Biophysical Stimuli: A Review of Electrical and Mechanical Stimulation in Hyaline Cartilage

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Abstract

Objective. Hyaline cartilage degenerative pathologies induce morphologic and biomechanical changes resulting in cartilage tissue damage. In pursuit of therapeutic options, electrical and mechanical stimulation have been proposed for improving tissue engineering approaches for cartilage repair. The purpose of this review was to highlight the effect of electrical stimulation and mechanical stimuli in chondrocyte behavior. **Design.** Different information sources and the MEDLINE database were systematically revised to summarize the different contributions for the past 40 years. **Results.** It has been shown that electric stimulation may increase cell proliferation and stimulate the synthesis of molecules associated with the extracellular matrix of the articular cartilage, such as collagen type II, aggrecan and glycosaminoglycans, while mechanical loads trigger anabolic and catabolic responses in chondrocytes. **Conclusion.** The biophysical stimuli can increase cell proliferation and stimulate molecules associated with hyaline cartilage extracellular matrix maintenance.

Keywords

hyaline cartilage, articular cartilage, growth plate, electrical stimulation, mechanical stimuli

Introduction

Hyaline cartilage is an avascular tissue characterized by having abundant extracellular matrix (ECM) that is mainly composed of collagen type II and proteoglycans (PG).^{1,3} This tissue is formed by a unique cellular type, the chondrocyte, which is responsible for maintaining the integrity of the ECM.^{4,5} Within the skeletal system, hyaline cartilage is found in two specialized structures of the long bones: the articular cartilage and the growth plate.^{1,5} The first acts as a smooth, lubricated, low-friction surface that receives mechanical loads and facilitates motion between opposing joints^{6,7}; while the second, which is located between the epiphysis and diaphysis, is responsible for the longitudinal growth and shape of long bones.^{8,9}

Histologically, both articular and growth plate cartilages are tissues stratified in different zones according to chondrocytes phenotype and spatial arrangement of the ECM. Accordingly, articular cartilage is organized in 5 zones: superficial, intermediate, medium, deep, and calcified,¹⁰ whereas the growth plate is arranged into 3 zones: reserve, proliferative, and hypertrophic.¹¹ In both tissues, the molecular structure of the ECM within the hyaline cartilage provides dimensionality, elasticity and strength to the tissue.^{3,10}

Given its location, hyaline cartilage is exposed to mechanical loads that generate different signals implicated

in the physiological regulation of chondrocytes behavior and several pathological processes, considering that chondrocytes sense and respond to different mechanical loads.^{2,12-14} For example, mechanical loading has been shown to affect cell deformation, fluid flow, nutrient and ion concentration gradients, pH changes, and anabolic and catabolic activity of ECM components and stimulation of growth factor synthesis.¹⁵⁻²⁰ In turn, overweight or strong impact loading have been proven to cause damage in the ECM and chondrocyte apoptosis, resulting in dysfunction of the tissue and subsequent damage.^{6,13,21,22} Several studies have shown that hyaline cartilage behavior is also affected by electrical and electromagnetic stimuli, resulting in changes in cell dynamics, such as migration,²³

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differentiation,²⁴⁻²⁶ morphology,²⁷⁻³⁰ proliferation,³¹⁻³⁶ and gene expression.³⁷⁻⁵⁰ This implies that mechanical, electrical, and electromagnetic stimulation may have an important role in the regulation of hyaline cartilage in both normal and pathological conditions.

Based on the above, understanding the influence of biophysical stimuli in the biology of hyaline cartilage not only provides useful information regarding their role in cartilage physiology *in vivo* but also supplies new tools for tissue engineering and regenerative medicine focused on the develop of new therapeutic approaches for the treatment of injuries in articular cartilage and growth plate. In this context, we performed a review of literature of the past 40 years of *in vivo* and *in vitro* studies, highlighting their results, advances, main limitations, and perspectives. The information presented summarizes the current understanding about the role of both the electrical stimulation over chondrocytes intended for articular cartilage recovery and the mechanical stimuli over growth plate and its influence on biological events during human bone development.

Electrical Stimulation for Tissue Engineering Hyaline Cartilage

Electric Fields

Electrical stimulation has been used to improve chondrocyte proliferation rate and the synthesis of characteristic ECM molecules. Electric fields (EFs) for cartilage regeneration have been used in *in vivo* and *in vitro* systems (Table 1).^{31,35,38,46,51-53}

In Vivo studies. The first reported use of electrical stimulation to influence cartilage was in 1974 by Baker *et al.*⁵² who assessed the influence of a bimetallic platinum electrochemical device in the recovery of full-thickness injuries of articular cartilage. The electrochemical device was implanted in the lateral femoral condyle at the location of the injury and voltages from 15 to 500 mV were applied. Results showed a 71% of increase in the production of ECM in treated animals.⁵² This finding set the basis for the application of electrical therapy in patients presenting with joint pathologies. The experience reported up to now in the literature has evidenced that the application of the EFs lead to pain improvement in patients with knee osteoarthritis. For instance, Farr *et al.*⁵³ used a transcutaneous electric stimulator in 288 patients with knee osteoarthritis. Patients who received electrical stimulation for more than 750 hours (73% of total population) reported both pain relief and a decrease in anti-inflammatory drug use (45.3%). Moreover, the therapy delays the need of a surgical intervention in chronic patients.⁵³

In Vitro Studies. The technique of delivering electrical stimulation to cell culture plates consists of an indirect coupling

system that uses external parallel electrodes connected to a power supply (Fig. 1A).^{31,38,39,54} The custom-designed devices are noninvasive capacitive coupling systems that can be used to stimulate any cellular type, and they are based on the same principle used in medical stimulation devices.^{51,53,54}

In vitro, the application of EFs to monolayer cultures has shown to increase cell population and to stimulate synthesis of main molecules that compound cartilage such as collagen type II, glycosaminoglycans (GAGs), and aggrecan. For instance, Rodan *et al.*⁵⁵ demonstrated that increased chondrocyte proliferation observed after EFs stimulation was triggered by a depolarization in the cell membrane, it means that the Na⁺ and Ca²⁺ fluxes generated by the EFs trigger DNA synthesis of chondrocytes. More descriptive studies were carried out by Brighton *et al.*,³⁴ in which they stimulated chondrocytes with different voltage values (10, 100, 250, and 1000 V) to a frequency of 60 kHz (sinusoidal-wave form). Their results indicated that voltages of 10 and 1000 V decreased cell proliferation; while a voltage of 250 V increased the GAGs synthesis.³⁴ In these studies, no data about the magnitude of the EFs were reported.

Similar studies were performed by Armstrong and Brighton where chondrocytes were stimulated with several EFs (15, 22.5, 30, and 45 mV/cm). Their results demonstrated an increase in cell population when EFs of 15, 22.5, and 30 mV/cm were applied. An increase in GAGs synthesis (66%) was obtained after stimulation with EFs of 45 mV/cm.^{31,33} From these studies, it was possible to conclude that an EF close to 20 mV/cm stimulates chondrocyte dynamics. Brighton *et al.*³² in later studies reported that an EF of 20 mV/cm increases the cell proliferation by 50%; while Wang *et al.*³⁹ in a more detailed study elucidated that this EF of 20 mV/cm increases aggrecan and collagen type II synthesis. On the contrary, researchers such as Nakasuji *et al.*³⁵ have applied EFs of greater magnitude (50, 100, 250, and 500 mV/cm) to chondrocytes cultured *in vitro*. The results showed that EFs of greater magnitude also affect cell proliferation and ECM synthesis.³⁵ Because of the discrepancy regarding the magnitude of the EFs required to stimulate the *in vitro* cultures, Vaca-González *et al.*⁵⁴ implemented a new methodology to calculate EFs and modulate proliferation and GAGs synthesis. Results allowed to establish the dielectric constant of the cell culture medium (complex permittivity and conductivity), and also showed that EFs of 4 mV/cm applied during 30 minutes increment cell population; while EFs of 8 mV/cm applied during 5 hours maintain a stable GAGs synthesis.⁵⁴

Studies Performed in Explants and 3-Dimensional Cartilage Constructs. The EFs have also been applied to cartilage explants and tridimensional constructs.⁵⁶ Brighton *et al.*³⁷ stimulated cartilage explants with EFs of 20 mV/cm. Results showed an increase in PG (34%) and collagen type

Table 1. Summary of Electric Field (EF) Application to *In Vivo* and *In Vitro* Systems.

Study Model	Chondrocytes Isolation Source or Joint Treated	Tension	Current	Frequency (kHz)	Electric Field	Stimulation Time	Effect on Cell Proliferation	Effect on Matrix Synthesis	Reference
<i>In vivo</i>	Femoral condyles of New Zealand white rabbits	15-500 mV	6 nA	—	—	1-9 weeks	Increase in cellular response, proliferation, and matrix production.	—	Baker et al. ⁵²
<i>In vivo</i>	Human knee with osteoarthritis	0-12 V	—	0.001	—	From 16 to more than 600 days	Pain relief and a decrease in anti-inflammatory drug use. Moreover, the therapy delayed the need of surgical intervention in chronic patients	—	Farr et al. ⁵³
<i>In vitro</i>	Epiphysis of embryo chicken tibia	1166 V for peer review	—	0.005	—	6 hours	Cell membrane depolarization controls proliferation process	—	Rodan et al. ⁵⁴
<i>In vitro</i>	Bovine costochondral joint	Part A (5-13, 5-33, 7-85) V _{rms}	105, 300, 750, and 1890 mA/cm ²	60 sinusoidal	7, 20, 50, and 126 V/cm	1 day	Increase in proliferation under an EF of 20 mV/cm	—	Brighton et al. ³²
<i>In vitro</i>		Part B (13, 5) V _{rms}	300 μ A/cm ²	60 sinusoidal	20 mV/cm	1 day	The cycles in an output signal (100%, 25%, 2%, and 0.25%) are no relevant parameters, hence changes in cell proliferation and matrix synthesis were no found	—	Wang et al. ³⁹
<i>In vitro</i>	Metacarpophalangeal epiphysis of a bovine	—	2, 4, and 24 mA/cm ²	—	—	6 hours	Increased levels of collagen type II and aggrecan with an EF of 20 mV/cm during 30 minutes	—	Vaca-González et al. ⁵⁵
<i>In vitro</i>	Distal and proximal extremities from humerus, scapulae and femurs of 2-day-old Wistar rats	50 and 100 V	—	60 sinusoidal	4 and 8 mV/cm	30 minutes, 1 and 5 hours during 1 week	Increase of proliferation with an EF of 4 mV/cm	Increase in glycosaminoglycans synthesis with an EF of 8 mV/cm	Brighton et al. ³⁷
<i>In vitro</i> (cartilage explant)	Articular cartilage explant from the patellae of adult cows	—	—	60 sinusoidal	20 mV/cm	1 hour and 30 minutes each for 6 hours	—	Increase in proteoglycan (34%) and collagen type II (74%)	Akanji et al. ⁴⁶
<i>In vitro</i> (3-dimensional culture)	Metacarpophalangeal joint of steers + agarose gel	0-12 V	—	0.1	—	6 and 12 hours	There is no influence over protein synthesis, cell proliferation and mRNA expression levels	—	Forgon et al. ⁵⁶
<i>In vivo</i> (growth plate)	Epiphyseal plate from rabbit femur	1.5 V	20 μ A	—	—	6 weeks	Increased thickness of the proliferative zone and also the columnar cells arranged was longer compared with the controls	—	Brighton et al. ³⁷
<i>In vivo</i> (growth plate)	Proximal tibia of the rabbit	2.5, 5, 10, 20 V	1.24, 1.19, and 1.53 mA	60 sinusoidal	16.5, 33, 66, and 132 mV/cm	48 hours	Increase in longitudinal growth of the proximal tibia with 3.3 mV/cm	—	—

Figure 1. Schematic diagram of an indirect coupling system to stimulate *in vitro* cell cultures. **(A)** Configuration to apply electric fields (EFs) to the culture medium between the parallel electrodes. **(B)** Configuration to apply electromagnetic fields (EMFs) to the culture medium between the parallel electrodes.

II (71%) after 14 days of stimulation.³⁷ Because EFs of 20 mV/cm have been shown to influence the cellular dynamics, the research group of Brighton studied the effect of the electrostimulation in osteoarthritic human cartilage explants. In this work, osteoarthritic explants stimulated for 14 days using 20 mV/cm EFs showed 1.4- and 1.5-fold increase in PG and collagen type II, respectively.³⁸ EFs have been also applied to 3-dimensional (3D) cartilage constructs used for cartilage repair; however, to our knowledge, the reports in literature are limited and the results have been contradictory. For instance, Szasz *et al.*⁴⁵ stimulated 3D chondrocyte-seeded agarose gels, obtaining increases in cell density and GAGs synthesis. In contrast, Akanji *et al.*⁴⁶ investigated the effects of direct current on cell proliferation and matrix synthesis, using a 3D chondrocyte-agarose model system. Their results demonstrated that EFs have no influence over protein synthesis, cell proliferation, and mRNA expression levels.⁴⁶

Effect of EFs Over the Growth Plate. Applying electrical stimulation across growth plate cartilage has little effect on composition,⁵⁷ but can alter the thickness of zones within the cartilage,^{57,58} and ultimately the length of the bone.^{57,59} However, high voltage reduces bone growth and maintains cartilage in a quiescent state.⁵⁹

Electromagnetic Fields

Similar to EFs, electromagnetic fields (EMFs) have been applied in *in vivo* and *in vitro* studies using cartilage and growth plate explants, and tridimensional structures

(Table 2).^{27,28,30,42,44,49,60} Similar to EFs, the technique of deliver EMFs consists of an indirect coupling system that uses external parallel coils connected to a function generator (Fig. 1B).^{28,29,47,60} This kind of noninvasive configuration has the advantage of generating small currents and potentials in the proximity of the targeted cells, and also it generates a homogeneous EMF across the cell culture.⁵¹

In Vivo Use of EMFs to Heal Osteoarthritis. *In vivo* animal models have been used to study the influence of EMFs in the recovery of knee osteoarthritis. Ciombor *et al.*²⁸ showed that articular cartilage morphology was preserved and the development of osteoarthritic lesions were retarded when EMFs were applied. EMFs have also been implemented in clinical studies to treat knee osteoarthritis. Results have evidenced that electromagnetic stimulation contributes to preserve articular cartilage morphology,^{60,61} improvement in joint motion, tenderness, and a reduction in pain.⁶²⁻⁶⁵

In Vitro Studies Using Cell Cultures. The application of EMFs in monolayer cultures have shown preservation of chondrocytes morphology,²⁹ increased DNA synthesis,³⁶ and enhancement of proliferation and GAGs synthesis in human chondrocytes.⁶⁶ It has also been demonstrated that EMFs significantly increase cell population, but decrease the synthesis of characteristic molecules of hyaline cartilage such as collagen type II, aggrecan, SOX 9, and GAGs.³⁰

Studies Performed in Explants 3-Dimensional Cartilage Constructs. Healthy and pathological tissue explants, have also been treated with EMFs.^{40,42,49} The use of EMFs over

Table 2. Summary of electromagnetic Field (EMF) Application to *In Vivo* and *In Vitro* Cartilage Explants and Scaffolds.

Study Model	Chondrocytes Isolation Source	Frequency (Hz)	EMFs	Stimulation Time	Effect on Cell Proliferation	Effect on Matrix Synthesis	Reference
<i>In vivo</i>	Knee of Hartley guinea pigs	1.5	1.0 G	1 hour/day for 6 months	Articular cartilage morphology was retarded the development of osteoarthritic lesions	The EMF was preserved. The EMF retarded the development of osteoarthritic lesions	Giombor et al. ²⁸
<i>In vivo</i>	Joint knee from Hartley guinea pigs	75	1.5 mT	6 hours a day for 3 months	The EMFs preserve the morphology of articular cartilage and the thickness of the medial tibia plateaus		Fini et al. ⁶¹
<i>In vitro</i>	Femoral condyles of human knee	10, 100, 370, 500	0.74, 0.85, 1.69, 3.14, 5.42 (mT peak)	6 h	Magnetic field strengths of 1.7 and 3.1 mT peak with frequencies of 500 and 100 Hz maintained chondrocyte spherical morphology		Jahns et al. ²⁹
<i>In vitro</i>	Human articular cartilage chondrocytes	75	2.3 mT	1, 6, 9, and 18 hours	—	9- and 18-hour stimulation leads to increased DNA and PG synthesis	De Mattei et al. ³⁶
<i>In vitro</i>	Porcine articular cartilage	5 pulses of 100 ns	10 or 20 kV/cm	1, 3, and 7 days	Significantly increase in cell proliferation	A decrease in glycosaminoglycan, collagen II, Sox9, and aggrecan gene expression was observed	Zhang et al. ³⁰
<i>In vitro</i> (cartilage explant)	Metacarpophalangeal joints of bovine	75	1.5 mT	24 hours	—	20% increase in proteoglycan synthesis	De Mattei et al. ⁴²
<i>In vitro</i> (cartilage explant)	Metacarpophalangeal joints of bovine	75	2.3 mT	24 hours	—	EMFs are able to promote anabolic activities and proteoglycan synthesis	De Mattei et al. ⁴⁷
<i>In vitro</i> (osteoarthritic cartilage explants)	Human femoral condyles	75	1.6 mT	4 hours daily for 4 days	Increment in DNA content	No changes in glycosaminoglycans were evidenced	Sadoghi et al. ⁴⁰
<i>In vitro</i> (3-dimensional culture)	Human chondrocytes cultured in a type-I collagen gel	16.7	2 mT	1, 3, 7, and 14 days	—	Positive staining for collagen II and proteoglycan in the immediate pericellular region	Schmidt-Rohlfing et al. ⁴³
<i>In vivo</i> (growth plate)	Male albino Wistar rats	900 and 1800 MHz	—	12 weeks	Stimulated rats experienced a more rapid weight gain and increase in length. Rats showed a loss in the reserve zone, with an increase in thickness in the proliferative zone		Nisbet et al. ⁶⁷
<i>In vivo</i> (growth plate)	Right hind limb of a rabbit	—	1.5 mT	10 minutes per day over 15 days	EMFs exert a suppressive influence on chondrocyte proliferation	—	Arsen'ev et al. ⁶⁸

cartilage explants have shown increase in PG synthesis and stimulation of anabolic activities,^{42,47} while the effect of EMFs in osteoarthritic explants have evidenced an improvement in osteoarthritis of grade I and III, increasing PG synthesis and counteracting catabolic activities.⁴⁹ However, it was also shown that EMFs have no effect on DNA and PG synthesis.⁴⁰ The application of EMFs has also been investigated in osteoarthritic chondrocytes cultured in 3D structures. Results showed that the PG concentration of osteoarthritic chondrocytes cultured in alginate scaffolds was stored in the cell culture medium after stimulation was finished.²⁷ Normal articular chondrocytes cultured in 3D constructs were also stimulated with EMFs. In studies performed by Schmidt-Rohlfing *et al.*⁴³ and Nicolin *et al.*,⁴⁴ the chondrocytes that were cultured in a collagen membrane showed that this scaffold is a useful bioengineering construct since allowed increases in cell proliferation, collagen type II synthesis, and PG in the immediate pericellular region.

Effect of the EMFs Over the Growth Plate. Studies have been carried out to study the effects of EMFs over growth plate development. A recent study showed that male albino rats stimulated with EMFs experienced a more rapid weight gain and increase in length. Moreover, the stimulated rats showed a loss in cartilage matrix density in the reserve zone, with an increase in thickness of the reserve and proliferative zones. In addition, measurements of the growth plate thickness showed that the trabecular zone was thinnest and the reserve and proliferative zones were thickest. There were no significant differences between the groups with respect to the thickness of the hypertrophic zone.⁶⁷ A similar study evidenced that EMFs exert a suppressive influence on chondrocyte proliferation in the growth plates and promote expansion of the differentiate fraction.⁶⁸ The epiphyseal plate has been also stimulated through *in vitro* cultures; for instance, the costochondral junction growth plate of a rat was exposed to different EMFs and changes of temperature. Results indicated that longitudinal growth of the costochondral junction was only observed when EMFs were applied in an environment where the temperature increased.⁶⁹

Mechanical Stimulation for Tissue Engineering Hyaline Cartilage

Compressive Loads

Because of the fact that hyaline cartilage, especially the one found in joints and growth plate, is mainly subjected to compressive mechanical stimuli,^{22,70,71} several *in vivo* and *in vitro* studies have been performed in order to identify the effects of mechanical compression in cell dynamics (proliferation, hypertrophy, and apoptosis),^{1,72-79} cell height,^{72,74,80} and the biosynthetic behavior of chondrocytes, assessed

mainly in terms of expression of molecules of the ECM (collagen types II and X and aggrecan) and some enzymes (MMP 13 and ADAMTS-4/5).^{81,82} In addition, changes in the expression of metalloproteinase inhibitors (TIMP-1 and TIMP-2), growth factors (VEGF), and other molecules are influenced by load (**Table 3**).^{16,79,83-85} Up to now the specific mechanisms involved in mechanotransduction are not well understood; however, 2 kinds of proteins have been involved in these processes: integral membrane proteins associated in ECM interactions (e.g., integrins) and mechanosensitive ion channels.⁸⁶⁻⁸⁹ Furthermore, signaling mediated by the primary cilium and MAPK pathway have also been implicated.⁹⁰⁻⁹⁵ Regarding the devices used to apply compressive loads, the technique of deliver mechanical loads consist of a system that uses loading actuators that exert an axial force (**Fig. 2A**).^{7,92,94} These devices present an advantage of maintain the cell cultures in atmospheric conditions of 37°C and 5% CO₂ while mechanical loads are been applied.⁹⁶

In Vivo Studies. To understand the effect of mechanical loading on chondrocytes behavior within the tissue, some *in vivo* studies have been performed in both articular cartilage and growth plate. The studies performed in articular cartilage have mainly focused in analyzing the synthesis of ECM molecules as PG, where a direct correlation between loading in the joint and PG levels within the tissue was observed.^{7,16,84,95} Several studies performed in the growth plate have assessed the effect of mechanical loading as a regulator of bone growth and ossification. Such behavior has been recognized by the Hueter-Volkman law, which establishes that static compression is crucial for bone development; however, excessive loads may inhibit the normal bone growth. Moreover, alterations of mechanical modulation have been associated to several clinical conditions, including the angular progression deformities in tibia (Blount disease), and the development of scoliosis.^{72,73} Studies using chicks and rodents have demonstrated that mechanical stimulation of bone rudiments during embryological development plays an important role in normal growth and bone morphogenesis.⁹⁷

At cellular level, studies using rodents have shown that mechanical loading induces histological changes within the growth plate, specifically in the width of the proliferative and hypertrophic zones.^{74-77,98} Additionally, it has been observed a decrease in collagen type II and aggrecan concentrations in growth plates subjected to long term static compressions.⁸²

In Vitro Studies: Tissue Cultures. As an alternative model to study the influence of mechanical loading over the chondrocytes behavior, *in vitro* protocols applying compressive loads to articular cartilage and growth plate explants

Table 3. Summary of Some Representative Studies Using Compressive Loads on Hyaline Cartilage.

Study Model	Chondrocytes Isolation Source	Load Magnitude	Frequency (Hz)	Application Scheme	Stimulation Time	Results	Reference
<i>In vivo</i>	Growth plates of young rats, rabbits, and calves	0.1 MPa	—	Compression and distraction were applied over 1 week	—	Reduced growth rate with compression and increased growth rate with distraction	Stokes et al. ⁷⁴
<i>In vivo</i>	Sprague-Dawley rats	0.2 MPa	—	Cyclic compression was applied at 1, 8, or 15 days after seeding	2 weeks	Loading reduced bone growth and no changes were observed in the synthesis of aggrecan, collagen II and collagen X	Cancel et al. ⁸²
<i>In vivo</i>	Adult female New Zealand White rabbits	Between 1 and 2 MPa	1	80 cumulative hours in 2-hour increments	3 days a week for 14 weeks	Increment in cartilage deep zone and PG synthesis. No changes in collagen were observed	Saadat et al. ⁸⁴
<i>In vivo</i> (growth plate)	Distal ulnae from 4-week-old swine	1 N	—	A prestrain (5%) was first applied, followed by a stress relaxation test (10% strain)	—	Under compression the cell/matrix volume ratio decreased in the reserve and hypertrophic zones, whereas it increased in the proliferative zone	Amini et al. ⁸⁰
<i>In vivo</i> (growth plate)	Distal ulnae of 4-week-old swine	Strain rate of $1.5E-03 \text{ s}^{-1}$	0.1	The samples were subjected to a 2% strain preload. The static loading samples underwent an additional 10% strain. The dynamic loading samples were submitted to an additional cyclic strain oscillating between 7% and 13% strain	48 hours of stimulation	With static loading cellular collagen organization was preserved, but a loss in aggrecan, type II and type X collagens synthesis was denoted. With dynamic loading, a loss of collagen arrangement was observed in the proliferative and hypertrophic zones, but it contributed to the synthesis of aggrecan and type II collagen	Sergie et al. ⁸¹
<i>In vitro</i> (cartilage explant)	Adult articular cartilage from the bovine humeral head	10 kg	0.001, 0.5, 1	At 0.5 Hz and the sine wave amplitude was varied between 5% and 20% of the articular cartilage. The sine wave amplitude was fixed at 10%, while the frequency was varied between 0.001 and 1.0 Hz	45 hours	The oscillatory loading increased protein synthesis, but had an inhibitory influence on PG synthesis. Static compression caused a significant increase in fibronectin synthesis	Wong et al. ¹⁰¹
<i>In vitro</i> (cartilage explant)	Articular cartilage explants from 18- to 24-month-old steers	0.1, 1.0, 2.5, or 5.0 MPa	0.001, 0.01, 0.1, or 0.5	Continuously applied, uniaxial cyclic loading was applied for 2 hours	1, 3, or 6 days	When the load was increased, the proteoglycan synthesis was decreased	Steinmeyer et al. ¹⁰²
<i>In vitro</i> (3-dimensional culture)	Femoropatellar from 1- to 2-week-old calves	—	0.001, 0.01, 0.1, and 1	6 hours under static compression; 10 hours under dynamic compression	From 2 to 43 days	Molecular synthesis decreased when static load was applied while with dynamic loading the synthesis was enhanced. Loading enhanced glycosaminoglycans production	Buschmann et al. ¹⁰⁴
<i>In vitro</i> (3-dimensional culture)	Metacarpal-phalangeal joints of 2- to 3-year-old free-range steers	—	0.3 and 1	Din unconfined compression for 30 minutes on and 90 minutes off for 16 hours	2 weeks	Contrarily, deposition of larger matrix. Molecules of aggrecan and collagen II were either not affected or inhibited by loading	Farnsworth et al. ¹⁰⁶

Figure 2. Schematic diagram of the devices used to apply mechanical loads to chondrocytes cultured in 3-dimensional (3D) structures. Both schemes have in common a loading cell, a cell culture well plate and the 3D construct. **(A)** Configuration to apply compressive loads to 3D cell cultures. This device uses a tissue culture incubator and a sterile box to maintain cell cultures in humidified atmosphere. **(B)** Configuration to apply compressive and shear stress to the chondrocyte culture. This device uses two loading actuators to apply loads in the X and Y axis.

have been performed. For articular cartilage, the reported results of ECM molecules synthesis under dynamic loading are contradictory with some studies showing an increase while others indicate either a decrease or no changes after loading.^{70,71,79,84,99-102} For growth plate, studies using tissue explants have shown differential morphological changes of chondrocytes in the different zones under compression.⁸⁰ Additionally, under short-term compressive loads, a decrease in aggrecan, collagen type II and X has been reported. In contrast, under dynamic loading no changes in ECM composition although changes in cell organization were observed.⁸¹

In Vitro Studies: Cell Cultures. Given the difficulties for analyzing the individual effects of mechanical stimuli to which cartilage is subjected *in vivo*, the response of chondrocytes has been evaluated in monolayer and 3D cultures.^{16,22,70,71,93,101,102} These analyses not only provide information about the cellular responses and the regulatory mechanisms associated, but also they allow to evaluate the use of mechanical stimuli in tissue engineering approaches.¹⁶ The effect of compressive loads in *in vitro* cultures of chondrocytes depends up on load's intensity and duration⁷. In this context, it has been observed that the application of static loads increased the production of several types of MMPs, and also inhibited the synthesis of collagen type II and PG.^{7,95} In turn, the effects of dynamic loading in cell proliferation and synthesis of PG, GAGs, and collagen type II are contradictory.^{7,70,71,79,84,95,96,99-118} This behavior may be related to the wide variability of the methodology used for each group, which can be related to different sources of chondrocytes, age of the animal model, characteristics of 3D matrix used, chondrocyte seeding density, frequency of the stimulus, load cycles, and initiation and duration of the stimulation.^{7,84,96,101,103-108,115}

Tension

The chondrocytes response to tensile loading have also been addressed using *in vitro* monolayer cultures. Such cultures have been performed by seeding cells in membranes that are submitted to uniaxial or biaxial dynamic stretching. Most of the studies have evaluated chondrocyte dynamics in terms of cell proliferation and molecular synthesis of collagens and PG. Similar to that observed for compressive loading, the evidence available suggests that the response of chondrocytes to tensile loading also depends on the duration and intensity of the stimulation.^{18,119-121} In fact, anabolic effects in chondrocytes are induced after short-term stimulation (less than 12 hours); while a decrease in the ECM molecules is only observed after a prolonged stimulation.¹⁸ In addition, some studies have reported an increase of other molecules such as proteases and their inhibitors (e.g., TIMPs, MMPs), soluble factors (e.g., TGF β , VEGF, PTHrP), and pro-inflammatory factors (e.g., nitric oxide, prostaglandin E, cyclooxygenase 2) after tensile loading stimulation.^{18,93,119,121-123}

However, it is difficult to extrapolate definitive conclusions regarding the stimulus characteristics and chondrocytes response considering that, similarly to compressive loading, there is a high variability in the stimulation protocol used among different studies in terms of strain magnitude, loading duration and frequency (reviewed in Bleuel *et al.*¹⁸).

Hydrostatic Pressure

In Vitro Studies: Tissue Cultures. Hydrostatic pressure (HP) has been extensively used as a stimulus to induce changes over hyaline cartilage such as protein expression and matrix production.¹²⁴ However, the mechanism by which HP

Table 4. Summary of Some Representative Studies Using Hydrostatic Pressure on Hyaline Cartilage.

Study Model	Chondrocytes Isolation Source	Load Magnitude	Frequency (Hz)	Application Scheme	Stimulation Time	Results	Reference
<i>In vitro</i>	Bovine chondrocytes	0.8 atm	—	5 minutes on and 30 minutes off	10 times	40% increase in proteoglycan synthesis, enhanced aggrecan mRNA	Suh et al. ¹²⁶
<i>In vitro</i>	Adult bovine chondrocyte	10 MPa	1	2, 4, 8, 12, and 24 hours	1 day	Increased aggrecan mRNA up to 24 h, maximal increase in collagen II mRNA with 4- and 8-h application.	Smith et al. ¹³²
<i>In vitro</i>	Adult bovine chondrocyte	10 MPa	1	4 hours	4 days	Significant increase in aggrecan and collagen II mRNA.	Smith et al. ¹³³
<i>In vitro</i>	Adult bovine chondrocyte	1, 5, 10 MPa	1	4 hours	4 days	Enhanced aggrecan expression with all treatments, enhanced collagen II expression only with 5 and 10 MPa, for 4 days	Ikenoue et al. ¹³⁴
<i>In vitro</i> (3-dimensional culture)	Adult P3 equine chondrocytes	3.44 or 6.87	0.25	20 minutes every 4 hours	3, 4, and 5 weeks	Both magnitudes increased glycosaminoglycan production; only 6.87 MPa increased collagen production.	Carver and Heath ¹²⁹
<i>In vitro</i> (3-dimensional culture)	Immature bovine chondrocytes	10	1	4 hours per day	8 weeks	Increased collagen production at 4 weeks and 8 weeks	Hu and Athanasiou ¹²⁵
<i>In vivo</i> (cartilage explant)	Adult bovine chondrocytes	5	0.5	1.5 hours	—	Enhanced sulfated glycosaminoglycan incorporation	Parkkinen et al. ¹³¹

produce those changes is not well understood. HP provides a robust method of chondrocyte stimulation since it can be applied over cells cultured in monolayer, 3D engineered constructs or cartilage explants (Table 4).^{125,126} Several studies have shown the relation between HP and biological response of hyaline cartilage, such as investigations performed by Parkkinen *et al.* have shown a microtubule-dependent compaction of the Golgi apparatus and decrease in GAGs synthesis¹²⁷. In a similar study, Lammi *et al.*¹²⁸ found that static HP resulted in a 37% decrease in GAGs synthesis, reduction of aggrecan mRNA levels and synthesis of atypically large aggrecan molecules. The alteration in aggrecan size demonstrates that HP may affect the production of ECM molecules at posttranslation levels.

Studies Performed in Cartilage Explants and 3-Dimensional Constructs. HP also has been applied over 3D scaffolds and explants. For instance, Carver and Heath^{129,130} showed that chondrocytes cultured in poly glycolic acid (PGA) meshes and stimulated with 6.87 MPa experienced an increase in GAGs and collagen production in adult cells, whereas the

same magnitude of HP had an increase only collagen synthesis in juvenile cells. There are no differences of applying HP either in cartilage explants or in chondrocytes cultured *in vitro*; however, the magnitude of the pressure and the frequency are relevant factors to stimulate PG synthesis.¹³¹

Shear Stress

Within the joint and growth plate chondrocytes are exposed either to compressive HP or deviatoric stresses such as shear stress.¹³⁵ Previous studies have indicated that chondrocytes are exposed to different levels of fluid flow within the tissue,^{136,137} suggesting that mechanical shear stress has a pathophysiologic relevance in cartilage biology.¹³⁸ Within this context, several studies have analyzed the influence of such stimuli on chondrocyte behavior. For instance, Mohtai *et al.*¹³⁹ applied shear stress over human articular chondrocytes seeded in a high monolayer culture. Cell cultures were stimulated with loads of 0.16, 0.41, 0.82, and 1.64 Pa at rotating velocities of 20, 50, 100, and 200 rpm. Results indicated that this stimulation scheme increased the release of

pro-inflammatory mediators and nitric oxide, decreased the expression of aggrecan and collagen type II, and induced molecular changes associated with apoptosis.¹³⁹ Additionally, it is known that hyaline cartilage tissue engineering constructs, are also affected by shear stress, revealing that this stimulus may alter the intercellular signaling pathways in chondrocytes.^{129,134} Some reports suggest that fluid shear stress reduces expression of aggrecan and collagen type II.¹⁴⁰

Other studies, like those developed by Waldman *et al.*¹⁴¹ used a stimulation scheme based on 2% of shear stress amplitude at 1 Hz for 400 cycles every second day, demonstrate that an intermittent application of dynamic shearing forces during 4-week periods improved the quality of the cartilaginous tissue formed *in vitro*. These data indicate that the nature and magnitude of shear stress may play a significant role in the homeostasis of the structure and function of hyaline cartilage. For this reason, the cellular mechanisms underlying the responses of articular chondrocytes to mechanical stresses are important for understanding the pathogenesis of several hyaline cartilage diseases.¹³⁸ Similar to compressive loads, the technique of deliver the shear stress consists of a loading actuator that generate forces along the *X* and *Y* axis at the same time (**Fig. 2B**).^{139,141} In this context, this configuration allows one to apply precise strains on multiple axis similar to the mechanical environment supported by the joint.¹⁴²

Cellular Mechanisms Involved in Transduction of Biophysical Stimuli

In chondrocytes, biophysical stimuli are sensed mainly by membrane proteins involved in ECM-cell interactions and ionic channels.^{7,143,144} In addition, some stimuli may induce changes in the cytoskeleton.^{23,145}

ECM-cell interactions are associated mainly with the transduction of mechanical stimuli, since they allow cells to sense conformational changes of the ECM that trigger, intracellularly, the activation of different cell signaling cascades leading to specific cell responses.^{7,143} The main proteins associated with ECM-cell interaction are integrins, integral membrane proteins that bind to several ECM molecules with different affinities.¹⁴⁶⁻¹⁴⁸ The main integrins expressed by chondrocytes are $\alpha 1\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 10\beta 1$, $\alpha V\beta 3$, and $\alpha V\beta 5$, which act as receptors for collagen types VI and II, matrilin-1, fibronectin, osteopontin, COMP, and vitronectin, respectively.^{87,88,147-149} Cytoplasmic domains of integrins are coupled to kinases that have been implicated in signal transduction through Ras, Rho, and Rac pathways.^{93,95} In addition to integrins, other membrane proteins like CD44 and annexin V (also known as anchorin CII) have also been associated in ECM-cell interactions in chondrocytes. CD44 is a transmembrane hyaluronan-binding glycoprotein that is involved not only in anchoring but also

in sensing and signaling functions.¹⁴⁹⁻¹⁵¹ Annexin V is a member of a family of calcium and phospholipid binding proteins that binds mainly with collagens present in the PCM and ECM, mainly type II and with lesser affinity to types V, IX, X, and XI.¹⁵⁰⁻¹⁵²

On the other hand, ion channels have been associated to both mechanical and electrical signal transduction. Although a plethora of ion channels have identified in chondrocytes membranes, calcium channels are the main type of ion channels associated to transduction of biophysical stimulation.^{86,89,95} The calcium channels most associated to these processes include voltage-gated sodium channels, voltage-gated calcium channels, and stretch activated ion channels. In addition, various members of the TRPV (transient receptor potential vanilloid) nonselective cationic channels family, especially TRPV4, have been associated to osmotic stress responses.^{86,89,95} In fact, it has been described that in chondrocytes, cell deformations caused either by changes in cell volume or due to mechanical loading, lead to activation of different kinds of ionic channels, and some studies have evidenced changes in intracellular calcium levels in chondrocytes after mechanical loading.^{86,91,92,94,95} Electric signals applied either in direct or indirect contact with the cells, exert their effect on the cell membrane by activating the voltage-gated calcium channels leading to increase in the intracellular Ca^{2+} levels.^{51,144,153}

Finally, as previously mentioned, some stimuli are associated to conformational changes of the cytoskeleton. That is the case of electrical stimulation, which has proven to induce changes in actin filaments causing cytoplasm elongation followed by a perpendicular alignment with regard to the applied EFs.¹⁴⁵ Furthermore, electric stimulation has shown to promote cell movement, process known as galvanotaxis.²³

Perspectives

This review focuses on the effect of biophysical stimuli in chondrocyte behavior, showing that electric and mechanical stimulation have important roles in physiology of hyaline cartilage. In addition, biophysical stimuli display interesting potential as additional tools for both tissue engineering and regenerative medicine, toward the development of new therapeutic alternatives for cartilage pathologies. The effects of biophysical stimulation on chondrocytes dynamics have been explored *in vivo* and using several *in vitro* approximations. In monolayer cell cultures, stimulation with EFs, EMFs, HP, and tension can promote chondrocyte proliferation and molecular synthesis.^{18,51,61,132,134} Taking this into account, with the current use of scaffolds combined with the application of biophysical stimuli it is possible to obtain tissue constructs that mimic *in vivo* characteristics of cartilage.

For both electric and mechanical stimuli, different frequencies, intensities, and duration of stimulation schemes

have been tested; however, the magnitudes of the stimuli that directly stimulate chondrocytes in those cultures have been poorly explored. Regarding electric stimulation, at present, calculations of cell culture media dielectric constants have been recently reported. These electric constants, such as permittivity and conductivity, are essential parameters to calculate electric and EMFs.^{54,154} Thus, this leads to a new research area that will allow to implement a better methodology to calculate the EFs and EMFs in a more detailed way. In electrical stimulations, there are many discrepancies in the obtained results because of the fact that one of the limitations is the required high voltage to generate the EFs, the stimulation time to achieve the best rate of proliferation and molecular synthesis, as well as the amount of days that cell cultures need to be under stimulation. For this reason, electrical stimulation for tissue engineering hyaline cartilage still is an open research area. On the other hand, little is known of the mechanical environment and the effects on fluid flow and nutrient availability within cartilage or *in vitro* 3D cultures of the different types of mechanical stimulation. Recently, there have been some approaches that use computational modeling to approach such issues.¹⁵⁵⁻¹⁵⁹ This is a promising field that may improve the understanding of the effects of mechanical stimuli on chondrocyte behavior as well as provide powerful tools for scaffold designs for 3D *in vitro* culture systems and mechanical stimulation scheme selection. Furthermore, biophysical stimuli could help to enhance cell proliferation, differentiation, and ECM deposition, opening up new possibilities in 3D articular cartilage and growth plate tissues, and allowing to design novel tissue-engineering constructs similar to real life.

Given the good results obtained using some protocols of electrical and mechanical stimulation independently for *in vitro* hyaline cartilage tissue engineering, the integration of these both methods is currently being studied.⁵⁰ This leads to a new field of research that will focus on stimulation of cartilage explants or 3D constructs with 2 biophysical stimuli simultaneously. As a novel and potential tool, it would be possible to create special devices that generate distinct electrical and mechanical intensities according to the necessity of a particular treatment. This contributes to tissue engineering because it is noteworthy that biophysical stimuli are the basis for the next generation of cartilage regeneration technology, since those electrical and mechanical stimuli help the development of biomimetic samples that recreate an environment where the functional, structural, and biological features of hyaline cartilage remain stable at the time of cartilage replacement.

Overall, the use of biophysical techniques provides not only new tools to create *in vitro* biomimetic tissues, but they can also be novel therapeutic approaches that may be implemented in patients with several chronic hyaline cartilage pathologies. The methodologies used to stimulate cartilage regeneration are getting closer and closer to develop

technologies that satisfy the requirements of a successful cartilage healing and make that the concept of cartilage tissue engineering can be extrapolated to a purely clinical environment.

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