

MECHANOBIOLOGY OF ENGINEERING ARTICULAR CARTILAGE

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INTRODUCTION

The term “mechanobiology” is relatively new. In 1997, Carter and his colleague provided the definition, “mechanobiology is the study of how mechanical or physical conditions regulate biologic processes.”¹ In the past, most of the research in biomechanics and biorheology were spent focusing on tissue and cellular properties. As a result of these investigations, methodologies and testing apparatuses have been developed and applied to the study of joint prostheses. As a result, many of the cellular and tissue responses to mechanical forces have been elucidated. An international symposium on the Mechanobiology of Cartilage and Chondrocyte was organized by the French Biorheology Society in 1999. The meeting addressed a new approach for determining cellular and tissue response to development of theoretical interpretation at the cellular level.² At the 2002 Orthopedic Research Society meeting, a session specifically addressing the topic of mechanobiology in cartilage was first organized. Under this heading, 12 papers were presented.

Based on mechanobiological means and information, we intend to improve upon the repair or replacement of damaged tissue. The biology of chondrocytes in response to physical, physicochemical, and mechanical stimuli are still unclear. This article focuses on my current work: 1) mechanosignal transduction and mass transfer stimulated with hydrostatic pressure and/or strain to elucidate the signal cascade at the cellular and tissue level, and 2) the development of an *in vitro* tissue processor for cell-based therapy, applied techniques and instruments used in mechanobiological investigation.

MECHANOSIGNAL TRANSDUCTION

During weight bearing and joint motion, hydrostatic stress in articular cartilage increases. The stress placed upon articular cartilage consists of both hydrostatic and octahedral shear components. Little is known about the effects of hydrostatic

fluid pressure on the mechanism of mechanosignal transduction. Furthermore, the dynamic effects of pure hydrostatic fluid pressure have not been evaluated in chondrocytes.

Cellular activities – such as proliferation, phenotypic expression, and metabolic activities – are altered due to mechanical changes. Mechanical signals are transduced through intracellular second messengers, such as cAMP, inositol phosphate, and calcium ions. Intracellular calcium is a widely used marker which can be measured using a fluorescent indicator.

Technical difficulties in measuring intracellular calcium concentration ($[Ca]_i$) have included the transient nature of changing calcium concentrations and the application of hydrostatic pressure to live cells using an optical instrument. The author developed a culture chamber in which a sapphire glass windows allows for an inverted microscope to non-invasively measure $[Ca]_i$ using a laser confocal microscope. The dynamic $[Ca]_i$ in chondrocytes may be measured using a fluorescent calcium indicator (X-rhod-1) and a laser confocal microscope system after applying pressure. This system allows hydrostatic fluid pressure application while minimizing fluid shear stress due to fluid flow and octahedral shear stress due to cell deformation.

Articular cartilage has longitudinally heterogenous histology, which can be divided into three zones: surface, middle, and deep. Traditionally, a homogenous pool of chondrocytes has been used in most experimental settings. Differences in cell and matrix components due to age, loading history, thickness, and varying pathophysiological conditions were ignored. In later experiments, the focus was placed on the loading history of each cell as cellular behavior and calcium concentration were measured.

In our experimental system, cartilage from each zone was harvested from the weight-bearing site of a bovine forelimb condyle. Cells were isolated enzymatically from the cartilage pieces and used for signal transduction experiments. The calcium indicator was loaded prior to pressure application. Hydrostatic fluid pressure was tested varying from 0 – 3 MPa and 0 - 0.025 Hz. Pressure was applied for 5 min with varying conditions. After releasing the pressure, fluid flow was stopped and images were acquired at atmospheric pressure. Imaging was scanned for 800 nsec at 10 sec intervals for 5 min. Relative fluorescent intensity was measured as the summation of the cells in a region of interest, 8 cells to one frame.

$[Ca]_i$ increased in chondrocytes harvested from all three

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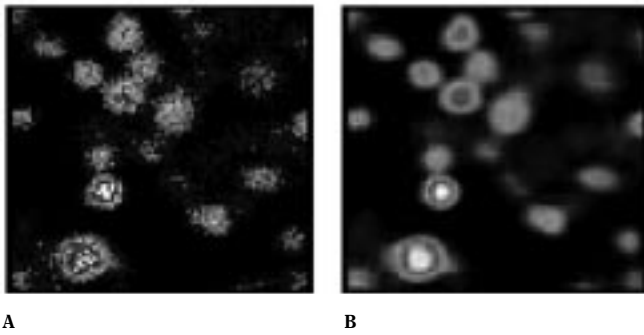


Figure 1. Fluorescent intensity indicating intracellular calcium concentration with pseudocolor in bovine articular chondrocytes. Hydrostatic fluid pressure was applied at cyclic 0.5 MPa, 0.025 Hz for 5 min. A) Basal intensity at time 0 after releasing pressure. B) Peak intensity 50 sec after releasing pressure.

zones.³ $[Ca]_i$ peak values of middle zone cells was two times higher than surface and deep zones (Figure 1). In order to avoid involvement of cytoskeletal motion, cytochalasin B, which depolymerizes actin filaments of cytoskeleton, was added prior to pressure application.⁴ Even without cytoskeletal effects, $[Ca]_i$ increased after releasing pressure, but the peak value was less than with cytoskeleton. This indicates that the cytoskeleton contributes to calcium signaling to some degree.

This project has been expanded to determine the location of the source of calcium and further characterize the signal cascade at the subcellular level. Most likely, pericellular matrix components should be included in a capacity of boundary water under pressure.

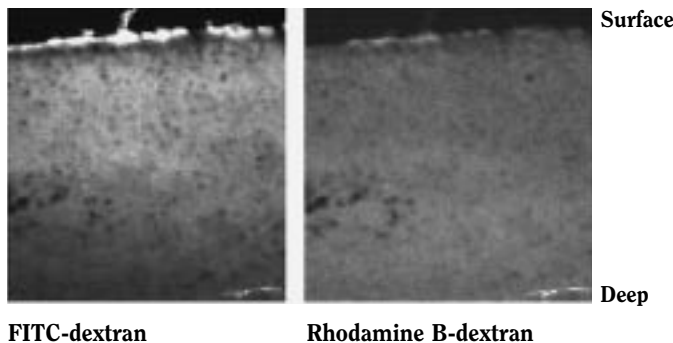


Figure 2. Interstitial distribution of fluorescent tracer in bovine articular cartilage *ex vivo*. Cartilage discs were incubated with FITC-dextran (70 kDa) and Rhodamine-dextrane (70 kDa) in culture dishes for 6 hr. Intense fluorescence was seen at the articular surface. Underneath of the surface layer, a dark band of fluorescence was seen with FITC and Rhodamine.

MASS TRANSFER IN CARTILAGE

Mass transfer in cartilage is a critical physical phenomenon which contributes to the infiltration of nutrients and chemical factors. In terms of its clinical application, all vehicles for delivery of nutrients to the target tissue need to be considered. When engineered cells or cell constructs used to replace damaged tissue are implanted, integration of implants with host tissue is also communicated through mass transfer. Because articular cartilage is an avascular tissue, dynamic hydrostatic

and distortional stress, occurring as the result of weight bearing and joint motion, contribute to active mass transfer. Even during the embryonic development, chemical factors are conveyed by interstitial fluid flow, which depend upon diffusion and convection.

Our hypothesis was that fluid exudation from the articular surface due to joint loading is minimized. We harvested cartilage discs from weight bearing sites of the forelimbs of 2 – 3 week old calves. These discs were incubated with fluorescent tracers, rhodamine-dextran or fluorescein-isothiocyanate (FITC)-dextran, each with a defined molecular weight. The discs were incubated for 6 and 12 hours. The cartilage was frozen immediately after samples were harvested. Sections were cut 20 μ m thick. Fluorescent intensity was acquired using a laser confocal microscope. Highest fluorescent intensity was detected in the middle zone. However, less intensity was detected from the longitudinal side of the disc (Figure 2).

From an engineering perspective, it is important to consider that once chondrocytes accumulate their own extracellular matrix, the permeability of the cell/construct decreases. We also have to consider molecular sheaving by the tissue. We found that FITC-dextran diffused into cartilage discs primarily from the articular surface and from the subchondral side, but far less from the site of the longitudinal cut (unpublished data). We suspect that the physicochemical environment of this tissue may influence cells to restrict molecular sheaving. This is a very important concept to consider when designing three-dimensionally organized articular cartilage.

CELLULAR AND TISSUE ENGINEERING FOR CELL-BASED THERAPY

The fundamental research described above has provided the basis for new bioengineered apparatus designs. For example, we have developed a cell culture apparatus for a cell-based therapy, which employs an *ex vivo* treatment process prior to introducing engineered cells into the body. In this system, we are able to apply our mechanobiological findings to the development of methods of tissue production. In order to retain original tissue characteristics and to replace damaged tissue in articular cartilage, cells need to multiply and to produce extracellular matrix. In the course of cell culture, medium change is a fundamental requirement for keeping cells alive. Our new apparatus enables us both to perfuse the tissue with medium and to apply hydrostatic pressure to the cells to mimic the hydrostatic stress and distortional stress to which articular cartilage is normally subjected. This methodology will result in the production of healthy articular neocartilage which may in turn be implanted into clinical articular cartilage lesions.

MEDIUM PERFUSION

Medium perfusion is a very popular technique used in bioreactors to promote metabolic function in mammalian cells and even bacterial organisms. Fluid flow generated with perfusion is expected to increase the mass transfer of nutrients and gases into cells. With the high cell density and relatively

large amounts of matrix accumulation seen in chondrocytes, these methods are useful because fluid flow generates fluid shear stress and, subsequently, the permeability of multiple layers of cell growth and extracellular matrix are increased. Indeed, our own bodies are bioreactors. When blood passes through capillaries as well as sinusoids in the liver, nutrients and gases are exchanged.

We tested a number of different cell types using a medium perfusion system designed by the author and his colleagues. We found that medium perfusion enhances viability and hematopoiesis in murine marrow cells⁵, osteogenesis in murine osteosarcoma⁶ and oral mucosa⁷. However, chondrocytes did not benefit in the same manner.⁸ Each perfusion culture system has different features. Thus, we must carefully choose a system and optimize conditions for the target cell type.

Fluid shear stress generated by fluid flow is a critical factor in cardiovascular cells such as endothelial cells. In cartilage, on the other hand, the interstitial fluid flow rate is extremely small. When we designed our perfusion culture system, we calculated the Reynold's number and defined laminar flow ($Re < 2000$). We did not intend to mimic interstitial fluid flow. With our perfusion system, cell constructs are freely suspended in the column, thereby creating obstacles to the fluid flow. Theoretically, this flow, called Bolus flow (axial-train model, Figure 3), accelerates diffusion and convection of molecules, subsequently increasing nutrient supply as well as releasing products such as unbound, free extracellular matrix. Construct materials must be chosen depending on their permeability, because we must take into account not only the construct material's properties, but also the effect of matrix accumulation on the cells themselves. By understanding these factors, we can control the fluid flow rate for the target cells and allow them to accumulate their own matrix.

PRESSURE/PERFUSION CULTURE SYSTEM

Hydrostatic stress promotes chondrogenesis in a novel fluid pressure culture system described by Glowacki and Mizuno et al.⁹ Hydrostatic fluid pressure at 2.8 MPa applied in either a constant or cyclic manner stimulated synthesis of not only sulfated but also non-sulfated proteoglycans (PGs) by chondrocytes cultured in 3-dimensional collagen sponges.¹⁰

Non-sulfated proteoglycan (chondroitin proteoglycan) is more abundant in embryonic and neonatal articular cartilage than the other sulfated PGs. The sulfation process – or sugar

Fluid (medium) flow

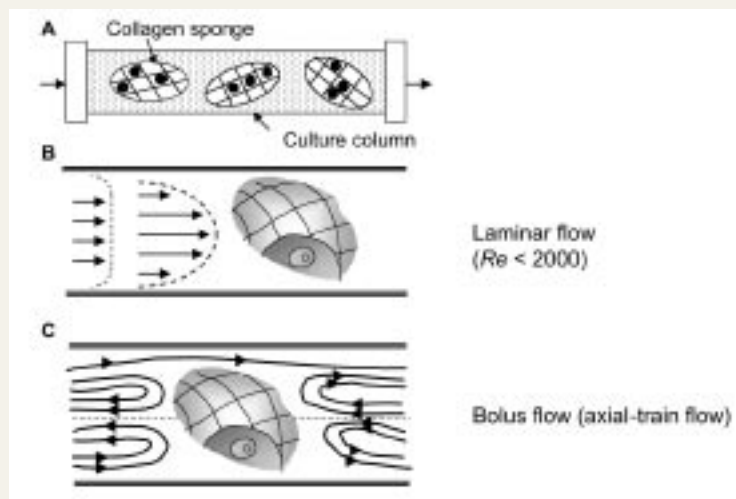


Figure 3. Theoretical fluid flow in a culture chamber. A) cell/collagen sponge constructs were suspended in a culture chamber. B) Fluid flow can be expected to be laminar flow, calculated assuming obstruction free conditions. C) Unbound, bound fluid flow, bolus flow can be expected in a column.

Hydrostatic fluid pressure culture unit

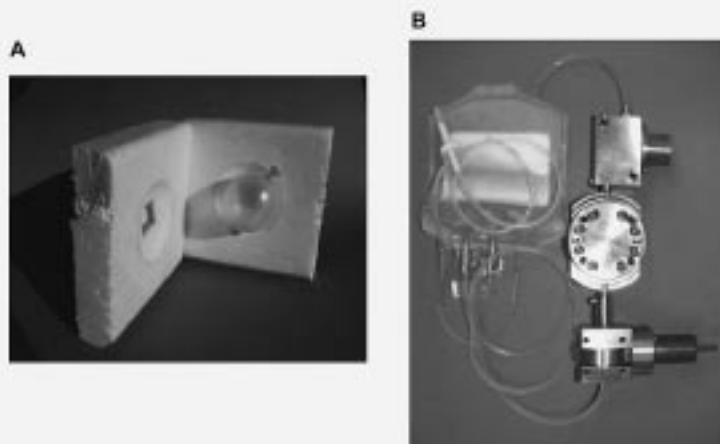


Figure 4. Tissue Engineering Processor: A) conceptual design of a disposable pressure proof culture unit. B) prototype of pressure proof chamber unit.

chain (glycosaminoglycan, or GAG) synthesis – may depend upon physical stimuli. We have not yet measured the absolute value of non-sulfated, 4-Sulfated, and 6-sulfated GAGs. This investigation is underway. Although sugar synthesis does not rely on gene products, we are studying how physical forces may affect GAG chain synthesis and how synthesis subsequently will modify chondrogenesis.

TISSUE ENGINEERING PROCESSOR

Based on knowledge attained from bench-top research, we have developed a computer-assisted tissue engineering support system (Figure 4). This system allows for cell culture at optimal culture conditions: hydrostatic fluid pressure (0 – 5 MPa), medium fluid flow rate (0.005 – 1 ml/min), and

constant or cyclic pressure (0 – 0.5 Hz) with varying gas concentration. After the cell or tissue construct is placed in the culture chamber, the system automatically cultures them with programmed conditions until the scheduled implant time is reached. We have used this processor to culture discarded cartilage obtained from patients undergoing orthopaedic procedures. By studying practical data from human cartilage, we hope to refine this protocol and propose new cell-based treatments.

In addition, this tissue processor allows us to attach a “compression and stretch” apparatus to further investigate the mechanobiology of skeletal tissue. We plan to apply other physical forces and alter physicochemical conditions for these and other target tissues.

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References

1. **Carter DG, Beaupre GS, Giori NJ, Helms JA.** Mechanobiology of skeletal regeneration. *Clin Orthop Rel Res* 1998; 355S:S41-S55.
2. **Urban JPG.** Present perspectives on cartilage and chondrocyte mechanobiology. In *Mechanobiology: Cartilage and Chondrocyte* (eds. Stolz JF). Netherland. 2001; 185-190.
3. **Mizuno S.** Hydrostatic fluid pressure stimulates intracellular calcuinin in bovine articular chondrocytes. Biomedical Engineering Meeting, Durham, NC, October 2001.
4. **Mizuno S.** Hydrostatic fluid pressure enhances intracellular calcium concentration in bovine articular chondrocytes. Orthopedic Research Society Meeting, Dallas, TX, February 2002.
5. **Glowacki J, Mizuno S, Greenberg JS.** Perfusion enhances functions of bone marrow stromal cells in three-dimensional culture. *Cell Trans* 1998; 83:2043-2051.
6. **Mueller, S.M., S. Mizuno, L.C. Gerstenfeld, & J. Glowacki.** Medium perfusion enhances osteogenesis by murine osteosarcoma cells in three-dimensional collagen sponges. *J Bone Mineral Res* 1999; 14:2118-26.
7. **Navarro FA, Mizuno S, Huertas JC, Glowacki J, Orgill DP.** Perfusion of medium improves growth of human oral neomucosal tissue constructs. *Wound Rep Reg* 2001;9:507-512.
8. **Mizuno S, Allemann F, Glowacki J.** 2001. Effects of medium perfusion on matrix production by bovine chondrocytes in three-dimensional collagen sponges. *J Biomed Mater Res* 2001;56:368-375.
9. **Mizuno S, Ushida T, Tateishi T, Glowacki J.** Effects of physical stimulation on chondrogenesis in vitro. *Mat Sci Eng C* 1998; 6:301-306.
10. **Mizuno S, Ushida T, Tateishi T, and Glowacki J.** Effects of hydrostatic pressure on chondrogenesis by bovine articular chondrocytes in three-dimensional culture. Orthopedic Research Society Meeting, San Francisco, CA, February 1999.