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Mini review

How best to preserve and reveal the structural intricacies of cartilaginous tissue

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ABSTRACT

No single processing technique is capable of optimally preserving each and all of the structural entities of cartilaginous tissue. Hence, the choice of methodology must necessarily be governed by the nature of the component that is targeted for analysis, for example, fibrillar collagens or proteoglycans within the extracellular matrix, or the chondrocytes themselves.

This article affords an insight into the pitfalls that are to be encountered when implementing the available techniques and how best to circumvent them.

Adult articular cartilage is taken as a representative *pars pro toto* of the different bodily types. In mammals, this layer of tissue is a component of the synovial joints, wherein it fulfills crucial and diverse biomechanical functions. The biomechanical functions of articular cartilage have their structural and molecular correlates. During the natural course of postnatal development and after the onset of pathological disease processes, such as osteoarthritis, the tissue undergoes structural changes which are intimately reflected in biomechanical modulations. The fine structural intricacies that subserve the changes in tissue function can be accurately assessed only if they are faithfully preserved at the molecular level. For this reason, a careful consideration of the tissue-processing technique is indispensable. Since, as aforementioned, no single methodological tool is capable of optimally preserving all constituents, the approach must be pre-selected with a targeted structure in view. Guidance in this choice is offered.

Contents

1. Introduction

"Believe only half of what you see": The morphological appearance of cartilage is a *fata morgana*. The structural organization of the tissue as we perceive it in the microscope depends greatly upon the nature of the technique that is implemented to preserve it for observation. No single processing technique is capable of optimally preserving each and all of

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the structural entities of cartilaginous tissue. Hence, the choice of methodology must necessarily be governed by the nature of the component that is targeted for analysis, for example, fibrillar collagens or proteoglycans within the extracellular matrix, or the chondrocytes themselves.

This article will begin with a general overview of the available preservation techniques (1. preservation techniques for cartilage: specific chemical and physical properties of the tissue are a source of multiple artifacts). The techniques that are best suited to preserve and reveal each of the structural components of cartilaginous tissue will then be delineated and briefly discussed in separate subsections: 2. chondrocytes and their membranous systems; 3. the network of fibrillar collagens; 4. the proteoglycans; 5. the tissue-water pool; and 6. the "best of the rest". Special techniques will be summarily dealt with at the end of the article in a separate subsection (7. special procedures), which will be followed by a few concluding remarks (8.conclusions).

In this review, adult hyaline articular cartilage will be taken as a representative *pars pro toto* of the different bodily types. In mammals, this layer of tissue is a component of the synovial joints, wherein it fulfills crucial and diverse biomechanical functions: it absorbs and distributes loads, transfers these to the opposing bony shafts, and ensures that the movements of the latter are executed in a practically frictionless manner (Hasler et al., 1999; Buckwalter et al., 2005). Since cartilaginous tissue owes 70% of its wet weight to its aqueous component, human beings may be envisaged as "walking on water" (Padalkar et al., 2013). Naturally, the water does not flow out of the tissue when we are walking. But this seemingly banal observation should by no means be taken for granted. On the contrary, it is a small wonder, the secret of which lies in the strong water-binding capacity of the highly-soluble proteoglycans, and in the fact that these macromolecules are trapped in an underhydrated state within an insoluble network of anisotropically-organized collagenous fibrils of high tensile strength (Heinegård et al., 1982; Tobias et al., 1992; McLeod et al., 2012; Padalkar et al., 2013). The fibrillar collagens and the proteoglycans, together with minor components, such as non-fibrillar collagens, glycoproteins and signaling peptides, constitute the remaining 30% of the wet weight of cartilaginous tissue (Anderson et al., 1964).

It is in the well-defined anisotropic organization of both the chondrocytes and the extracellular matrix of adult joint cartilage that the key to its capacity to counteract the deleterious effects of externally-applied mechanical forces lies. An externally-applied mechanical force induces a flow of water within and through the extracellular matrix. The densely-packed glycosaminoglycan-chains of proteoglycanous aggrecans resist this fluid flow in a manner that is proportional to the rate of loading. In practical terms, this means that water cannot move through the matrix with sufficient rapidity to relieve the hydrostatic pressure that builds up during the compression of the tissue. The consequence is, that the compressive stiffness of the cartilaginous tissue increases. It has recently been shown that the loss of glycosaminoglycans—which is a hallmark of the early stages of osteoarthritis—causes a dramatic increase in the hydraulic permeability of the tissue. This finding suggests that early osteoarthritic cartilage may be more vulnerable to loading rate than to loading magnitude, which is the conventionally-studied parameter (Nia et al., 2013). Hence, over the wide frequency-range of joint motion that is the norm during daily activities, hydraulic permeability would appear to be the most sensitive marker of early tissue degradation.

Compression-propagated fluid flow also induces the movement of mobile positive counterions relative to the positions of the fixed, negatively-charged groups on the immobilized glycosaminoglycan-chains of the aggrecan molecules. This separation of the mobile from the fixed charges generates localized electrical streaming-potential fields, which slow down the movement of the counterions and thus also the flow of water (Frank and Grodzinsky, 1987). The generation of the localized electrical streaming potentials is an energy-absorbing process. This absorption of energy further limits the flow of fluid through the tissue during its compression—in such a manner that its dynamic stiffness is maintained and its effective hydraulic permeability reduced by 10 to 40% (Frank et al., 1990). Although the dominant protective effect of the proteoglycans is attributable to the impeding influence of the closely-packed glycosaminoglycan-chains on fluid flow (Nia et al., 2013), the generation of electrical streaming-potential fields is also a contributing factor (Frank and Grodzinsky, 1987).

By virtue of the unique sequestered microenvironment that is generated by the entrapment of underhydrated proteoglycans within a network of collagenous fibrils of high tensile strength, the internal osmotic pressure of adult articular cartilage is permanently maintained at a level of about two atmospheres (Maroudas, 1976; Urban et al., 1979; Maroudas and Bannon, 1981; Horkay, 2013). Other features that are characteristic of the tissue include the absence of a blood-vascular supply, of lymphatic vessels and of nerve endings (Eggli et al., 1988; Hunziker et al., 2007).

2. Preservation techniques for cartilage: specific chemical and physical properties of the tissue are a source of multiple artifacts

The preparation of blocks of cartilaginous tissue for preservation necessitates a violation by cutting of the integrity of the sequestered internal microcompartments. By this mechanical act of excision, the high internal pressure of the tissue drops explosively to the atmospheric level. Water gushes into the cartilage in a tsunami-like fashion, drawn there by the hydrophilicity of the proteoglycans, whose state now changes from an underhydrated to a hydrated one (Hunziker and Graber, 1986). The tissue swells (Hunziker and Schenk, 1989). Even maintenance in a humid aeric atmosphere suffices to induce this phenomenon, which is accelerated in an aqueous environment (Thyberg et al., 1973). Molecules of the extracellular matrix undergo dislocation and disruption, thereby leading to a destruction of the native structural intricacies of the tissue (Hunziker et al., 1983; Hunziker and Schenk, 1987).

Upon immersion in an aqueous solution, irrespective of whether this is a cell-culture medium or a chemical fixative, such as formaldehyde or glutaraldehyde, or whether it is buffered or not, low-molecular-weight proteoglycans within the extracellular matrix of cartilaginous tissue are immediately extracted; the higher-molecular-weight ones first undergo a process of shifting, which has a knock-on effect, leading to the secondary dislocation of other macromolecules that lie in their wake and of those with which they interact chemically (Hunziker et al., 1983; Hunziker and Schenk, 1987). 10 to 30% of the proteoglycan-population is extracted from the tissue during the first 10 to 15 minutes of its immersion in an aldehyde-based medium (Hunziker and Graber, 1986). The processes of molecular dislocation and extraction lead to changes in the structural organization of the tissue of such a high order of magnitude as to yield a morphological image that bears little resemblance to that of native cartilage (Hunziker et al., 1982, 1983). This "image" of articular cartilage is the one that has been impressed on our mind's eye since the advent of the classical textbook reproductions in the 19th century (Davies et al., 1962).

Since the simple routine chemical fixation of cartilaginous tissue in an aldehyde-based medium leads to such a severe distortion of its macromolecular organization, this mode of proceeding is totally inadequate for a fine structural analysis in the transmission electron microscope. The lateral resolution is so greatly reduced—being at a par with that achieved in the light microscope (micrometer-range), even on thin (30- to 40-nm-thick) sections—as to render any measurements other than coarse, elementary ones completely spurious (Poole et al., 1982). Tissue that has been preserved in this manner could be used to estimate the numerical density of chondrocytes, or to ascertain whether a network of collagenous fibrils is present or not, but information of a more sophisticated nature would not be forthcoming (Aszodi et al., 1998, 2001; Gustafsson et al., 2003). An example will illustrate the dangers