Age-related degeneration of lumbar intervertebral discs in rabbits revealed by deuterium oxide-assisted MRI


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Summary

Objectives: Intervertebral disc (IVD) degeneration is associated with a loss of disc water content and change in biochemical composition of the disc. Rabbit is a frequently used model to evaluate the efficacy of therapeutics for disc degeneration. This study addresses whether rabbits undergo age-related disc degeneration, assessed using deuterium oxide-assisted magnetic resonance imaging (MRI) of the lumbar IVDs.

Materials and methods: The lumbar spines of adolescent, adult, and aged rabbits (6–36 months) were subjected to T2-weighted/short-tau inversion recovery (STIR) MRI scan along with water–deuterium oxide (H2O:D2O) dilutions. The total and maximum H2O:D2O index (HDI) of the lumbar IVDs were determined and compared between disc levels at different ages.

Results: Adolescent rabbit lumbar discs had similar total HDI, suggesting the hydration and biochemical composition was similar among the lumbar levels. With the use of H2O:D2O reference, the discs were shown to undergo continual decrease in signal with aging which non-calibrated measurement method could not reveal. The HDI decrease rate was higher at the caudal than cranial levels.

Conclusion: This study provided in vivo evidence of age-related progressive disc degenerative change in rabbit lumbar discs, suggesting aged rabbits can be considered as a natural disc degeneration model in disc regeneration studies. However, it is important to select proper disc levels as intra-subject controls due to different rates of degenerative changes between caudal and cranial levels.

Key words: Magnetic resonance imaging, Intervertebral disc, Deuterium oxide, Aging, Rabbit.

Introduction

Degeneration of intervertebral discs (IVDs) has been characterized by changes in extracellular matrix composition as well as loss of proteoglycan and hence water content. The changes have been suggested as a consequence of up-regulated inflammatory mediators and metalloproteinases. Loss of notochordal cells in nucleus pulposus has also been associated with disc degeneration and an invasion of chondrocytes from endplate has been suggested to cause a change of notochordal phenotype of nucleus pulposus to fibrocartilaginous one. This change of phenotype will result in a change of extracellular matrix composition, in particular reduction of proteoglycans and water, and hence T2-weighted signal in magnetic resonance imaging (MRI) assessment.

Rabbit is a popular animal model for IVD research, particularly in the generation of degeneration models and testing of biological therapies. However, there is a lack of information on potential disc degenerative changes in aging rabbits. Although cytomorphological analysis of nucleus pulposus argued against age-related loss of notochordal cells in skeletally matured rabbits, previous studies have provided some evidence of degenerative changes in aged rabbit discs. For instance, annulus fibrosus cells from aged rabbits display an increased interleukin-1-related inhibition of proteoglycan synthesis in vitro, while annulus fibrosus and nucleus pulposus in aged rabbits have higher levels of mRNA expression of anabolic cytokines such as bone morphogenetic protein-2 and -7 (BMP-2, 7) and transforming growth factor-beta 1 (TGF-β1), analogous to activation of chondrogenic signals in cartilage. Another study has shown an altered mRNA ratio of ADAM metallopeptidase with thrombospondin type 1 motif, 4 (ADAMTS4) to TIMP metallopeptidase inhibitor 3 (TIMP3) in the aged rabbits, which may associate with change of extracellular matrix metabolism. Thus, like humans, IVDs in rabbits may also undergo progressive degenerative changes with age.

Compared to disc height measurement, MRI has high sensitivity in detecting disc degeneration. In particular, continuous scale of MRI evaluation has been desirable because of its objectivity when compared to conventional discontinuous scales such as Thompson or Pfirrmann scale grading frequently used to diagnose IVD degeneration as well as to investigate the effectiveness of regenerative therapies in animal models. T(1rho)-weighted imaging has recently emerged as a technique to evaluate disc degeneration through quantification of glycosaminoglycans (GAGs). Nonetheless, the technique may require...
modification of hardware together with implementation and optimization of sequence for particular scanners, and its effectiveness on small laboratory animals are yet to be investigated.

Deuterium is an isotope of hydrogen. It has a different magnetic moment from hydrogen and therefore does not contribute to the nuclear magnetic resonance (NMR) signal at the hydrogen resonance frequency. Known water molecule concentration can be produced by mixing water and heavy water (deuterium oxide) and generate specific signal intensity for a calibration of the MRI or NMR analysis, such as in the quantification of water content in bone, cartilage, and muscle. Similar calibration method has not been attempted in the MRI assessment of IVD degeneration in animal models or humans. This study aims to assess signs of age-related degenerative changes in the lumbar IVDs of rabbits by using deuterium oxide-assisted MRI to investigate the relative water content and biochemical status in the discs at different ages.

Materials and methods

MRI IMAGE ACQUISITION

The animal experiments were performed according to the protocols approved by the local health department and institutional ethics committee. Six New Zealand White rabbits across each of the age of 6 months, 1 year, 1.5 year, and 3 year of age were anesthetized by intramuscular injection of ketamine (Alfasan Woerden, Holland). Sagittal T2-weighted images of their lumbar spines were acquired with a Siemens Magnetom Trio scanner (3 T) and knee coil receiver with the following settings: 40-mT/m gradient strength; maximum density out of all disc slices to the calibration curve. The mean density values of the H2O:D2O phantoms were also plotted as in the quantification of water content in bone, cartilage, and muscle. The background subtracted mean density of the standards was measured and plotted against HDi (HDi assigned to the dilution ratios) to establish a calibration curve for each scan.

MEASUREMENT OF H2O:D2O INDEX (HDi)

Look-up table (LUT) was applied to the images and the signal area in the disc was then outlined manually under an 8× zoom-in view in Scion Image (release alpha 4.0.3.2). The background subtracted mean density (d) and the normalized mean density of the water (e) extracted from each disc slice (i) available were measured and multiplied to determine the total pixels (or as the integrated intensity in the program) within the slice. The total integrated density (I) for the disc of interest, which represented the total MRI signal of the disc, was calculated by summation of the pixels across all the available slices of that disc, where

\[ I = \sum_i d \times e \]

The background subtracted mean density of the H2O:D2O phantoms was measured from the center of phantoms and multiplied by the total area measured for the disc of interest to obtain the integrated density of the phantoms, which were then plotted against the HDi (0, 20, 40, 60, 80, 100 HDi) assigned to their corresponding dilution ratios to establish a calibration curve for the disc of interest. Total HDi of the disc of interest was determined by interpolating the total integrated density value of the disc to the calibration curve.

The mean density values of the H2O:D2O phantoms were also plotted against their corresponding HDi to establish another calibration curve. The maximum HDi of the disc of interest was determined by interpolating the maximum density out of all disc slices to the calibration curve.

STATISTICAL ANALYSIS OF VARIANCE

Calibration curve establishment and the total HDi determination of the four disc levels were performed three times from a selected scan of 6-month-old rabbit and the intra-class coefficient was calculated to determine the intra-observer repeatability. Two-tail paired T test assuming unequal variances was independently performed on total HDi or total integrated density values between age groups to calculate P values. Analysis of variance (ANOVA) and Bonferroni’s post-hoc tests were performed at 95% confidence level to test inter-level differences. Variance of the total HDi (Var(HDi)) was determined for the disc levels at all ages to test if the discs underwent abnormal rate of HDi changes during aging. Pearson product-moment correlation coefficients were computed between total HDi and age, or between total integrated density and age, for each disc level.

Results

To obtain higher sensitivity from areas of fluid, STIR was applied in T2-weighted processing in order to remove signal from fat. Signal from fish oil capsule under STIR [Fig. 1(B)] was suppressed preferentially from about 80 to 40% H2O when compared to that of without STIR application [Fig. 1(A)], indicating that fat suppression had been effective.

Examination of the calibration curve established by the normalized mean density of the water–deuterium oxide (H2O:D2O) phantoms from representative scan indicated a monotonic relationship between dilutions and MRI signal (Fig. 2). The relationship is linear in general with slight parabolic deviation in the extreme values (<20% or >80% H2O). Comparative analysis of the calibrations among all scans indicated an existence of ±14 to 32% variation (data not shown).

![Fig. 1. T2-weighted/STIR MRI on deuterium oxide dilutions. H2O:D2O mixture and fish oil capsule were scanned without (A) or with (B) STIR sequence. Signal of fish oil capsule under STIR was suppressed preferentially.](image)

![Fig. 2. Calibration curves from H2O:D2O phantoms. The normalized mean density of the standards was measured and plotted against HDi (HDi assigned to the dilution ratios) to establish a calibration curve for each scan.](image)
To test if disc hydration varied with disc levels and age of animal, sagittal MRI scans of the 6–36 months (0.5–3 years) animals were obtained along with the H$_2$O:D$_2$O dilutions [Fig. 3(A)] and the total HDi values of T12/L1, L1/L2, L3/L4, and L5/L6 IVDs were determined by summation of signals from the image series [Fig. 3(B)].

A comparison of different measurement methods (Fig. 4) was first performed base on computation of L3/L4 signal with (calibrated) or without (non-calibrated) the use of HDi calibration curves, or by normalization to the signal from a single phantom (40% H$_2$O) (semi-calibrated). Normalization of the calibrated and semi-calibrated data to the non-calibrated data was performed for the purpose of comparing the three methods. Measurement using the calibration curves indicated that disc signal decreased continually upon aging. Non-calibrated measurement resulted in an underestimation of signal change relative to the calibrated measurement, while normalization to a single phantom led to an overestimation of signal change.

Analysis of various lumbar levels using the HDi calibration method showed that all lumbar discs had similar HDi at 0.5 year (Fig. 5). T12/L1 level showed relatively lower HDi than the lumbar levels at 0.5 year. From 0.5 to 3 year, all disc levels showed continuous decrease in HDi. T12/L1, L1/L2 and L3/L4 showed similar decrease rate in HDi, while L5/L6 displayed the highest rate of decrement than the other levels. The intra-class coefficient of the HDi computation was 0.83 and the ANOVA $F = 1.52$, implying high level of intra-observer concordance. Similar analysis without the use of calibration curves resulted in trends of mild or even no changes at the same disc levels (Fig. 6).

Statistical analysis indicated that HDi decrement was highly significant at L3/L4 and L5/L6 at and beyond maturity (12 months) (Table Ia). The decrement became significant at all lumbar levels in the aged rabbits (36 months).
Correlation analysis demonstrated a negative relationship between HDI and age (Table Ib). An increasing trend of correlation from cranial to caudal segments was observed, with a maximum of about 57% co-variation in L5/L6. Similar analysis for the non-calibrated measurements resulted in no significant differences for inter-age comparison (Table IIa). Little relationship was observed between the measurements and age in the correlation test, with a maximum of about 7% co-variation (Table IIb). By ANOVA and post-hoc analysis, the inter-level differences were found to be not significant ($P > 0.05$) at all ages using either calibrated or non-calibrated method (data not shown).

Maximum HDI values were also determined to find out any correlation with the HDI decrement. At 6-month-old, maximum HDI was highest in L3/4 (Fig. 7). It was found that the maximum HDI of all discs decreased with age. Caudal levels in general have higher drop rate than cranial levels. Maximum HDI of L3/L4 and L5/L6 dropped at nearly twice the rate of L1/L2 and T12/L1.

To investigate whether the discs underwent abnormal rate of HDI changes, as in the case of disc degenerative disease in human, the Var(HDI) was analyzed based on the assumption that the measurement would have similar variance in all disc levels or age under the same methodology. Interestingly, our analysis showed that the Var(HDI) is not similar (Fig. 6). Var(HDI) was found higher in cranial than caudal levels at 0.5 year. All levels attained a low Var(HDI) at 1 year, which then increased dramatically from 1.5 year onward. Var(HDI) of L5/L6 dropped at 3 year.

**Discussion**

**EFFECTIVENESS OF H$_2$O:D$_2$O CALIBRATION IN DISC STATUS EVALUATION**

MRI is commonly used to evaluate IVD hydration while absolute quantification of water content often requires sophisticated control of instrumental and experimental parameters in MRI. The methodology described in this study is by no means a measurement of water content as in the absolute proton density determination with $^1$H MRI. Rather, it aims to relate the disc signal, which determined by water content and T1/T2 relaxation time of biochemical compositions such as proteoglycans and collagens of the disc, to the H$_2$O:D$_2$O controls. This study has demonstrated that the method can significantly detect as small as 10% difference of signal (mean HDI) of IVDs under a relatively small sample size (Table Ia, 6 vs 12 months in L3/L4). Importantly, the incorporation of H$_2$O:D$_2$O calibration in the MRI scans allows more objective evaluation of disc status as it corrects intra-scanner variation and provides a mean of normalization to substantiate the comparison of measurements from different MRI scans.

Currently there has been no particular model that can parallel the nature of human disc degeneration. Rabbit IVD differs from that of human in terms of structure and cellular content as in other animals. However, disc degeneration can be induced in rabbits in a controllable, reproducible, and cost-effective manner and hence they are relevant in being an experimental model. Use of MRI in evaluation of disc degenerative status has been recently adopted in establishing rabbit or other animal models of induced disc degeneration and a number of disc regeneration studies. These investigations have demonstrated a positive correlation of the T2-weighted MRI signal with the degenerative status of IVD evaluated through histology and disc height measurement. These methods, however, have some limitations such as: (1) analysis based on signal intensity from one disc slice rather than whole disc volume, (2) discontinuous grading scale without signal quantification, (3) use of non-standardized internal control such as signal from neighbor tissue, or...
use of standard of unknown composition, which does not allow signal calibration to compensate for system variation.

$\text{H}_2\text{O}:\text{D}_2\text{O}$ dilutions have been used as calibration phantoms to quantify water content of cortical bone in vitro by NMR spectroscopy$^{14}$, articular cartilage in vivo by $^1\text{H}$ MRI$^{15}$, and skeletal muscle in vivo by MRI$^{15}$. A pilot test of deuterium oxide on calculation of water content of rat IVDs has also been previously reported$^{23}$. Our findings suggest that a combination of $\text{H}_2\text{O}:\text{D}_2\text{O}$ calibration with 3-T MRI has the sensitivity to assess disc degenerative changes in rabbit IVDs.

Water and fat molecules are the major sources of signal in MRI. Fat suppression protocol provides higher contrast between vertebral bodies and the discs as well as allows more accurate determination of relative water content in the disc to evaluate degeneration (although the discs generally have negligible fat content)$^{17,24}$. The STIR protocol has resulted in effective fat signal suppression without compromising the $\text{H}_2\text{O}:\text{D}_2\text{O}$ phantom signals (Fig. 1) and the monotonic relationship between $\text{H}_2\text{O}:\text{D}_2\text{O}$ dilutions and MRI signal (Fig. 2). There is a moderate degree of intra-scanner variation, justifying the need of standards for disc signal normalization.

The comparison of different measurement methods suggests that the use of calibration phantoms enables more precise measurements of T2-weighted disc signal (Fig. 4) and is indispensable in revealing the age-related change in IVDs (Figs. 5 and 6). Although being able to detect the age-related changes, normalization to a single phantom or otherwise an unknown standard is less desirable as it may overestimate such changes (Fig. 4).

### DEGENERATIVE CHANGE IN LUMBAR DISCS OF MATURED AND AGED RABBITS

NZW rabbits have seven, sometimes only six, lumbar vertebrae and normally have a life-span of around 5 years. Our data suggest that the rabbit lumbar discs continually undergo gradual HDi decrement when the animals get mature and aged, with a higher rate at the caudal end compared to the cranial levels (Fig. 5 and Table Ia). There is also an increasing correlation of HDi decrement with age towards the caudal levels (Table Ib). This provides evidence that rabbit lumbar discs undergo age-related degenerative change with a preferentially higher rate at caudal levels. In addition, it indicates that the degenerative change may start as early as during the maturation stage (1-year-old).

Changes in either signal volume (related to disc geometry) or signal density may be accounted for a difference in total HDi. Our data have shown that the change in maximum HDi correlated well with the change in total HDi in the lumbar levels (Fig. 7). This suggests that the degenerative change is associated with an alteration of disc composition instead of signal volume alone. Thus, we propose that age-related progressive changes of extracellular components, leading to the gradual degeneration in rabbit lumbar discs are reflected in a decrease in HDi.

Our data further suggest that the rabbit lumbar discs have various extents of water content and/or biochemical composition at different ages and levels (Fig. 8). The HDi values at cranial levels tend to have a higher variance at adolescence which drops at maturity, followed by an increment of variance beyond 1 year of age. We reason that it may be due to a variation of disc status in the population as a result of different growth rate of the animals. The increase of variance after maturity may on the other hand reflect that the discs have various rates of degenerative changes upon aging.

### CONSIDERATIONS IN USING SUBJECT CONTROLS AND FALSE-NEGATIVITY OF DEGENERATION

Contemporary investigations of rabbit lumbar disc, including disc biology, regeneration, and biomechanics, tend to use multiple disc levels as intra-animal controls. Our study...
suggests that such comparison should be limited to the cranial levels as they have more comparable HDI during growth and aging. Furthermore, the observed HDI variations within the lumbar discs implied that care should be taken to avoid mis-interpretation whenever inter-animal comparison, or pooling data from distanced disc levels, is involved in studies that use matured/aged rabbits as models.

On the other hand, investigations using this methodology shall unlikely associate with the issue of false-negativity of degeneration due to abnormal vascularization or edema in IVD because of the following reasons. Firstly, neo-vascularization has been reported only at the periphery of the degenerated human IVD and we also have never observed vascularization or edema inside human or rabbit IVD. While vasculization can occasionally happen in large Schmorl's nodes along with adjacent edematous bone marrow, it is, however, related to a developmental rather than a degenerative problem. Secondly, blood has a much shorter T2 than water in IVD and the signal from blood is virtually insignificant in T2-weighted imaging at 110 ms TE. Therefore, vascularization, even if it exists, will have negligible effect on the measurement.

Conclusion and perspective

This study has provided the first in vivo evidence of progressive degenerative change in rabbit lumbar discs, indicating rabbits undergo progressive disc degeneration with age. With the use of conventional scanner and acquisition parameters, the deuterium oxide-based calibration can easily supplement and enhance the effectiveness of the protocol by providing relatively robust and sensitive signals to evaluate IVD degeneration. Theoretically, this methodology can be adapted to other animal models or even clinical applications for disc degeneration and regeneration studies. Scanners with a magnetic field above 3 T may be desirable for higher precision measurements in small animals. Method that depletes proteoglycan or GAG of the disc, such as intradiscal injection of chondroitinase ABC, which has been used to induce disc degeneration, may potentially help testing the relationship between the calibrated-MRI signal and GAG content.

Conflict of interest

There is no conflict of interest to disclose.

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References


