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Isolated effects of external bath osmolality, solute concentration, and electrical charge on solute transport across articular cartilage

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ABSTRACT

The metabolic function of cartilage primarily depends on transport of solutes through diffusion mechanism. In the current study, we use contrast enhanced micro-computed tomography to determine equilibrium concentration of solutes through different cartilage zones and solute flux in the cartilage, using osteochondral plugs from equine femoral condyles. Diffusion experiments were performed with two solutes of different charge and approximately equal molecular weight, namely iodixanol (neutral) and ioxaglate (charge = −1) in order to isolate the effects of solute's charge on diffusion. Furthermore, solute concentrations as well as bath osmolality were changed to isolate the effects of steric hindrance on diffusion. Bath concentration and bath osmolality only had minor effects on the diffusion of the neutral solute through cartilage at the surface, middle and deep zones, indicating that the diffusion of the neutral solute was mainly Fickian. The negatively charged solute diffused considerably slower through cartilage than the neutral solute, indicating a large non-Fickian contribution in the diffusion of charged molecules. The numerical models determined maximum solute flux in the superficial zone up to a factor of 2.5 lower for the negatively charged solutes (charge = −1) as compared to the neutral solutes confirming the importance of charge-matrix interaction in diffusion of molecules across cartilage.

1. Introduction

Articular cartilage is an avascular tissue with highly inhomogeneous organization that lines the end of long bones and enables low-friction joint mobility [1,2]. The avascular nature of articular cartilage means that it has to rely mainly on diffusion for transport of vital signaling molecules, nutrients and oxygen. Cyclic loading and the associated fluid flow augment the transport of large molecules through an additional transport mechanism, namely convection [3–5]. Transport of small solutes such as ions, however, cannot be significantly amplified via convection [5]. Extracellular matrix (ECM) of cartilage consists mostly of collagen type II, proteoglycans (PGs) and water. Fragments of PG and collagen type II are continuously transported within ECM as a result of enzymatic digestion and remodeling. Collagen type II and PGs account for the major components of cartilage that provide the ECM with its shear and tensile properties as well as with resilience [2,6]. Articular cartilage is characterized by a zonal architecture where water content, as the major parameter influencing solute diffusion, varies from 80% in the superficial layer to 60% in the deep layer [7–10]. The orientation, thickness, and concentration of collagen type II fibrils together with uneven distribution of PGs in various zones of articular cartilage play significant roles in the solute diffusion across the tissue [1,11]. Solute diffusion depends predominantly on the nature of the interaction that can vary as a consequence of different density and morphology of the tissue at the molecular level (steric hindrance) and ion–ion interactions. The latter is believed to take place when the solute is charged and thereby repulsion/attraction interaction with the negatively fixed charges of the glycosaminoglycans chains (GAGs) is dominant [12–14]. The combined effects of electric phenomena with the steric hindrance make the diffusion process across the articular cartilage extremely complicated.

To better understand the above-mentioned complexities in the transport of solutes across cartilage, the current study aims to separate the various physical mechanisms as much as possible by using a carefully designed set of diffusion experiments and associated finite element modeling.

First, we address the question whether the solute transport in cartilage has a Fickian nature. This would be the case if the

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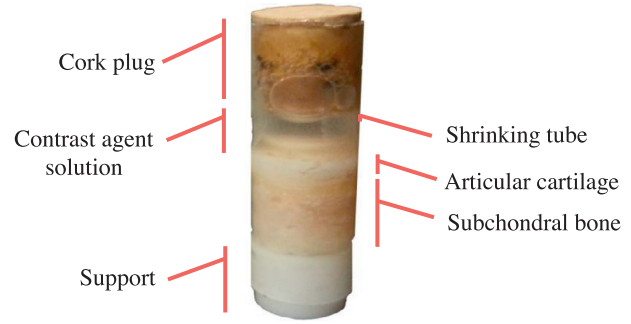

Fig. 1. The sample consists of an osteochondtal plug, a cork plug, a shrinking tube, contrast agent solution and a support.

concentration-time curves remain unchanged for different levels of osmolality and different levels of solute concentration in case the solute is non-charged. Osmolality difference between bath and cartilage deforms the cartilage and consequently alters the molecular morphology and thus the interaction between the solute and the extracellular matrix that causes deviation from Fickian diffusion. For instance, it has been shown that Fickian models could poorly predict the diffusion of cryoprotective agent (CPA) through articular cartilage primarily due to local osmolality variation within the extracellular matrix [15].

Second, we address the contribution of electrical charges to the transport of solutes across cartilage. In the majority of previous studies, the charged solute's diffusion attributes have been obtained by adopting Fickian-based models [16–18]. Answering the second research question allows us to quantify the effect of this simplification on the accuracy of determined solute fluxes and the described diffusion behavior. We therefore not only isolate the effects of external bath osmolality, concentration and charge from each other but also quantify those effects using equilibrium curves and zonal concentration curves as well as solute fluxes. The present study features a unique experimental approach through which the aforementioned effects could be separated. Moreover, biphasic-solute and multiphasic models [9,19] that we had previously developed have been used to determine solute fluxes.

2. Materials and methods

2.1. Sample harvest and conditioning

Equine knees for this study were obtained from the Equine Clinic in Utrecht University (approved by *Animal Experiments Committee in Utrecht University*). Using a custom-made hollow drill bit, four osteochondral plugs (8.5 mm diameter, cartilage thickness = 2.57 ± 0.27 mm) were harvested from the medial femoral condyle of two 6-year old equine femurs (samples 1 and 2 from one donor and samples 3 and 4 from the other). To prevent sample overheating and to keep the drilling site moist during drilling, we continuously sprayed phosphate buffer saline (PBS, 290 mOsmol/kg H_2O, pH 7.4, Life technologies) on the drilling site. We stored the osteochondral plugs in a large bath of a solution comprising PBS, protease inhibitors (*cOmplete tablets, EDTA free, Roche, Germany*) and 5 mM Ethylenediaminetetraacetic acid (*EDTA*) at −20 °C before the diffusion experiments. Upon thawing, the samples were tightly wrapped laterally using heat-shrinking sleeves to prevent lateral contrast agent leakage (Fig. 1). The sample was protected from heat during heat-shrinking process by optimizing the heat source distance from the sample, constantly spraying cold PBS on the sample surface, and mounting small wet cotton pieces on the boundaries.

Table 1
Specification of the baths used in the diffusion experiments: Four different bath conditions were used to investigate the effects of concentration (*Iodix 320,290 vs. Iodix 160,290*), external bath osmolality (*Iodix 320,290 vs. Iodix 320,600*) and solute's charge (*Iodix 320,600 vs. Ioxag 320,600*) on the diffusion in cartilage.

Bath	Solute	Charge	Concentration (mgI/ml)	Osmolality (mOsm/kg H_2O)
Iodix 320,290[a]	Iodixanol	0	320	290
Iodix 320,600[a]	Iodixanol	0	320	600
Iodix 160,290[a]	Iodixanol	0	160	290
Ioxag 320,600[a]	Ioxaglate	−1	320	600

[a] The conditions are described by their abbreviated solute name (iodixanol (*Iodix*) and ioxaglate (*Ioxag*)), solute concentration in the bath and bath osmolality.

2.2. Contrast agent solutions

The transport of two clinical contrast agents with similar molecular weights was investigated: iodixanol (*Visipaque*, 1550 g/mol, *charge* = 0, *GE Healthcare, Netherlands*) and ioxaglate (*Hexabrix*, 1269 g/mol, *charge* = −1, *GE Healthcare, Netherlands*). In order to study the effects of bath concentration, osmolality, and electrical charge on solute transport, we prepared four different contrast agent baths (Table 1). We adjusted the osmolality of each solution at the required level by adding sodium chloride. Enzymatic digestion was prevented during the diffusion experiments through the addition of protease inhibitors (*cOmplete, Roche, Netherlands*) and *EDTA* to the baths. A freezing point osmometer (*Advanced® Model 3320 Micro-Osmometer, Netherlands*) was used to measure osmolalities.

2.3. CECT imaging

We placed the wrapped samples on a custom-made holder and fixed the holder inside micro-CT (*Quantum FX, Perkin Elmer, USA*). In each condition, we loaded approximately 650 µL from the baths onto the cartilage surface. A cork plug and proper micro-CT chamber humidification were used to minimize solution evaporation during micro-CT scans (Fig. 1). We scanned the samples at room temperature using micro-CT under a tube current of 180 µA and a tube voltage of 90 kV, resulting in a scan time of 2 min and a voxel size of $40 \times 40 \times 40\ \mu m^3$. The resolution was chosen such that the field of view included the cartilage specimen, the contrast agent bath, and the subchondral bone. We acquired images during diffusion process at time points t_{-1} (before adding the bath), t_0 (bath injection), $t = 5$, 10, 30 min and $t = 1$, 2, 3, 4, 5, 7, 10, 12, 24 and 48 h. When the experiment on every specimen was finished using one bath and before starting a new experiments with another bath (Fig. 2), we washed the osteochondral plugs in series of large desorption baths [20] (PBS+protease inhibitor+EDTA (5 mM) for 48 h (4 °C) which proved to be effective (less than 5% difference between the gray values). The average gray values for each sample and each condition were recorded at t_0 to make sure of the efficacy of the contrast agent washout process. The equilibrium concentration of ioxaglate (inversely related to GAG content [21]) did not vary after performing the aforementioned experiments during our pilot studies suggesting no cartilage degeneration (data not shown). Furthermore, the remaining of each bath was tested after each experiment to search for any clues of GAG leakage using Dimethylmethylene Blue assay (DMMB), which proved no visual sign of GAG loss.

2.4. Image acquisition and pre-processing

Image acquisition included 3D image reconstruction that was undertaken automatically using the built-in micro-CT software. The

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