

# The limitation of acute necrosis in retro-patellar cartilage after a severe blunt impact to the in vivo rabbit patello-femoral joint

S.A. Rundell, D.C. Baars, D.M. Phillips, R.C. Haut \*

*Orthopaedic Biomechanics Laboratories, College of Osteopathic Medicine, Michigan State University, A414 East Fee Hall,  
East Lansing, MI 48824, USA*

## Abstract

We have previously shown that surface lesions and acute necrosis of chondrocytes are produced by severe levels of blunt mechanical load, generating contact pressures greater than 25 MPa, on chondral and osteochondral explants. We have also found surface lesions and chronic degradation of retro-patellar cartilage within 3 years following a 6 J impact intensity with an associated average pressure of 25 MPa in the rabbit patello-femoral joint. We now hypothesized that cellular necrosis is produced acutely in the retro-patellar cartilage in this model as a result of a 6 J impact and that an early injection of the non-ionic surfactant, poloxamer 188 (P188), would significantly reduce the percentage of necrotic cells in the traumatized cartilage. Eighteen rabbits were equally divided into a 'time zero' group and two other groups carried out for 4 days. One '4 day' group was administered a 1.5 ml injection of P188 into the impacted joint immediately after trauma, while the other was injected with a placebo solution. Impact trauma produced surface lesions on retro-patellar cartilage in all groups. Approximately 15% of retro-patellar chondrocytes suffered acute necrosis in the 'time zero' and '4-day no poloxamer' groups. In contrast, significantly fewer cells (7%) suffered necrosis in the poloxamer group, most markedly in the superficial cartilage layer. The use of P188 surfactant early after severe trauma to articular cartilage may allow sufficient time for damaged cells to heal, which may in turn mitigate the potential for post-traumatic osteoarthritis. Additional studies are needed to improve the efficacy of this surfactant and to determine the long-term health of joint cartilage after P188 intervention.

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*Keywords:* Osteoarthritis; Necrosis; Cartilage; Impact trauma; Cell viability

## Introduction

Osteoarthritis (OA) affects over 21 million Americans and is the leading cause of disability in the United States [32]. While the mechanisms responsible for this disease are unknown, the risk of OA is increased significantly in joints suffering a major injury [11]. Establishing a cause and effect relationship between joint injury and OA can be difficult as after an osteochondral injury joint disease may not be diagnosed for 2–5 years, while OA

from less severe joint injuries may not be diagnosed for 10 or more years after the trauma [33]. OA is characterized clinically by joint pain and narrowing, as diagnosed by radiological examination [12]. Pathologically, the disease exhibits a loss of cartilage and sclerosis of underlying bone.

Experimental studies with animal models have sought to understand the potential association between acute joint injury and the chronic pathogenesis of OA. We developed a model of post-traumatic OA using the Flemish Giant rabbit [14]. In a recent study using this model, a rigid impact mass was dropped with 6 J of energy onto the flexed patello-femoral joint, resulting in acute surface fissures, progressive degradation of

\* Corresponding author. Tel.: +1 517 355 0320; fax: +1 517 353 0789.

*E-mail address:* haut@msu.edu (R.C. Haut).

retro-patellar surface cartilage, and thickening of underlying subchondral bone after 3 years [10]. In another study, this impact was found to produce patello-femoral contact pressures of approximately 25 MPa [22]. Another laboratory, using the New Zealand white rabbit subjected to a 10 J impact, found advanced OA-like changes in the patello-femoral joint, with fibrillation, ulceration, and erosion of retro-patellar cartilage within 6 months post-contusion [19]. Early OA-like changes were also described using the flexed canine patello-femoral joint subjected to approximately 2.2 kN of impact force [30]. Surface fissures were found on retro-patellar cartilage with clefts in the underlying subchondral bone and calcified cartilage. After 6 months the fissures had loss of safranin-O staining, and new bone formation occurred in the subchondral bone. While these studies found an association between blunt impact trauma and subsequent OA-like changes, the mechanisms of acute damage leading to these pathological changes are yet unclear.

A single blunt impact resulted in surface fissures and necrotic cell death in chondral [8,31] and osteochondral explants [16,21]. Significant fissuring and about half the cells acutely necrotic was found with 40 MPa of unconfined compression on bovine chondral explants [8]. Necrotic cells were found in the superficial zone of the cartilage (upper 20%) located near fissures and in the middle zone (next 50%), with a few dead cells found in the deepest zone, especially in osteochondral explants [16]. A critical threshold stress of 15–20 MPa was found for cell death and collagen matrix damage in unconfined compression using the bovine chondral explant [31]. A later study using osteochondral explants found that significant surface fissuring with adjacent cellular necrosis occurred when impact loading rates exceeded the gel diffusion rate of the cartilage [20]. Cell death occurred largely in the superficial zone adjacent to fissures. Necrotic cell death was also found *in situ* using the bovine patella subjected to a single impact of 53 MPa [17]. Cell death occurred around surface cracks, but not in impacted areas away from these cracks. The authors suggested “that early stabilization of damaged areas of the cartilage may prevent late sequelae that lead to OA.” Other studies have also associated articular cartilage physiopathology in OA with chondrocyte death [13].

Few *in vivo* experimental studies, however, have been conducted that specifically associate cell death with the development of OA. In one study significant chondrocyte necrosis was induced in rabbit patello-femoral joints by localized freezing *in vivo* [28]. After 1 year the affected cartilage had changes consistent with early OA. These changes were similar to those documented by our laboratory using the impacted joint model [10]. A hypothesis of the current study was that a 6 J blunt impact onto the rabbit patello-femoral joint would induce acute chondrocyte necrosis in retro-patellar cartilage.

Defining features of necrosis are damage to the plasma membrane and the inability of the cell to maintain ionic gradients across its membrane, resulting in swelling and subsequent rupture of the cell [7]. Due to the nature of necrosis, mild surfactants have been used to restore integrity to cells after physical and chemical stresses [4,24]. Specifically, poloxamer 188 (P188) was found to ‘save’ neurons from early necrosis after severe mechanical loading [2,18]. We recently found that P188 surfactant was able to ‘save’ chondrocytes from acute necrosis in bovine chondral explants subjected to 25 MPa of unconfined compression [25]. A second hypothesis of the current study was that injection of P188 surfactant into the *in vivo* patello-femoral joint shortly after a 6 J blunt impact would significantly reduce the percentage of necrotic cells in retro-patellar cartilage in the acute setting (4 days post-trauma).

## Methods

Eighteen skeletally mature, Flemish Giant rabbits (aged 6–8 months) were used in this study, which was approved by the All-University Committee on Animal Use and Care. The blunt impact experiments have been described previously [14]. Briefly, a 1.33 kg mass with a flat 25 mm diameter aluminum impact interface was dropped from 0.46 m (6 J of impact energy) onto the right patello-femoral joint of anesthetized animals (2% isoflurane and oxygen). The opposite limb was not impacted and used as a paired, unimpacted control. A load transducer (2.225 kN capacity, Model 31/432, Sensotec, Columbus, OH) was attached behind the rigid interface to record impact loads. Peak contact load, time to peak, and total contact duration were collected at 10 kHz. The mass was arrested electronically after the first impact, preventing multiple impacts.

Six rabbits were randomly selected as ‘time zero’ animals, and were sacrificed immediately after impact. The remaining 12 animals were sacrificed 4 days post-impact. During these 4 days the animals were exercised 10 min a day at 0.3 mph on a treadmill [23]. The exercise protocol was initiated approximately 4 h after impact. Animals were housed in individual cages when not exercising. Six of the 4-day-old animals received a single 1.5 ml injection of an 8 mg/ml concentration of P188 surfactant in sterile phosphate-buffered saline (PBS) injected into the traumatized patello-femoral joint capsule within about 2 min after impact. The remaining six animals received a sham injection of 1.5 ml sterile PBS into the joint shortly after trauma. The combination P188 in PBS and the PBS sham solutions were filter sterilized prior to injection using a 0.2 mm vacuum filter (Nalgene, Nalge Nunc Int., Rochester, NY). After injection, the limb was exercised manually to distribute the P188 surfactant and PBS solutions in the joint.

Patellae from the impacted and the opposite unimpacted limbs were excised immediately after sacrifice. The retro-patellar surface was wiped with India ink to highlight surface defects and was photographed using a digital camera (Polaroid DMC2, Polaroid Corporation, Waltham, MA) under a dissecting microscope (Wild TYP 374590, Heerbrugg, Switzerland). Each patella was then wrapped in PBS soaked gauze.

Osteochondral sections from both patellae from each animal were prepared for cell viability analyses. A low speed bone saw (model# 11-1180-170, Buehler, Inc., Lake Bluff, IL) was used to remove trabecular bone from the overlying retro-patellar cartilage, leaving approximately 0.5 mm of subchondral bone. Full depth sections (0.5 mm thick) of retro-patellar cartilage and subchondral bone were cut using a specialized cutting device [8] from the area of known patello-femoral contact during blunt impact [14]. Sections were stained with Calcein AM and Ethidium Homodimer, according to the manufacturer’s specifications (Live/Dead Cytotoxicity Kit, Molecular Probes, Eugene, OR). Approximately eight sections taken near each patella’s mid-line were

viewed using a fluorescence microscope (Leitz Dialux 20 (frequency: 50–60 Hz), Leitz Mikroskopie und Systeme GmgH, Wetzlar, Germany) and were photographed with the digital camera. Each photographic section was divided into two zones: superficial (top 20% of the cartilage depth) and deep (bottom 80%), using an established protocol [25]. A blinded observer (A.S.) manually counted live and dead cells in each section using image analysis software (Sigma Scan, SPSS INC., Chicago, IL). The percentage cell death in each zone and the total percentage cell death were determined for each section. Individual section data were averaged to yield the percentage of cell death in each zone and the total percentage of cell death for each limb. The length of surface fissuring was measured on 4 randomly selected impacted and unimpacted pairs of patellae from each group using the same image analysis software [8].

Two factor repeated measures ANOVA and Student–Newman–Keuls post-hoc tests were used to detect differences in the percentage of cell death, percentage of cell death in each zone, and the average total fissure length between impacted and unimpacted limbs and between treatment groups. A two factor (time and limb) ANOVA was performed to detect differences between and within the ‘time zero’ and ‘4-day no P188 group’, while a separate two factor (P188 treatment and time) ANOVA was performed to detect differences between and within the ‘4-day no P188’ and ‘4-day P188’ groups. A single factor ANOVA was used to detect differences in peak impact loads and time to peak loads between treatment groups. Statistical significance was set at  $\alpha < 0.05$ . All experimental data are presented as mean  $\pm$  one standard deviation.

## Results

No statistical differences were found in the times to peak impact load or the magnitudes of peak load between the treatment groups. The peak impact load was  $676 \pm 134$  N, and the time to peak load was  $3.5 \pm 1.0$  ms. The animals did not favor one limb over the other during the 4 days of treadmill exercise, per observations by the licensed veterinary animal technician (J.A.).

Gross inspections of the impacted and unimpacted patellae indicated surface fissures on the retro-patellar surfaces (Fig. 1). On the impacted patellae more fissures having a proximal to distal orientation were located

near the mid-line of the patella (Fig. 2). No significant differences in the length of surface fissures were detected between treatment groups for either impacted or unimpacted patellae; however with the small sample size, statistical power between groups was below the desired level of 0.8.

The stained osteochondral sections showed necrotic cells in both the superficial and deep zones of impacted and unimpacted retro-patellar cartilage (Fig. 3). Analyses of cell viability across the depth of the retro-patellar cartilage showed statistically significant differences in the percentages of dead cells between the impacted and unimpacted patellae for the ‘time 0’ ( $p < 0.001$ ) and for the ‘4-day no P188’ groups ( $p = 0.002$ ) (Fig. 4). In contrast, no significant difference ( $p = 0.072$ , power = 0.947) was found in the percentage of total cell death between the impacted and unimpacted patellae for the ‘4-day P188’ group. The effect of P188 injections into the joint shortly after impact trauma was also evident in a significant difference ( $p = 0.027$ ) in the percentage of total cell death between the impacted limbs of the ‘4-day no P188’ group and the ‘4-day P188’ group. In contrast, no significant difference existed between the percentage of total cell death in the impacted ( $p = 0.195$ ) or the unimpacted ( $p = 0.899$ ) retro-patellar cartilage between the ‘time 0’ and the ‘4-day no P188’ groups.

Gross inspection of the cartilage sections indicated a large amount of cell death in the superficial zone (Fig. 3b). While significant differences were found between the percentages of cell death in the superficial zones of impacted versus unimpacted retro-patellar cartilage for the ‘time zero’ ( $p = 0.002$ ) and the ‘4-day no P188’ ( $p = 0.008$ ) groups, no significant difference (power = 0.994) was recorded in the percentage of cell death in this zone for the ‘4-day P188’ ( $p = 0.095$ ) group (Fig. 5). In contrast, significant differences did occur in

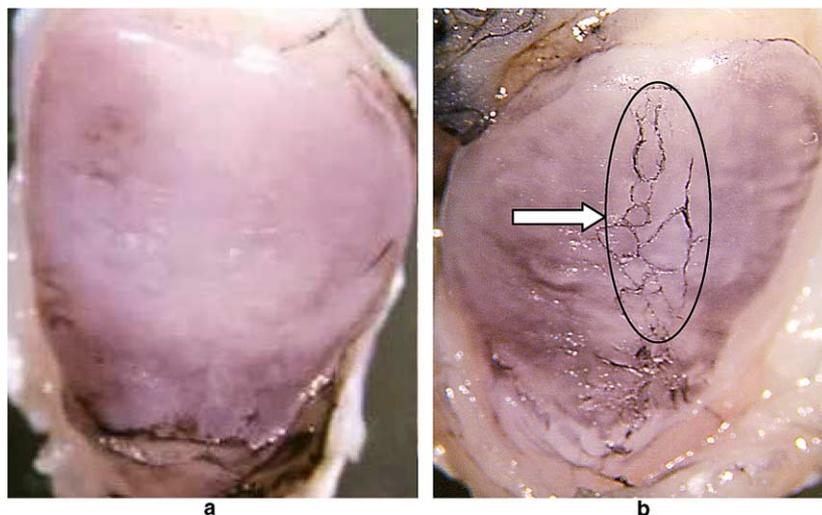


Fig. 1. Digital photographs (25 $\times$ ) of unimpacted (a) and impacted (b) retro-patellar surfaces. Impact induced fissures were stained with India ink. The fissures were digitally measured and recorded with digital imaging software.

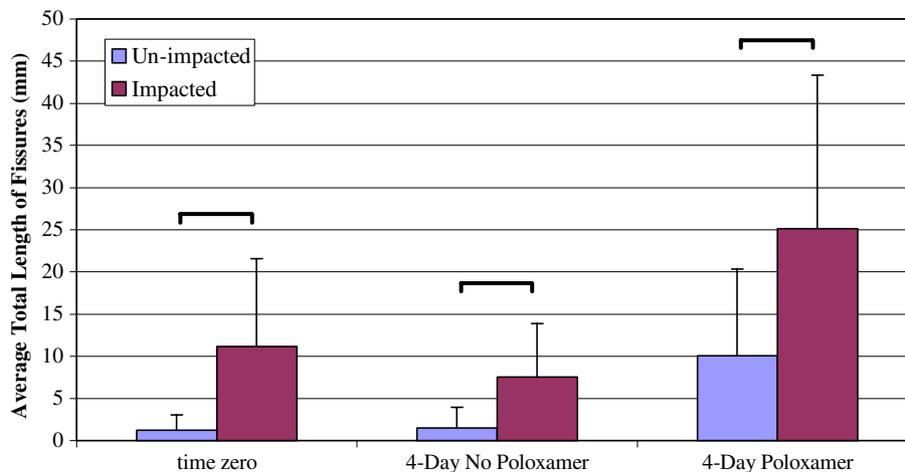


Fig. 2. Digitally measured and recorded surface fissure lengths were averaged. Brackets indicate significant differences between groups. A significantly greater average length was found in impacted versus unimpacted patellae in all of the groups.

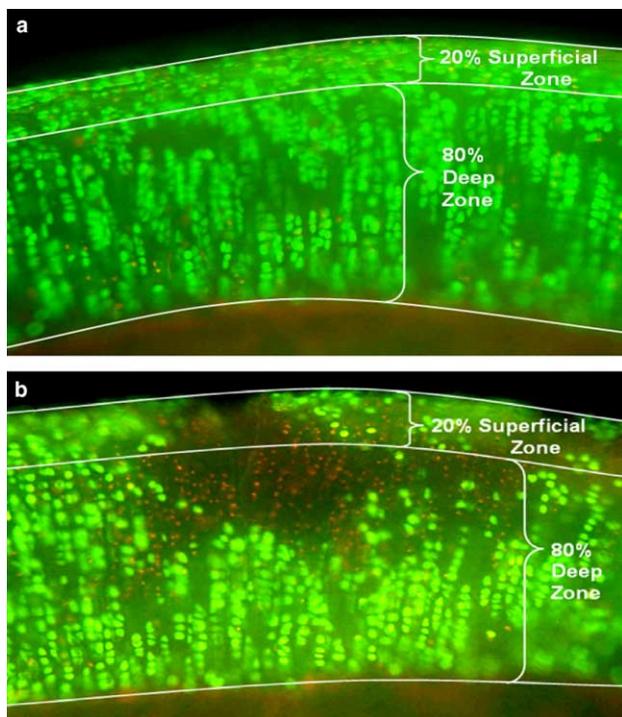


Fig. 3. Unimpacted (a) and impacted (b) patellar cross-sectional slices were stained for cell viability. Live cells were stained green; dead cells were stained red. The impacted patellae were stained with calcein AM and ethidium homodimer either immediately following impact or 4 days post-impact. Images (a) and (b) show patellae that were untreated and viewed immediately after impact. The impacted patella (b) shows extensive areas of cell death in the surface zone after trauma.

the percentages of cell death in the deep zones between impacted and unimpacted patellae in all treatment groups. Statistical differences were not detected for the superficial or deep zones between groups in this study, but with the small sample size, the statistical power between groups was again less than 0.8.

## Discussion

A 6 J impact to the flexed rabbit patello-femoral joint produced surface fissures on the retro-patellar cartilage. The proximal to distal orientation of the fissures was previously associated with the impact event [14]. Impact trauma resulted in acute death of chondrocytes throughout the depth of the retro-patellar cartilage by approximately 15% more in the impacted versus the opposite unimpacted patella. Furthermore, the percentage of total dead cells in the impacted versus the unimpacted retro-patellar cartilage was not significantly different for the '4-day P188' group. While the zonal layer data showed significant differences in the percentage of dead cells in the superficial and deep zones of impacted versus unimpacted patella for the 'time zero' and '4-day no P188' groups, in the '4-day P188' group the effect was only significant in the deep zone. These data suggest that P188 was most effective in 'saving' cells from necrotic death in the superficial zone.

A defining feature of cell death by necrosis is swelling, due to the injured cell being unable to maintain ionic gradients across a damaged plasma membrane [7]. We measured the percentage of dead cells by membrane disruption; membrane damage was documented by the ability of EthD-1 (ethidium homodimer) to only pass through a damaged cell membrane. This damage mechanism was also supported by the efficacy of P188 surfactant to repair these cells. A previous study showed that this surfactant specifically inserts only into damaged areas of a cell membrane [18].

In a similar *in vivo* study using New Zealand White rabbits, a 3 J blunt impact to the patello-femoral joint resulted in a 14% increase in apoptotic (TUNEL positive) cells in retro-patellar cartilage versus the unimpacted joint at 4 days post-trauma [6]. The study

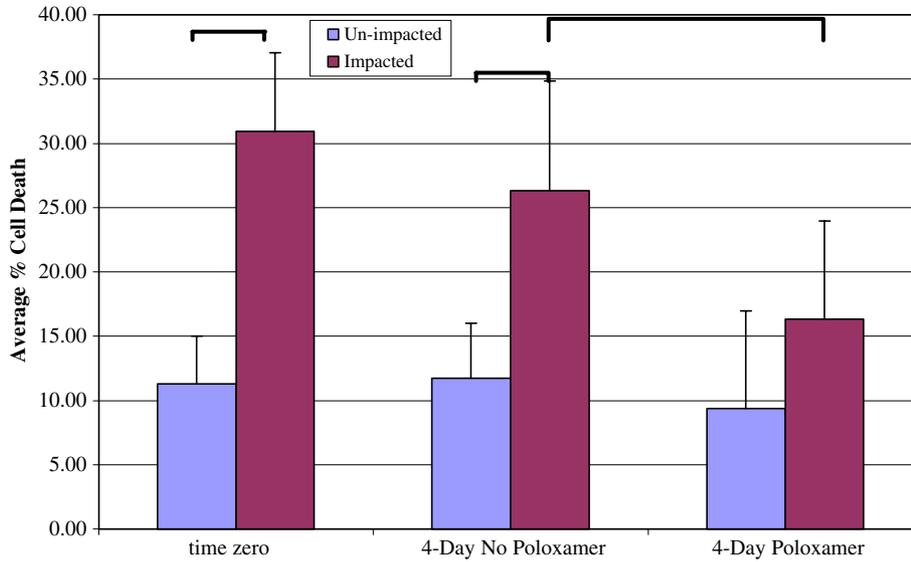


Fig. 4. Significantly greater amounts of cell death occurred in the impacted versus unimpacted patellae of both the ‘time 0’ and the ‘4-day no P188’ groups. A significantly greater amount of cell death was recorded in the impacted patellae of the ‘4-day no P188’ group versus the ‘4-day P188’ group. The brackets denote statistical significance by a two factor repeated measures ANOVA.

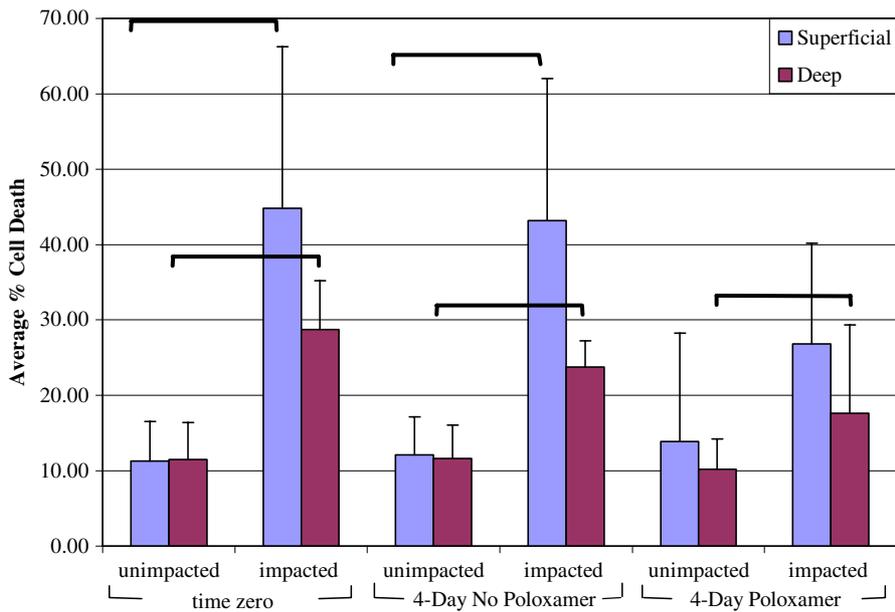


Fig. 5. A greater percentage of cell death occurred in the superficial and deep zones of the impacted versus unimpacted patellae in both the ‘time 0’ and ‘4-day no P188’ groups. A significant difference was found in the total percentage of cell death in the deep zones of impacted versus unimpacted patellae in the ‘4-day P188’ group. The brackets denote statistical differences using a two factor repeated measures ANOVA performed separately for the superficial and deep zones.

verified cellular apoptosis by examining attributes of nuclear morphology. Chondrocyte apoptosis has also been shown in human biopsy tissue near sites of chondral fracture [15]. Canine cartilage explants subjected to 5 MPa of cyclic compression at 0.3 Hz exhibited the development of both necrosis and apoptosis, increasing progressively with load duration and time. Necrosis was observed 2 h after cessation of loading, whereas apoptosis (TUNEL-positive cells) was not significant until 48

or more hours after loading stopped. Apoptosis was verified in some cells using transmission electron microscopy [3]. A study that induced osteochondral wounding of a joint also found significant percentages of necrotic and apoptotic cells in the tissue [29]. These data suggest that mechanical injury to a joint may result in both necrotic and apoptotic cell death. Human chondral explants exposed to 14 MPa of unconfined compression revealed that 34% of chondrocytes died via apoptosis

[6]. Administration of z-VAD.fmk, a pan-caspase inhibitor, reduced cell death to 25% in these explants.

A limitation of the current study was that cell death by other mechanisms, for example by apoptosis, was not examined following P188 intervention. An influx of  $\text{Ca}^{2+}$ , for example, into the chondrocyte prior to membrane resealing by P188 may trigger an early programmed cell death [1]. A previous study on neuronal cells has also found P188 to be effective in limiting apoptosis as detected by TUNEL staining [27]. After trauma, interventions like z-VAD.fmk could likely be used in combination with P188 to more effectively reduce chondrocyte death or dysfunction in the longer term [5].

We found that early administration of P188 surfactant into the joint resulted in a decrease in the percentage of total dead cells in retro-patellar cartilage from approximately 15–7%. In a previous study, we showed that in bovine chondral explants, 25 MPa of unconfined compression applied in 1 s resulted in approximately 34% cell death 24 h after impact [25]. Immediate treatment of these explants after impact with an 8 mg/ml concentration of P188 surfactant reduced cell death to approximately 14% at 24 h. Differences in total cell death between the previous and current studies may have been due to the presence of underlying bone in the *in vivo* model that stiffened the articular cartilage and prevented excessive deformation during impact to the joint [16]. Furthermore, in contrast to the current study in which P188 surfactant appeared more effective in ‘saving’ cells in the superficial zone of the retro-patellar cartilage, the treatment of chondral explants in the previous study with this same concentration of surfactant significantly reduced cell death in all layers of the tissue.

One explanation for the more limited efficacy of the treatment in the current study may relate to the penetration of the P188 surfactant. In the previous study the chondral explants were ‘pumped’ immediately after administration of the surfactant and approximately 22 h after impact with 10 cycles of unconfined compression at 1 MPa pressure and a frequency of 1 Hz. In the current study the animal joint was flexed approximately 10 times immediately after injection of the surfactant, and the animals were exercised daily beginning on the day of impact. We assumed that post-trauma exercise would help ‘pump’ the surfactant into the cartilage. The ‘pumping’ of surfactant into the tissue may have been less effective in the *in vivo* joint due to differences in the pressure intensity and the unknown joint loading during treadmill exercise. Penetration of the surfactant into the cartilage may have also been limited by the underlying subchondral bone in the *in vivo* joint. The efficacy of surfactant treatment, on the other hand, may be enhanced by a reduction in the concentration of the surfactant solution, after the relationship between efficacy and solution concentration is established in fu-

ture *in vitro* studies with both chondral and osteochondral explants.

Another limitation of the current study was that the quantity of surfactant solution was limited to 1.5 ml. This quantity was chosen to limit the amount of fluid in the small rabbit knee and to match the quantity of polysulfated glycosaminoglycan solution used in a previous study [9]. The relationships between optimization of the surfactant concentration, the quantity of fluid, and the timing of the intervention post-impact should be determined in future studies.

We used a small sample size ( $n = 6$ ) and an *in vivo* model which yielded large variations in both surface fissure and chondrocyte viability data. This resulted in insufficient statistical power between treatment groups. The average total length of surface fissures in the ‘4-day P188’ group was approximately 4 times greater in the unimpacted and about 2 times greater in the impacted patellae than the ‘4-day no P188’ group, but a significant difference was not detectable. The greater length of surface fissuring in the ‘4-day P188’ group resulted from two animals that exhibited large amounts of base-line surface fissuring on their unimpacted retro-patellar surface. These animals were not disregarded due to the already small sample size. Furthermore, previous studies documented chondrocyte death around surface cracks [8,17], but the ‘4-day P188’ group exhibited significantly less percentage of dead cells on average in the impacted patellae versus the ‘4-day no P188’ group. These data suggest that the increased, base-line surface fissuring documented in the ‘4-day P188’ group did not adversely affect the results of the study.

In summary, 6 J of blunt impact to the flexed rabbit patello-femoral joint resulted in a significant percentage of necrotic cells in the retro-patellar cartilage immediately after insult. We hypothesize that the chronic cartilage degradation, which we documented in a previous 3 year post-trauma study [10], may be partly due to the death of these chondrocytes. We also found, in concert with earlier studies on chondral explants [25], that immediate administration of the traumatized joint with P188 surfactant resulted in a significant decrease in the percentage of necrotic cells. The above hypothesis on a mechanism of post-traumatic osteoarthritis may be tested in the future with injection of P188 surfactant into the joint immediately after blunt insult. The long term consequences of ‘saving’ these cells from early necrotic death, in terms of them ultimately becoming apoptotic and producing degradative enzymes [26] must be investigated in future studies. This intervention with P188 surfactant, however, may also allow sufficient time to evaluate the biological condition of these and other cells in the traumatized tissue and to utilize additional pharmacological treatments, such as the caspase inhibitor mentioned earlier, if needed, for the long term survival of the joint cartilage.

## Acknowledgements

This study was supported by grants from The Centers for Disease Control and Prevention, The National Center for Injury Control and Prevention (R49/CCR503607), and The TRW Automotive Fund. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the CDC or the TRW Corporation. The authors wish to gratefully acknowledge the help of Clifford Beckett, Aaron Stewart (A.S.), and Jean Atkinson (J.A.) for technical assistance during this study.

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