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Comparison of three methods for nucleus pulposus volume measurement in rabbit lumbar spines: a preclinical model for measurement of the effectiveness of prophylactic intervertebral disk fenestration in dogs

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OBJECTIVE
Compare 3 methods of nucleus pulposus (NP) volume measurement using the rabbit lumbar spines as a preclinical model to determine the effectiveness of prophylactic intervertebral disk fenestration in dogs.

ANIMALS
Twelve 9-month-old, skeletally mature female entire New Zealand White rabbits weighing between 3.5 to 4.5 kg.

METHODS
NP volume measurements of dissected rabbit lumber spines between L1 and L6 were made and compared using gross measurements, reconstructed MRI images, and water volumetry based on Archimedes' principle. Water volumetry was used as the true gold standard volume measurement in this study.

RESULTS
The true volume (mean ± SD) of the nucleus pulposus NP as measured by water volumetry increased caudally from L1/L2 (16.26 ± 3.32 mm^3) to L5/L6 (22.73 ± 6.09 mm^3). Volume estimates made by MRI were significantly higher than those made using water volumetry at all sites (L1/L2 [$P = .044$], L2/L3 [$P = .012$], L3/L4 [$P = .015$], L4/L5 [$P < .001$], and L5/L6 [$P < .001$]). Gross measurements also significantly overestimated volume when compared to water volumetry at all sites; L1/L2 ($P = .021$), L2/L3 ($P = .025$), L3/L4 ($P = .001$), L4/L5 ($P < .001$), and L5/L6 ($P < .001$). MRI and gross volume estimates were significantly different at L4/L5 ($P = .035$) and L5/L6 ($P = .030$).

CLINICAL RELEVANCE
The findings of this preclinical model might be relevant to veterinary surgeons who perform prophylactic fenestration for which there is no reliable method to determine the amount of NP to be removed. Preclinical ex vivo and in vivo fenestration studies with pre- and postoperative NP volume assessment are required.

Keywords: intervertebral disk, fenestration, nucleus pulposus, volume, intervertebral disk disease

Intervertebral disk disease (IVDD) is the most common spinal disease that results in neurological dysfunction in dogs.[1,2] Hansen Type 1 IVDD, common in chondrodystrophic dog breeds, is characterized by chondroid metaplasia of the nucleus pulposus (NP). Extrusion of NP into the vertebral canal causes compression and/or contusion of the spinal cord and subsequent neurological dysfunction.[3,4] Fenestration is a surgical technique that involves the complete mechanical removal of NP by making a window in the annulus fibrosus and is described as a prophylactic procedure to reduce reoccurrence of IVD extrusion (IVDE).[3,5]

Multiple veterinary studies strongly support the prophylactic effect of fenestration in dogs with previous episodes of symptomatic IVDE.[3,6,7] Published information suggests reoccurrence rates of 0% to 24.4% with prophylactic disk fenestration[8–13] and 2.67% to 41.7% without fenestration.[10,12,14–17] With around 90% of IVDEs predictably occurring between T13 and L3,[18] prophylactic fenestration can be targeted to those disks most likely to cause problems in the future.[19] Despite the reported benefits of fenestration, the

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effectiveness of the procedure is determined by the amount of NP removed,[19] which can be variable and is dependent on the surgical approach, technique used, and surgeon experience.[3,20,21] Further, it has been shown that the effectiveness of disk fenestration is governed by the amount of nucleus pulposus removed.[22] Finally, it has been reported that reoccurrences of extrusion at a previously fenestrated disk space may be related to incomplete removal of disk material from the disk space at the time of fenestration.[12] Therefore, the development of a method to reliably estimate the in situ volume of NP in clinical patients using advanced imaging such as MRI would be useful for veterinary surgeons to assess the effectiveness of fenestration in terms of the amount of NP removed and also to calculate the amount of extruded material within the spinal canal.

The rabbit lumbar spine was chosen as a preclinical model and proof of concept in this study for NP volume measurement in the in vivo clinical setting for canine patients. The objective of this study was to compare NP volume measurement from rabbit lumbar IVDs using 3 different methods: (1) MRI scans, (2) gross dimensions of the NP space, and (3) water volumetry of the dissected NP. We hypothesized that there would be no significant difference in NP volume measurements between the 3 methods.

Methods

Sample collection

Lumbar spines were harvested from 9-month-old, skeletally mature female entire New Zealand White Rabbits (n = 12), weighing 3.5 to 4.5 kg, that were euthanized as part of other ethically approved studies conducted by a single institution. Skeletal maturity was confirmed before animal recruitment by a radiographic screening of physes; commonplace at our institution. Lumbar spines were dissected en bloc from the thoracolumbar junction to the sacrum, ensuring the L1/L2 through to L5/L6 (inclusive) were included in each dissected specimen. Only L1/L2 through to L5/L6 were used for analysis given New Zealand White rabbits have variable numbers of lumbar vertebrae.[23] Lumbar IVDs were chosen for this model given they are a common site for IVDE in dogs clinically and our familiarity with rabbit lumbar spine anatomy at our institution.

MRI volume measurement region of interest (ROI) calculation

Immediately after humane euthanasia, lumbar spines were harvested and scanned by a 3T MRI scanner (Philips Ingenia CX, Philips Healthcare) using a 16-channel transmit-receive (T-R) knee coil. T2-weighted modified Dixon turbo spin echo (TSE) sequences were acquired in the sagittal plane with an anterior-posterior phase direction and right-to-left slice direction. Slices were acquired at 0.6 X 0.6 X 1.5 mm voxels, reconstructed to 0.2 to 0.25 mm. Scan time was 5 minutes 37 seconds, TR 2852 ms, TE 95 ms. Images were reviewed in third-party viewing software (Horos, Nimble Co LLC d/b/a Purview) by a single experienced observer to allow estimation of the NP volume for disk spaces L1/2 through to L5/6 inclusive. The volume of each NP was calculated using the closed polygon region of interest (ROI) tool, where all slices in the sagittal plane were measured, followed by the calculation of volume **(Figure 1)**.

Gross measurement of volume

After MRI scanning, L1/2 through to L5/6 disk spaces were dissected from the lumbar spine specimens. Each disk space was transected transversely to expose the cranial **(Figure 2)** and caudal endplates (Figure 2). The gross volume of the NP was calculated by multiplying the length, width, and height of the space occupied by the NP. All measurements were made using digital calipers and measured in millimeters (mm). Due to the ovoid shape of the cavity, 3 measurements for height were taken across the space and averaged (Figure 2). The craniocaudal length of the NP space was measured at the most lateral aspect of the IVD as the distance between the 2 adjacent vertebral bodies. Volume (mm^3) was then calculated for each NP cavity by multiplying length, width, and height.

Water volumetry measurement

After each NP space was exposed and gross measurements taken, each NP was completely dissected out of the cavity with fine dissecting equipment. The suspension technique was then used to determine the volume of each specimen. This was done by Archimedes principle and a variation of the hydrostatic weighing technique, whereby measuring

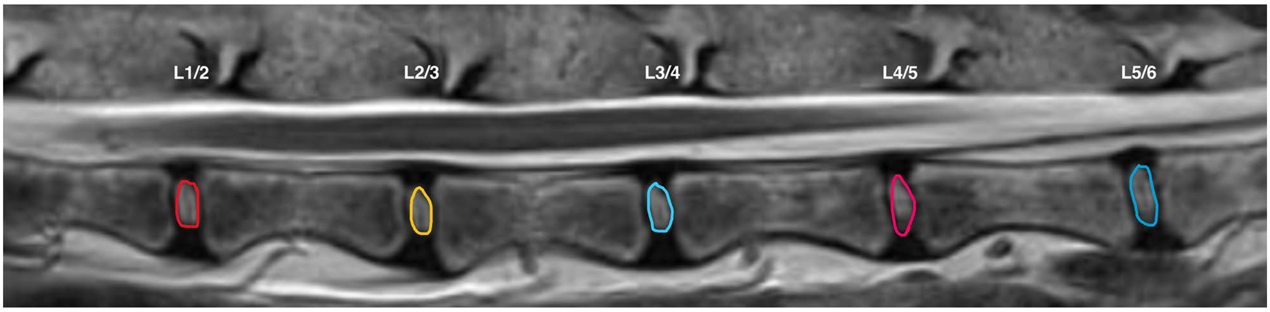

Figure 1—Representative T2 sagittal MRI sequences of L1/2, L2/3, L3/4, L4/5, and L5/6 intervertebral disk spaces in a rabbit. The nucleus pulposus for each disk space has been outlined using the region of interest (ROI) tool to calculate volume.

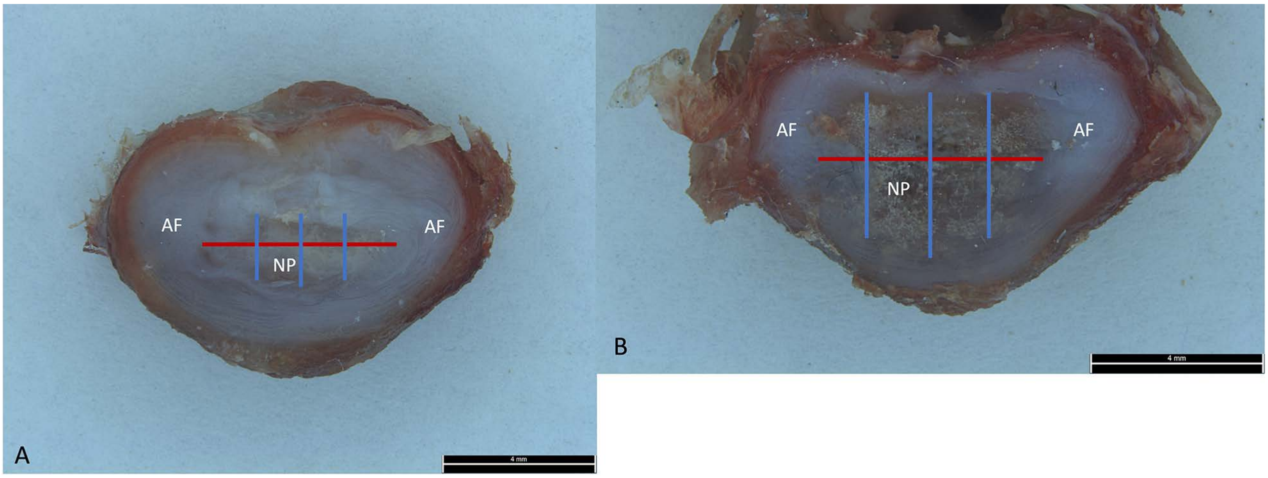

Figure 2—Cranial (a) and caudal (b) endplates of L1/2 sectioned transversely through the disk space. The red line represents the width of the NP space. The height was averaged across 3 measurements (blue line). AF = annulus fibrosus; NP = nucleus pulposus.

the weight of an object in 2 mediums of known density, the density, and hence the volume of the object can be determined. All measurements were made using a Mettler Toledo precision electronic microbalance **(Figure 3)**.

Figure 3—Setup for suspension method of water volumetric analysis using Mettler Toledo microbalance. Sample pictured is a dissected nucleus pulposus suspended in distilled water on hydrostatic weighing scale apparatus. The weight of the nucleus pulposus is displayed in grams.

The density (ρ) of an object can be calculated using the mass of the object in air (m_a) and distilled water (m_w) as well as the known density of the distilled water ρ_w (0.99997g/cm^3), and air ρ_a (0.0012g/cm^3), using the following equation.

$$\rho = \frac{m_a}{m_a - m_w}(\rho_w - \rho_a) + \rho_a$$

The volume V of an object can then be calculated using its mass and density by the following equation.

$$V = \frac{m_a}{\rho}$$

Statistical analysis

Descriptive data is presented as mean ± SD unless otherwise stated. Statistical differences between mean volumes of each of the 3 measurement methods for each IVD were evaluated by a repeated measured 1-way ANOVA and Tukey post hoc test. Statistical analysis was performed using SPSS for Windows (SPSS). For all analyses, statistical significance was set at $P < .05$.

Results

The calculated NP volumes are presented for each IVD for the 3 measurement methods **(Table 1)**. For all 3 measurement methods, NP volume increased for disk spaces moving from cranial to caudal. NP volume using gross measurements of the NP space and the ROI tool from MRI scans were significantly higher than the water volumetry technique for all disk spaces in all samples (L1/2 through to L5/6). Gross measurement estimates provided the highest values at each site. Volume estimates made by MRI were significantly higher than those made using water volumetry at all sites; L1/L2 ($P = .044$) L2/L3 ($P = .012$), L3/L4 ($P = .015$), L4/L5 ($P < .001$), and L5/L6 ($P < .001$). Gross measurements significantly

Table 1—Nucleus pulposus (NP) volume measurements for intervertebral disk (IVD) spaces of rabbit lumbar spines using gross measurement, MRI, and water volumetry techniques. Volumes are presented as mean ± SD in mm^3.

IVD space	Measurement technique	NP volume (mm^3)
L1/L2	Gross	21.18 ± 6.01[a]
	MRI	19.7 ± 4.48[a]
	Water volumetry	16.26 ± 3.32
L2/L3	Gross	22.92 ± 6.01[a]
	MRI	21.4 ± 2.84[a]
	Water volumetry	18.42 ± 2.46
L3/L4	Gross	29.28 ± 5.19[a]
	MRI	25.73 ± 3.67[a]
	Water volumetry	21.02 ± 4.95
L4/L5	Gross	37.96 ± 9.83[ab]
	MRI	30.77 ± 5.71[a]
	Water volumetry	22.32 ± 4.32
L5/L6	Gross	41.64 ± 11.02[ab]
	MRI	33.5 ± 5.01[a]
	Water volumetry	22.73 ± 6.09

[a]Statistically significant compared to water volumetry ($P < .05$). [b]Statistically significant compared to MRI ($P < .05$).

overestimated volume when compared to water volumetry at all sites; L1/L2 ($P = .021$), L2/L3 ($P = .025$), L3/L4 ($P = .001$), L4/L5 ($P < .001$), and L5/L6 ($P < .001$). MRI and gross volume estimates were significantly different at L4/L5 ($P = .035$) and L5/L6 ($P = .030$). NP volume using gross measurements of the NP space were significantly higher than MRI measurements for the L4/5 and L5/6 disk spaces. There was no significant difference in volume measured by MRI and gross measurements for the L1/2, L2/3, and L3/4 disk space.

Discussion

This study compared NP volume measurement from rabbit lumbar IVDs using 3 different methods as a preclinical model for the in vivo canine setting. We hypothesized that NP volume measurements by MRI, gross dimensions of the NP space, and water volumetry of the dissected NP, would not be significantly different. In this model, MRI and gross dimensions estimates were greater than the water volumetry method for all disk spaces between L1/2 and L5/6 inclusive. Therefore, we rejected the null hypothesis and accepted the alternate hypothesis. The results of this study are useful as a proof-of-concept study and are relevant to the in vivo clinical setting where MRI might be used to estimate in situ NP volume in dogs and, therefore, to assess the efficacy of surgical removal of NP via fenestration and potentially the volume of extruded material into the spinal canal.

Multiple studies advocate for prophylactic fenestration in at-risk breeds to prevent future occurrence of IVDE; however, it is not clear how much NP needs to be removed to prevent extrusion.[21,24] Shores et al (1985) reported that the effectiveness of disk fenestration is determined by the amount of nucleus pulposus removed. Knapp et al (1990) speculated that IVDE at previously fenestrated disk spaces is likely related to the incomplete evacuation of NP at the time of fenestration. Therefore, there is a clinical need for a repeatable method of NP volume estimation before and after prophylactic fenestration to determine efficacy and guide the likelihood of future extrusion at that disk space. Advanced diagnostic imaging modalities such as MRI would seem the most likely means to achieve this in the in vivo setting, hence the need for evaluation in this preclinical study.

MRI is considered the gold standard imaging modality for the spine in human and veterinary medicine. MRI allows for differentiation between the NP and annulus fibrosus soft tissue structures of the disk.[2,3,25] On T2-weighted MRI images of the spine, the native IVD exhibits high signal intensity in the inner water- and proteoglycan-rich NP, with low signal intensity in the fibrocartilaginous annulus fibrosus. In this study, rabbit lumbar NP volume was consistently over-estimated on MRI compared to the water volumetry method. Despite this, MRI estimation for NP volume is obviously the only possible method in the in vivo setting and was more accurate than the gross measurements technique in this study. As long as the method for volume estimation using MRI and associated software tools is repeatable, then a comparison of volume assessment pre- and postfenestration would be valuable to the clinician, with the knowledge that the actual number may be an overestimation of the true volume.

The findings of this study suggest that gross measurements provide the least accurate estimate of NP volume, and MRI, as the most theoretically accurate imaging tool of soft tissue volume calculation, is indeed an estimate in the rabbit. This is likely applicable to the dog's lumbar spine. MRI scans can distinguish between soft tissue structures based on scanning protocol. In extruded degenerative disks the NP is distinguishable as it has lower water content and altered proteoglycan composition resulting in a lower signal intensity on T2-weighted scans.[2,26] In healthy nondegenerative disks, however, studies have shown both the NP and inner part of the annulus fibrosus to be hyperintense, due to their higher water component.[27] This could support the finding in this study which used healthy spine samples, demonstrating it may be difficult to obtain completely accurate volume estimates when differentiating between tissue borders on MRI reconstructions. Similarly, in acutely herniated nondegenerative disks due to trauma, differentiating between NP and annulus fibrosus may be difficult.

While this study used MRI as the diagnostic imaging modality of choice, computed tomography (CT) has also been used to estimate NP volume and volume of extruded material in clinically affected dogs. CT scans are commonly used to diagnose IVD extrusion, most useful for mineralized extruded disk material within the spinal canal. CT is unable to differentiate between the soft tissue structures of the IVD. Despite this, some studies have used CT to report on the amount of extruded material into the canal in dogs presenting with thoracolumbar T3-L3 myelopathies. A recent study by Sakaguchi et al (2023) reported that the volume of extruded materials is correlated with neurologic severity in dogs with type I IVDE. In that study, the authors used the ROI tool to estimate the volume of extruded material in the canal relative to the nonextruded adjacent disk space and reported the affected disk had a greater volume than the native disk. This method is unreliable as the IVD and extruded material were counted twice and considered "one disk" and then compared to the immediate adjacent disks, as CT could not differentiate the NP from the annulus for volume measurement. Further, when extrusion occurs it is seldom the annulus fibrosus that extrudes but rather the degenerate, calcified NP. Improved accessibility to MRI in modern-day veterinary medicine means that this imaging modality would be useful and more widely available to clinicians for the estimation of NP volume, although the time taken to complete postoperative scanning is a logistical challenge that should be considered.

The severity of spinal cord injury is multifactorial, with the rate of extrusion, degree of contusive injury suffered, and the duration of spinal cord compression 3 of the major contributors to neurological dysfunction.[28] Multiple studies have reported that the maximal transverse spinal cord compression ratio is not correlated with neurologic severity for dogs with type I thoracolumbar IVDE.[29–31] The most recent of these studies by Sakaguchi et al (2023) investigated if the volume of compression might affect neurological severity. The authors concluded that the volume of extruded material is at least partly responsible for the presenting neurologic severity in dogs with type I thoracolumbar IVDE. Residual disk material is also not uncommonly found postoperatively in the spinal canal after decompressive surgery.[19] Aside from evaluating the effectiveness of fenestration, this proof-of-concept study may also be useful for estimation of the amount of extruded material within the vertebral canal causing spinal cord compression in dogs with thoracolumbar IVDE. This would be of benefit to veterinary clinicians for the estimation of compression at the affected site of extrusion which may assist with case management.

Water volumetry based on Archimedes' principle was considered the gold standard reference for NP volume measurement in this study and likely reflects the "true volume" of the NP. Archimedes' principle states that the volume of any object submerged in a fluid is equal to the volume of the fluid it displaces.[32] Any submerged object experiences an upward-acting buoyancy force equal to the weight of the fluid displaced causing the phenomenon.[32,33] Water volumetry is commonly used to calculate the true volume of irregularly shaped objects and validate the accuracy and reliability of advanced imaging modalities.[32,34–37] In human medicine, water displacement is often used when determining subtle, early volumetric changes to oedematous peripheral limbs.[38,39] Many studies have also used water volumetry to validate the accuracy and reliability of 3D imaging modalities in volumetric assessment of various tissue types including thyroid glands,[40] liver,[37] bones,[36] and dissected soft tissue.[34] Water displacement becomes challenging when the volume of the object measured is so small,[41] such as the rabbit NP. True volume measurements in this study were taken using the suspension technique, a variation of the hydrostatic weighing technique that incorporates Archimedes' principle of water displacement and buoyancy forces to provide a more accurate and precise volume measurement.[41] The technique states that the difference in weights of an object measured in 2 mediums of known density allows the observer to calculate the density of the object and the volume.[41] Our model shows even current gold-standard imaging techniques may not provide us with the actual volume of the NP. However, water volumetry has little clinical practicality as even if NP material is removed and measured, there is no reference point preoperatively for comparison. In the clinical setting, MRI will still remain the most practical option when comparing pre- and postoperative volume measurements. Although MRI gives an estimate, provided the measurement method is the same, and subject to the same limitations, the comparison is relative and still useful for the assessment of the effectiveness of NP removal.

The volume of NP for all rabbit lumbar IVDs sequentially increased from the cranial L1/2 IVD to L5/6 as measured by all methods. Without further studies repeating this method for other species, no direct comparisons can be made. However, we suspect that moving caudally along the lumbar spine, surgeons could expect to remove a greater volume of NP from nonextruded in situ NP when performing prophylactic fenestration. As stated previously, postoperative MRI would be required to evaluate the effectiveness of fenestration by volume measurement compared to preoperative imaging.

We acknowledge some limitations in this study. Rabbit and canine lumbar spines have some anatomical differences. Rabbit lumbar spines were selected as a preclinical model for the dog due to greater sample accessibility. Further, age and sex-matched rabbits from a closed breeding colony were used to minimize variation between samples to provide a more accurate means for comparison between measurement methods. Additionally, this study did not assess cervical, and caudal thoracic disks where IVDE is also common in dogs. Despite anatomical differences in size and shape of the vertebrae, intervertebral disks, and spinal cord, the principles behind the imaging modalities and volume estimate techniques used to identify these structures remain the same for dogs and other species.[42] Future studies could use our model of volume estimation to more accurately evaluate the effectiveness of fenestration in cadavers with pre- and postoperative MRI evaluation. The results of that study may then be extrapolated to the in vivo preclinical setting and finally to our canine patients, first in native, nondegenerate disks before considering assessment of pathological disk spaces. This method would allow for an objective measurement of the effectiveness of fenestration that is determined by how much NP has been removed, as reported by Shores et al (1985).

NP volume measurements in rabbit lumbar IVDs made grossly and by MRI significantly overestimated the true volume as measured by water volumetry. NP volume increases with each IVD space caudally. Despite being an estimate, MRI will likely remain the most commonly used tool for NP volume estimates due to practicality. The findings of this preclinical model may be useful for veterinary surgeons when estimating the volume of in situ NP and during surgical planning for the removal of extruded material or performing prophylactic fenestration.

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