Material and Methods**

**Specimen Preparation**
Fresh human tali of both right and left ankle joints were removed en bloc from 10 human organ donors with no documented history of joint diseases within 24 hours of death through the Gift of Hope Organ & Tissue Donor Network (Elmhurst, IL). Each joint was graded with a modified Collins scale for morphological appearance as described\(^15\) and only normal joints of grade 0–1 were used. The mean age of donors was 78.9 (range 72–84); 6 were males and 4 females. Specimens kept moist at all times were individually potted in polymethylmethacrylate and
mounted in a custom fixture. Six 8mm circles were drawn on the surfaces of each talus with India ink (Fig.1A), thus that the main weight bearing region was covered and the circles were at least 1mm apart. Before impaction, the cartilage surface contained within each circle was centered and aligned flush to the flat front end of the 4mm diameter cylindrical indentor with rounded edges. Using a pneumatic, pressure controlled impaction device, the cartilage surface was hit once with the front of the indentor (Fig 1B). The transferred impulse was 1Ns generating forces of up to 600N within 2ms. It has been shown that the coefficient of variation in force transfer is approximately 3% (assuming constant cartilage height and quality)\(^{16}\). Immediately after the impact, full thickness 8mm cartilage plugs that consisted of impacted region (4mm diameter core) and surrounding adjacent 4mm ring (Fig.1C) were removed with a scalpel from the bone and weighted before culture. Non-impacted control cartilage was obtained from the subtalar joint. The explants were randomly assigned to 6 experimental groups: 1) non-impacted control; 2) impacted untreated control; 3) impacted explants pre-treated with P188 (Pluronic F68, Sigma-Aldrich, St Louis, MO; 8mg/ml) for 1 hour before the impact; 4) impacted explants treated with P188 for 48 hours after the impact; 5) impacted explants treated with caspase-3 inhibitor (Ac-DEVD-CHO; BIOVISION; Mountain View, CA; 10μM) for 48 hours after the impact; and 6) impacted explants treated with caspase-9 inhibitor (Ac-LEHD-CHO; BIOVISION; 2μM) for 48 hours after the impact. Cartilage explants were cultured individually in 24-well plates in 1ml culture media (Dulbecco’s modified Eagle medium supplemented with 100U/ml penicillin, 50ug/ml gentamicin, and 5% fetal bovine serum) at 37°C and 5% CO\(_2\) atmosphere. The effect of treatments was assessed at days 0 (immediately after injury), 2, 7, and 14 post-injury. The culture media were changed every 48 hours. At each time point, wet weight of the cartilage explants was measured; tissue was bisected through the center to assure that each part contains both core and ring. One half was processed for histology and Tunel stain for apoptosis; another half was used to detect cell survival by live/dead assay.

**Cell Survival**

Cell survival was measured as previously described in details\(^{17}\) using calcein AM and ethidium bromide homodimer-1 (Molecular Probes, Eugene, OR). Live cells emit a green fluorescence after metabolizing calcein by ubiquitous intracellular esterases, while ethidium bromide homodimer-1 enters cells upon the compromise of plasma membrane integrity after cell death and stains nuclear DNA red. Two regions of interest were chosen for image analysis, superficial and middle/deep zones. The superficial zone was defined as the first 100μm of the tissue from the articular surface to ensure that the zone lay within the top 10% of the articular cartilage regardless of the total thickness of the fragment. The middle/deep zone was defined as the tissue located in the lower 80%\(^{18}\). These layers were analyzed separately within the core (directly impacted area) and the ring (the area immediately adjacent to the impacted core). For a given region of interest, the percentage of live cells was manually counted as the number of live cells divided by the total number of cells (live and dead) multiplied by 100%. Quantitative analysis was performed by two independent observers in a blinded manner.

**Tunel assay for chondrocyte apoptosis**

Apoptotic cells were labeled \textit{in situ} using commercial Tunel assay (ApopTag™ Plus peroxidase detection kit, #S7101 Chemicon International, Temecula, CA). After fixation in 4% paraformaldehyde, the specimens were dehydrated, embedded in paraffin, and sectioned at 5μm. Tissue sections were then deparaffinized and incubated in proteinase K for 10 minutes (20μg/ml). The ends of the fragmented DNA in the tissue were labeled with terminal deoxynucleotidyl transferase (TdT) in the presence of digoxigenin-conjugated and unlabelled nucleotides followed by the incubation with the anti-digoxigenin antibody conjugated to a horseradish peroxidase reporter. Immunohistochemical detection of this antibody-peroxidase complex was carried out by the exposure to a chromogenic substrate diaminobenzidine and hydrogen peroxide. Slides were then counterstained with Methyl Green and mounted under a
coverglass. Light microscopy was used to determine the percentage of apoptotic cells. Brown nuclei indicated apoptotic cells and blue nuclei indicated viable cells. Four tissue sections from each sample were analyzed by two independent observers in a blinded manner to calculate an average number of apoptotic and viable cells in each area and within each cartilage zone. The regions were designated as described before considering superficial and middle/deep layers in the core and ring areas. Calculations were performed as described above.

**Histological Assessment**

Paraffin-embedded sections adjacent to those used for Tunel assay were utilized for histology with Safranin O/fast green staining\(^{19}\). Histological grading was conducted based on modified criteria originally established by Mankin et al\(^{20}\). Specimens were analyzed primarily for abnormalities in cellularity, Safranin O stain distribution, and surface fibrillation; no grades were given for cracks since they were created by impaction and their random appearance greatly depended upon characteristics of cartilage and donor’s age though forces were always applied at constant rate (Table 1). Four tissue sections from each sample were analyzed considering both core and ring area separately as described before.

**Statistical Analysis**

If data were not normally distributed, a logarithmic data transformation was performed. Prior to ANOVA, Levene’s Test for Equality of Variances was performed; \(p<0.05\) was considered statistically significant and a Student-Newman-Keuls test for pairwise comparison of subgroups was applied. A Student’s t-test was used to compare each group with the non-treated control group.

**Results**

All experiments were repeated at least four times with each treatment at each time point. In this study core and ring regions were cultured together as one piece in order to investigate whether cellular responses induced directly by injury caused changes in the areas which had not experienced the impact but were immediately adjacent to the impacted core.

**Cell Survival (Live/Dead Assay)**

The percentage of live cells in the non-impacted control remained relatively unchanged throughout the 14-day culture and constituted about 80–87% in the superficial layer and more than 90% in the middle/deep layer, (\(n=4\); Fig. 2A,a&b; Fig. 2B–D). Impaction caused significant cell death especially in the superficial layer of the core which was identified immediately after trauma, where only 1/3 of all cells remained alive. Dead cells were present on the surface of cartilage explants and around the cracks produced by injury (Fig.2A,c&d; arrows). Cell survival in the superficial layer of the non-treated damaged core continued to progressively decrease with culture (by about 40%); at day 0 it constituted (36%±19%), while at day 14 there was only 22.4%±12% (\(p<0.05\)) of viable cells (Fig.2B). Chondrocyte survival in the middle/deep layer of the core remained steady during culture (about 80%±7% from day 2 through day 14); though immediately after the impact (day 0) only 67%±14% of cells were viable (Fig. 2C). In the ring, initial cell death detected at day 0 was insignificant; but by day 2 it was considerably increased although to much lesser levels than in the core (Fig.2A,e&f; Fig.2B). With continued culture no additional cell death occurred in the ring. In the middle/deep layers, no statistical differences were detected between experimental groups (Fig. 2B). When cell survival was quantified throughout cartilage depth (Fig. 2D), the highest cell death was found in the core region with the ring being in the middle.

Amongst treatments, a 48-hour culture with P188 after injury appeared to be the most effective. Already at day 2, the percentage of dead cells was significantly reduced in the superficial layer.
of the core in comparison with the corresponding non-treated group and day 0 control (Fig. 3B). This effect was maintained throughout the entire culture despite the fact that P188 was only present initially (65%±15%, 70%±10% and 47%±15% of live cells at day 2, 7 and 14 compared with 35%±18%, 32%±20% and 22%±12% in the non-treated group; p<0.05 at all time points). Though by day 14 the percent of live cells was decreased in the presence of P188, the differences with the non-treated group still remained significant (Fig.3A,c, Fig.3B). Cell survival in the middle/deep layer of P188 treated core explants did not differ from that in the non-treated group (data not shown) with the exception of day 7, where the difference achieved significance and constituted 95%±2% of viable cells in the P188 treated group vs 80%±4% in the control (p<0.05).

Although the ring regions did not experience the impact, only about 50% of cells in the superficial layer were alive (if untreated) by day 2 in comparison to 35%±18% in the impacted core (Fig.3). Culture for 14 days did not cause further cell death in the non-treated control ring (Fig.3C). Addition of P188 immediately after the impact prevented or reduced cell death in the superficial layer of the ring (82%±9%, 75%±9% and 75%±12% of live cells respectively at day 2, 7 and 14 vs 56%±9%, 55%±20% and 53%±18% of live cells in the non-treated ring; p<0.05 at all time points; Fig.3A,b&d, Fig.3C). No significant differences in cell survival were detected in the middle/deep layer of the ring between P188 and non-treated control. One-hour pre-treatment with P188 prior to injury was not able to prevent cell death at any time point in comparison with the impacted non-treated group (data not shown).

Caspase inhibitors improved cell survival in the impacted core; however, in contrast to P188 the effect was seen only in the first 7 days of culture (Fig.3). At day 7, the percent of live cells constituted 59%±10% for caspase-3 inhibitor, 61%±16% for caspase-9 inhibitor vs 32%±20% in the non-treated control (p<0.05; Fig.3B). In the ring, caspase inhibitors were not able to enhance cell survival (Fig.3C). Comparing all treatments only P188 appeared to be the most efficient in inhibiting cell death in the superficial layer of both core and ring (ANOVA P=0.038, Kruskall Wallis <0.05; Fig.3). No significant differences in cell survival were detected in the middle/deep layer of the ring in the impacted non-treated control and thus, no significant differences were found between treatments (data not shown).

**Chondrocyte Apoptosis**

There were less than 5% of apoptotic cells detected in the non-impacted control during the entire culture. As expected, a single impact to cartilage caused cell death by apoptosis, which was observed in both superficial and deeper layers of the impacted core. In the superficial layer of the non-treated core, apoptosis was statistically increased immediately after injury (day 0) from 5%±1% in the non-impacted control to 34%±4% in the impacted core (p<0.01) and by day 14 it gradually increased to 60%±6% (Fig.4A). In the middle/deep layers of the impacted core, apoptosis at early time points (day 0 and 2) was significantly lower (6%±3% and 13% ±4% respectively) than in the superficial layer; while with culture it was elevated up to 42% ±10% by day 7, after which it reached a plateau (Fig.4B). Surprisingly, at day 0 about 25% of the cells appeared Tunel-positive in the superficial layer of the non-treated ring (Fig.4C) and this number gradually increased by day 7 (46%±2%); no additional apoptosis was observed between days 7 and 14. In the middle/deep layer of the ring, there was a statistical increase in apoptosis from 5%±3% at day 0 to 27%±11% at day 7, p<0.05.

While present in culture media, all treatments inhibited apoptosis in the core (compare day 0 and 2 time points, Fig.4A). After their removal, the number of apoptotic cells remained lower in all treatment groups in comparison with non-treated control at the corresponding time points, though significant differences were observed in P188 group throughout the culture and in caspase-3 and -9 groups between days 2 and 7 (Fig.4A). Anti-apoptotic agents also prevented expansion of cell death from the superficial down to the deeper layers of cartilage; the strongest
effect was induced by P188 (48 hours) and caspase-3 inhibitor (Fig.4A&B), where apoptosis was significantly lower at all time points (p<0.01). More importantly, P188 blocked an expansion of cell death by apoptosis to the adjacent, non-impacted ring region (Fig.4C). Its effect was evident until day 7 of ex vivo culture, despite the fact that P188 was only present for the first 48 hours. At day 7, the percent of apoptotic cells in the P188 treated rings was lower by more than 6-fold in comparison with the corresponding non-treated control (p<0.01). After day 7, the rate of apoptosis in P188 group increased suggesting perhaps that the surfactant delayed the onset of cell death by apoptosis rather than blocking apoptosis per se. No major differences were seen in the middle and deep layers of the ring (Fig.4D). Neither inhibitor of caspases was able to prevent the spread of apoptosis to the adjacent non-impacted ring (Fig.4C&D). Of note, at day 14 cartilage explants treated with caspase-9 inhibitor contained many empty lacunae suggesting that cell death occurred at earlier time points (data not shown).

Pre-treatment with P188 for 1 hour before the impact significantly reduced the amount of apoptotic cells in the superficial layer of both core and ring at days 0 and 2 (34±3% non-treated core vs. 8±1% treated core, p<0.01, and 21±2% non-treated ring vs. 8±1% treated ring at day 0; p<0.05) but was not able to maintain the effect throughout the entire culture.

**Histology**

Control, non-impacted articular cartilage had a smooth surface, normal structural organization and uniform Safranin O staining throughout a 14-day culture (Fig.5A,a&b). In contrast, impaction produced a disruption to the superficial layer with fissures, loss of chondrocytes and a decrease in Safranin O staining (Fig.5A,c&d), which progressed with culture and at day 14 Mankin score was 7.8±4 (Fig.5B) vs. 1±0.5 in the non-impacted control (p<0.05). In the adjacent ring, Mankin score also progressively increased: at day 0 it was 1±0.6 and by day 14 it reached 4.63±1.77 (p<0.05; Fig.5C)

A 48-hour treatment with P188 after an impact prevented degenerative changes in the core (Fig.5A,e&f; Fig.5B), in which Mankin score was statistically lower at every tested time point (p<0.05) in comparison with non-treated control. P188 was also able to delay cartilage degeneration in the adjacent ring up to day 7 (1±0.5 vs 3.1±1.9 in the non-treated ring; p<0.05). Caspase-3 inhibitor was more effective in blocking degenerative changes in both core and ring at all time-points than the inhibitor of caspase-9, which was unable to prevent changes in histological appearance of cartilage (Fig.5).

**Discussion**

The objective of this study was to determine if therapeutic interventions with inhibitors of necrosis, P188 surfactant, or apoptosis, caspase-3 or -9 inhibitors, could decrease cell death caused by an impact to articular cartilage and thus prevent or delay the development of cartilage degeneration and post-traumatic OA.

Here we focused on a model in which damage is limited to cartilage surface through a direct single impact of 1Ns. Such impact produces matrix disruptions and cell death but allowing cellular responses to injury to be studied over time. As the impaction was performed while the cartilage was still confined and attached to the bone it realistically mimics the in vivo load transfer and damage pattern of cartilage layers.

The findings of the present study demonstrate that a single impact load of human articular cartilage ex vivo resulted in cell death by necrosis and apoptosis. Initially, dead cells were mainly localized in the directly impacted core area. Whereas after day 7, cell death by apoptosis propagated centrifugally in to the surrounding non-impacted region, defined here as the “ring”, suggesting that cellular responses induced within the injury site advance to the unaffected...
adjacent areas and stimulate analogous signals. Similar observations were reported \textit{in vivo} in animal models\textsuperscript{1,14,23} and \textit{in vitro} in studies with cyclic impacts\textsuperscript{22}, where an impact induced a predictable progressive degeneration of articular cartilage starting from the impacted area and moving peripherally towards non-impacted articular surface.

Analyzing cell death separately within each cartilage layer, we found that the majority of these cells were localized in the superficial layer, which is consistent with previously published reports\textsuperscript{13,14,25}. The reason for a higher cell death in the superficial layer (in addition to obvious ones) may lie in structural differences between cartilage layers. The superficial zone is characterized by a higher level of cell and tissue strains on the surface and significantly lower compressive modulus, both of which make this zone more susceptible to injury than the middle or deep zones\textsuperscript{26}.

As reported previously\textsuperscript{8,14}, progressive cartilage degeneration in both core and ring was documented here histologically by the depletion of Safranin O staining, chondrocyte death and surface damage closely resembling degenerative changes observed in OA cartilage. Although cartilage degradation was initiated by the impact, it expanded in 14-day culture suggesting that this model is a suitable model for studying the mechanisms responsible for the development of post-traumatic OA.

In regards to the interventional therapy, our results showed that a 48-hour treatment after injury was sufficient to inhibit the progression of cartilage degeneration. Consequently, P188 was the most effective among tested agents in decreasing cell death and reducing cartilage destruction in the impacted explants. More importantly, it was also able to reduce (at least within the first 7 days) or delay pathological propagation from the core to the adjacent ring region. This transient effect of P188 may be explained perhaps by pharmacodynamics and mode of action of P188 in interacting with disrupted cellular membranes. It is suggested by the manufacturer that P188 inserts into the membrane holes and “seals” ruptured membrane thus preventing cell death by necrosis. Apparently by protecting membrane integrity P188 surfactant reduces not only necrosis but also delays the onset of a secondary response, apoptosis, initiated by signals from necrotic cells. This makes P188 a very attractive treatment for post-traumatic events; especially since it is not cytotoxic and is much cheaper than any other existing inhibitor of apoptosis.

The effect of P188 was the most pronounced in the superficial layer of cartilage; while no significant changes were induced in the middle and deep layers. This could be attributed to either the fact that the damage primarily affected cartilage surface or to the possible limited penetration of the surfactant into the matrix. In this study the strongest effect of P188 was observed at 48-hour post-trauma treatment, while exposures for 1 or 24 hours (before and after the impact) were ineffective. Contrary, in a closed joint injury model\textsuperscript{12} and \textit{in vitro} in a blunt trauma model\textsuperscript{10} P188 significantly increased the percentage of live cells in the superficial zone already at 1 hour after the impact and in all three zones at 24 hours after the impact suggesting that P188 could help restoring the integrity of cell membranes and protecting chondrocytes from early necrotic death. Encouraging results of \textit{in vivo} animal studies and our \textit{ex vivo} experimentations warrant extensive examination of P188 as potential agent for prevention/delay of cartilage degeneration in post-traumatic OA.

Though caspase inhibitors decreased cell death, their effect was less pronounced and was primarily limited to the impacted core. No statistical improvement was observed in the adjacent ring as well as no prevention of centrifugal progression of cell death was noticed. Distinct responses documented for the core and ring regions to the treatment with caspase inhibitors may suggest different signaling mechanisms responsible for cell death induced directly by mechanical injury vs cell death initiated by diffused signaling from necrotic chondrocytes\textsuperscript{22}.

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Alternatively, the response of chondrocytes within the ring may be delayed and may require more frequent or prolong application of these or other specific inhibitors\textsuperscript{27}. Histologically, only caspase-3 inhibitor demonstrated statistical superiority in comparison with the non-treated group. A stronger effect of caspase-3 inhibitor than caspase-9 inhibitor could be explained by specific role of each caspase in the apoptotic pathway. Caspase-3 is the main switch in the pathway and its activation is required for most responses associated with apoptosis; whereas, caspase-9 is induced by cytotoxic agents and mitochondrial stress\textsuperscript{8}.

Nonetheless, since the effect of caspase inhibitors is limited to apoptotic signaling, it may be impossible to investigate within a 14-days period of our study whether decreased cell death observed under the treatment with inhibitors would eventually result in the restoration of cartilage cracks induced by the impact or even in the delay or prevention of cartilage degeneration. It is also unlikely that a single treatment with the inhibitors is sufficient to induce a long-lasting effect keeping in mind a short-term activity of any protease inhibitor. Longer follow-up, alternative treatment schema and \textit{in vivo} studies are needed to address these questions.

In summary, to the best of our knowledge the present study is the first to assess the effect of P188 surfactant and caspase-3 and -9 inhibitors in an acute \textit{ex vivo} impact model of human cartilage injury. The results indicate that early intervention with P188 and caspase-3 inhibitor can decrease cell death and cartilage degradation after the injury; however, P188 was the only agent that was able to delay the progression of cell death and thus cartilage destruction in the adjacent to the impact but non-impacted regions. The results of this study suggest that the right combination of factors controlling cell death and cartilage catabolism with agents capable of stimulating cartilage repair have a future as novel therapeutic approaches in treatment of post-traumatic or secondary OA.

\section*{Acknowledgments}

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\section*{References}


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Figure 1.
Experimental set-up. A, Fresh human tali (cartilage with bone attached) individually potted in polymethylmethacrylate with cartilage surface aligned parallel to the front end of a 4mm diameter cylindrical impactor. Areas that will be subjected to injury are outlined by Indian ink; B, Pneumatic, pressure controlled impaction device; C, Schematic depiction of tested areas (core, ring, and control) in the polymethylmethacrylate potted human tali.
Figure 2.
Cell survival detected by Live/dead assay in the control, non-treated samples. A, Representative photomicrographs of cartilage sections at days 2 (left column) and 14 (right column) of culture. a&b, Non-impacted cartilage; c&d, Impacted core regions; e&f, Adjacent ring regions; Arrows indicate the areas of impact. Original Magnification 100×. B–D, quantitative assessment of cell survival in the superficial layer (B), middle/deep layer (C), and in the entire cartilage section (D) corresponding to photomicrographs shown in A. Day 0 means immediately after injury. (*) Represents statistical difference between impacted and non-impacted controls (t test p<0.05).
Figure 3.
Comparison of cell survival detected by Live/Dead assay between treated and non-treated impacted samples. A, Representative photomicrographs of core and ring cartilage sections shown at day 14 of culture. a & b, non-treated impacted controls; c & d, treatment with P188; e & f, treatment with caspase 3 inhibitor; g & h, treatment with caspase 9 inhibitor. Original Magnification 100×. B–C, Quantitative assessment of cell viability corresponding to photomicrographs shown in A at various time points of culture in the superficial layer of the core (B) and the ring (C). (*), represents statistical difference between treated and non-treated groups at the corresponding time points (p<0.05).
Figure 4.
Quantitative assessment of cell death by apoptosis in the superficial (A & C) and middle/deep (B & D) cartilage layers of the impacted core (A & B) and adjacent ring (C & D). Values are shown as the percentage of Tunel-positive cells; comparison is made between treatments. (*) Represents statistical difference between treated and non-treated groups at corresponding time points when p<0.01; (^) represents statistical difference when p<0.05.
Figure 5. 
Histological assessment of cartilage explants. A. Representative photomicrographs of cartilage explants stained with Safranin O/fast green taken at day 2 (left column) and day 14 of culture (right column). a&b, non-impacted control; c&d, impacted, non-treated control; e&f, explants treated with P188 for 48 hours; g&h, explants treated with caspase-3 inhibitor; i&j, explants treated with caspase-9 inhibitor. Original Magnification 40×. 
Mankin score of the core (B) and ring (C) areas of the impacted, non-treated control or cartilage explants treated with P188, caspase-3 or caspase-9 inhibitors. (*) Represents statistical difference between impacted non-treated control and treated groups at corresponding time points when p<0.05.
### Table 1
Modified Mankin Score used for histological analysis.

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| Maximum Score                     | 13    |