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The use of a non-ionic surfactant (P188) to save chondrocytes from necrosis following impact loading of chondral explants

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Abstract

While current injury criteria for the automotive industry are based on bone fracture, the majority of knee injuries suffered in collisions each year do not involve fracture of bone. Instead, clinical studies of traumatic joint injury often document early pain and development of chronic diseases, such as osteoarthritis. Previous studies suggest chronic disease can be initiated by cell death that occurs in articular cartilage during mechanical trauma to the joint. In the current investigation early necrosis of chondrocytes was investigated after blunt trauma to chondral explants. A non-ionic surfactant (P188) was explored as a potential tool for early intervention into the disease process, as this surfactant has been shown to repair damaged membranes in other cell lines. Three groups of adult bovine chondral explants were equilibrated for 48 h in culture media. Two groups were then loaded to 25 MPa in unconfined compression. Half the specimens in each group were incubated in media supplemented with 8 mg/ml P188 immediately after loading, while the other half was returned to standard media. At 1 and 24 h the percentages of live and dead cells in compressed and control groups were determined with a cell viability stain. At 1 h post-trauma, P188 incubated specimens had a significantly increased percentage of live cells in the superficial zone versus the no-P188 group. At 24 h the percentages of live cells in all three zones of the P188-treated explants were significantly greater than in the no treatment group. This study showed that P188 surfactant could help restore the integrity of cell membranes in cartilage damaged by blunt mechanical trauma. With the ability of P188 to "save" chondrocytes from early necrotic death using in vitro chondral explants, its role in prevention of a post-traumatic osteoarthritis in a diarthrodial joint should be further explored using in vivo animal models.

Keywords: Cartilage; Cell death; Necrosis; Apoptosis; Cartilage injury

Introduction

Lower extremity injuries occur in nearly 25% of automobile accidents in the US each year [2]. In the automobile industry, certification testing of new automobiles involves the measurement of lower extremity injury potential using instrumented anthropomorphic dummies. The current injury criteria are based on the force needed to fracture bone from human cadaver studies that were performed in the 70s [1]. While the knee is injured in approximately 10% of lower extremity injury cases, less than 50% of these injuries are severe and involve bone fracture [2]. The majority of knee injuries are, in fact, less severe "sub-fracture" injuries such as contusions, abrasions, and lacerations. Furthermore, clinical studies of traumatic joint injury often document early pain and development of chronic diseases, such as osteoarthritis, without the occurrence of bone fracture in a traumatized joint [13,27,31–33]. While joint injury has been associated with knee osteoarthritis, these studies often reflect a great disparity in the risk factor because of inconsistencies in study designs, different exposure definitions, and the age and geographic locations of the study populations [33].

Osteoarthritis (OA) affects more than 21 million Americans and is the leading cause of disability in the US each year [33]. Clinically the disease is characterized by joint pain and narrowing of the joint as diagnosed by radiological examination [12]. Pathologically, the disease exhibits a loss of cartilage and sclerosis of underlying subchondral bone. Histologically, fragmentation of the cartilage surface is observed in the diseased joint, together with cloning of chondrocytes and eventually

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violation of the tidemark by blood vessels. Various studies have associated the physiopathology of articular cartilage and the time coarse of OA with the existence of apoptotic chondrocytes [4,14,15,18,20]. A recent study also showed that a single impact loading on the rabbit patella-femoral joint induced significant apoptosis in retropatellar cartilage without bone fracture [7]. The same study showed that the extent of chondrocyte apoptosis increased with the intensity of the insult in in vitro experiments on adult bovine chondral explants. In contrast, canine cartilage explants subjected to low levels of cyclic compression exhibited the development of cellular necrosis progressively increasing in time, whereas apoptosis (TUNEL-positive cells) was not seen until 48 or more hours after loading [5].

Necrosis of chondrocytes produces degradative changes chronically in an in vivo animal model [30]. Cellular necrosis has also been documented, using cell viability stains, following unconfined compression impacts on chondral explants in low (1 s to peak) and high (50 ms to peak) rate of loading experiments from our laboratory [9]. The percentage of cellular death is higher in the low versus the high rate of loading experiments. In another study, using the bovine chondral explant, the percentage of necrotic cell death following severe blunt impact loading significantly increased in the first 24 h post-trauma [10]. These data suggest that a 'window of opportunity' may exist to intervene in a progressive disease process, if cartilage cell membrane damage could be controlled or repaired early after traumatic loading of the joint.

A defining feature of cellular death by necrosis is swelling, due to the injured cell being unable to maintain ionic gradients across a damaged plasma membrane. Ultimately, the necrotic cell ruptures [8]. Because of their amphiphilic properties, some mild surfactants are able to interact with the lipid bilayer of cell membranes to restore their integrity after injury from physical stress [6,28], electroporation via electrical trauma [22], and chemical and thermal stresses [17]. Poloxamer surfactants belong to a class of water-soluble multi-block copolymers that have important 'surface-active' properties. Poloxamer 188 (P188) is a tri-block copolymer having an average molecular weight of approximately 8400 daltons that is often abbreviated as POE–POP–POE with POE and POP representing poly(oxyethylene) and poly(oxypropylene), respectively. The POE chains are hydrophilic due to their short carbon unit between oxygen bridges, whereas the POP center is hydrophobic due to the larger propylene unit. This surfactant directly inserts into electropores after electroporation of muscle cells and after Joule heating-induced loss of membrane integrity. Recent studies on brain trauma also suggest that this group of amphiphilic tri-block copolymers, and particularly P188, can help 'save' neurons from developing early necrotic death following severe mechanical loading [3,25].

The hypothesis of the current study was that incubation of chondral explants in culture media supplemented with the non-ionic synthetic surfactant P188 would be effective in limiting the development of chondrocyte necrosis within the first 24 h after a severe blunt trauma. These data would then serve as a rationale to study the efficacy of early P188 intervention in a severely traumatized, in vivo joint to potentially delay or even mitigate the development of post-traumatic OA.

Methods

Six bovine forelegs from three mature animals (18–24 months of age) were obtained from a local abattoir within six hours of slaughter. The legs were skinned and rinsed with water prior to exposing the metacarpal joint under a laminar flow hood. A biopsy punch (Miltex Instrument Company, Bethpage, NY) was used to make eighty 6 mm diameter chondral explants from the lower metacarpal surface of the limbs. Each explant was separated from the underlying bone with a scalpel. All specimens were washed three times in Dulbecco's Modified Eagle Medium: F12 (DMEM, Invitrogen Co., Grand Island, NY, #12500-062). The explants were then equilibrated for 48 h in the DMEM:F12 media (2 explants per well, approximately 1 mg of cartilage per 1 ml of media) supplemented with 10% fetal bovine serum (Invitrogen Co.), 50 μg/ml ascorbic acid, 21.9 mg/ml glutamine and additional amino acids and antibiotics in a humidity controlled incubator (37 °C, 5% CO_2, 95% humidity).

The explants were randomly assigned to three treatment groups, un-impacted controls with no P188 (Pluronic F-68, catalog number P-1300, Sigma-Aldrich Co., St. Louis, MO) treatment, an impacted P188-treated group, and a group impacted with no P188 treatment after trauma. In pilot experiments, a group of un-impacted explants were incubated for 24 h in culture media supplemented with 8 mg/ml P188 and compared to a group of no-P188 control explants. These studies indicated that the cell viability was not altered by incubation in P188, so subsequent studies did not include additional groups of P188-treated control explants. This helped increase the statistical power of the study.

All impacted explants were taken to 707 N (~25 MPa), following a 5 N preload, in unconfined compression between two highly polished stainless steel plates. A 0.5 Hz (1 s time to peak) haversine loading protocol was programmed for application onto the explants in a servo-controlled hydraulic testing machine (Instron, model 1331, retrofitted with 8500 plus electronics, Canton, MA). Peak load, time to peak, and maximum explant compression were documented in each experiment. Immediately after impact loading the P188-treated explants ($n = 32$) were returned to culture media supplemented with 8 mg/ml P188. Each explant was immediately compressed manually with a hand-held stainless steel platen for 10 cycles of pressure at approximately 1 MPa. This manual loading protocol was established in pilot studies that showed without pumping the explant, the P188 treatment was not statistically effective in preventing impact-induced cell death.

Sixteen explants from each impact group and eight controls were used to determine cell viability at 1 h. All remaining explants, including the controls, were washed three times in DMEM:F12 at 1 h. The P188-treated group ($n = 16$) was then incubated for 24 h post-impact in supplemented DMEM:F12 media. The 24 h no-P188 ($n = 16$) and control ($n = 8$) groups were incubated in DMEM:F12 media alone. Both impacted groups and the un-impacted controls were pumped again approximately 22 h after trauma. Following incubation for either 1 or 24 h after trauma, each explant was cut through its entire thickness with a specialized cutting tool with parallel blades spaced 0.5 mm. These thin slices were then stained with calcein AM and ethidium homodimer (EthD-1), according to the manufacturer's specifications (Live/Dead Cytotoxicity Kit, Molecular Probes, Eugene, OR).

Three slices were viewed across approximately 2 mm near the center of each explant in a fluorescence microscope (Leica DM LB, Leica Mikroskopie und Systeme GmgH, Wetlzar, Germany) and photographed using a digital camera (Spot Digital Camera, Diagnostic

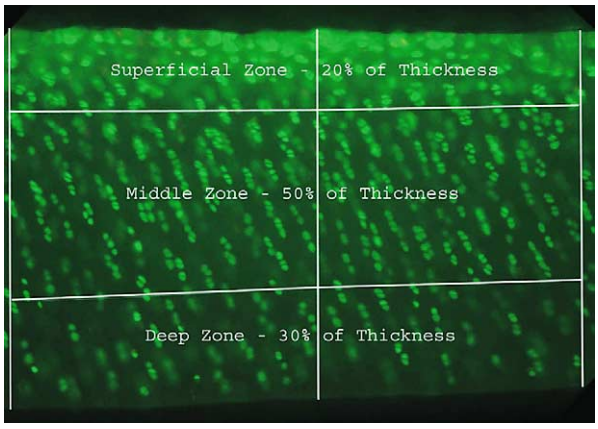

Fig. 1. The digitally photographed cartilage explants were divided into three explant zones: superficial (top 20%), middle (50%), and deep (bottom 30%). Two parallel lines were drawn approximately 2 mm apart and perpendicular to the explant surface to outline an area for determination of viable and non-viable cells. Each line was divided into its upper 20%, middle 50% and lower 30%. Another line was drawn centrally on the explant to help construct the horizontal lines that demarcated each zone of the explant.

Instruments Inc.). Viable cells were distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of virtually non-fluorescent cell-permeant calcein AM to intensely fluorescent calcein. EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence. EthD-1 is excluded by the intact plasma membrane of viable cells.

Each slice was then photographically divided into three zones: superficial (top 20% of the explant thickness), middle (middle 50%), and deep (bottom 30%) (Fig. 1). Two parallel lines were drawn approximately 2 mm apart and perpendicular to the explant to outline an area for determination of viable and non-viable cells. Each explant was divided into its upper 20%, middle 50%, and lower 30% using each line as a guide. Another line was drawn centrally on the photograph to help construct the horizontal lines that demarcated each zone of the explant. This photographic processing was performed using a commercial program (Corel Draw, Corel Inc., Dallas, TX). A blinded observer, using image analysis software (Sigma Scan, SPSS INC., Chicago, IL), quantified the density of cells in each zone and the number of viable and dead cells in each zone for the various groups. This program was also used to measure explant thickness using the central line drawn on each explant photograph.

The number of total cells and the percentages of viable cells were determined in each zone of the various groups and compared using a commercial package (Sigma Stat, SPSS Inc., Chicago, IL). Two-factor repeated measures ANOVA were performed to determine differences in the density of total cells and the percentages of viable cells in each zone between groups. In this statistical model, treatment and zone (repeated) were factors. Student–Newman–Keuls (SNK) post hoc tests were used to determine differences between zones and groups. A one-factor ANOVA was used to test differences between groups for explant thickness and each biomechanical factor measured in the study. Statistical significance was determined for $p < 0.05$. All experimental data are expressed as mean ±1 standard deviation.

Results

An analysis of the biomechanical data from the four groups of impacted explants (1 and 24 h, with and without P188 treatment) indicated no significant differences between groups. The average maximum applied load, maximum compression, and time to peak load were 644.0 ± 70.6 N, 0.298 ± 0.054 mm and 0.928 ± 0.017 s, respectively, for all impacted explants ($n = 64$). A measurement of explant thickness from the cell viability photographs also indicated no significant differences between the six groups of explants, namely, the controls, impacted with P188 treatment and impacted without treatment groups at both 1 and 24 h. The average thickness of these explants was 0.74 ± 0.19 mm.

The primary objective of this study was to show that P188 treatment after blunt trauma to chondral explants resulted in fewer dead cells than in explants without treatment. The total density of cells between the various groups of explants (controls, impacted with P188, and impacted without P188 treatment after trauma) was not different. Furthermore, no statistical differences were noted in the density of cells between zones or between groups for each zone (Fig. 2). There was a trend, however, for fewer cells in the deep zone than in the superficial and middle zones of the explants.

The percentage of live cells in control explants was not different between 1 and 24 h (Fig. 3a and b). The average percentage of live cells was 96 ± 3.9 ($n = 16$). This was also not different than the P188-treated controls at 24 h (Fig. 3c). The percentage of live cells was dramatically decreased with compressive loading of the explant (Fig. 4). The extent of cell death in the impacted explants was most evident in the superficial zones of the various groups. This was documented by a 45% reduction ($p < 0.001$) and a 62% reduction ($p < 0.001$) in percentage of live cells in the superficial zone for explants without P188 treatment following the mechanical trauma at 1 and 24 h, respectively (Fig. 5a). Extensive cell death was specifically noted around impact-induced surface fissures, which were limited to the superficial zone of the explants at both 1 and 24 h after impact (Fig. 4a and b). The percentage of live cells in the middle zone of the impacted explants without treatment was not reduced ($p > 0.05$) at 1 h, but was reduced 28% ($p < 0.001$) after 24 h (Fig. 5b). The percentage of live cells in the deep zone was not significantly different than controls at 1 or 24 h after trauma (Fig. 5c).

Reductions of 25.8% ($p < 0.001$) and 20.5% ($p < 0.001$) occurred in the percentages of live cells in the superficial and middle zones of impacted explants without treatment between 1 and 24 h following trauma (Fig. 5a and b). In contrast, there was no significant change ($p = 0.079$) in cell viability for the deep zone between 1 and 24 h after impact trauma (Fig. 5c).

In contrast with the impacted, no treatment groups the percentage of live cells in the P188-treated explants was only significantly reduced by compressive loading in the superficial zone. Specifically, reductions of 24.3% ($p = 0.004$) and 39.6% ($p < 0.001$) in cell viability were documented in the superficial zones of the treated explants at 1 and 24 h, respectively. Yet compared to the

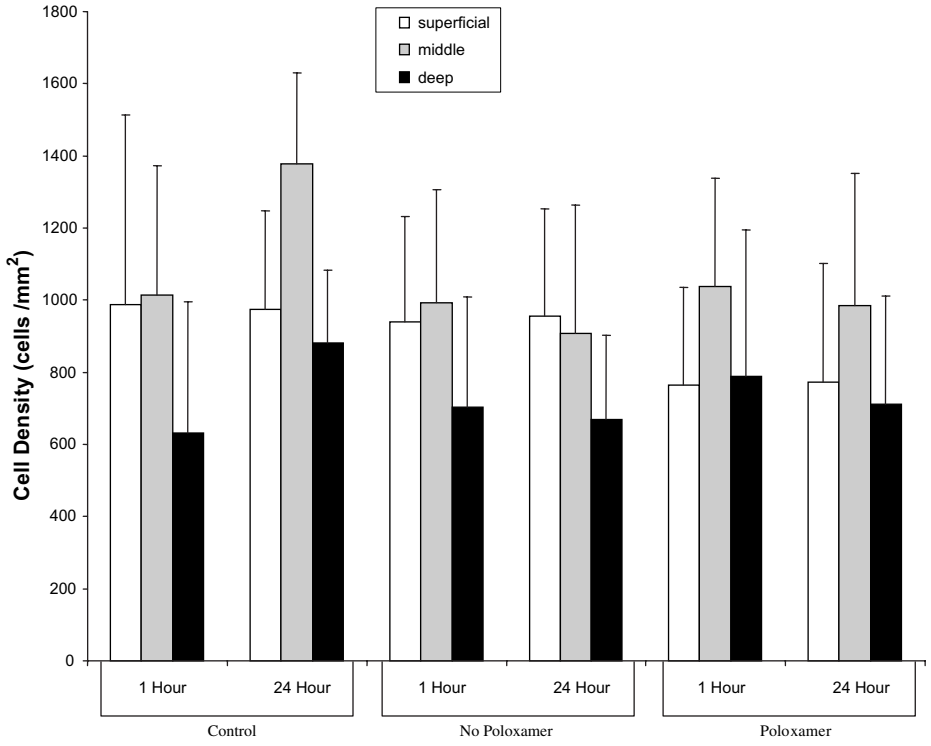

Fig. 2. The density of total cells (cells/mm^2) was determined by measuring the total explant area and quantifying total cells, within the zonal guidelines using image analysis software. No statistically significant differences were found between the explant zones (superficial, middle, deep), time (1 and 24 h), or treatment (with or without P188) as determined by two-way repeated measures ANOVA. The plot shows mean ±1 standard deviation ($n = 32$ for each group with $n = 16$ at 1 h and $n = 16$ at 24 h for the impacted explants and $n = 8$ at 1 h and $n = 8$ at 24 h for controls).

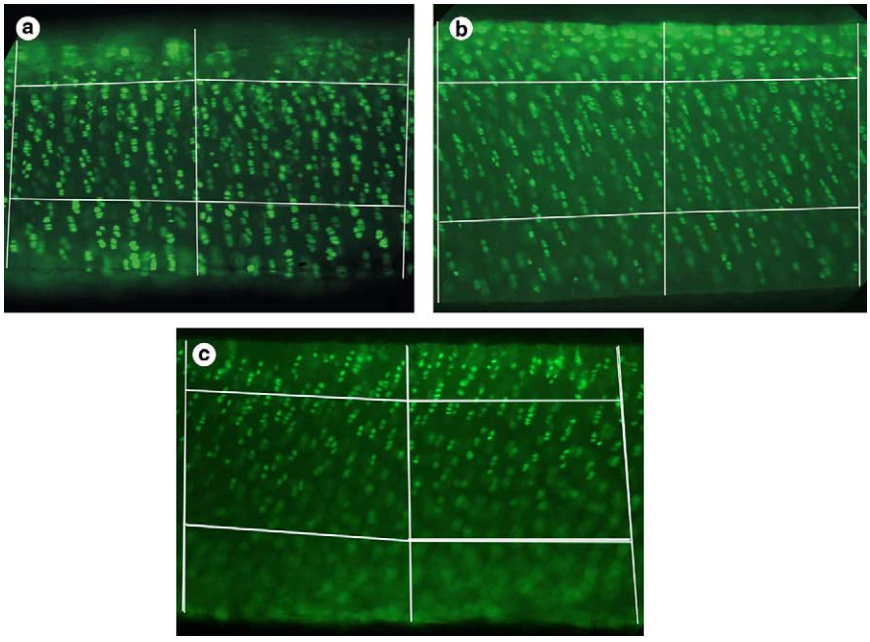

Fig. 3. Images of cartilage explants stained for cell viability. The cartilage explants were stained with calcein AM and ethidium homodimer at either 1 or 24 h. Live cells stained green and necrotic cells stained red. Images (a) and (b) show explants that were untreated and used as non-impact controls. Cell viability was determined at 1 h (image (a)) and 24 h (image (b)). Image (c) was treated with 8 mg/ml P188 and used as a non-impacted control with cell viability determined at 24 h. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

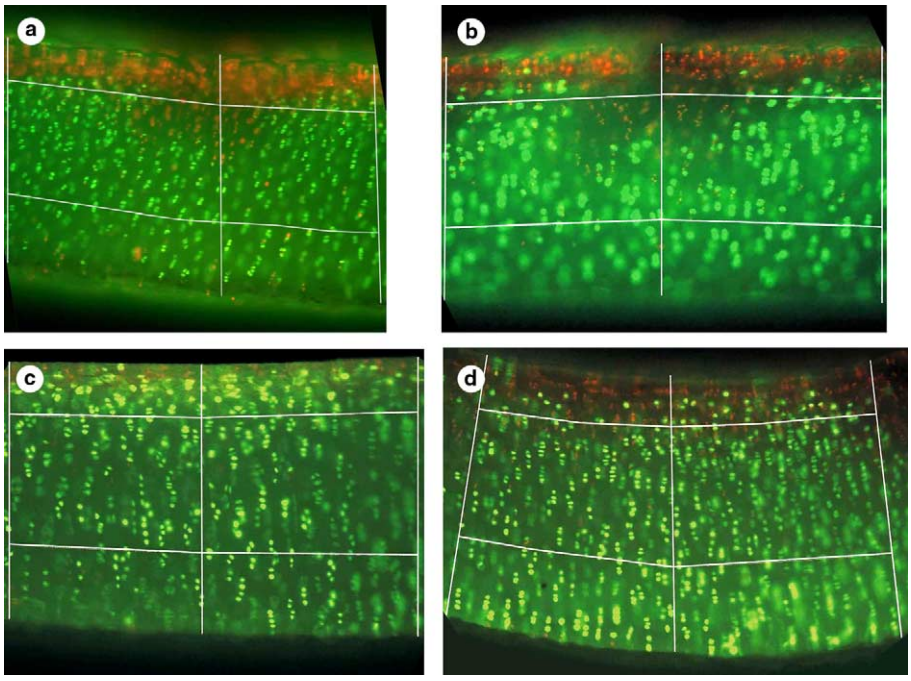

Fig. 4. Images of loaded cartilage explant specimens stained for cell viability. Live cells were stained green and dead cells were stained red. The cartilage explants were loaded to 25 MPa at a low rate of loading (1 s to peak) and stained with calcein AM and ethidium homodimer at either 1 or 24 h post-impact. Images (a) and (b) show explants that were untreated and viewed at 1 and 24 h, respectively. They show extensive areas of cell death after trauma. Note the surface fissures in the center of each of these figures with localized cell death in that area. Images (c) and (d) were treated with 8 mg/ml P188 following blunt impact and show reductions in cell death versus untreated explants at 1 and 24 h, respectively. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

no treatment group, significant increases in cell viability of 38.8% ($p < 0.001$) at 1 h and 49.1% ($p < 0.001$) 24 h after mechanical trauma occurred in the superficial zones of the impacted explants with P188 treatment (Fig. 5a). While there were no significant reductions in cell viability within the middle zone of the P188-treated explants versus controls at 1 ($p = 0.90$) or 24 h ($p = 0.49$) after trauma, a significant increase of 28.8% ($p < 0.001$) in the percentage of viable cells still occurred in this zone for the treated versus no treatment group at 24 h (Fig. 5b). Likewise, no significant changes were noted in cell viability in the deep zones of impacted and treated explants versus control explants at 1 ($p = 0.69$) and 24 h ($p = 0.71$). There still was a significant increase of 10.7% ($p = 0.049$) in the percentage of live cells in the impacted, treated group versus the no treatment group 24 h after trauma in the deep zone (Fig. 5c).

Discussion

The objective of the current study was to determine the potential for 'saving' traumatized chondrocytes with the mild, non-ionic surfactant P188. The hypothesis was that chondrocytes damaged by unconfined compression of chondral explants would be 'saved' from necrosis by early administration of P188. P188 surfactant did significantly increase the percentage of live cells in the superficial zone of impacted chondral explants within 1 h after 25 MPa of compressive loading. The surfactant was also effective for increasing the cell viability in all zones of the traumatized cartilage by 24 h. Other studies have documented the ability of P188 to repair damaged plasma membranes [3,6,17,22,28]. Controlled laboratory experiments indicate that P188 inserts into lipid monolayers at surface pressures equal to or lower than approximately 22 mN/m at 30 °C; this pressure corresponds to the maximal surface pressure attained by P188 on a pure water subphase [26]. Because the equivalent surface pressure of a normal bilayer is on the order of 30 mN/m, the lack of P188 insertion above 22 mN/m helps confirm that P188 selectively adsorbs into damaged portions of plasma membranes [26]. The 'patch' can then arrest the leakage of intracellular materials and help maintain ionic concentrations across this semi-permeable layer to prevent necrosis of the cell [26].

In the current study, the action of P188 treatment on mechanically traumatized chondrocytes was explored for the first time. The study also confirmed a 'window of opportunity' to save cells from necrotic death, as the percentage of live sells decreased from approximately 83% to 67% between 1 and 24 h following 25 MPa of unconfined compression in the chondral explant. While the surfactant was only effective in the superficial zone

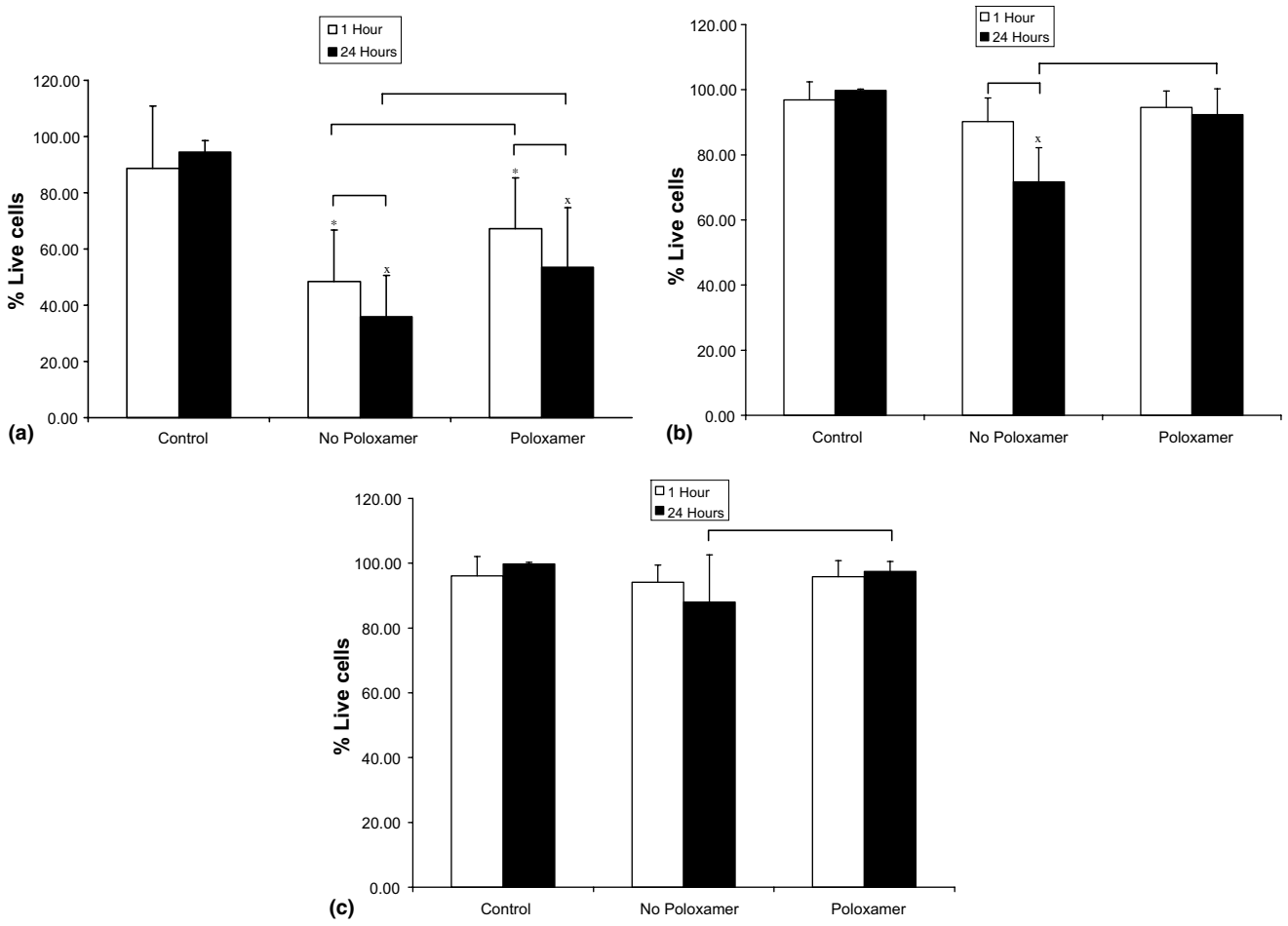

Fig. 5. Cell viability in the superficial (a), middle (b), and deep (c) zones of cartilage explants following blunt loading. The explants were loaded to 25 MPa at a low rate of loading (1 s to peak) and stained for cell viability at either 1 or 24 h post-impact. P188 was added (8 mg/ml) immediately following blunt impact. Bars linking two separate columns indicate a statistical difference. A (*) indicates a statistical difference versus its 1 h control, while an (x) indicates a statistical difference versus its 24 h control. The plot shows mean ±1 standard deviation ($n = 32$ for each group with $n = 16$ at 1 h and $n = 16$ at 24 h for the impacted explants and $n = 8$ at 1 h and $n = 8$ at 24 h for controls).

of the chondral explant at 1 h, at 24 h P188 effectively increased the percentage of live cells in all zones of the explant. This was likely due to limited penetration of the surfactant into the matrix in the first hour after trauma. The observation of decreased cell density in the deep zone of the explant was consistent with previous work with human cartilage [19]. This connection provides support for the methods of the study and suggests that similar results might be obtained with human tissue.

In pilot studies we found the need to cyclically compress the chondral explants for P188 to have a statistically significant effect on cell viability after this level of trauma. A limitation of the study was that this protocol was not further investigated. We currently hypothesize that this 'pumping' procedure helped the surfactant penetrate the cartilage matrix. Ten cycles of compression at approximately 1 MPa was simply an early attempt that was effective, so it was used for this initial study. It will be important to further investigate the protocol as it may relate to the efficacy of this proposed intervention in a clinical setting.

Only one level of applied loading was also investigated in the current study. The explants were compressed approximately 40% in this study, based on photographic measurements of explant thickness. These data compare favorably, however, with earlier studies in our laboratory where explant thickness was measured prior to traumatic loading [9,10,21]. While approximately 25 MPa of pressure has been previously recorded on retro-patellar cartilage during blunt impact loading of the human knee [16], this level of pressure applied to a chondral explant likely generated significantly larger cellular strain than might occur in an intact joint. A recent study from our laboratory indicates a significant reduction in the extent of impact-induced cell death in osteochondral versus chondral explants subjected to the same applied load [21]. This result might suggest that more cells in the cartilage will be less severely damaged

and improve the effectiveness of P188 treatment in the intact joint scenario.

With the ability of P188 to repair damaged membranes in chondral explants following severe blunt loading, the potential use of this surfactant should be explored as an intervention following blunt force trauma to a diarthrodial joint. In studies of chemical damage to cells with *N*-methyl-D-aspartate, P188 treatment has been shown to normalize membrane receptor and intracellular functions with fast (within 20 s) action [25]. If similar capabilities exist in the P188 repaired chondrocytes, these cells may function normally in the chronic setting. The surfactant has also been shown to be 'squeezed out' of the cell membrane after it heals, and unmetabolized P188 will be excreted in the urine of the patient [29].

P188 repaired chondrocytes might anyway die soon after traumatic loading of the joint by apoptosis. This issue needs future exploration using an in vivo animal model, such as that currently being developed in our laboratory [11]. However, a pan-caspase inhibitor, z-VAD.fmk [benzyloxycarbonyl-val-ala-asp (Ome) flouromethylketone] can help prevent chondrocyte apoptosis in human articular cartilage explants compressed 30% [7]. Caspase inhibition reduces chondrocyte apoptosis in human chondral explants from 34% in non-treated specimens to 25% with z-VAD.fmk intervention at 48 h. Thus, P188 repaired chondrocytes could still be 'saved' from apoptotic death by an additional intervention with this caspase inhibitor.

The mechanism of cell death following traumatic loading of articular cartilage is largely unknown, but the reduction of early necrosis in the tissue may further reduce or mitigate the spread of apoptosis in adjacent cells. Recent studies showed that apoptotic cells damaged by cyclic mechanical load can signal cells in adjacent areas of the cartilage and encourage cellular apoptosis away from the impact site [24]. This type of signaling from damaged cells may be enhanced following severe impact loading by the leakage of intracellular constituents into the tissue matrix from the necrotic cells in the area of direct impact loading.

In addition to the clinical issue of surfactant penetration into the matrix of traumatized cartilage, the potential toxic effects of this surfactant in a joint must be addressed. Studies to date, however, have shown P188 to be non-toxic in a variety of clinical settings [23]. These include the cleansing of skin wounds, as a laxative, and as a hemorheologic and antithrombotic agent used to reduce blood viscosity for the reduction of painful episodes in patients suffering from sickle cell anemia. Cellular necrosis in articular cartilage has been shown to generate early OA-like changes in the tissue of a chronic animal model [30]. While this might suggest a direct role of acute cell death in the development of a post-traumatic OA in the joint, additional studies are needed. Furthermore, the potential for an early intervention of the disease process with P188 or other surfactants needs additional study.

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