Macromolecular diffusion in intact, degraded and crosslinking-augmented intervertebral discs

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Received: 22 November 2016; Revised: 26 January 2017; Accepted: 27 February 2017

Abstract
Injection of macromolecular anabolic enzymes is a promising treatment for disc degeneration. However, macromolecular diffusion within the degraded or crosslinking-augmented discs remains unclear. In this study, healthy porcine anular fibrosus (AF) and nucleus pulposus (NP) were prepared. After a 24 hr trypsin-induced matrix degradation and a following 24 hr genipin-induced crosslinking augmentation, the diffusion of 0.37 kDa fluorescein sodium, 4.4 kDa dextran and 40 kDa dextran in the AF and NP were evaluated. Regardless of molecular weight, macromolecular diffusion was highest along the circumferential AF followed by the radial AF and NP. Matrix degradation and the crosslinking-augmentation decreased macromolecular diffusion. Scanning electric microscopic (SEM) images revealed that the intact circumferential AF contained oval-shaped pores, while the intact radial AF included long and narrow cavities. These porous diffusion paths collapsed after matrix degradation. The following crosslinking augmentation recovered matrix hydration and the area encircled by pore contour. Nevertheless, the diffusion paths were still occluded by detached collagen fibrils. In conclusion, pore shapes regulate macromolecular diffusion in discs. The decrease of macromolecular diffusion after matrix degradation is due to pore deformation. The following crosslinking augmentation makes the macromolecules more easily trapped within the diffusion paths.

Key words: Molecular therapy, Disc degeneration, Macromolecular diffusion, Genipin, Crosslinking augmentation
1. Introduction

Disc degeneration is one of the leading causes of lumbar disorders. Removal of the prolapsed disc materials or the whole degenerative disc followed by stabilizer implantation is the standard surgical treatment. However, disc removal, stabilizer implantation and collateral muscle injuries degrade spinal functions. A substantial percentage of patients suffer from adverse post-surgery complications, including adjacent disc diseases, (Gao, Lei, He, Liu, Xiao, Wen, Liang and Li; Kepler and Hilibrand; Prasarn, Baria, Milne, Latta and Sukovich) muscle atrophy or chronic soreness (Sangala, Nichols and Freeman; Tsutsumimoto, Shimogata, Ohta and Misawa). Recently, molecular therapies have been proposed to treat disc degeneration. Recombinant growth factors, such as epidermal growth factors, transforming growth factors, bone morphogenetic protein and platelet-derived growth factors (Bae and Masuda; Masuda) are injected into degenerative discs to enhance anabolism without breaking disc integrity.

Molecular transportation within discs can be through diffusion mechanisms, by which molecular movement is passively driven by the solute concentration gradient. Macromolecular diffusion is dependent on porous structure and water flow of disc matrix. Porous structures form the molecular diffusion channels. An example of a molecular pathway is through the micro pores found in the anulus fibrosus (AF) (Jackson, Yuan, Huang, Travascio and Yong Gu). Studies have been focused on diffusion of small solutes, such as glucose, lactate, and ions, (Chiu, Newitt, Segal, Hu, Lotz and Majumdar; Drew, Silva, Crozier and Peary; Hsu and Setton; Jackson, Yuan, Huang, Travascio and Yong Gu; O'Hare, Winlove and Parker; Roberts, Urban, Evans and Eisenstein; Selard, Shirazi-Adl and Urban; Travascio and Gu; Urban, Holm and Maroudas; Yuan, Jackson, Huang and Gu) while the studies of macromolecular diffusion are relatively limited. However, most of the recombinant growth factors are macromolecules. Due to their larger size, the interactions between macromolecules and the disc matrix are more frequent and complicated. As a result, macromolecular diffusion in discs cannot be accurately predicted according to the diffusive patterns of small solutes.

The porous structure and water flow of degenerative discs are very different from those of intact ones, because the up-regulations of degrading enzymes deplete disc compositions. The overall effect of matrix degradation on macromolecular diffusion in discs is inconclusive. Unfavorable influences include matrix dehydration and diffusion pathway obstructions by degraded tissues. On the other hand, damages to matrix structures may create new fissures, which facilitate macromolecular transportation. Injection of genipin solution has been shown to be effective in disc augmentation by the formation of crosslinks between peptides (Sung, Chang, Ma and Lee). These exogenous crosslinks restore integrity of injured AF and increase water inflow during rest.(Chuang, Lin, Tsai and Wang; Chuang, Popovich, Lin and Hedman) Based on these results, genipin injections prior to molecular therapies may prevent leakage of growth factors and accelerate growth factor distribution through the enhancing water inflow. However, this therapeutic efficacy has not been clarified. Therefore, the objective of this study was to investigate the macromolecular diffusion within the intact, degraded and crosslinking-augmented discs.

2 Material and Methods

2.1 NP Specimen preparation. The lumbar discs of healthy 6-month-old juvenile pigs were harvested within 4 hrs after sacrifice and assigned to the intact, degraded and crosslinking-augmented nucleus pulposus (NP) groups. For the degrade NP group, trypsin solution (0.25%, 0.5 ml) was injected into the discs to deplete proteoglycan and collagen fibers (Eyre, Weis and Wu; Jim, Steffen, Moir, Roughley and Haglund). Degradation underwent at 4°C for 12 hrs and then at room temperature for another 12 hrs. For the crosslinking-augmented NP, the degraded discs were further injected with genipin solution (0.33%, 0.5 ml). Reaction proceeded at 4°C for 24 hour. Each NP specimen weighted around 0.1 g, and each group included 24 specimens.

2.2 AF specimen preparation. Cubes of AF specimens (4 mm) were dissected from porcine lumbar discs, and assigned into intact (n=48), degraded (n=48) and crosslinking-augmented AF group (n=48). Degradation was resulted from immersion of trypsin solution (0.25%) at 4°C for 12 hrs followed by a 12 hr room temperature incubation. For the crosslinking-augmented AF group, the degraded AF specimens were further crosslinked within genipin bath (0.33 %) for 24 hrs.

2.3 Fluorescently-labeled macromolecular solutions. Three fluorescently-labeled macromolecular solutions (100µM) were prepared, i.e., 0.37 kDa fluorescein sodium (FS), 4.4 kDa dextran (label: tetramethylrhodamine isothiocyanate,
TRITC) and 40 kDa dextran (label: fluorescein isothiocyanate, FITC). These macromolecules were chosen for their similar weight to those of large nutrients and recombinant growth factors, ex. glucose (0.18 kDa), EGF (6 kDa), BMP (16-28 kDa) and PDGF (33 kDa).

2.4 Fluorescence photographic system. A continuous fluorescence photography system was developed to monitor the time-dependent molecular diffusion in biological tissues. The system consisted of a cooling digital SLR camera (Canon Kiss X2 450D), light sources, a filter wheel and an object stage (Fig. 1A). To decrease the thermal noise of the image, a cooling chip (TEC1-127030-30, Hebei IT Co., Ltd) and a fan was attached onto the CMOS sensor in the camera. The light sources were white, blue, and green light emitting diodes (3528 SMD LED FPCB, LD Trade Co., LTD). The blue light could excite FS and FITC, while the green light could excite TRITC. The scattering red light was blocked by a low-pass dichroic filter (cut-off: $\lambda_{550nm}$) (Fig. 1B). To avoid overlapping of excitation light, a green light filter (cut-off: $\lambda_{550nm}$, B+W 092, Schneider Optics, Inc., CA) was installed for FS/FITC, while a red light filter was installed for TRITC (cut-off: $\lambda_{660nm}$, B+W 092) (Fig. 1C).

![Fluorescence photographic system](image)

Fig 1. (A) Schematic depiction of the custom-designed fluorescence photography System; (B) Light spectrum of blue, green and white light sources. The red dash-dot encircled the filtered light spectrum. GP-RR=green light pass, red light reflect; (C) The overlapping of excitation light (blue) and emission light (green) for FS/FITC, and the overlapping of excitation light (green) and emission light (red) for TRITC

2.5 Diffusion test protocol. An acrylic diffusion measurement apparatus was designed (Fig. 2) (Jackson, Yuan, Huang, Travascio and Yong Gu). The specimen was placed in the transportation channel and confined by 2 steel porous nets, while the macromolecule solution and the saline solution were filled in the source chamber and the receiving chamber, respectively. The fluorescence images of the acrylic apparatus were taken every 5 min during the diffusion test. The fluorescence intensity of the receiving chamber was transformed to gray scale, and < 0.1% change was defined as the equilibrium state. The solution concentration of the receiving chamber was calculated from the fluorescence intensity (gray scale) based on a linear regression function calibrated by 5 pairs of fluorescence intensities and the solution concentrations, 6.5 / 12.5 / 25 / 50 / 100 µM (Fig. 3).
2.6 Diffusion coefficient. The apparent diffusion coefficient of the investigated macromolecule was calculated according to Ficks’ first law,

\[ J = -D \frac{dC}{dx} \]  

where \( J \) is the diffusion flux (g/sec/mm²), \( D \) is the diffusion coefficient (mm²/sec) and \( C \) is the solution concentration (g/mm³). At a steady state, the diffusion coefficient is equal to the apparent diffusion coefficient \( (D_a) \) (Jackson, Yuan, Huang, Travascio and Yong Gu):

\[ D_a = \ln \frac{C_s - C_r(t_0)}{C_s - C_r(t)} \frac{V_r}{(t - t_0) A} \]  

where \( C_s \) and \( C_r \) is the solution concentration in the source chamber and the receiving chamber, \( V_r \) is the receiving chamber volume, \( A \) and \( h \) are the cross-sectional area and the width of the specimen, \( t \) is the diffusion time and \( t_0 \) is the initial time of the diffusion test. The \( C_r(t) \) was constant throughout the diffusion test. The \( C_r(t) \) was zero at the beginning of the diffusion test and then increased with the diffusion time.

2.7 Water content measurement. The specimen hydration was expressed as the weight difference of specimen after lyophilization divided by specimen wet weight (%).
2.8 Scanning electronic microscopic (SEM) images. The intact, degraded and crosslinking-augmented AF specimens were dissected and fixed in gluteraldehyde solution (2.5%) for 2 hrs. After freezing, the specimens were sliced into 300 μm thick samples, fixed in OsO4 solution at 4°C for 12 hr, dehydrated sequentially in 30, 50, 70, 85, 90, 95, 100% ethanol solution bath. After a 30 min immersion of propanone solution, the samples were dried by a critical point dryer (Hitachi HCP-2) and then sputter-coated with gold. The SEM images were taken from circumferential and radial views with a scanning electron microscope (FEI Inspect S).

2.9 Statistics analysis. The mean (standard deviation) diffusion coefficient of each investigated macromolecules in intact, degraded and crosslinking-augmented AF/NP tissues was respectively determined by 8 specimens. A three-way ANOVA was performed to find the factors among macromolecular weight, disc regions and tissue conditions, which significantly affected the macromolecular diffusion coefficient. A post-hoc Bonferroni test was conducted to find the influential level of the significant factors. Significance was considered at p<0.05.

3. Results

3.1 Diffusion coefficient. On average, diffusion of the investigated macromolecules in AF/NP specimens of each condition reached an equilibrium state by 60 min (Fig. 4). Representative fluorescence images of 40 kDa FITC diffusion in each of the investigated disc regions are illustrated in Fig. 4. The diffusion coefficient of the macromolecules decreased with the increasing molecular weight (p<0.001) and altered with different disc regions (p<0.001). Regardless of molecular weight, macromolecular diffusion coefficients were highest along circumferential AF and reduced along the radial AF and in NP. Diffusion coefficients decreased after matrix degradation (p<0.001) and the following crosslinking-augmentation (Table 1).

![Fig 4](image)

Fig 4. Representative fluorescence images of FITC diffusion in the intact NP, circumferential AF and radial AF before and after 60 min diffusion.

Table 1. Mean value (standard deviation) of diffusion coefficient ($10^{-4}$ mm$^2$/sec) of each investigated macromolecules in the circumferential AF (AFC), radial AF (AFR) and NP in intact, degraded, and crosslinking-augmented conditions.

|        | AFC | AFR | NP | AFC | AFR | NP | AFC | AFR | NP |
|--------|-----|-----|----|-----|-----|----|-----|-----|----|
| 0.37 kDa | 5.46 | 3.27 | 3.62 | 1.37 | 1.26 | 1.26 | 1.08 | 0.78 | 0.90 |
| FS     | (0.65) | (0.90) | (1.23) | (0.31) | (0.20) | (0.33) | (0.15) | (0.10) | (0.14) |
| 4.4 kDa | 4.21 | 2.33 | 2.27 | 1.15 | 1.09 | 0.97 | 0.88 | 0.73 | 0.82 |
| dextran | (1.04) | (0.60) | (0.70) | (0.04) | (0.07) | (0.20) | (0.07) | (0.04) | (0.16) |
| 40 kDa  | 2.50 | 1.07 | 1.43 | 1.03 | 0.85 | 0.90 | 0.79 | 0.54 | 0.65 |
| dextran | (1.11) | (0.84) | (0.43) | (0.09) | (0.19) | (0.22) | (0.07) | (0.09) | (0.07) |

3.2 Water content. The water content of AF was significantly less than that of NP in the intact status (p<0.001). The water content of AF/NP decreased after degradation (p<0.001 for NP) and recovered after crosslinking-augmentation (p<0.001) (Table 2).
Table 2. Water content (%) of the AF and NP in the intact, degraded and crosslinking-augmented status.

|        | Intact     | Degraded  | Crosslinking-augmented |
|--------|------------|-----------|------------------------|
| AF     | 82.1 (5.2) | 80.5 (4.6)| 91.2 (2.5)             |
| NP     | 91.4 (4.7) | 85.4 (2.9)| 91.7 (0.7)             |

3.3 SEM Images. The intact circumferential AF revealed distinct parallel collagen lamellae, with one layer containing oval-shaped pores and one layer composed of dense collagen mass. The diameters of those pores ranged from less than 1 µm to more than 4 µm (Fig. 5a, 5g). The intact radial AF included dense longitudinal collagen fibrils embedded with some long and narrow cavities (Fig. 5d, 5j). After matrix degradation, the oval-shaped pores in the circumferential AF collapsed or deformed. The dense collagen mass in the nearby layers were compressed and twisted. Distinction between laminar layers was not clear (Fig. 5b, 5h). In the degraded radial AF, collagen lamellae distorted and crimped. Collagen fibers were dissociated into irregular and disrupted fibrils. Cracks presented within collagen fibril bundles (Fig. 5e, 5k). After crosslinking-augmentation, oval-shaped pores reappeared in the circumferential AF. The surrounding collagen wall aggregated together. Boundaries between laminar layers were distinguishable again (Fig. 5c, 5i). In the crosslinking-augmented radial AF, the crimped collagen fibers were straightened. Space between collagen fibril bundles was enlarged and clogged by numerous fine fibrils collagen (Fig. 5f, 5l).
Fig 5. SEM image of the intact (left column), degraded (middle column) and crosslinking-augmented AF (right column) from the circumferential (AFC) and the radial (AFR) view at the magnification of 200x and 500x.

4 Discussion

This study evaluated the effect of matrix degradation and crosslinking augmentation on macromolecular diffusion in discs using self-established fluorescence photographing techniques. The diffusion coefficient of FS, measured with validated fluorescence recovery after photobleaching (FRAP) techniques, was reported to be $1.22 \times 10^{-4}$ mm$^2$/sec in circumferential AF and $0.79 \times 10^{-4}$ mm$^2$/sec in radial AF of bovine discs (Travascio and Gu). In this study, diffusion coefficient of FS was $5.46 (0.65) \times 10^{-4}$ mm$^2$/sec in circumferential AF and $3.27 (0.90) \times 10^{-4}$ mm$^2$/sec in radial AF of porcine discs. The diffusion coefficients of FS in these two studies were comparable. The differences may be resulted from different animal specimen. The feasibility of the techniques established in this study was clarified.

4.1 Effect of disc region. Anisotropic pore shapes in cubic AF specimens accounted for different molecular diffusions (Jackson, Yuan, Huang, Travascio and Yong Gu; Travascio and Gu). The present study presented consistent results, with higher macromolecular diffusion along the circumferential AF than the radial AF. Macromolecular diffusion is regulated by the mutual compatibility of the molecule’s shapes and the diffusion channel contour. Round-shaped macromolecules diffuse through articular endplates more easily than the chain-shaped ones (Roberts, Urban, Evans and Eisenstein). Higher macromolecular diffusion in circumferential AF indicates that the reverse is also valid, in which oval-shaped pores are more favorable for macromolecular diffusion. Compared to circumferential AF, lower macromolecular diffusion in NP could result from the loose aggregation of abundant proteoglycan and the lack of distinct porous channels. Macromolecules are trapped within NP during diffusion due to water bond to the proteoglycan. Without distinct porous channels, macromolecules easily collide with NP compositions due to long radius. The distance between branches of proteoglycan is around 3-4 nm, (Roberts, Urban, Evans and Eisenstein) shorter than the hydrodynamic radius of the 4.4 kDa dextran ($\approx 4.5$ nm) or the 40 kDa dextran ($\approx 4.78$ nm). Frictional heat produced by the molecule-matrix collisions reduces the kinetic energy of moving macromolecules.

4.2 Effect of matrix condition. Macromolecular diffusion decreases after matrix degradation. The decrease in macromolecular diffusion in the degraded AF is contributed by deformations and occlusion of the matrix pores, which is caused by weak collagen fibers and detached collagen fibrils. Even without SEM images as evidence, the decrease of
macromolecular diffusion in the degraded NP can still be explained by the similar mechanism that decreases macromolecular diffusion in the degraded AF. The disrupted matrix compositions disperse and block the macromolecular diffusion route in the NP. The water content of AF and NP decreases after matrix degradation due to proteoglycan hydrolysis. Macromolecules are less trapped in the disc matrix with the water molecules. However, this favorable effect is overcome by the unfavorable effect of pore deformation and occlusion. Macromolecular diffusion further decreases after crosslinking augmentation. Genipin-induced exogenous crosslinking condenses delaminated collagen. The deformed shape of pores is partially recovered with enlargement of cross-sectional area, whereas the detached collagen fibrils clogging the matrix pores are fixed (which is more manifested in radial AF SEM images). The water absorption in degraded disc is increased, as it is in the intact discs, (Chuang, Popovich, Lin and Hedman) either due to the cease of proteoglycan leakage or due to the supplement of negative charges by exogenous crosslinkings. The increase of water retention makes the macromolecules more easily trapped in the diffusion route by the clogging of collagen fibrils. Therefore, the genipin injection prior to molecular therapies would be more preferable for the limited target area.

4.3 Limitations. Some limitations in this study should be addressed. Macromolecular diffusion within biological tissue is a complex mechanism affected by immobile carriers, molecular crowding and binding interactions (Sanabria, Kubota and Waxham). Complicated mathematical models have been created in combination with fluorescence spectroscopy to describe individual effect of aforementioned factors on macromolecular diffusion (Sengupta, Garai, Balaji, Periasamy and Maiti; Travascio and Gu; Travascio, Jackson, Brown and Gu). This study used a one-dimensional diffusion model, which may neglect the implicit molecular diffusion characteristics but clearly reveal the gross responses of macromolecular diffusion to disc region and matrix condition. Another limitation is that the reaction time of digestive enzyme was only 24 hrs. In in-vivo condition, disc cells may continuously secret digestive enzymes once the degeneration process is initiated. Both pore deformation and cavity occlusion will progress and decrease macromolecular diffusion until degeneration is suppressed.

5 Conclusions

This study revealed that macromolecular diffusion in discs is region-dependent, with higher diffusivity along circumferential AF followed by radial AF and in NP. Enzyme-induced matrix degradation causes collagen fiber delamination and proteoglycan depletion. The diffusion paths are deformed and occluded, which decreases macromolecular diffusion. The following crosslinking augmentation would further reduce macromolecular diffusion, because occlusion of diffusion pathway is not removed, but water retention of disc is recovered.

Acknowledgement: This study was supported by National Health Research Institute (NHRI-EX98-9733EI) and National Science Council, Taiwan (NSC 97-2628-E-002-224-MY2).

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