The analysis of residual enzyme in the cartilage tissue after partial digestion

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Statement of Purpose: Several studies have reported that enzymatic treatment could increase chondrocyte bioactivity and improve integrative cartilage repair [1]. The partial digestion of cartilage appears to be one promising technique for integrative cartilage repair. However, most of those studies were interested in the comparison of the property changes between enzymatically treated and untreated cartilage tissue without reporting the effect of residual enzyme in the tissue. The residual enzyme in the treated cartilage fragments could further degrade the tissue and may result to impair their integrative capacity. In this work, we aimed to evaluate a method to detect the residual enzyme in pulverized and enzymatically treated cartilage tissue. The residual enzyme concentration of the partial-digested cartilage tissue was determined by measuring the radiolabeled (^{125}I) enzyme. Meanwhile, a confocal laser scanning microscopy (CLSM) study was performed to visualize the penetration of fluorescent-labeled enzyme into cartilage.

Methods: Articular cartilage was harvested from porcine knees and minced into fragments by a tissue pulverizer. Subsequently, 100 mg of the pulverized tissue was treated with an enzyme mixture of collagenase and thermolysin at 37° for 20 min, then washed in normal saline for 3 or 6 times. The treated tissue and the buffer for washing were collected. Prior to experiment, the enzyme and cartilage tissue were labeled respectively with red fluorescent dye (Alex Fluor 555) and green fluorescent dye (5-DTAF) to visualize the penetration depth in the tissue under CLSM. Alternatively, the enzyme was radiolabeled with Iodine-125 (^{125}I) using the iodogen precoated tubes in order to detect the residual enzyme in the treated tissue or washing buffer. Their radioactivity was measured by a gamma counter to estimate the residual enzyme in the partially digested tissue. The enzyme activities in the washing buffer were also measured by EnzChek® gelatinase/collagenase assay kit as a control.

Results: The residual enzyme was visualized by CLSM and the depth of penetrations were noted in 10 min, 20 min, 30 min and 60 min, as measured by the width of the red fluorescent band extending from the surface into the cartilage (Fig. 1a). Visual measurement found a linear increase in the penetration depth of Liberase with increasing incubation time. Furthermore, the penetration depth of enzyme was estimated by measuring the emission bandwidths of red fluorescence (Fig. 1b), and the penetration depth after 10, 20, and 30 min of enzymatic treatment were, respectively, 35.8 μm, 67.4 μm, and 77.2 μm. The residual enzyme in the washing buffer and treated tissue were measured by detecting the radioactivity directly without further processing. Concentrations were calculated by interpolation from a standard curve, which was prepared by mixing 5 μl (0.5 mg/ml) of ^{125}I labeled enzyme and 10 ml of radio-free enzyme (0.5 mg /ml), and yielded concentrations of 5.0 ~ 5×10^{-5} mg/ml, to ascertain linearity of the procedure (R^2: 0.99). As shown in Fig. 2a, the residual enzyme in washing buffer was decreasing with increasing time of washing, and the lowest amount of residual enzyme in the washing buffer was reached after 5 washes. The residual enzyme in the digested tissue was 95.663 ± 12.388 μg/ml after 3 washes, and dropped to 46.931 ± 9.184 μg/ml with 3 more washes (Fig. 2b)

Conclusions: The enzymatic treatment partially degraded cartilaginous extracellular matrix (ECM) components and released embedded cells to improve integrative cartilage repair [2]. However, the residual enzyme in the tissue could further degrade the tissue and may affect their integrative capacity. Traditional methods for measuring enzyme activity in tissue typically involve homogenization or grinding tissue, and subsequent determination of enzyme activity after addition of a fluorogenic or otherwise labeled substrate [3]. In this work, the residual enzyme and penetration depth of immobilized enzyme after different enzymatic treatment duration were also directly and non-destructively visualized by CLSM. Meanwhile, the characterization of residual enzyme in the treated tissue was also performed without any further processing. Consequently, this work has shown obvious advantage in the characterization of residual enzyme in the enzymatically treated tissue with minimal manipulation. The effect of residual enzyme on integrative cartilage repair will be studied in the future work.

References: [1] Jarno BB. , Arthritis Res Ther. 2004;6:469-476. [2] Liao CJ, J Biomed Mater Res A. 2007;81A;3: 567–577. [3] Zhenrui C. Mar Chem. 2011;123(1-4):23-31.

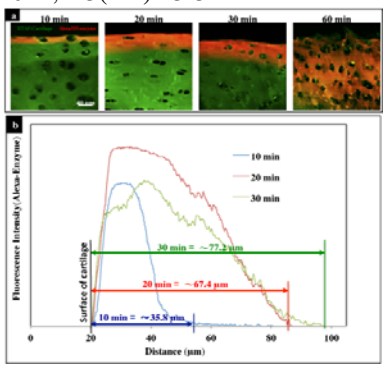

Figure 1. CLSM observation

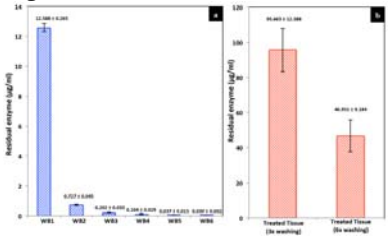

Figure 2. Residual enzyme in (a) wash buffer and (b) treated tissue.