[Orthopaedic Surgery]

Subphysiological Compressive Loading Reduces Apoptosis Following Acute Impact Injury in a Porcine Cartilage Model

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Background: Acute cartilage injuries induce cell death and are associated with an increased incidence of osteoarthritis development later in life. The objective of this study was to investigate the effect of posttraumatic cyclic compressive loading on chondrocyte viability and apoptosis in porcine articular cartilage plugs.

Hypothesis: Compressive loading of acutely injured cartilage can maintain chondrocyte viability by reducing apoptosis after a traumatic impact injury.

Study Design: In vitro controlled laboratory study.

Level of Evidence: Level 5.

Methods: Each experiment compared 4 test groups: control, impact, impact with compressive loading (either 0.5 or 0.8 MPa), and no impact but compressive loading (n = 15 per group). Flat, full-thickness articular cartilage plugs were harvested from the trochlear region of porcine knees. A drop tower was utilized to introduce an impact injury. The articular plugs were subjected to two 30-minute cycles of either 0.5 or 0.8 MPa of dynamic loading. Cell viability, apoptosis, and gene expression of samples were evaluated 24 hours postimpaction.

Results: Cell viability staining showed that 0.5 MPa of dynamic compressive loading increased cell viability compared with the impact group. Apoptotic analysis revealed a decrease in apoptotic expression in the group with 0.5 MPa of dynamic compressive loading compared with the impact group. Significantly higher caspase 3 and lower collagen II expressions were observed in impacted samples without compressive loading, compared with those with. Compressive loading of nonimpacted samples significantly increased collagen II and decreased caspase 3 expressions.

Conclusion: In this porcine in vitro model, dynamic compressive loading at subphysiological levels immediately following impact injury decreases apoptotic expression, thereby maintaining chondrocyte viability.

Clinical Relevance: Therapeutic exercises could be designed to deliver subphysiological loading to the injured cartilage, thereby minimizing injury.

Keywords: compressive loading; osteoarthritis prevention; cartilage gene expression; chondrocyte viability; impact injury

Articular cartilage is a connective tissue composed of chondrocytes in a proteoglycan-rich extracellular matrix with type II collagen. Because of the avascular nature of cartilage, it has limited access to self-repair mechanisms (such as progenitor cells), limiting its regenerative healing properties. Acute cartilage injuries frequently sustained during athletics, combat, motor vehicle accidents, and falls induce cell death.[5,6,17,18] Injured articular cartilage is unable to heal, leading to cartilage loss, and ultimately is associated with an increased incidence of osteoarthritis later in life.[3,11,27,42]

Following injury, chondrocytes can survive, undergo apoptosis, or experience necrosis. Necrotic chondrocytes are so severely damaged by the impact that they die shortly after the injury (seconds to hours).[7] Apoptotic chondrocytes experience a programmed cell death controlled by signaling factors and die within hours to days of injury.[7,21] Since the apoptotic process

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The following authors declared potential conflicts of interest: Lee Kaplan, MD, has patents or patents pending and received royalties from Smith & Nephew.

DOI: 10.1177/1941738113504379

occurs over time,[11] there may be time to intervene, thereby reducing cell death.

Conservatively, physiological loading conditions of knee cartilage are forces between 1 and 2 MPa,[20,38,39,41] with regional peak stresses reaching 3 to 4 MPa during walking[16,45] and as high as 7 MPa during running.[16] In healthy cartilage, exercise (physiological loading) is recommended as a preventative measure used to minimize osteoarthritis development.[1,13,26,31] Similarly, subphysiological loading, such as minimum weightbearing and continuous passive motion, has been shown to attenuate joint injury–induced inflammation,[14] increase the quality of cartilage,[24] and stimulate proteoglycan metabolism[32,33] and is used clinically in physical therapy for orthopaedic patients recovering from surgery.[22,28,33,36,40] However, limited evidence-based research exists to explain the beneficial effects of subphysiological loading. Mechanical loading plays a key role in joint homeostasis and potentially in reversing damage; however, mechanical loading has not been evaluated as a potential early intervention to prevent osteoarthritis development after cartilage injury. Therefore, the objective of this study was to investigate the effect of cyclic compressive loading on acutely injured articular plugs and establish an in vitro pig model that may be used to investigate different biochemical agents to halt or deter cartilage degeneration. We hypothesized that compressive loading of injured cartilage could increase chondrocyte viability by reducing apoptosis at injured sites after a traumatic impact injury.

METHODS

Porcine Model

Porcine articular cartilage was utilized, as it exhibits a similar morphology to that of human cartilage.[30,37] Mature 1.5- to 2-year-old pigs were obtained from a local slaughterhouse and butchered within 4 hours of sacrifice, and the cartilage explants were harvested within 2 hours of tissue recovery.

Tissue Harvest

In a porcine knee, the trochlear region has relatively flat surfaces on either side of the patellar groove; however, the porcine condyles have a high degree of curvature. To investigate the effects of mechanical injury and subsequent compressive loading, it was important to use relatively flat articular cartilage plugs, allowing the entire surface to be loaded equally. Flat, full-thickness osteochondral plugs, 8 mm in diameter, were harvested from the trochlear region of porcine knees using an osteochondral graft harvester according to the manufacturer's guidelines (Smith & Nephew, Andover, Massachusetts). The first experiment compared 4 test groups: control, impact, impact with 0.5 MPa of compressive loading, and no impact but 0.5 MPa of compressive loading. The second experiment compared another 4 test groups: control, impact, impact with 0.8 MPa of compressive loading, and no impact but 0.8 MPa of compressive loading. Between 10 and 12 plugs were harvested from each knee depending on the size of the knee. For each experiment, the plugs from the 3 pigs (6 knees) were randomly assigned to the 4 test groups, with at least 2 samples from every knee in each test group (randomized complete block design), resulting in 15 plugs per experimental group.

Following harvest, the osseous surface of the plugs was trimmed to 4 mm (full height of the osteochondral section) using an oscillating autopsy saw with a half-inch blade. The plug's osseous surface was leveled, creating a sample that would sit flat in the impaction drop tower and bioreactor systems.

The plugs were soaked for 2 hours in a 1% antibiotic saline solution. The plugs were then rinsed in serum-free culture medium (Dulbecco's Modified Eagle Medium; Invitrogen Corp, Eugene, Oregon) with 1% ITS-Premix (BD Biosciences, San Jose, California) and 50 µg/mL of ascorbic acid (Sigma-Aldrich Corp, St Louis, Missouri) and 1% antibiotic-antimycotic (Atlanta Biological, Lawrenceville, Georgia). The plugs were cultured individually for 24 hours in 24-well plates with 2 mL of serum-free culture medium in an incubator at 37°C with 5% CO_2 to habituate the tissue to the new environment.

Impact Injury

Following a 24-hour habituation period, individual cartilage plugs were placed (articular surface up) in the tissue dish of a custom drop tower (Figure 1a), which featured a recessed well tissue dish equipped with a force transducer that enabled dynamic force measurements. The initial drop height was measured from the surface of the tissue dish to the impact surface of the weight. Impact loads of 25 MPa are associated with chondrocyte death and extracellular matrix fissures,[15,29] with cell death occurring at as low as 14 MPa.[11] Pilot testing was performed to determine the optimal weight and height for creating partial-thickness articular cartilage lesions using 20 to 25 MPa of impact pressure (with variability due to individual plug variations). A 500-g weight released from a height of 15 cm was used to create impact injuries (mean impact pressure, 25.22 ± 6.05 MPa). Following impaction, the cartilage plug was placed in 2 mL of fresh culture medium in a 24-well plate.

Mechanical Loading

Mechanical loading was performed in a custom bioreactor system (Figure 1b). The bioreactor system had 10 culture chambers, each connected to an air cylinder that used air pressure in parallel to deliver equal force to the plugs simultaneously; at 0.5 MPa, the load varied 1.5% (7.27 kPa) between chambers. The air cylinder's pistons were equipped with a porous plate, which allowed nutrients to reach the surface of the cartilage. Assuming that the surfaces of the cartilage plugs were perfectly flat, the system provides uniform uniaxial compressive loading to the entire surface. The cartilage plugs were housed in an unconfined culture chamber filled with culture medium during cyclic compressions, which allowed the cartilage to swell and compress freely and minimized frictional heat generation.

One hour postimpaction, plugs from the mechanically loaded test groups were subjected to two 30-minute rounds

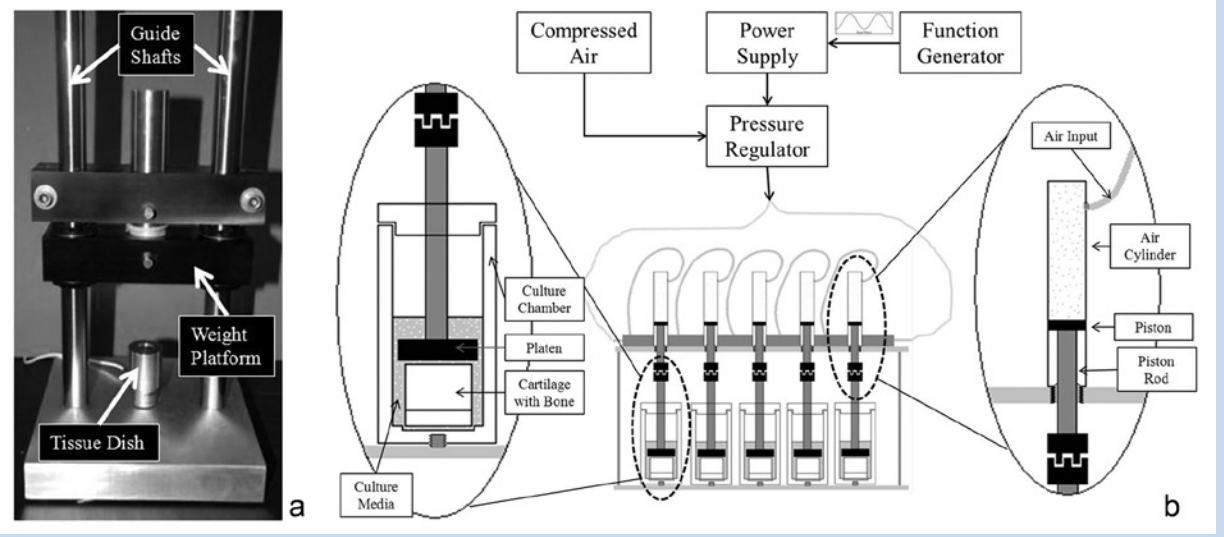

Figure 1. Custom-built equipment. (a) Drop tower used to create reproducible impact injuries. (b) Custom-designed bioreactor used to apply dynamic forces to 10 cartilage samples simultaneously. The cartilage samples were housed in custom-designed chambers attached to air cylinders. The bioreactor system utilizes an air pressure regulator and function generator to deliver forces with a variable range of magnitudes and frequencies.

of load-controlled sinusoidal pressure (0.5 MPa in experiment 1 and 0.8 MPa in experiment 2) at 1 Hz (equivalent to an able person's walking pace[41]) 6 hours apart. The time points were selected on the basis of a previous study that showed cell death is initiated between 2 and 6 hours following injury.[20] The bioreactor was placed in a 37°C incubator with 5% CO_2 for the duration of the experiment.

Further details on sample processing are available in Appendix 1 (available at http://sph.sagepub.com/content/suppl).

Statistical Analysis

For the total number of cell comparisons, each sample was first normalized to control samples from the same pig so that comparisons could be made across multiple animals, with control groups representing 100%. For cell viability and TUNEL analysis, no normalization was performed, since the data were presented as a percentage. A 1-way analysis of variance was conducted, followed by least significant difference and Student-Newman-Keuls post hoc tests to examine differences between multiple experimental groups using SPSS (IBM, Armonk, New York). Findings were reported as means, with error bars representing the standard deviations. Findings were considered significant with a confidence interval of 95% or higher ($P \leq 0.05$).

RESULTS

Effect of Impact Injury on Articular Cartilage

In this in vitro culture model, control tissue was nonimpacted and nonmechanically loaded and showed 64.46% ± 16.16% cell viability 48 hours postharvest (24-hour habituation culture + additional 24 postexperimental injury culture) (Figures 3a and 4a). Compared with control samples, impacted articular cartilage showed a decrease in cell viability (5.52%) (Figure 3b and 4a), a decrease in the total number of cells (4.56%) (Figure 4b, Table 1), and a significantly higher number of apoptotic cells (from 18.2% to 63.3%, $P < 0.001$) (Figure 4c). Hematoxylin and eosin staining revealed surface damage in the superficial tangential zone in the impacted, nonmechanically loaded group (Figure 3d and 3e). Although no significant gene expression differences were found between impact and control samples, impacted samples tended to exhibit lower COL2 (Figure 5a) and higher CASP3 (Figure 5b) compared with control samples.

Effect of Compressive Loading on Articular Cartilage

Normal Cartilage

The nonimpacted 0.5-MPa compressive loading group exhibited a significantly increased total number of live cells (17.35%) compared with baseline control samples ($P = 0.002$) (Figure 6a). However, no significant difference was found between the 0.8-MPa compressive loading group (cultured without impaction) and controls (Figure 6a). Significant differences (31.50%) in the total number of live cells were observed when comparing cartilage cultured with 0.5 and 0.8 MPa of compressive loading ($P < 0.001$) (Figure 6a). Gene expression analysis of 0.5-MPa mechanically loaded, nonimpacted cartilage in culture showed significantly higher

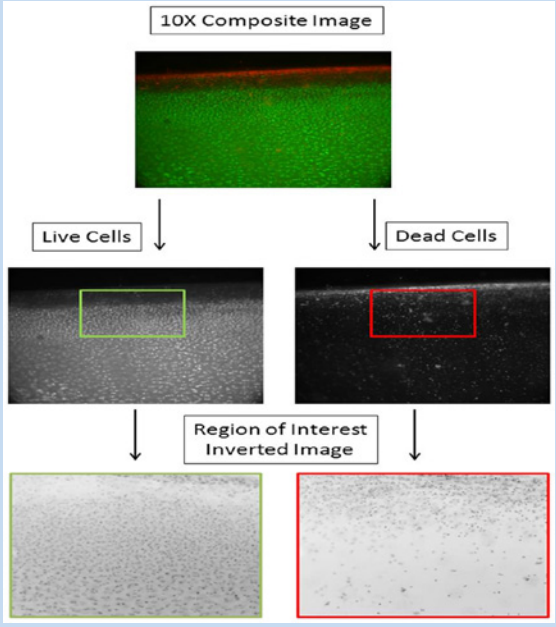

Figure 2. Schematic of quantification of fluorescent-stained images. The example shown is of impacted cartilage (without mechanical loading). A 10× image is taken and broken into its component images: live cells and dead cells for cell viability (as shown) and total cells and TUNEL positive cells for apoptotic analysis. A region of interest is defined in the center of the image, from the surface of the cartilage down (in the superficial tangential region). The image is black/white inverted, and the cells are counted using ImageJ software. For cell viability analysis (as shown), total number of cells represents the live cells plus the dead cells, and cell viability is calculated as follows: (live cells × 100) / total number of cells. For apoptotic analysis, the DAPI stain counts total number of cells directly; therefore, percentage apoptotic is as follows: (TUNEL – positive cells × 100) / DAPI-stained cells.

COL2 expression (107.08%, $P = 0.008$) (Figure 5a) and lower CASP3 expression (37.65%) (Figure 5b) compared with controls.

Injured Cartilage

In the impacted, compressive loading group with 0.5 MPa of pressure, a significant increase (33.29%) in the total number of live cells was observed compared with the impacted, nonmechanically loaded group ($P = 0.001$) (Figure 6b). Cell viability staining showed that the application of 0.5 MPa of compressive loading to impacted cartilage significantly increased cell viability compared with injured cartilage that did not receive mechanical loading ($P = 0.004$) (Figures 3c and 4a). There was no significant difference in the number of live cells between the 0.8-MPa mechanically loaded group and the injured cartilage (Figure 6b); however, there was a significant difference between the 0.8- and 0.5-MPa injured cartilage groups ($P = 0.010$), demonstrating that the magnitude of compressive loading has an effect on the therapeutic benefits.

Apoptotic TUNEL analysis revealed a 41.0% decrease in apoptotic staining (the number of apoptotic cells) from the impacted group without compressive loading to the impacted group with 0.5 MPa of compressive loading (from 63.3% to 22.4%, respectively—a 90.8% reduction in injury-related apoptosis; $P < 0.001$) (Figure 4c): ([impact – control] – [mechanical loading – control]) / (impact – control) × 100. In addition, no significant differences were observed between the control group (18.3%) and the injured + mechanical loading group (22.4%).

Gene expression analysis of impacted 0.5-MPa mechanically loaded cartilage showed a significant decrease in CASP3 expression (67.15%, $P = 0.012$) (Figure 5b) compared with impacted samples.

No significant differences were observed in cell viability, total number of cells, or apoptotic expression between the impacted and nonimpacted 0.5-MPa compressive loading groups. Gene expression analysis revealed a significantly higher COL2 expression in the nonimpacted 0.5-MPa compressive loading group than the 0.5-MPa compressive loading group with impact (107.59%, $P = 0.003$) (Figure 5a).

DISCUSSION

The present study investigated the effects of compressive loading on chondrocyte viability of porcine cartilage explants in vitro. Results showed that compressive loading at 0.5 MPa, immediately following impact injury, decreases apoptosis but not at 0.8 MPa.

Following an injury, apoptotic chondrocytes begin to die within hours; over time, the tissue is compromised and loses its ability to maintain and restore itself. The present findings suggest that application of compressive loading at low levels (such as minimum weightbearing or continuous passive motion) to acutely injured cartilage has a positive effect on cell viability following injury. Furthermore, the COL2 expression in the impacted and mechanically loaded samples was comparable with control samples, indicating that the metabolic activity of the cells was still normal and producing relevant matrix molecules. Therefore, this study verified the hypothesis that mechanical loading of injured cartilage may increase chondrocyte viability by reducing apoptosis at injured sites after an impact injury, subsequently increasing the number of cells that survive the injury long term.

In a previous study by Torzilli et al, 0.5 MPa of mechanical loading was used to mitigate interleukin-1–induced proteoglycan and collagen loss caused by aggrecanses and matrix metalloproteinases.[38] Similarly, these findings show that 0.5 MPa of mechanical loading has a positive effect on cartilage following acute injury. In the present study, the application of 0.5 MPa of compressive loading to injured cartilage resulted in a significant decrease (64.64%) in apoptotic expression (apoptotic TUNEL analysis [Figure 4c] and CASP3 gene expression analysis [Figure 5b]), significantly increased total number of live cells (12.05%,

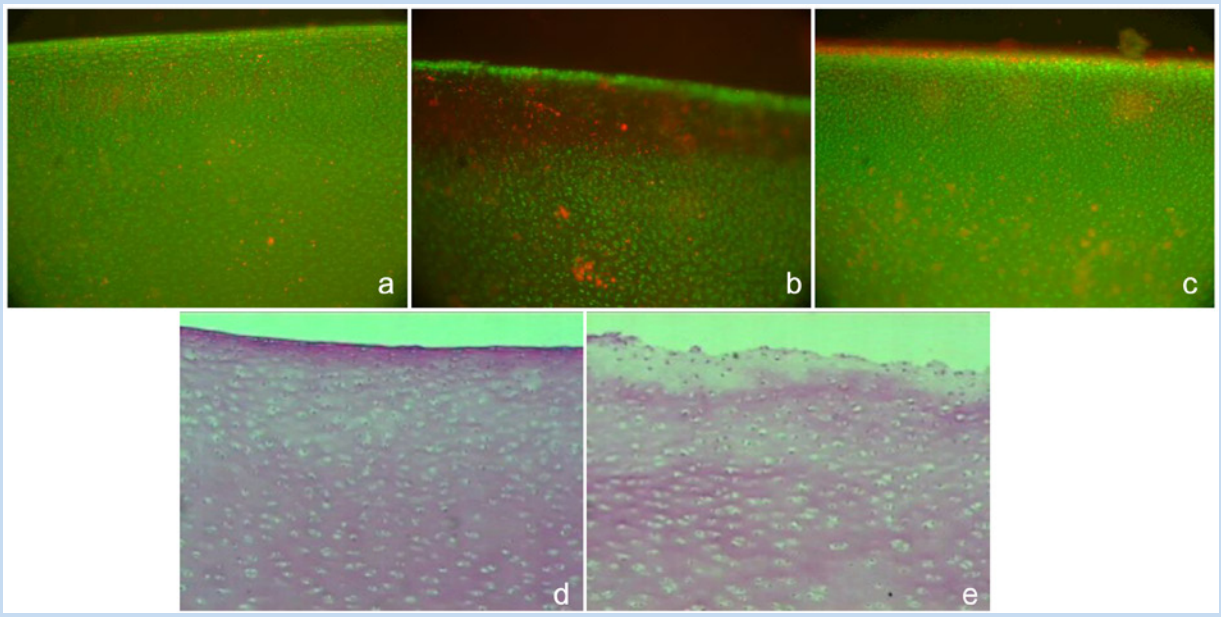

Figure 3. Cell viability and hematoxylin and eosin analysis. (a to c) Cell viability–stained images (10×): overlap images of the live cells (green) and dead cells (red). (a) Control (no impact, no mechanical loading); (b) impact (no mechanical loading); (c) impact with 0.5 MPa of mechanical loading. (d and e) Histologic analysis, hematoxylin and eosin–stained images. (d) Control sample; (e) impacted sample displaying damage on the surface of the cartilage.

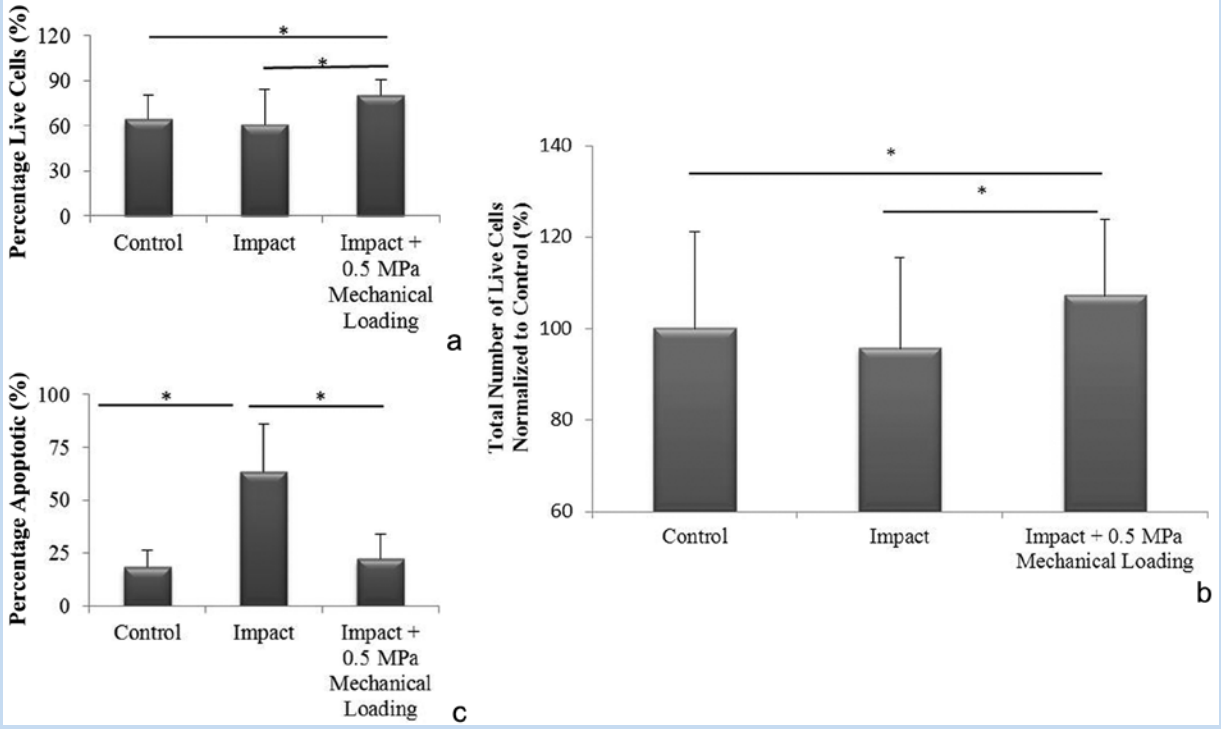

Figure 4. Effect of 0.5MPa compressive loading on impacted cartilage: (a) Cell viability (the number of live cells x 100%/ total number of cells), control: 64.46 ± 16.16 %; impact: 60.90 ± 23.46%; impact + 0.5MPa mechanical loading: 80.19 ± 10.73 % (n = 15, $*P = 0.006$); (b) Comparison of the total number of live cells, normalized to the control ($*P = 0.011$); (c) Apoptotic Stained Images (percent of cells staining positive for apoptotic expression; n = 15, $*P \leq 0.001$).

Table 1. Average numbers of live cells versus all cells (live + dead)

	Live Cells	Live + Dead Cells
Control	1259.21 ± 313.49	1921.52 ± 387.88
Impact	1164.43 ± 437.27	1833.65 ± 389.37
Impact + compressive loading (0.5 MPa)	1536.63 ± 216.98	2037.07 ± 327.46

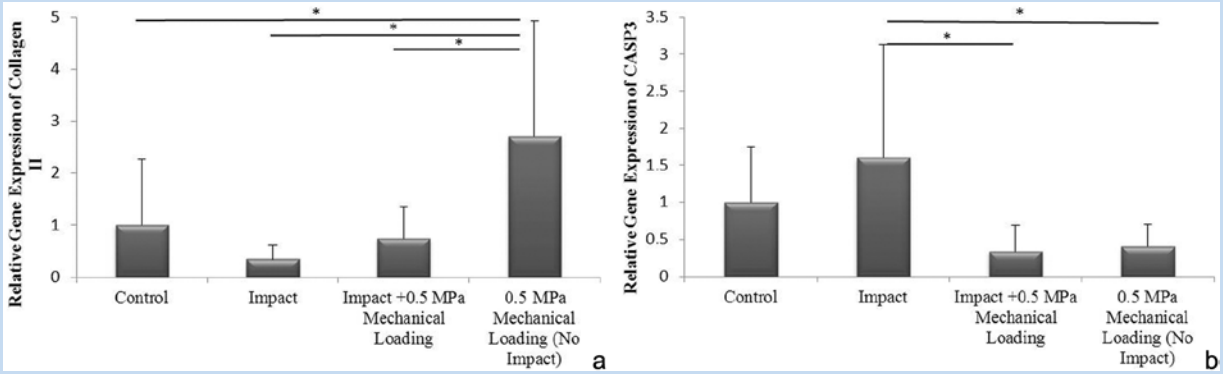

Figure 5. Gene expression analysis. Samples expressed as fold change of the control; (a) Collagen II expression (*$P = 0.003$); (b) Caspase 3 expression (*$P = 0.051$).

$P = 0.05$) (Figure 4b), and significantly increased chondrocyte viability (31.68%, $P < 0.01$) (Figure 4a) compared with impacted cartilage without compressive loading. All findings (decreased apoptosis, increased total number of live cells, and cell viability) indicate that compressive loading at low levels has a positive effect on cartilage following acute injury. Furthermore, the higher CASP3 expression (Figure 5b) and apoptotic staining (Figure 4c) in the impacted group compared with the control at the 24-hour postimpaction time point suggests that the viability and total number of live cells of the injured cartilage may continue to decrease over time.

The long-term survival of chondrocyte in cartilage explants in vitro decreases over time.[2,44] In the present study, 0.5 MPa of compressive loading had a beneficial effect on normal nonimpacted tissue. Specifically, a 17.4% increase in the total number of live cells was observed in the nonimpacted mechanical loading group compared with controls (Figure 4b), suggesting that subphysiological loading (0.5 MPa) of cartilage sustains chondrocyte viability better than culturing without loading. Articular cartilage is avascular, so chondrocyte survival relies on anaerobic metabolism and nutrient diffusion from the synovial fluid.[25] Cartilage survival in vitro may be maximized by dynamic loading to promote large solute nutrient transport into the avascular tissue.

This study suggests that mechanical loading at subphysiological levels may be protective to cartilage. Therapeutic exercises of this magnitude may minimize the effects of injury. The benefits of mechanical loading have been well documented.[20,34,35,38,39,43] This study mechanically loaded the injured cartilage 1 hour following impact (the time frame may not be clinically relevant) and evaluated the effects 24 hours postinjury with a 90.8% reduction in injury-related apoptosis. The early intervention in this study may be ideal for mitigating an apoptotic response. Previous studies have shown that the percentage of apoptotic chondrocytes in humans increases progressively from 6 hours to 1 week following injury,[11] suggesting that therapeutic treatments to halt apoptosis may be beneficial.

There are several limitations in this study including the bioreactor system, which features only uniaxial unconfined compression, which does not fully mimic the complex mechanical loads applied to the cartilage surface in vivo. Another limitation is that it was conducted in an in vitro environment.

CONCLUSION

This study showed that subphysiological levels of mechanical loading (0.5 MPa) immediately following impact injury decrease apoptotic expression and maintain chondrocyte viability in this in vitro porcine model. Specifically, subphysiological mechanical loading may be a useful early intervention for maintaining cartilage health after injury.

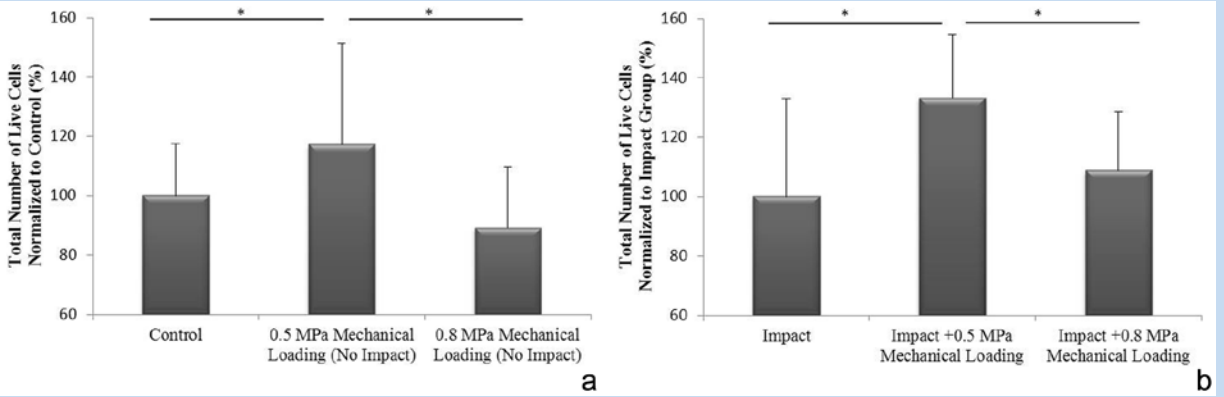

Figure 6. (a) The effect of different magnitudes (0.5 vs 0.8 MPa) of mechanical loading on nonimpacted cartilage in culture (*P = 0.001). (b) The effect of different magnitudes (0.5 vs 0.8 MPa) of mechanical loading on impacted cartilage (*P = 0.002).

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