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A *single application* of cyclic loading can accelerate matrix deposition and enhance the properties of tissue-engineered cartilage

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Summary

Objective: Mechanical stimulation is a widely used method to enhance the formation and properties of tissue-engineered cartilage. While studies have evaluated the responsiveness of chondrocytes to mechanical stimuli, little is known about how much stimulation is actually required. Thus, the purpose of this study was to investigate the effect of a *single application* of cyclic loading to chondrocytes on the formation and properties of *in vitro*-formed tissue.

Design: Isolated bovine articular chondrocytes were seeded on ceramic substrates in 3D culture and subjected to a *single application* of compressive cyclic loading at 1, 8 or 15 days after seeding. Once the time at which the chondrocytes were most sensitive to mechanical loading was determined, the effect of a *single application* on the synthesis and accumulation of matrix molecules as well as the mechanical properties of the *in vitro*-formed cartilage tissue was evaluated.

Results: Chondrocytes were more responsive to cyclic loading applied early in culture. Cyclic forces applied 24 h after the cultures were established increased collagen and proteoglycan syntheses ($48 \pm 11\%$ and $49 \pm 11\%$, respectively). This *single application* of cyclic loading also increased the accumulation of collagen (stimulated: 207 ± 20 μg, control: 173 ± 9 μg) and proteoglycans (stimulated: 302 ± 24 μg, control: 270 ± 14 μg) as well as improved the mechanical properties of the *in vitro*-formed tissue (twofold increase in equilibrium stress and modulus) determined 4 weeks after the applied stimulus.

Conclusions: A *single application* of cyclic loading to chondrocytes early in culture increased matrix accumulation and enhanced the mechanical properties of the *in vitro*-formed tissue. This suggests that mechanical forces do not have to be applied intermittently over long periods of time to accelerate *in vitro* tissue formation.
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Key words: Tissue engineering, Articular cartilage, Calcium polyphosphate substrates, Chondrocytes, Mechanical stimulation.

Introduction

Resurfacing damaged joints with cartilaginous tissue formed *in vitro* is a promising new approach to repair articular cartilage. Isolated chondrocytes grown in culture can synthesize cartilaginous extracellular matrix (ECM) macromolecules indicating that these cells maintain their phenotype under these conditions[1–4]. However, it has proven difficult to accumulate enough ECM to produce a tissue with mechanical properties that mimic native articular cartilage[1–4].

It is well known that chondrocytes can respond to mechanical stimuli[5–7] and it has become generally recognized that mechanical forces play an important role in both the development and maintenance of healthy cartilage *in vivo*[8–10]. For this reason, mechanical stimulation of chondrocytes during *in vitro* tissue formation has been investigated as a strategy to produce functional cartilaginous tissue. Previous studies have shown that cyclic mechanical forces applied to isolated chondrocytes grown in scaffolds[2,11,12] or on the surface of substrates[13,14] can stimulate the synthesis of cartilaginous ECM macromolecules. The effect of specific loading conditions (frequency, amplitude, and duration) has also been investigated by observing the change in biosynthetic response of the cells[2,7,13–15]. When exposed to these forces intermittently over a long period of time, the cultures accumulate greater amounts of ECM, which results in the improved mechanical performance of the *in vitro*-formed cartilaginous tissue[13,14,16,17]. Interestingly, similar changes in both ECM accumulation and mechanical properties were observed between these studies even though there was a large variation in the amount of stimulus applied (ranging from hours to minutes each day). A similar effect has been observed in other connective tissue cell types. Rubin and Lanyon[18] demonstrated that only 36 cycles of intermittent stimulation (2 min) were required to increase bone formation in an *in vivo* turkey ulnar model and their results also indicated that no further increase in bone

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formation was observed between 2 and 60 min of stimulation each day. This suggests that relatively little stimulation may actually be required to increase matrix accumulation by chondrocytes in culture. Thus, the purpose of this study was to investigate the effect of a *single application* of compressive cyclic loading to isolated chondrocytes at various times in culture on the synthesis and accumulation of ECM and the subsequent mechanical properties of tissue-engineered cartilage.

Materials and methods

CHONDROCYTE CULTURES

Primary articular chondrocytes were grown using a scaffold-free culture system and cells isolated from the calf metacarpal−phalangeal joint of 6−9-month-old calves by sequential enzymatic digestion[19]. Cells were obtained from two to three animals and pooled together in order to have sufficient cells for each experiment. The isolated chondrocytes were seeded in multi-layer culture (160,000 cells/mm^2, approximately up to 10 cell layers thick) on the top surface of porous calcium polyphosphate (CPP) ceramic substrates (4 mm diameter $\times$ 6 mm length)[20] which have been previously shown to support the formation of cartilaginous tissue[4]. The cells were incubated at 37 °C and 5% CO_2 in Ham's F12 medium supplemented with 25 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (HEPES) and 20% fetal bovine serum (Sigma Chemical Co., St. Louis, MI), and 100 μg/mL ascorbic acid (Sigma)[19]. The medium (2 mL per sample) was changed every 2−3 days with fresh ascorbic acid added at each change. Each experiment was done in triplicate and the results pooled yielding a minimum sample size of $n = 6$ per group.

MECHANICAL STIMULATION

To first establish the time early in culture at which the chondrocytes were most sensitive to mechanical loading, cultures were subjected to a *single application* of compressive cyclic loading at either 1, 8 or 15 days after seeding and then harvested to determine the effect on proteoglycan and collagen syntheses (see below). Cyclic compressive forces of 9.81 mN (1 g) amplitude for 30 min at 1 Hz were applied to the cultures using a Mach-1™ mechanical tester capable of stimulating up to four samples simultaneously (Biosyntech, Laval, PQ). The device used custom designed titanium alloy (Ti6Al4V) platens[13,14] to apply mechanical forces simultaneously to four samples which were monitored using a 9.81 N (1 kg) tension−compression load cell (minimum resolution of 98 μN or 10 mg). Since relatively little matrix accumulation occurs over these time-periods[19] and as not to disperse the chondrocytes during cyclic loading, mechanical forces were applied to the layer of cells on the top surface of the cell culture through the use of compliant agarose gel cylinders (3.5 mm diameter $\times$ 8 mm length; Sigma) rested on top of the culture (Fig. 1). Prior to mechanically stimulating the cultures, several different gels of varying weight percents (0.5−5% agarose) were mechanically tested (cyclically loaded between 0 and 30 mN at 1 Hz for 30 min) to select an appropriate gel to apply dynamic loading to the cultures. Low-weight percent gels ($<$1%) were unable to achieve the desired load magnitude without failing and high-weight percent gels ($>$2%) all fractured (presumably by fatigue) before the test was completed. For this reason, we opted to use a 2 weight percent agarose gel (compressive stiffness: 10 ± 1 kPa, $n = 6$). Load transmission through the agarose cylinder was confirmed as similar amounts of deformation were required to achieve the desired load amplitude with or without the culture situated underneath the gel ($\sim$2 mm for a 30 mN applied load). To mechanically stimulate the cells, the agarose cylinder was situated on the surface of the culture and then cylinder-cell layer construct was first preloaded to 2.5 mN (0.25 g) which was then defined as the zero-strain state. The agarose cylinder-cell layer construct was then deformed under displacement-control with the amplitude of cyclic (sinusoidal) deformation (less than 5% total harmonic distortion) adjusted manually each time to apply sinusoidal loading to the construct of the desired magnitude. Agarose cylinders were removed immediately after stimulation and selected cylinders were screened for the presence of cells (see below). To ensure that each of the four simultaneously tested samples was properly loaded, only CPP substrates and agarose cylinders of similar heights were used (e.g., CPP heights showing less than 10 μm variation).

Once the time point at which the chondrocyte cultures were more responsive to cyclic loading was determined (as described earlier), additional cultures were established and mechanically stimulated (at this time point) to determine the sensitivity of the cultures to specific loading conditions (i.e., cyclic load amplitude and duration). In these experiments, chondrocyte cultures were subjected to a *single application* of compressive cyclic loading at different amplitudes: 9.8, 19.6 or 29.4 mN (1, 2 or 3 g, respectively)

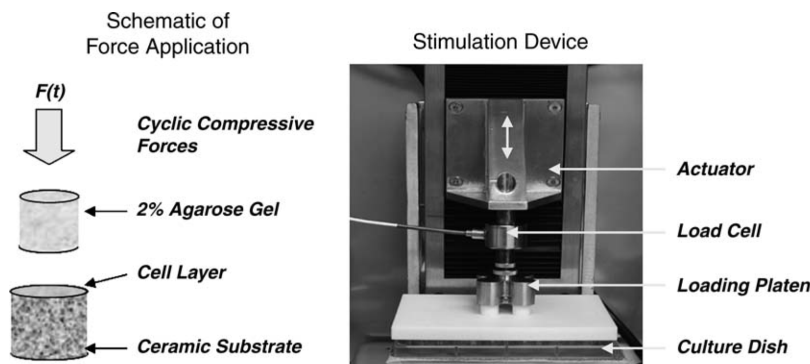

Fig. 1. Cyclic compressive forces were applied via 2% agarose gels so as not to disperse the cells seeded on the surface of the ceramic substrate (left). Mechanical forces were applied using a Mach-1™ mechanical tester with custom designed loading platens and culture plates (right).

and durations: 15, 30 or 60 min each at a frequency of 1 Hz. Cultures were then either immediately harvested to determine the effect on proteoglycan and collagen syntheses or further cultured under static (no load) conditions for a period of 4 weeks to determine the effect on ECM accumulation. All stimulation experiments were conducted aseptically in culture media at 37 °C. Control cultures were treated similarly but did not receive any stimulation.

ASSESSMENT OF PROTEOGLYCAN AND COLLAGEN SYNTHESES

After the *single application* of cyclic loading, agarose cylinders were removed and the chondrocytes (stimulated and matched unstimulated controls) were incubated in the presence of both [^{35}S]SO_4 (4 μCi/culture) to label proteoglycans and [^{3}H]proline (5 μCi/culture) to label collagen for a period of 24 h. Although proline can be incorporated into different proteins, in chondrocyte cultures approximately 90% of proline becomes incorporated into collagen[21,22]. Since synthesized ECM components can either be incorporated into the culture or released to the media, matrix synthesis was determined by evaluating both fractions. The conditioned medium was collected and the radiolabeled ECM molecules were precipitated. Proteoglycans were precipitated by adding 100% cold ethanol (3:1 ratio)[23] whereas collagen was precipitated by adding 70% ammonium sulphate solution (3:4 ratio)[24,25] overnight at 4 °C. Samples (precipitated proteoglycan and collagen samples) were each centrifuged at 14,000 rpm for 30 min at 4 °C. The pellets were washed three times in 70% ethanol and centrifuged after each wash at 14,000 rpm for 10 min at 4 °C. Pellets were then resuspended in either 4 M guanidine hydrochloride to solubilize the proteoglycans or 10% sodium dodecyl sulfate (SDS) in Tris buffer (0.1 M, pH 7.0) to solubilize collagen. Radioisotope incorporation in the resuspended media pellets was then determined using a β-liquid scintillation counter (Beckman LS6000TA, Beckman Instruments, Mississauga, ON). The unincorporated isotope from the tissue cultures was removed by gently washing the samples three times in phosphate-buffered saline. Cultures were then digested by papain (40 μg/mL in 20 mM ammonium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), and 2 mM dithiothreitol (DTT)) for 48 h at 65 °C. The accumulation of newly synthesized proteoglycan and collagen in the matrix was then estimated by quantifying radioisotope incorporation from aliquots of the papain digest using a β-liquid scintillation counter. The amount of synthesized molecules in each fraction (culture or media) as well as the total matrix synthesis (culture + media) was calculated relative to the DNA content of the tissue and expressed as a percentage of the unstimulated controls.

BIOCHEMICAL ASSESSMENT OF THE ACCUMULATED ECM

After 4 weeks of static culture following the *single application* of cyclic loading, the tissue that had formed on the top surface of the ceramic substrate was removed, lyophilized and weighed to determine the dry weight of the tissue (AT-250 Balance, Mettler Instrument Corporation, Hightstown, NJ). The tissues were digested with papain (as described above) and stored at −20 °C until analysis. Aliquots of the digest were assayed separately for the proteoglycan, collagen and DNA contents[4]. Briefly, the proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycans using the dimethylmethylene blue dye binding assay[26]. Collagen content was estimated from the determination of the hydroxyproline content. Aliquots of the papain digest were hydrolyzed in 6 N HCl at 110 °C for 18 h and the hydroxyproline content of the hydrolyzate was then determined using chloramine-T/Ehrlich's reagent assay[27]. The DNA content was also determined from aliquots of the papain digest using the Hoechst dye 33258 assay[28]. Similarly, agarose cylinders used to apply mechanical forces to the cultures were screened for the presence of adherent cells using the Hoechst dye 33258 assay after digesting the cylinders with papain according to the procedure described by Buschmann *et al.*[1].

HISTOLOGICAL ASSESSMENT OF THE ACCUMULATED ECM

Cultures were also established to perform histological assessments of the *in vitro*-formed tissues following long-term culture after the *single application* of cyclic loading. After 4 weeks of culture, the tissue that had formed on the top surface of the ceramic substrate was removed using a scalpel blade (no. 11), fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections (5 μm thick) were cut, stained with either hematoxylin−eosin (H&E) or toluidine blue, and examined by light microscopy.

COMPRESSIVE MECHANICAL PROPERTIES

Additional cultures were also established to determine the effect of the *single application* of cyclic loading on the compressive mechanical properties of the *in vitro*-formed tissues following long-term culture. Immediately upon removal after 4 weeks of static culture, the thickness and diameter of the tissue above the CPP substrate (determined from the average of several positions around the circumference of the sample as not to deform the tissue) were measured using a digital micrometer (Mitutoyo American Corporation, Aurora, IL). Equilibrium compressive stress−strain properties of the cartilaginous tissue-CPP substrate constructs were assessed using a Mach-1™ mechanical tester (Biosyntech, Laval, PQ), as described previously[4]. Briefly, samples were first preloaded to 9.8 mN (1 g) (which was defined as the zero-strain state) and then subjected to sequential step compressions of 2.5% strain to a maximum of 25% strain. At each step, the force decay was recorded until equilibrium was reached as defined by a change in force less than 2 mN/min (0.2 g/min). The equilibrium stress was calculated and plotted as a function of the applied strain. The equilibrium modulus was then determined from the backwards first-order numerical derivative of the equilibrium stress−strain curve at 25% compressive strain. Mechanical testing of the *in vitro*-formed cartilaginous tissues was conducted in uniaxial, unconfined compression while submersed in culture media at 37 °C.

STATISTICS

All results were expressed as the mean ± standard error of the mean (S.E.M.) and analyzed using statistical software (SigmaStat 1.0, SPSS Science, Chicago, IL). Matrix synthesis and tissue formation results were analyzed using one-way analysis of variances (ANOVAs) and the Fisher's least-square differences (LSD) *post hoc* test to determine the effect of the time of exposure, loading amplitude, or loading duration. Mechanical property results were analyzed using a *t* test to determine the difference between stimulated and unstimulated cultures. The data were checked prior to performing the tests for both normality and equal-variance. Significance was assigned at *P*-values less than 0.05.

Results

EFFECT OF MECHANICAL LOADING AT DIFFERENT TIMES IN CULTURE

To determine the most appropriate time to commence the mechanical stimulation of articular chondrocytes, isolated cells were subjected to a *single application* of compressive cyclic loading applied at either 1, 8 or 15 days after the cells were seeded. Cells stimulated 24 h after seeding were the most responsive to cyclic mechanical loading as demonstrated by the increased accumulation of newly synthesized ECM macromolecules (48% greater for both collagen and proteoglycans) compared to the unstimulated controls ($P < 0.01$). As the culture aged, the cells were not as responsive to mechanical loading. This was more pronounced for proteoglycans as synthesis was inhibited when the cells were stimulated 15 days after seeding (33% decrease, $P < 0.01$) (Fig. 2). Quantification of the total proteoglycan content of the cultures demonstrated that the cells were accumulating increasing amounts of ECM over this time period (1 to 15 days after seeding) (day 1: 32 ± 9, day 8: 105 ± 15, day 15: 179 ± 19 μg sulphated GAG; $n = 8$ per group). DNA assays performed on the cultures and the retrieved agarose cylinders immediately after stimulation showed neither loss of cells from the culture as a result of mechanical stimulation nor were any cells seen adherent to the agarose cylinders after loading (data not shown).

EFFECT OF MECHANICAL LOADING CONDITIONS ON MATRIX SYNTHESIS

To determine the sensitivity of these early cultures to mechanical loading, isolated articular chondrocytes were subjected to a *single application* of cyclic loading under different conditions 24 h after the cultures were established. Under all of the loading conditions investigated, chondrocytes showed an increase in the accumulation of newly synthesized collagen and proteoglycans compared to the unstimulated controls ($P < 0.05$). Although there was no effect of load amplitude ($P = 0.3$) (Fig. 3), additional experiments demonstrated that this lack of response was evident even with 5 g load amplitude (data not shown). There was, however, a significant effect of the duration of exposure ($P < 0.05$) (Fig. 4). Cyclic forces applied for 30 min elicited the greatest response ($P < 0.04$) and increased the accumulation of newly synthesized ECM macromolecules by 48% relative to the unstimulated controls. To determine whether these changes were due to increased ECM synthesis vs the increased retention of the newly synthesized molecules, the amount of newly synthesized ECM macromolecules that was released into the media was also quantified. There was little effect of mechanical stimulation on the amount of newly synthesized ECM macromolecules released into the conditioned media as only chondrocytes stimulated for 60 min released more proteoglycans, as opposed to collagen (Table I). Total ECM synthesis (combination of both the

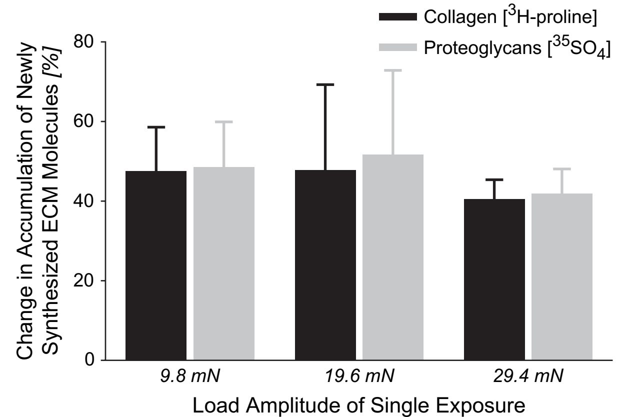

Fig. 3. Percent change in newly synthesized ECM macromolecules (collagen and proteoglycans) following a *single application* of cyclic compressive loading (30 min duration at a frequency of 1 Hz) under different load amplitudes of 9.8, 19.6 or 29.4 mN (1, 2 or 3 g) applied 24 h after seeding. Results were corrected for DNA content and expressed relative to the controls (unstimulated) as described under the Materials and methods. Results are expressed as the mean ± S.E.M. ($n = 6$ all groups).

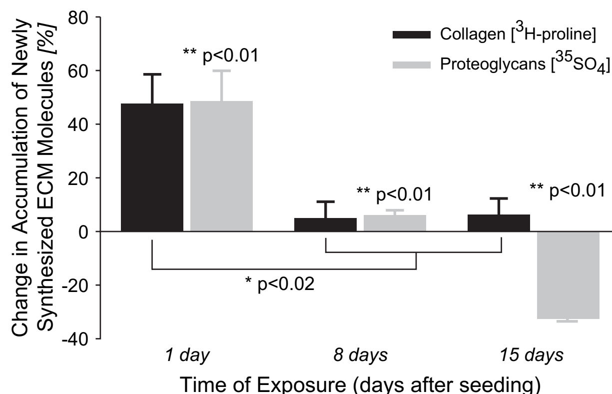

Fig. 2. Percent change in newly synthesized ECM mlacromolecules (collagen and proteoglycans) following a *single exposure* of cyclic compressive forces (9.8 mN amplitude for 30 min at 1 Hz) applied at either 1 (24 h), 8 or 15 days after seeding. The results obtained were normalized for DNA content and expressed relative to the controls as described under the Materials and methods. Results are the mean ± S.E.M. ($n = 6$ all groups). *Significantly different from the other groups ($P < 0.02$); collagen only. **Significantly different from the other groups ($P < 0.01$); proteoglycans only.

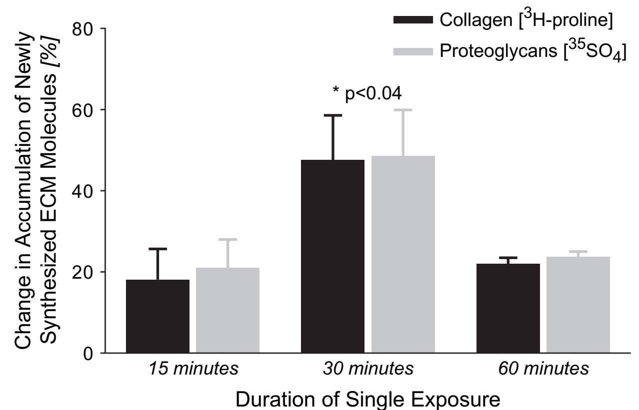

Fig. 4. Percent change in newly synthesized ECM macromolecules (collagen and proteoglycans) following a *single application* of cyclic compressive loading (load amplitude of 9.81 mN at a frequency of 1 Hz) for different durations (15, 30 or 60 min). Results were corrected for DNA content and expressed relative to the controls as described under the Materials and methods. Results are expressed as the mean ± S.E.M. ($n = 6$ all groups). *Significantly different from the other groups ($P < 0.04$).

Table I
Percent change in synthesized ECM macromolecules after a single exposure to cyclic forces

	Released into the media		Total (culture + media)	
	Collagen (%)	Proteoglycans (%)	Collagen (%)	Proteoglycans (%)
Effect of load (mN)				
9.8	19 ± 14	12 ± 14	40 ± 11*	32 ± 12*
19.6	13 ± 15	5 ± 16	44 ± 21*	36 ± 18*
29.4	25 ± 12	4 ± 12	39 ± 4*	23 ± 9*
Effect of duration (min)				
15	−15 ± 13	13 ± 15	16 ± 8*	18 ± 10*
30	19 ± 14	12 ± 14	40 ± 11*,†	32 ± 12*
60	5 ± 16	34 ± 6*	21 ± 1*	28 ± 3*

Percent change in newly synthesized ECM molecules (determined by radioisotope incorporation) released into the media and the total ECM synthesis (retained in tissue + released into media) following a *single application* of cyclic compressive loading for the different load amplitudes (19.8, 19.6 and 29.4 mN for 30 min at 1 Hz) and durations (15, 30 and 60 min at 1 g and 1 Hz) applied 24 h after seeding. Results were corrected for DNA content and expressed relative to the controls as described under the Materials and methods. Results are expressed as the mean ± S.E.M. ($n = 6$ all groups).

*Significantly different than control ($P < 0.05$).

†Significantly different from all other groups ($P < 0.05$).

incorporated and released radiolabeled molecules) (Table I) was similar to the accumulation of newly synthesized ECM macromolecules retained in the cultures amongst the different loading protocols (Table I). Thus, the percentage of the total newly synthesized ECM macromolecules that became incorporated into the culture (i.e., percent ECM retention) was unaffected by mechanical stimulation (proteoglycans: 60.4 ± 1.2%, collagen: 94.8 ± 0.3%; $n = 36$).

EFFECT OF THE SINGLE EXPOSURE TO MECHANICAL LOADING ON TISSUE FORMATION

Selected cultures were mechanically stimulated and then grown in static (no load) culture for a period of 4 weeks to determine the effect of a *single application* of cyclic loading on tissue formation. These cultures were only exposed to different durations of stimulation (at 9.8 mN load amplitude) since no observed influence of load magnitude on matrix synthesis was previously observed. Four weeks after the single exposure to cyclic loading, the chondrocytes had formed a continuous disk-shaped layer of cartilaginous tissue on the top surface of the substrate (Fig. 5) (diameter: 4.09 ± 0.01 mm, thickness: 1.0 ± 0.1 mm, $n = 12$). The ECM stained with toluidine blue indicated the presence of sulphated proteoglycans (data not shown). There were no obvious differences in the histological appearances of the two tissues. Cells exposed to cyclic forces for 30 min or greater accumulated more ECM (18% increase in dry weight) compared to the static (no load) controls (Table II). The ECM of these tissues also contained significantly more collagen (29% increase) and proteoglycans (14% increase) compared to the cultures grown under static (no load) conditions. Although cells may have pushed into the ceramic substrate as a result of mechanical loading, the *single application* of cyclic loading did not appear to affect the cellularity of the *in vitro*-formed tissue as there was no significant change in DNA content as a result of stimulation ($P = 0.2$) (Table II). The cartilage tissue that formed also displayed significantly improved mechanical properties. Cultures subjected to a *single application* of cyclic loading displayed in excess of a twofold increase in both the maximum equilibrium stress (control: 0.9 ± 0.2 kPa, stimulated: 2.1 ± 0.7 kPa; $n = 6$ per group; $P < 0.05$) and equilibrium modulus (control: 5 ± 2 kPa, stimulated: 12 ± 5 kPa; $n = 6$ per group; $P < 0.05$) (Fig. 6) compared to the unstimulated controls. For comparison purposes, the mechanical properties of the *in vitro*-formed tissues were inferior to native bovine articular cartilage harvested from the same joint (max. equilibrium stress: 53 ± 12 kPa, max. equilibrium modulus: 817 ± 170 kPa; $n = 8$[4]).

Discussion

A *single application* of compressive cyclic loading to articular chondrocytes significantly affected both the accumulation and properties of cartilaginous tissue formed *in vitro*. Chondrocytes subjected to dynamic loading 24 h after the

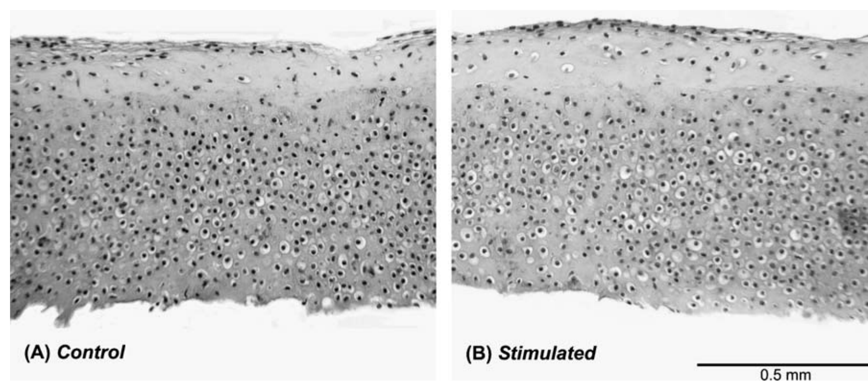

Fig. 5. Histological appearance of the *in vitro*-formed tissues after 4 weeks of static culture following a *single application* of cyclic compressive loading applied 24 h after seeding. (A) Cartilaginous tissue formed in the absence of mechanical loading (control). (B) Cartilaginous tissue formed following a single exposure to cyclic forces (stimulated) (9.81 mN amplitude, 30 min, 1 Hz) which was similar in appearance to cartilage formed in the absence of stimulation (A and B: H&E; original magnification 100×).

Table II
Effect of the single exposure to cyclic forces on in vitro *tissue formation*

	Loading duration			
	Control ($n = 11$)	15 min ($n = 6$)	30 min ($n = 6$)	60 min ($n = 6$)
Dry weight (mg)	1.1 ± 0.1	1.2 ± 0.1	1.3 ± 0.1*	1.3 ± 0.1*
DNA (μg)	11 ± 1	11 ± 1	10 ± 1	14 ± 2
GAG (μg)	270 ± 14	262 ± 23	302 ± 24*	309 ± 29*
GAG/DNA (μg/μg)	25 ± 2	23 ± 4	31 ± 2*	23 ± 4
OH-pro (μg)	17 ± 1	20 ± 2	21 ± 2	22 ± 1*
OH-pro/DNA (μg/μg)	1.6 ± 0.1	1.7 ± 0.1	2.1 ± 0.2*	1.6 ± 0.2

Biochemical evaluation of cartilaginous tissue formed after 4 weeks of static culture following a *single application* of cyclic compressive loading (load amplitude of 9.81 mN at 1 Hz) applied for different durations (15, 30 and 60 min) on cultures 24 h after seeding. Results are expressed as mean ± S.E.M. and n = the number of samples per group.

*Significantly different from all other groups ($P < 0.05$).

cells were seeded resulted in the greater accumulation of ECM (by 15–30%) which was more mechanically functional (twofold increase in compressive properties) as observed 4 weeks after the applied stimulus. Although several studies have demonstrated that chondrocytes can upregulate expression of ECM genes[29], increase ECM synthesis[30–32] and develop a mechanically functional ECM[13,14,16,17] in response to short-term or intermittent cyclic mechanical loading, this is the first study, to our knowledge, to show that both the accumulation and mechanical properties of *in vitro*-formed cartilage can be improved by only a *single application* of cyclic loading similar to that seen with long-term intermittent stimulation (4 weeks)[13,14,16]. A similar beneficial effect of short-term stimulation has also been shown for bone. In an *in vivo* bone model, Rubin and Lanyon[18] demonstrated that only 36 cycles of mechanical stimulation (approximately 2 min) each day increased bone formation in the turkey ulna.

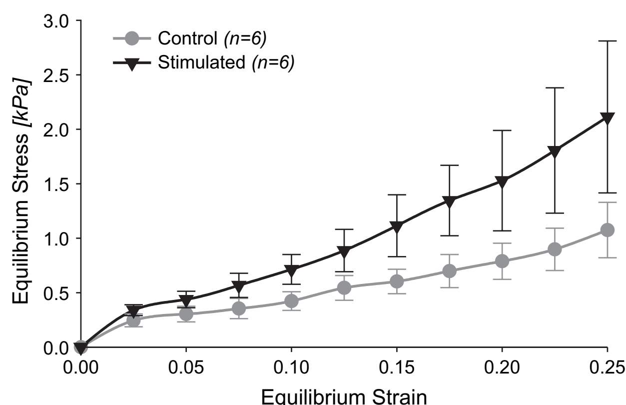

Fig. 6. The equilibrium compressive stress–strain behavior of the *in vitro*-formed tissues after 4 weeks of static culture following a *single application* of cyclic compressive loading applied 24 h after seeding. Gray circles: Cartilaginous tissue formed in the absence of mechanical loading (control). Black triangles: Cartilaginous tissue formed following the single exposure to cyclic forces (stimulated) (9.81 mN amplitude, 30 min, 1 Hz). Equilibrium mechanical testing was conducted between 0 and 25% strain at 2.5% strain increments. Results are expressed as the mean ± S.E.M. (n = 6 both groups).

In this study it was also found that articular chondrocytes maintained in scaffold-free 3D culture were more sensitive to cyclic mechanical forces early in the culture period. The syntheses of collagen and proteoglycans were both increased by approximately 48% (compared to the unstimulated controls) when cyclic loading was applied 24 h after the culture was established. When the same stimulus was applied to cells later in culture, the cells became unresponsive to the applied forces and did not synthesize elevated amounts of matrix macromolecules. There are several potential explanations for these observations. The mechanical properties of the accumulating cartilaginous ECM (i.e., increasing ECM stiffness) may affect cellular sensitivity to mechanical stimuli. It has been speculated that the chondrocyte pericellular matrix primarily serves a biomechanical role[33,34] which has been supported by recent finite element (FEA) models[35,36]. These models have shown that the increasing compliance of the cartilage ECM from the interterritorial to the pericellular region modifies the local stress-state and deformation perceived by the cell thereby affecting its biosynthetic response to mechanical stimuli. Another potential mechanism could be due to the possibility that dynamic loading early in culture leads to consolidation of the freshly seeded cells and this favors chondrogenesis *in vitro* similar to that seen when chondrocytes or mesenchymal stem cells are grown in pellet or micro-mass culture[19,37,38]. It also may account for the unanticipated inhibition of proteoglycan synthesis later on in culture (beyond 8 days) as the cells in these older cultures would then be essentially stimulated under load-control (as opposed to displacement-control) which can result in the inhibition of proteoglycan synthesis as has been observed for cartilage explants[39,40]. Consolidation of the newly seeded cells would explain the observed differences between the present study and that of Buschmann *et al.*[2]. In that study, chondrocytes embedded in agarose were less sensitive to mechanical loading early in culture and become increasingly more sensitive as further ECM is accumulated. Encapsulated cells cannot interact with one another to the same extent as in scaffold-free culture where cells are in direct contact. However, it is likely that other factors may also contribute. For example attachment to different materials can influence the types of integrin(s) expressed on the cell surface, which would then affect how the cells interact with their matrix or respond to mechanical stimuli[41].

In this study, the chondrocyte response to a *single application* of cyclic loading at various times during cartilage tissue formation was examined. Chondrocytes were found to be more responsive to cyclic forces applied early in the culture period. A *single application* of cyclic forces applied 24 h after the cells were seeded was sufficient to increase cartilage tissue formation and mechanical properties. It should be noted that the approach taken here (single stimulus followed by 4 weeks of static culture) does not necessarily reflect the optimal conditions to maximize ECM accumulation to more closely mimic native cartilage. The results, however, do suggest that mechanical forces may not have to be applied continuously, or even intermittently, over long periods of time to accelerate *in vitro* tissue formation. Although the actual mechanism leading to improved tissue is presently unknown, future studies will be directed to determine whether this effect is due to the biomechanical properties of the cartilaginous ECM or from the consolidation of the newly seeded cells.

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