

Aquaporin-1 Expression in Herniated Human Lumbar Intervertebral Discs

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Abstract

Study Design: Case series.

Objective: Intervertebral disc (IVD) degeneration is the cause of spondylosis. The pathogenesis is poorly understood, but disc dehydration often plays a role. In this study, we aim to identify and quantify aquaporin-1 (AQP1) in ex vivo human degenerated IVDs obtained intraoperatively and to investigate the relationship between AQP1 levels and magnetic resonance imaging (MRI) T2 intensity of the disc.

Methods: Ex vivo samples of nucleus pulposus (NP) tissue from lumbar IVDs were obtained from 18 consecutive patients who underwent surgery for disc herniation at L4/5 and L5/S1 level. Immunohistochemistry was performed to determine the presence of AQP1 expression, and this was quantified by Western blot analysis. AQP1 expression was compared to preoperative IVD signal intensity on T2-weighted MRI.

Results: NP tissue was obtained from 18 patients (9 for L4/5 level and 9 for L5/S1 level). AQP1 expression was detected in all samples by Western blot and immunohistochemistry. AQP1 expression had a linear correlation with the preoperative IVD signal intensity on T2-weighted MRI at L4/5 level ($R^2 = 0.90$) and at L5/S1 level ($R^2 = 0.92$). AQP1 expression was 52.2 ± 59.0 at L5/S1 level and 15.9 ± 20.6 at L4/5 ($P = .10$).

Conclusions: Our results show that AQP1 can be detected in IVD obtained from live human subjects. Increased AQP1 expression is associated with greater disc hydration as measured by signal intensity on T2-weighted MRI. AQP1 may have a role in the dehydration associated with disc degeneration.

Keywords

aquaporin-1, disc herniation, degenerative disc disease, T2 intensity, MRI

Introduction

Millions of Americans suffer from low back pain (LBP).^{1,2} When considering medical management and lost wages, the overall cost of LBP exceeds \$100 billion per year in the United States.^{1,3-7} Degeneration of the intervertebral disc (IVD) leading to degenerative disc disease (DDD) and disc herniation is thought to be one of the major sources of LBP.^{5,6,8} Dehydration of the IVD contributes to the pathogenesis of DDD.^{3,9} T2-weighted magnetic resonance imaging (MRI) can measure the water content of IVDs and is the primary imaging technique for evaluating disc degeneration.

Previous studies have identified aquaporin-1 (AQP1) in IVD from cadaveric specimens.¹⁰ AQPs as a class of transmembrane protein is responsible largely for water molecule

transport across the membrane. AQPs may play a role in the natural history of disc degeneration and more understanding may offer a potential for novel therapeutic targets. However, there is still no literature demonstrating the existence of AQP1 in ex vivo specimens obtained from live humans.

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The aims of our study were (1) to demonstrate AQP1 expression in human tissue obtained during surgery for DDD and (2) to determine the relationships between AQP1 expression and preoperative T2-weighted MRI findings. We hypothesized that there would be a direct relationship between AQP1 expression and T2 intensity of the IVD. An improved understanding of IVD degeneration at a molecular level could contribute to novel targets for the treatment of DDD.

Methods

Patient Enrollment

With institutional review board (IRB) approval (IRB # 11-002685), a total of 18 consecutive adult patients who underwent surgery on the lumbar spine at L4-5 and L5-S1 for spondylosis were enrolled into this study. Specimens were obtained intraoperatively from University of California, Los Angeles, Medical Center, Santa Monica. Noncontrast MRIs of the lumbar spine were performed preoperatively for all patients.

Tissue Preparation

Within 30 minutes after obtaining the disc sample, the nucleus pulposus (NP) from the herniated IVDs that was removed surgically was isolated and washed 3 times in phosphate-buffered saline to remove erythrocytes and other contaminants that would influence AQP1 quantification. Tissue samples were rotated in a tube of phosphate-buffered saline at 4°C for 5 minutes and repeated a total of 3 times.

Immunohistochemistry

The NP was treated with 4% (v/v) paraformaldehyde overnight. The tissues were then embedded in paraffin blocks. Seven-micrometer sections were cut for immunohistochemistry (IHC). Mouse anti-AQP1 polyclonal antibody was used to confirm AQP1 expression in human IVD and control tissues. Seven-micrometer sections of disc tissue biopsies were incubated for 16 hours at 60°C and subsequently deparaffinized in xylene for 15 minutes. Tissue samples were rehydrated in graded alcohols and incubated in water. Once hydrated, slides were treated with 3% hydrogen peroxide for 10 minutes. After rinsing with water, citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) was used for antigen retrieval. Next, the tissue samples were blocked with normal horse serum for 1 hour. AQP1 antibody was diluted 1:150 in Dako Antibody Diluent with Background Reducing (S080981-2) and incubated for 1 hour at room temperature. Slides were rinsed with TBS-T, and Secondary Probe and Polymer (BioCare Medical Mach 4 Mouse) was used to label AQP1 primary antibody. DAB (BioCare Medical Cardassian DAB) was used to visualize AQP1 and counterstained with Mayer's hematoxylin, dehydrated, and mounted with XAM. The percentage of immunopositive cells in each sample was determined by histological analysis.

Western Blot

Western blot analysis also utilized anti-AQP1 polyclonal antibody. NP tissue was homogenized in radioimmunoprecipitation assay buffer. Protein concentration of each lysate was determined using a bovine serum albumin protein concentration kit. One milligram of each lysate was run on a sodium dodecyl sulfate polyacrylamide gel electrophoresis gel by electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked by 5% nonfat dry milk for 3 hours. The membrane was incubated with antibodies for AQP1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) overnight at 4°C. The membrane was then incubated in a horse-radish peroxidase-tagged secondary antibody, and the AQP1 and GAPDH bands were visualized by a chemiluminescent substrate onto an X-ray film. The resulting X-ray film was scanned and the AQP1 and GAPDH bands were quantified using ImageJ (National Institutes of Health [NIH] freeware). Normalized AQP1 expression values were determined using ImageJ to quantify the intensity of the AQP1 and GAPDH bands on the X-ray film. AQP1 band intensities were divided by GAPDH band intensities to obtain normalized values.

MRI Analysis

Preoperative lumbar spine MRIs of all 18 patients were reviewed by the senior author (DCL) and an additional observer. MRI scans were obtained on a Siemens MAGNETOM Avanto 1.5 tesla scanner using a field of view of 200 cm (Siemens Healthcare, Erlangen, Germany). T2 sequences were obtained using an echo time (TE) of 113 ms and a repetition time (TR) of 5330 ms. NIH MRI analysis software (NIH IMAGE; <http://rsb.info.nih.gov/nih-image/about.html>) was used to measure the T2 intensity of each patient's diseased IVD NP that was obtained intraoperatively. All measurements were performed in the sagittal plane. The value was then compared to the T2 signal intensity of water/cerebrospinal fluid to achieve a normalized T2 intensity index for each IVD. Neither observer had access to the laboratory data while making their measurements. The intraclass correlation coefficient was used to assess the degree of agreement between the senior author and the additional observer's measurements.

Data Analysis

Standard statistical analyses were performed for both L4-5 disc group and L5-S1 disc group. These include means and standard deviations. For each group separately, normalized AQP1 expression was plotted as the dependent variable and linear regression line was calculated with normalized T2 MRI intensity as an independent variable. The coefficient of determination (R^2) was calculated for each regression. Student *t* test was performed to compare AQP1 expression between the L4-5 group and the L5-S1 group. *P* value was calculated and reported.

Table 1. Demographic Information, Normalized AQP1 Expression, Normalized T2 Intensity for Patients With L4/5 IVDs.

Patient	Gender	Age (Years)	IVD Tissue	Normalized AQP1 Expression	Normalized T2-Weighted MRI Values	Surgery
1	Female	24	L4-L5	2.9	10	Discectomy
2	Female	38	L4-L5	9.4	15	Discectomy
3	Female	40	L4-L5	7.5	14	Discectomy
4	Female	30	L4-L5	0.3	10	Discectomy
5	Male	38	L4-L5	13.7	5	Discectomy
6	Male	65	L4-L5	5.2	20	Discectomy
7	Male	48	L4-L5	1.2	10	Discectomy
8	Male	38	L4-L5	55.0	80	Discectomy
9	Female	47	L4-L5	47.6	55	Discectomy

Abbreviations: AQP1, aquaporin-1; IVD, intervertebral disc; MRI, magnetic resonance imaging.

Table 2. Demographic Information, Normalized AQP1 Expression, Normalized T2 Intensity for Patients With L5/S1 IVDs.

Patient	Gender	Age (Years)	IVD Tissue	Normalized AQP1 Expression	Normalized T2-Weighted MRI Values	Surgery
1	Male	43	L5-S1	2.3	8	Discectomy
2	Female	40	L5-S1	42.7	40	Discectomy
3	Female	39	L5-S1	1.3	10	Fusion
4	Male	64	L5-S1	110.3	48	Fusion
5	Male	34	L5-S1	6.0	4	Discectomy
6	Male	68	L5-S1	36.1	20	Fusion
7	Female	52	L5-S1	118.1	50	Discectomy
8	Male	26	L5-S1	151.7	60	Discectomy
9	Female	51	L5-S1	1.0	10	Discectomy

Abbreviations: AQP1, aquaporin-1; IVD, intervertebral disc; MRI, magnetic resonance imaging.

Results

A total of 18 patients (9 females and 9 males) were enrolled into this study, and specimens were successfully obtained from all patients (Tables 1 and 2). There were 9 patients each in the L4-5 disc group and the L5-S1 disc group. The mean age for the L4-5 group was 40.9 ± 11.7 years and for L5-S1 group was 46.3 ± 13.7 years. Fifteen patients underwent minimally invasive decompressive surgery without fusion consisting of hemilaminotomy and medial facetectomy with removal of herniated disc. Three patients underwent fusion procedures; 2 patients had minimally invasive transforaminal lumbar interbody fusion, and 1 patient had anterior lumbar interbody fusion.

IVD samples were obtained from all 18 patients. Quantification of AQP1 Western blot bands and normalization to GAPDH expression yielded the normalized AQP1 expression values shown in Tables 1 and 2. Figures 1 and 2 demonstrate the Western blot analyses for the L4-5 and L5-S1 groups, respectively. The mean normalized quantity of AQP1 expression for L4-5 IVD was 15.9 ± 20.6 , and for L5-S1 IVD it was 52.2 ± 59.0 . IHC was used to visualize AQP1 expression by NP cells within IVD and to confirm Western blot data (Figure 3). The presence of positive AQP1 staining by IHC matched the Western blot results of the corresponding samples. Quantification of the NP intensity on preoperative T2-weighted MRI revealed a mean normalized T2 intensity for L4-5 IVD to be 24.3 ± 25.6 and for L5-S1 IVD to be 27.8 ± 21.6 . For both L4-5 ($R^2 = 0.90$) and L5-S1 ($R^2 = 0.92$) IVD groups, there

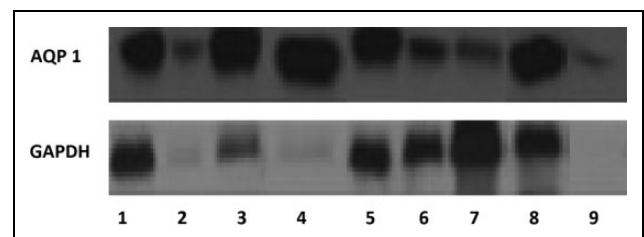


Figure 1. Western blot analysis of aquaporin-1 and GAPDH expression in whole tissue lysate of L4-5 nucleus pulposus from 9 patients.

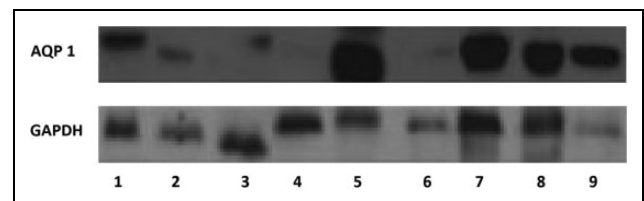


Figure 2. Western blot analysis of aquaporin-1 and GAPDH expression in whole tissue lysate of L5-S1 nucleus pulposus from 9 patients.

appeared to be a direct linear relationship between AQP1 expression and normalized T2 intensity signals (Figures 4 and 5). The intraclass correlation coefficient for the senior author and additional observer's T2 intensity measurements was 0.98.

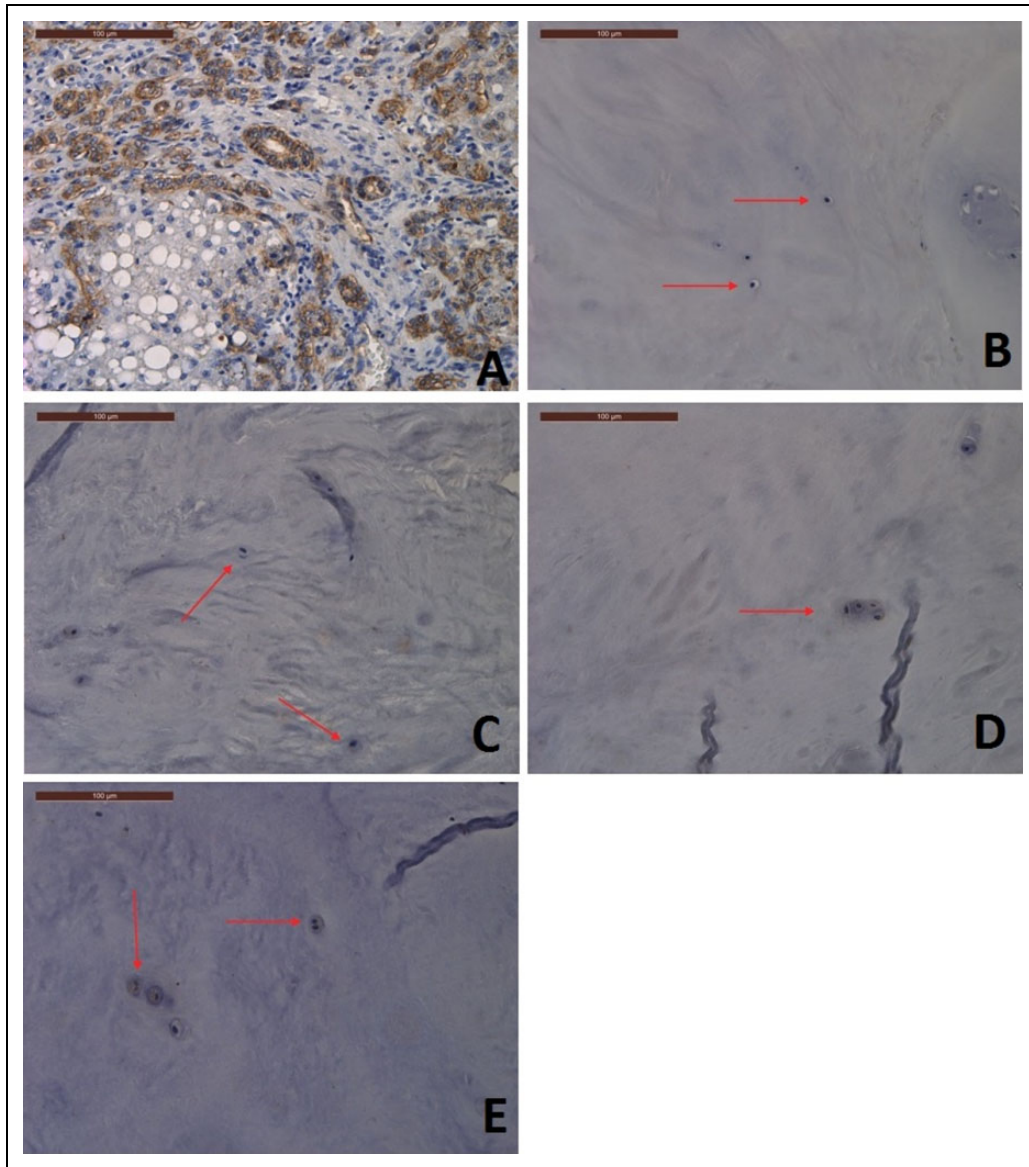


Figure 3. Immunohistochemical localization of aquaporin-1 in human liver control (a) and nucleus pulposus (b-e). Samples from L4-5 IVD patient # 4 (b) and L5-S1 IVD patient # 3 (c) show little to no AQP1 localization and samples from patients 12 (d) and 16 (e) show high AQP1 expression (stain: Mayer's hematoxylin, magnification: 20 \times).

Discussion

The IVD consists of 3 morphologically unique regions with specialized extracellular matrices (ECM) that promote different mechanical and physical properties.^{9,10} The NP is a gelatinous region centrally located in the IVD that consists of a disorganized meshwork of proteoglycans and collagens.^{10,11} The ECM of the NP can consist of around 80% water and chondrocyte-like cells residing in the NP maintain homeostasis. The IVD is subject to daily dynamic compressive loads causing constant fluid flow, which can affect cell volume. The control of cell volume is achieved by altering the cytoskeleton and regulating active plasma membrane transport mechanisms that are not well characterized.¹²

Membrane transport in the IVD may be affected by aquaporins, a family of bidirectional water-permeable transmembrane channel proteins.^{10,13,14} Most aquaporins transport water molecules (AQP0, 1, 2, 4, 5, 6, and 8), while some can also transport small molecules such as glycerol and urea (AQP3, 7, and 9).¹⁵⁻¹⁷ The analysis of aquaporin expression patterns in multiple tissues indicates that aquaporins play a vital role in the water transport system, specifically where rapid water movement for reabsorption and secretion is critical (kidney and lungs).^{13,14} A study by Richardson et al of postmortem IVDs without degeneration showed that chondrocyte-like cells in the NP express AQP1 and AQP3.¹⁰ Trujillo et al demonstrated upregulation of AQP1 in articular chondrocytes and

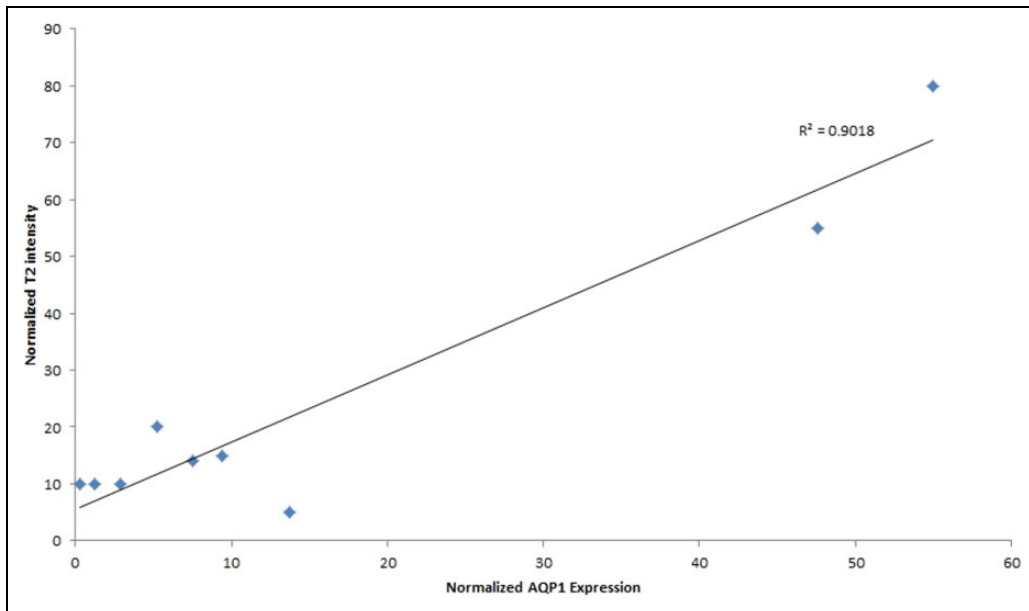


Figure 4. Normalized aquaporin-I expression and normalized T2-weighted MRI intensity from 9 patients in the L4-5 IVD group showed a linear positive correlation ($R^2 = 0.90$).

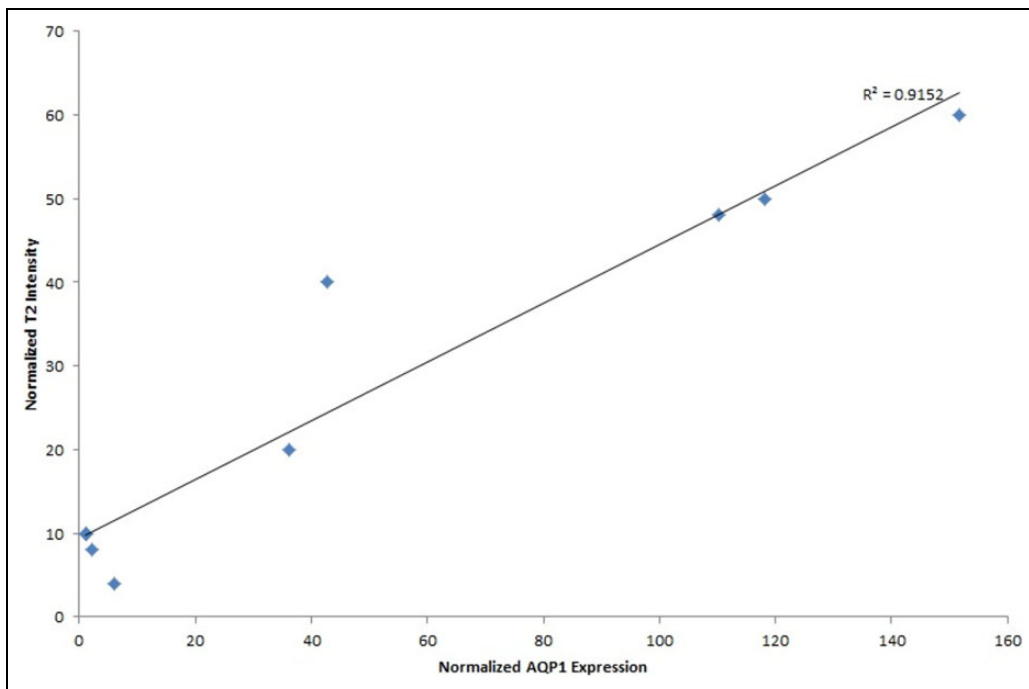


Figure 5. Normalized AQP1 expression and normalized T2-weighted MRI intensity from 9 patients in the L5-S1 IVD group showed a linear positive correlation ($R^2 = 0.92$).

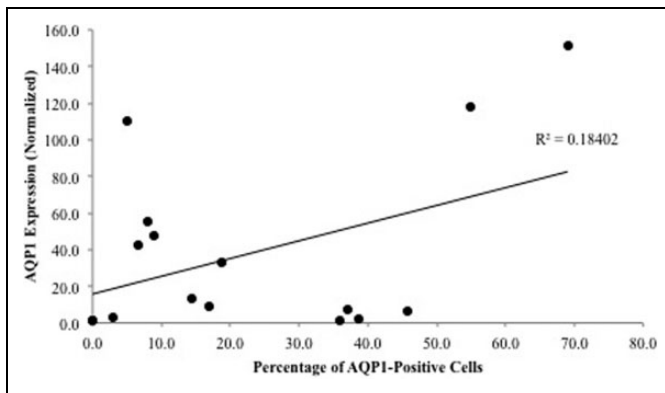
synoviocytes affected by rheumatoid arthritis,¹⁸ and Musumeci et al showed strong AQP1 expression in fibrochondrocytes of experimentally induced osteoarthritic knee menisci.¹³ Additionally, AQP1 has been shown to be upregulated in human synovitis and suggested to contribute to joint swelling and edema.¹⁹ However, AQP expression in the lumbar spine has not been evaluated in patients with DDD or compared to radiographic findings.

In addition, although loss of water in IVDs has been associated with disc degeneration,^{4,10} the pathogenesis is poorly understood. Previous studies have suggested the importance of water transport across the cell membrane in resident chondrocyte-like cells for volume regulation in response to mechanical stimuli and changes in osmolarity.¹⁰ Richardson et al have shown through IHC that AQP1 is expressed in the chondrocyte-like cells in the NP of IVDs in human cadavers.¹⁰

Table 3. Statistical Comparisons Between L4/5 IVD Group and L5/S1 IVD Group. Nonpaired Student *t* Tests Were Performed.

	L4/5 IVD	L5/S1 IVD	<i>P</i>
Age (years)	40.9 ± 11.7	46.3 ± 13.7	.38
Normalized AQP1 expression	15.9 ± 20.6	52.2 ± 59.0	.10
Normalized T2 intensity	24.3 ± 25.6	27.8 ± 21.6	.76

Abbreviations: AQP1, aquaporin-1; IVD, intervertebral disc; MRI, magnetic resonance imaging.

**Figure 6.** Quantity of aquaporin-1 expression on Western blot analysis did not correlate with percentage of aquaporin-1 immunopositive cells seen on immunohistochemistry ($R^2 = 0.184$).

Here, we have been able to confirm AQP1 expression by the chondrocyte-like cells of fresh NP by IHC and Western blot with samples obtained *ex vivo*. Quantitative measurement of AQP1 content in NP cells by Western blot could indicate a role of the transport molecule in the pathogenesis of disc degeneration. We focused our study in patients with L4-5 and L5-S1 disc herniation and spondylosis because these 2 segments are the most common sites of disc degeneration in adults.^{20,21}

We found that AQP1 expression in the NP varied among disc samples. L5-S1 discs had a higher average AQP1 expression than the L4-5 IVDs ($P = .10$), which approached statistical significance (Table 3). Our results support a direct linear correlation between AQP1 expression and T2 intensity of the disc on MRI scan. This relationship was observed in both L4-5 ($R^2 = 0.90$) and L5-S1 ($R^2 = 0.92$) groups. The increase in AQP1 expression likely leads to increased water transport into the cells and hence increased T2 intensity on MRI scan. Therefore, our results suggest that decreased AQP1 expression within cells located in the NP is associated with decreased water content in degenerated discs. Since there was no correlation observed between percentage of cells immunopositive for AQP1 and quantity of AQP1 expression on Western blot (Figure 6), the lower AQP1 expression in L4-5 is most likely not due to cell loss but rather downregulation. Identifying a potential promoter or regulator involved in AQP1 downregulation would aid in the understanding of the pathogenesis of disc degeneration. Decreases in NP AQP1 content have been associated with increasing age in rat discs^{22,23} and inflammation in bovine

discs.²³ Thus, disc degeneration may represent an inability of the NP to upregulate AQP1 expression and thereby increase the water content of the NP for protection against biomechanical forces. Indeed, dehydration of the IVD is observed in aging and degeneration.^{24,25}

Pearce et al reported no correlation between water content and NP signal intensity on T2-weighted MRI,²⁶ but others have described significant correlations between water content and T2 relaxation times.^{27,28} There are likely additional factors governing NP hydration, including the expression of other aquaporins such as AQP3 that Richardson et al identified in postmortem NP tissue.¹⁰

Our preliminary study is limited by the small sample size and lack of nondegenerated tissue controls to compare with pathological AQP1 expression. Additionally, disc hydration is dynamic and can be affected by recent loading as well as the disc's position in relation to the patient's line of gravity. We did not control for the time of day that each MRI was obtained. Since the patients in our cohort had chronic LBP averaging over 1 year in duration, we do not believe their activity was likely to change significantly prior to the surgery or imaging. Only 3 patients had straightening of their lumbar lordosis and none had major listhesis at the level of their surgery. One technical issue was the variability in the amount of GAPDH present across the samples in the Western blot. The extent of disc degeneration causes the cellular density within the NP to vary from patient to patient. Therefore, loading an equal amount of total protein from each sample's protein lysate gave variable levels of GAPDH. A common technique utilized in many studies is real-time polymerase chain reaction (RT-PCR), which allows RNA to be used as a normalizing factor. However, RNA extraction from NP samples yielded insufficient RNA samples that were also too impure to use for RT-PCR. With these obstacles identified, we utilized a semiquantitative approach to examine AQP1 expression by Western blot and quantifying AQP1 expression as a ratio to GAPDH expression for each sample.

The study by Richardson et al showed that the chondrocyte-like cells of the NP from cadavers without disc pathology expressed AQP1 and AQP3.¹⁰ AQP3 is part of the aquaporin family of proteins and actively transports glucose as well as water molecules. Initially, we sought to examine both AQP1 and AQP3 expression in pathological NP samples. However, the lack of AQP3 antibodies that could target human AQP3 in a Western blot forced us to pursue AQP1 alone. The use of AQP3 antibodies that were not optimized for Western blot produced blots that could not be used for our semiquantitative approach of protein expression.

To our knowledge, this is the first study to demonstrate AQP1 expression in human IVDs that were obtained *ex vivo* from live human subjects. Since AQP1 expression was associated with water content, a deficiency of AQP1 may contribute to the pathogenesis of DDD. This deficiency could be due to changes in the protein leading to degradation. Alternatively, an inability to sense or respond to compression-induced ionic and osmotic stimuli that normally direct change in cell volume

could result in AQP1 underexpression. While AQP1 upregulation is associated with the joint edema of rheumatoid arthritis and osteoarthritis,^{18,19,29} it may be beneficial in the IVD where hydration is necessary to withstand static and dynamic compressive loads.

Conclusion

In conclusion, our pilot study demonstrates that AQP1 exists in herniated IVDs from ex vivo specimens taken from live humans. Furthermore, AQP1 expression might be lower in L4-5 IVDs than L5-S1 IVDs. There appears to be a linear direct correlation between AQP1 expression and T2 intensity of IVDs for both L4-5 and L5-S1 discs. We theorize that decreased AQP1 expression leads to decreased water content in the IVDs, which results in degeneration.

Again, we recognize that this is an early pilot study with numerous limitations. Further research utilizing healthy discs as controls and larger sample sizes will expand our understanding of DDD and potentially lead to novel therapeutic methods.

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Declaration of Conflicting Interests

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