Influence of maturity on nucleus–endplate integration in the ovine lumbar spine

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Abstract

Purpose Recent investigations using an ovine spine model have established that the disc nucleus contains a highly convoluted fibre network with endplate-to-endplate connectivity, this connectivity being achieved via distinctive nodal attachment points. The purpose of this study was to investigate how this nodal anchoring system might be influenced by maturation.

Methods Lumbar motion segments were dissected from newborn, 3, 12 months and fully mature ovine animals, subjected to a novel annular ring-severing procedure to remove the strain-limiting influence of the annulus, then either mechanically tested to destruction or examined microstructurally and ultrastructurally. The morphology of the nodes and their linear density within the relatively thin section planes were analysed to provide a basis for comparison between the four age groups.

Results Mechanical testing following ring severing revealed that the remaining nuclear material in all samples, irrespective of maturity, had the ability to transmit a substantial load from endplate to endplate. Imaging of the ring-severed samples from all age groups in their stretched, but unruptured state revealed the presence of axially aligned fibrosity in the nucleus region consistent with endplate-to-endplate connectivity. Endplate insertion nodes were observed in all age groups. Ultrastructural

examination revealed that the fibrillar architecture of these nodes in the newborn discs was similar to that observed in the nodes of mature discs. However, there was a rapid increase in their linear density between birth and 3 months, after which this remained constant.

Conclusions The nodal attachment points identified previously in mature ovine discs are also present in newborn, and 3- and 12-month-old animals with an initial rapid increase in their linear density between birth and 3 months, after which it remained constant. The size and morphology of the attachment points were similar for all ages. Our study suggests that the increase in nodal density in the ovine disc endplate is part of an adaptive response to the loading environment that the disc is exposed to from birth to maturity.

Keywords Ovine model · Nucleus–endplate connectivity · Insertion nodes · Influence of maturity

Introduction

Recent investigations using an ovine spine model have established the existence within the nucleus of a heavily convoluted fibre network integrated structurally with the endplates [1, 2]. The experimental demonstration of this anchorage system was established using a novel annular ring-severing technique which effectively decoupled the nucleus from the constraints of the annular fibres [1]. It was found that by axially extending the endplates, the nucleus could be stretched up to four to five times its original height before a limiting extension was reached, above which actual rupture of the nuclear fibres occurred. Chemical fixation in this extended state followed by decalcification then enabled us to show microscopically and ultrastructurally the existence of

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discrete nodal insertions into the cartilaginous endplate and confirmed the existence of actual structural integration between the nucleus and endplate. These findings contrast with the more widely held view that the inner nucleus is a largely unstructured gelatinous substance which possesses little or no structural cohesion with its surroundings [3–5].

We further argued that these nodes provided a form of tethered mobility for the nucleus fibres, allowing them to move with relative freedom as the disc flexes, compresses and extends. The fibres themselves are assumed to provide a substrate for the proteoglycans (PGs), requiring some measurable affinity between them. Certainly, the nucleus samples in our previous study [1] were shown to remain intact as a hydrated, swollen entity even following removal of the annulus, thus indicating that some form of connectivity exists between the proteoglycans and the fibre network.

The above-noted studies were conducted using ovine discs and it is well accepted that there are important developmental differences between human and ovine spines. In humans the ring apophysis presents as a cartilaginous rim around the edge of the vertebral body from which the vertebra grows [6, 7]. By contrast, the sheep spine possesses a separate growth plate which fuses at maturity [8]. However, given that this difference is associated with the vertebral body, it would not be expected to greatly affect the nodes which are associated with the cartilaginous endplate. Given that the ovine spine remains widely accepted as a viable model for exploring structure–function relationships due to its biochemical and biomechanical similarities to the human spine [8–11], it is a suitable choice for this new study which is aimed at investigating how the nodal anchoring system in the endplate might change with maturation. Since it is accepted that the nucleus plays a key role in disc function, a deeper understanding of how it develops may well provide insights into possible pathways for disc degeneration. This study is a first step towards this goal.

Methods

Tissues

Ovine lumbar spines, dissected from freshly slain newborn, 3 and 12 months old lambs, and mature animals (≥ 3 years) were wrapped in plastic film and stored at -20°C for no longer than 3 months. It has been shown that this storage technique has a minimal effect on the mechanical and structural properties of the tissue [12–16]. Only those spines with healthy discs (based on appearance) [17] were included.

In preparation for testing, the extraneous soft tissues and posterior elements were removed from each spine. While maintaining each lumbar spine in its frozen state, discs and

Fig. 1 Schematics showing sample preparation. Using pairs of off-set sagittal and coronal cuts, composite vertebra–nucleus–vertebra blocks were sawn from the central regions of the motion segment as indicated by the *dashed lines* on the whole disc in (a). The nucleus was then completely isolated by ring severing any residual annular elements—see *dashed lines* in schematic (b). Note that for the sake of clarity, schematic (b) shows only the posterior and anterior residual annular elements. The sample with its isolated nucleus was then axially stretched, as shown in (c)

their adjacent vertebral portions were isolated. Using a series of axial saw cuts a composite block consisting primarily of vertebra–nucleus–vertebra was extracted from each spine segment as shown in Fig. 1a and then thawed. It should be noted that this initial preparation did, unavoidably, leave some residual annular elements that were subsequently removed by a simple ring-severing technique (see Fig. 1b, c) and described in detail in [1].

Mechanical assessment

An assessment of the tensile properties of the extracted vertebra–nucleus–vertebra sample was conducted on

samples from each age group using the following procedure: the sample was mounted between the plinths of an Instron 5543 testing machine fitted with a 1,000 N load cell. Tissue glue was used for this mounting procedure whilst ensuring that the sample was not subjected to any bending moment, i.e. an axial tensile load only was applied. All tests were carried out with a crosshead displacement rate of 0.5 mm/min. Loading was terminated at approximately 30–50 N due to the limitations of the sample attachment method; over this loading range, axial extensions within the disc tissue of no more than 1.5 mm were achieved. Each sample was kept hydrated with physiological saline throughout the testing procedure.

The sample was unloaded and then temporarily detached from the testing machine whilst retaining the ability to relocate it in the same position for further testing. The residual annular elements were then carefully ring severed, with their complete severance indicated by a sudden, large increase in both extensibility and mobility of the remaining nucleus material. The ring-severed sample was then remounted in the testing machine and re-tested at the same displacement rate used previously. All tests on these ring-severed samples were taken to failure. The results from the tensile tests were presented as raw load–displacement curves (as opposed to stress–strain curves) due to the difficulty of obtaining an accurate measurement of the cross-sectional area of the remaining nuclear material in the ring-severed sample. Employing this procedure meant that any quantitative comparison of tensile response was confined to the unsevered versus ring-severed state within each sample, rather than between samples.

Microstructural assessment

For the microstructural studies, vertebra–nucleus–vertebra samples prepared as described above were subjected to ring severing [1] and then manually stretched. From prior experimentation it was found that the remaining nuclear mass could be extended up to five times its original axial height (i.e. up to 500 % strain) before reaching a limiting extension beyond

which progressive failure occurred. The ring-severed sample was maintained in this unruptured, but highly extended state by applying a minor load of approximately 1 N. It was then fixed in 10 % formalin for 7 days in this strained state.

Following fixation, each sample was fixed, and then decalcified for 14 days in 10 % formic acid. The decalcified sample was trimmed and 30 µm-thick sagittal sections were then obtained by cryosectioning. These sections were wet mounted, unstained on coverslipped slides and examined using either standard light or differential interference contrast (DIC) optical microscopy. It should be noted that the above method of fixation under a slight tensile load provided a much clearer microscopic view of the nodal anchoring system as previously described [1].

Evaluation of nodal density

The optical images were merged using Adobe Photoshop, scale bars were added to the composite image and the nodal attachment points carefully counted. It was possible to count nodes in both the stretched and unstretched samples, thus allowing control discs to be included in the statistical analysis. For the purpose of this counting, any evidence of a node (usually denoted by a characteristic pattern of the cells or visible fibrosity) was recorded as a single node. The length of the cartilaginous endplate adjacent to the nucleus was then carefully measured using ImageJ (ImageJ 1.45s, Wayne Rasband, National Institutes of Health, USA) by tracing freehand along the length of the curved endplate. This procedure enabled the nodal lineal density within a given section plane to be determined in each image and expressed as number of nodes per mm. The data were analysed using appropriate statistical methods in SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). While the main focus of this study was to evaluate the effect of age on nodal density, the influence of disc level and endplate aspect (superior versus inferior) was also analysed within each age group to quantify what, if any, effect these factors had on node density.

Table 1 Number of samples from each disc level in each age group subjected to mechanical testing and microstructural analysis. Mature data have been previously published [1]

Level	Age group							
	Newborn		3 Months		12 Months		Mature	
	Mech.	Image	Mech.	Image	Mech.	Image	Mech.	Image
L12	8	2	8	0	6	2	3	9
L23	–	7	–	7	–	5	2	3
L34	–	8	–	4	–	5	3	7
L45	8	0	8	0	6	0	1	2
L56	–	8	–	6	–	6	3	2
L67	–	2	–	1	–	0	0	0
Total	16	27	16	18	12	18	12	23

Fig. 2 Representative load versus displacement curves from each of the four age groups tested showing the differences in response between the unsevered (*curve A*) and ring-severed (*curve B*) states.

Scanning electron microscopy (SEM)

Additional ring-severed samples from the newborn spine segments were examined using SEM to provide a comparison of node architecture at the fibril level with previously published data from mature spines [2]. Again, the samples were fixed in their extended state and decalcified, then trimmed in the sagittal plane with a sharp blade to localize regions of the nucleus–endplate comparable to those examined under light microscopy. To enhance fibrillar clarity at their exposed cut surfaces, these specimens were digested in bovine testicular hyaluronidase (Sigma Type I–S, 400–1,000 U/mg, at a ratio of 1.25 mg/mL in 0.1 M sodium acetate and 0.1 M sodium chloride pH 5 buffer solution giving approximately 875 U/ml) for 3 days at 37 °C to remove the proteoglycan component. The samples were dehydrated in ethanol, critical point dried, vacuum coated with platinum and then examined using a Phillips XL30S FEG SEM and an imaging voltage of 5 kV.

Sample numbers

Table 1 shows the number of samples examined mechanically or microstructurally with respect to disc level and

Note that all samples exhibit an initial “J-type curve” response followed by an irregular drop in load to final failure

age. A further seven samples from six newborn spines were examined at the fibril level using SEM.

Results

Mechanical analysis

Representative load–displacement curves obtained for samples tested from each maturity group, prior to and following the ring severing of their residual annular elements, are shown in Fig. 2. Despite the variation in responses there are some clear similarities between the

Table 2 A summary of the peak load data from the ring-severed samples for each age group

Age group	Number of samples	Average load (N)	Load range (N)
Newborn	16	6.1	3.1–11.5
3 Months	16	17.3	7.3–30.3
12 Months	12	24.8	8.5–38.8
Mature	12	20.2	10.3–25.0

Mature data have been previously published [1]

Fig. 3 Lower-magnification DIC images of untested L3–4 discs from newborn, 3-month-old, 12-month-old and mature ovine spines. The red boxed regions in each image are shown enlarged in Fig. 6. All

discs are oriented as indicated in the *top image*. Also, note that where it is visible the growth plate is indicated by X

samples. Firstly, prior to ring severing all samples were relatively inextensible, exhibiting a near-linear increase in load (see curve A in each plot). Following ring severing, the response of the nucleus is clearly visible: there is an

initial and variable phase of easy extension, followed by rapid stiffening up to a peak load via a characteristic “J-type” response typical of many compliant biological tissues [18]. Following attainment of this peak load, for all

Table 3 A summary of average disc and nucleus diameter and nucleus height from measurements along the midline of sagittal sections through control samples

Age group and number (<i>N</i>)	Disc length (mm)	Nucleus sagittal diameter (mm)	Nucleus height (mm)	% Nucleus
Newborn (<i>N</i> = 4)	5.9 (5.0–6.9)	3.7 (3.3–4.1)	1.0 (0.7–1.3)	62
3 Months (<i>N</i> = 3)	9.3 (8.7–10.1)	4.1 (4.0–4.1)	2.1 (1.8–2.4)	44
12 Months (<i>N</i> = 4)	9.1 (8.6–9.4)	4.0 (3.8–4.3)	2.4 (2.0–2.5)	44
Mature (<i>N</i> = 3)	10.0 (9.9–10.3)	4.4 (4.3–4.5)	2.6 (2.4–2.8)	44

The percentage of disc diameter occupied by the nucleus was calculated from these diameter measurements

levels of maturity there was a progressive, though irregular reduction in load to ultimate failure: the results are summarized in Table 2.

Disc morphology under optical microscopy

Representative lower-magnification images of controls from each maturity group are shown in Fig. 3. In the newborn discs, the vertebral bodies are largely cartilaginous and can be seen to ossify with advancing age. The growth plate is unfused in the newborn, and 3- and 12-month groups, appearing as a narrow band running below the vertebral endplate (see X in Fig. 3), whereas it is fused in the mature tissue.

Defining the nucleus very approximately as the lamella-free region in the centre of the disc, measurements taken on controls from each age group (see Table 3) indicated that whereas in the newborn where the nucleus occupied ~62 % of the total diameter, this reduced to ~44 % at 3 months and remained approximately constant from thereon. Similarly, the nucleus height increases substantially between the newborn and 3-month-old discs, with a more gradual increase from 3 months to the fully mature state. Further, in the 12-month and mature discs, the nucleus was shifted posteriorly and the endplates became progressively more convex. These findings are in general agreement with previously published results [19–22].

In all samples examined microscopically in their ring-severed and stretched but unruptured state, the appearance of an axially aligned fibrosity in the nucleus, and especially nearer the edges of the stretched nucleus region (Fig. 4), implied endplate-to-endplate connectivity. By contrast, the central nucleus region remained largely disordered in all discs of all maturities, thus indicating that this had not been unravelled by the axial stretching following ring severing. This is consistent with the earlier study by Wade et al. [1] showing that unravelling of the central nucleus occurs only in the final phase of nucleus rupture.

Higher-magnification DIC imaging of the nucleus–endplate regions at all stages of maturity (see Fig. 5) revealed the presence of endplate insertion nodes of similar morphology to those previously reported in fully mature

animals. These nodes are further delineated by a characteristic conical pattern of endplate cells: this latter feature considerably aided their detection and thus the quantification of nodal linear density which was required to be conducted at a much lower magnification (see next).

Influence of age on nodal density

The boxed regions in Fig. 3 are shown enlarged in Fig. 6 and provide a direct visual comparison of nodal linear density with respect to maturity. Preliminary data analysis using the Shapiro–Wilk and Levene tests showed that although the data were normally distributed, the variance between the groups was unequal. Therefore, the Kruskal–Wallis test was used to compare the lineal density between the age groups. This showed that the newborn discs were significantly different from the other age groups and that there were no significant differences between the 3- and 12-month-old and mature age groups (see Fig. 7 for details). A similar analysis was performed to identify any possible trends within the age groups, both between disc levels and with respect to the superior versus inferior endplates. These analyses showed that there were no statistically significant differences in nodal linear density within age groups for the main levels examined (i.e. L2–3, L3–4, L5–6).

Fibril-level morphology

Due to the similarity in nodal morphology across the entire maturity spectrum at the microstructural level (see Fig. 5), a fibril-level analysis was carried out on the newborn tissues to provide a comparison with previously published ultrastructural data obtained from the fully mature tissue (see Wade et al. [2]): the aim was to cover the immaturity/maturity extremes.

The characteristic nodal integration between nucleus and endplate in the newborn tissue is shown in Fig. 8 for both the newborn and mature discs and an overall similarity is evident even at this lower magnification. Figure 9a provides a higher-magnification view of the boxed region shown in the newborn sample. At still higher magnification Fig. 9b shows the generally axial alignment fibrils in the

Fig. 4 Representative lower-magnification views of nucleus–endplate continuity imaged in ring-severed, axially stretched samples from newborn, 3 months, 12 months, and mature groups. Note the

similar appearance of these samples; the fibres of the nucleus have been drawn into alignment in all cases. All samples are orientated as in the *top left* image

upper region of the node (region X in 9a). Figure 9c captures a view of the fibrils near the tip of the node (region Y in Fig. 9a) where they have turned from their near-axial orientation into the plane of the cartilaginous endplate (region Z in Fig. 9a) whose densely woven fibrillar structure is imaged in Fig. 9d. Again, this fibril-level structure of the node is entirely consistent with that of the nodes in the fully mature tissues described earlier [2].

Discussion

The tensile responses obtained prior to and following ring severing at all maturity levels confirm the relatively high stiffness of the sample prior to severing (see all curves marked A in Fig. 2) and the characteristic large-strain “J-type” response of the isolated nucleus which, as explained previously [1], is associated with the

Fig. 5 High-magnification views showing similar nodal insertions (indicated by yellow arrows) in newborn, 3 months, 12 months, and mature discs

unravelling of the nuclear fibrosity. While the absolute magnitudes of the peak loads and displacements vary between samples, these differences can be readily explained by the size differences between the samples and the variability of the ring-severing process which was required to be conducted at a macro rather than micro level. The change in both overall disc size and annular wall thickness with age (see Fig. 3; Table 3) meant that ring severing was always performed in a somewhat

‘blind’ manner, the required endpoint at which the annular layers were fully severed being indicated by a sudden ease of axial extension. Due to this unavoidable variation in sample preparation, it was considered inappropriate to perform any normalization of the peak loading data and thus any comparative stress determination.

As has been previously suggested, the age-related changes in the overall structure of the disc are assumed to

Fig. 6 Examples of the nucleus–endplate region from newborn, 3 months, 12 months, and mature animals. Nodal insertions are identified by *yellow arrows*. It should be noted that the location of

these nodes was established by careful inspection of these same images in their greatly enlarged state

reflect an adaptive response to loading [7, 19, 23]. However, less attention has been given to the age-related variations occurring within the disc at the microstructural level.

This new study has demonstrated a clear similarity in nodal size and micro-level morphology across the full spectrum from newborn to fully mature. Further, both the newborn

Fig. 7 Box plots summarising the nodal densities for the four age groups. The trends identified by the statistical analysis are clearly visible here. Note that the inner fences on the box plots are either the maximum/minimum or 1.5x the interquartile range, and the *boxes* show the upper and lower quartiles with the median within these

and fully mature tissues show a similarity of interweaving between the nucleus fibrils and the endplate fibrils, thus indicating that fibril-level integration is consistent between these two age extremes. This multilevel structural uniformity suggests a consistency in the functional role played by these nodes throughout the life of the healthy disc.

Although this paper has not analysed the composition of the nodes, the nucleus and inner annulus consist mostly of type II collagen [24–26] and thus is likely to be a primary nodal component. Further, in view of the more recent discovery of elastin in the nucleus [27, 28], this too could also form part of the nodal structure but is outside the scope of the present study.

While these nodes must play a primary role in structurally integrating the nucleus–endplate region as demonstrated by its ability to bear substantial tensile loads (see Table 2), we are not suggesting that these nodes contribute directly to the impressive load-bearing properties of the disc–endplate system *in vivo*. Clearly, the functional strength of the disc is derived from both the hydrostatic containment of the hydrated nucleus by the highly ordered structure of the annulus–endplate system and the ability of this same system to sustain direct compression, tension and minor torsion forces.

By contrast, the very convoluted nature of the nuclear fibrils that are, in turn, gathered to form the distinct insertion nodes in the cartilaginous endplate is entirely consistent with these nodes having a role other than contributing to the primary mechanical strength of the disc. The large-strain extensibility of the convoluted fibres would be expected to contribute little to the primary

strength of the disc which can clearly undergo only relatively limited deformations when the annulus is intact.

Flexion would of course increase the displacement strains in the nucleus (as observed in previous investigations [29–31]) but these would certainly not approach the levels of strain shown to be achievable in the isolated nucleus [1, 32]. Further, previous studies have shown that the annulus will fail at axial tensile strains of around 50–60 % [33, 34], i.e. much lower than the 200–300 % tensile strain indicated by the loading curves in Fig. 2 (i.e. by using the average disc dimensions in Table 3).

We have proposed previously that the nodal insertions provide the nucleus with a form of tethered mobility enabling it to both maintain its structural integrity and, crucially, to act as a relatively mobile binding substrate for the proteoglycans while accommodating the range of disc deformations associated with normal physiological loading [1]. Further, this node-mediated integration between nucleus and endplate could account for the relatively common clinical observation that nucleus and attached endplate material can be seen to migrate when a disc prolapse/herniation occurs [35, 36].

The absence of any significant difference in the linear density of the nodes both between disc levels and between inferior and superior aspects of the same disc for any given degree of maturity suggests that the overall pressure within the disc and the loading regime that the disc is subjected to is roughly similar between levels.

However, the observed trend of a rapid increase in nodal density between the newborn and 3-month-old samples (see Fig. 7) is consistent with a process of biological adaptation arising from the natural increase in disc loading as both body mass and physical activity increase—the reduced nodal density in the newborn disc is consistent with the animal yet to be exposed to post-birth loading levels. Following this rapid adaptation and increase in overall disc size (see Table 3), the nodal density seems to plateau, indicating that the environment of the nucleus remains fairly constant in the healthy disc through into maturity. Thus, although it is difficult to separate the effects of development and loading between the newborn and 3-month-old lambs, the similarity in nodal density in the 3-month-old, 12-month-old and mature discs indicates that the environment of the disc nucleus remains relatively constant during these ages. It seems logical to suggest that as the disc grows and is subjected to increased loading, new ‘infilling’ nodes are created to maintain nodal density at the required level.

Any loading of the insertion nodes will therefore be an indirect rather than a direct consequence of disc loading. The higher nuclear pressures and displacements generated with more intense spinal loading will increase the forces experienced by the nucleus–endplate junction and thus

Fig. 8 Low-magnification SEM images of the nucleus–endplate region from newborn and mature samples. Readily identified node tips are marked by arrows in both images and confirm the overall

similarity between these two extremes of maturity. Note that the mature image has been taken from Wade et al. [2] and used with permission of Journal of Anatomy

would be expected to translate into higher forces experienced by the nodes. This, in turn, would presumably provide the adaptive driving force for an increase in nodal density so as to limit the magnitude of force acting on any one node. This interpretation is consistent with the increase in nodal density with increasing maturity as demonstrated in the present study.

Conclusions

The nodal attachment points identified previously in healthy, mature ovine lumbar discs are also present in newborn, and 3- and 12-month-old animals. The morphology of the nodes at both the micro- and ultrastructural levels was similar for all ages, but there was a rapid increase in their

Fig. 9 **a** Enlarged SEM view of node in the boxed region of the newborn image in Fig. 8. Images in **b–d** are enlarged views, respectively, of regions marked *x*, *y*, and *z* in **a**

linear density between birth and 3 months, after which it remained constant. It is proposed that the nodes adapt rapidly to the changing loading environment they are exposed to from birth to approximately 3 months, after which they are maintained into maturity.

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Conflict of interest None.

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