Spine Deformity

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Spine Deformity 1 (2013) 161–170

Biomechanics

Metabolic Effects of Angulation, Compression, and Reduced Mobility on Annulus Fibrosis in a Model of Altered Mechanical Environment in Scoliosis

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Received 5 October 2012; revised 5 December 2012; accepted 4 February 2013

Abstract

Study Design: Comparison of disc tissue from rat tails in 6 groups with different mechanical conditions imposed.
Objectives: To identify disc annulus changes associated with the supposed altered biomechanical environment in a spine with scoliosis deformity using an immature rat model that produces disc narrowing and wedging.
Background: Intervertebral discs become wedged and narrowed in a scoliosis curve, probably partly because of an altered biomechanical environment.
Methods: We subjected tail discs of 5-week-old immature Sprague-Dawley rats to an altered mechanical environment using an external apparatus applying permutations of loading and deformity for 5 weeks. Together with a sham and a control group, we studied 4 groups of rats: A) 15° angulation, B) angulation with 0.1 MPa compression, C) 0.1 MPa compression, and R) reduced mobility. We measured disc height changes and matrix composition (water, deoxyribonucleic acid, glycosaminoglycan, and hyaluronic acid content) after 5 weeks, and proline and sulphate incorporation and messenger ribonucleic acid expression at 5 days and 5 weeks.
Results: After 5 weeks, disc space was significantly narrowed relative to internal controls in all 4 intervention groups. Water content and cellularity (deoxyribonucleic acid content) were not different at interventional levels relative to internal controls and not different between the concave and convex sides of the angulated discs. There was increased glycosaminoglycan content in compressed tissue (in Groups B and C), as expected, and compression resulted in a decrease in hyaluronic acid size. We observed slightly increased incorporation of tritiated proline into the concave side of angulated discs and compressed discs. Asymmetries of gene expression in Groups A and B and some group-wise differences did not identify consistent patterns associating the discs' responses to mechanical alterations.
Conclusions: Intervertebral discs in this model underwent substantial narrowing after 5 weeks, with minimal alteration in tissue composition and minimal evidence of metabolic changes.

Keywords: Intervertebral disc; Growth; Deformity; Rat model; Metabolism

Introduction

A scoliosis deformity involves wedging asymmetry of both the vertebrae and intervertebral discs [1,2]. Whereas the pathomechanism of the progressive wedging of the vertebrae is thought to involve asymmetrical loading that modulates vertebral growth (Hueter-Volkmann principle), the mechanism by which the discs become wedged is poorly understood, and the relative timing of vertebral and discal wedging is unclear [1,2]. The progression of a scoliosis deformity slows markedly or ceases at skeletal maturity

Author disclosures: IAFS (grant from National Institutes of Health [NIH] R01 AR 052132; support for travel to meetings for the study from NIH R01 AR 052132; consultancy for Kspine, Inc.); CM (grant from NIH R01 AR 052132); DDA (grant from NIH R01 AR 052132); PJR (none).

This work is supported by Grant R01 AR 052132 from the National institutes of Health, Bethesda, Maryland. Haddon Pantel provided technical support.

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2212-134X/$ - see front matter © 2013 Scoliosis Research Society.
http://dx.doi.org/10.1016/j.jspd.2013.02.001

when the vertebral growth plates become inactive, but it is not clear why wedging of the discs would also cease.

It has been reported [3] that intervertebral discs subjected to compression, reduced mobility, and angulation in a young, immature rat tail model of supposed altered spinal biomechanics in scoliosis were narrowed and stiffer, with structural wedging resulting from tail angulation. The structural nature of the disc deformity was evidenced by its minimum lateral bending stiffness being at an angle close to the in vivo (deformed) value, indicating that angulated discs had remodeled to the imposed deformity [3]. This implies that wedging of intervertebral discs during progression of a scoliosis deformity may result from a combination of imposed angulation, asymmetrical compression, and reduced mobility.

The overall aim of the present study was to explore the underlying mechanism of this reported loss of disc space in immature rats by identifying how the disc tissue responded to different components of the altered mechanical environment implicated in scoliosis. We measured annulus tissue composition after 5 weeks and investigated the short-term (5-day) and long-term (5-week) effects on tissue synthesis and expression of genes associated with tissue synthesis and degradation. We hypothesized that the loss of disc height would be associated with decreased water and glycosaminoglycan (GAG) content, decreased hyaluronic acid (HA) size, reduced synthesis of collagen and GAGs, and increased expression of genes associated with disc tissue degradation and degeneration.

Methods

We imposed permutations of altered biomechanical conditions on different groups of immature rat tails using the model reported previously [3]. We measured water, deoxyribonucleic acid (DNA), and GAG content and hyaluronic acid (HA) size after 5 weeks to identify compositional changes. We assessed synthesis of collagen and GAG and changes in gene expressions of key matrix proteins and their degradative enzymes at 5 days and 5 weeks, to determine whether metabolic changes were occurring. Rat tail discs were selected because the rat tail is easily accessible for controlled application of compression [3-8] and because of the rapid growth of these animals [9,10]. We chose the initial age of the animals (5 weeks) and the duration of the experiment (5 weeks) to simulate human adolescent growth. In rats, the rate of growth increases between 1 and 5 weeks, then declines until skeletal maturity, which is achieved by 11.5−13 weeks [11]. Thus, peak growth velocity occurred early in the experimental period.

Animal groups and procedures

We attached external rings to adjacent caudal levels (Caudal 7 and Caudal 8 vertebrae) of 5-week-old (mean, 140-g body mass) Sprague-Dawley rats by 2 percutaneous, 0.5-mm-diameter pins, transfixing the vertebral bodies. This was done under general anesthesia (80 mg/kg ketamine and 10 mg/kg xylazine) with postoperative pain control (0.05 mg/kg buprenorphine). We installed the pins with fluoroscopic (X-ray) visualization of the vertebrae and apparatus. The apparatus remained in place for the duration of the experiments (5 weeks or 5 days).

We modified the method and apparatus described by MacLean et al. [7] for this study so that rings could be installed either parallel or with an initial 15° relative angulation (by rotating the rings each by 7.5° to the transverse plane of the vertebrae). Angulation of 15° has been shown to cause substantial disc space loss [3] and is comparable to the apical wedging in a clinically significant scoliosis curve. We used threaded rods linking the rings to control the angulation between the rings, along with springs that could be mounted on the rods to apply a sustained compressive force (Fig. 1). We adjusted the connecting rods and springs to align the rings provisionally parallel to each other, or at a small angle to compensate for misalignment measured from post-surgery computed tomography images. Compression was imposed by compressing the calibrated springs to produce a force corresponding to the desired stress [12]. We measured the lengths of the springs and adjusted them weekly. The stress was the applied force divided by vertebral metaphyseal cross-sectional areas, as reported in Stokes et al. [12].

We compared 4 intervention groups of animals with different permutations of imposed mechanical conditions, as well as a sham group and control group (Fig. 1). In Group A (angulation) we imposed 15° angulation by adding springs and locknuts to the rods but not compressing the springs. In Group B, we imposed 0.1-MPa compression stress compressing the calibrated springs, along with 15° angulation. In Group C, we imposed 0.1-MPa compression without angulation. In Group R, there was no imposed angulation and the

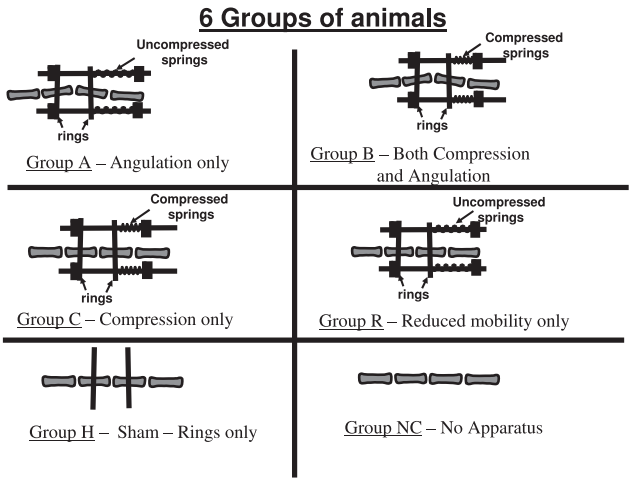

Fig. 1. Diagrammatic representation of tail instrumentation for the 6 groups of animals studied. In Groups A and B, the imposed angulation was 15°.

springs were not compressed. This was termed the reduced mobility group, because we observed the apparatus to limit tail motion in the instrumented region. In the sham group (Group H), we attached the rings to the adjacent vertebrae without angulation but employed no rods or springs. In the control group (Group NC), no apparatus was applied.

There were 5 sets of animals in each of the 4 intervention groups (A, B, C, and R) for 1) polymerase chain reaction (PCR) at 5 days; 2) proline and sulphate incorporation at 5 days; 3) PCR at 5 weeks; 4) proline and sulphate incorporation at 5 weeks; and 5) composition (water, GAG and DNA content, and HA size) at 5 weeks. Initially, there were 11 animals per set (10 in Set 2), for a total of 216 intervention animals. We based this number of animals on a power analysis to identify group differences greater than twice the variance of mean measurements with 80% power and probability $<.05$. There were 11 sham animals (Group H) and 8 control animals (Group NC), used for disc space and compositional studies only. We monitored disc dimensions by micro–computed tomography scans with animals under sedation 2 days after installation of the apparatus, and again immediately before euthanasia at 5 weeks (Sets 3 and 5). Radioactive tracers (^{35}S sodium sulphate and tritiated proline; PerkinElmer, Waltham, MA; 0.5 and 1 mCi/kg, respectively) were injected intraperitoneally (Sets 2 and 4) after installation of the tail apparatus. The institutional animal care and use committee reviewed and approved all live animal procedures.

After euthanasia, we removed sections of the tails, including 4 vertebrae and 3 discs (the intervention level disc [Level L] and adjacent control discs [Level C]), for analysis. In Groups A and B, we cut the discs into 2 halves, corresponding to the convex and concave sides of the tail, and processed them separately. In the data analyses, values from the adjacent control discs (Level C) were averaged.

Analysis of composition

We dissected discs free of adjacent tissues, removed the nucleus pulposus, and immediately weighed, lyophilized, and reweighed the remaining annulus tissue to obtain dry weight and hydration. We then rehydrated tissue and digested it in 0.5 mL 1 mg/mL α-chymotrypsin in 50 mmol/L Tris-HCl containing 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L iodoacetamide, and 10 mg/mL pepstatin-A overnight at 37°C, then in 1 mL 1 mg/mL proteinase K in the same buffer for 1 week at 56°C. We then centrifuged samples and removed the supernatant. We used aliquots of this digest to measure GAG content by the dimethyl methylene blue dye-binding method [13] and DNA content as a surrogate for cellularity by the Hoechst 33258 assay.

Analysis of hyaluronic acid size

The disc digests used for composition analysis were also used for analysis of HA size differences. We pooled digests (0.5 mL total) from 5 animals and heated them at 100°C for 5 minutes to inactivate proteinases. We then mixed the sample with 0.5 mL 50 mmol/L Tris/HCl, 150 mmol/L NaCl, pH7.4, containing 0.5 % bovine serum albumin and fractionated it by gel filtration chromatography through Sephacryl S1000 (95 × 1-cm column from VWR, Radnor, PA, USA) in 50 mmol/L Tris/HCl, 150 mmol/L NaCl, pH 7.4, at 6 mL/h. We collected fractions (1 mL) and analyzed them for HA content by a competitive binding assay using a biotinylated HA binding protein [14]. To compensate for differences in HA content between the samples and to facilitate comparison of the chromatograms, results are expressed as a percentage of the fraction with the highest HA content.

Incorporation of sulphate and proline

We digested the annulus tissue in 0.5 mL proteinase K (1.0 mg/mL, at 58°C to 60°C) until digestion was complete and dialysed (8- to 10-kDa size Spectra/Por Float-A-Lyzer; VWR (Radnor, PA, USA) to remove unincorporated isotopes. Four times the sample volume of scintillation fluid (111195/T; Research Products International, Mt. Prospect, IL) was added and 2 repeated counts of 600-seconds duration were performed using a Wallac Model 1409 (Perkins Elmer, Waltham, MA, USA) scintillation counter.

Real-time reverse-transcriptase polymerase chain reaction

We assayed gene expression evaluated by messenger ribonucleic acid (mRNA) levels for matrix proteins (collagen I, collagen II, and aggrecan) and degradative enzymes and their inhibitors (matrix metalloproteinase 13 [MMP13], MMP3, tissue inhibitor metalloproteinase 1 [TIMP1], TIMP3, and "A disintegrin and metalloproteinase with thrombospondin motifs 4 and 5" [ADAMTS4, and ADAMTS5]) by real-time reverse-transcription polymerase chain reaction (RT-PCR). We employed ribonucleic acid isolation, probes, primers, reverse transcription, and amplification as described by MacLean et al. [7]. The relative amount of mRNA in the instrumented disc compared with the internal control discs for each of the 9 genes was computed according to the comparative Ct method [7,15]. For each sample, we recorded the number of PCR thermal cycles producing fluorescence that reached a predetermined threshold level as the Ct value. We computed the relative gene expression for each of the 9 genes in tissue from the instrumented level by subtracting the Ct value of an endogenous control gene (18S rRNA) from the Ct value of the experimental gene, yielding the ΔCt value. Then, we further normalized ΔCt values of the instrumented samples to the ΔCt values of the internal control discs, yielding the ΔΔCt value. Finally, assuming that each strand of cDNA in the sample is copied exactly once per PCR cycle (giving an exponential relation between the cycle number and quantity of

cDNA), we used the base-2 antilog of the ΔΔCt value in statistical analyses.

Statistical analyses

We made group-wise and level-wise comparisons by analyses of variance of individual measurements of intervertebral discs, or of paired differences (between sides or levels) with post hoc *t* tests (Bonferroni correction). We made analyses with SPSS software (SPSS Inc., Chicago, IL). p = .05 was considered significant.

Results

Animals' body mass averaged 140 g when instrumentation was installed and 440 g when they were euthanized after 5 weeks. There was a substantial and highly significant narrowing of the disc space in all intervention animals. (Fig. 2). The loaded disc values as a percentage of the control disc values were 70%, 42%, 39%, and 66% in Groups A (angulated), B (angulated and compressed), C (compressed), and R (reduced mobility), respectively, whereas the corresponding percentages for the sham and control groups were 97% and 102%, respectively (Fig. 3).

Composition

Neither hydration (water content) nor DNA content (as a surrogate for cellularity) of discs at the intervention levels was different from those of the internal control or of the sham (Group H) animals at 5 weeks. There was no evidence of a difference between disc tissue hydration or DNA content on the convex and concave sides of angulated tails (Groups A and B) (Fig. 4). The GAG content was increased in compressed discs compared with control discs in Groups B and C (Fig. 4). The intervention level contained significantly more GAG than control levels in Group C and in Group B on both the concave and convex sides of the tail. However, there were no significant convex–concave side differences in the angled discs (Groups A and B).

Hyaluronic acid size

In the instrumented angulated discs (Group A), there was a small decrease in HA size on the concave side relative to the convex side (Fig. 5A). In the compression group (Group C), we observed a decreased HA size in the instrumented discs relative to the distal control discs (Fig. 5B). The magnitude of this difference was greater than between the sides of the angulated discs. In both cases, the decrease in HA size appeared to be related to increased disc compression.

Incorporation of sulphate and proline

We observed a slightly increased incorporation of tritiated proline into the disc tissue of compressed discs and into the concave side of angulated tails (Fig. 6), but differences were not statistically significant. Values of incorporated isotope at the intervention level expressed as a percentage of the control level were not significantly different in any of the groups. In Groups A and B, none of the convex–concave side differences were significant. In the case of sulphate

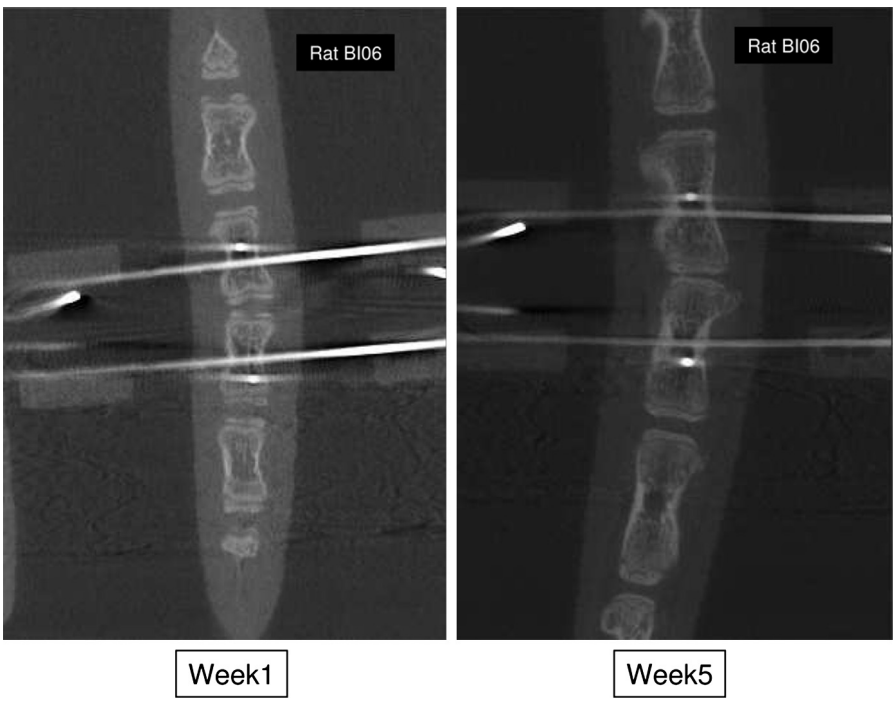

Fig. 2. Mid-coronal plane sections from micro–computed tomography scans showing instrumented level of an animal in the B group at weeks 1 and 5.

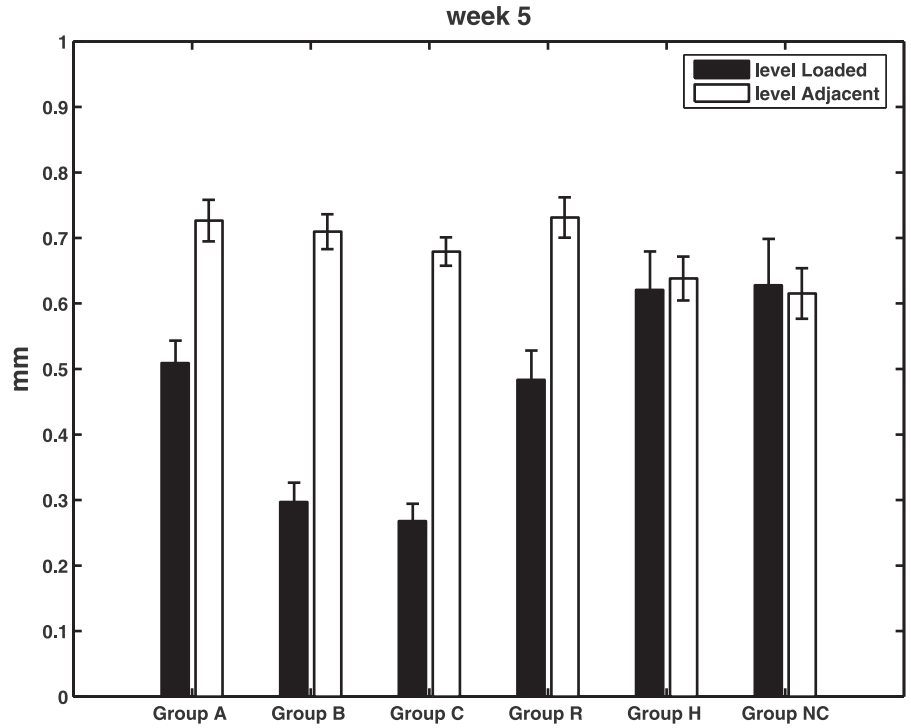

Fig. 3. Disc space measurements (in millimeters) at week 5 (mean and standard error of the mean). For each group, left (filled) column indicates the intervention level; right (unfilled) column indicates the average of 2 adjacent internal control levels.

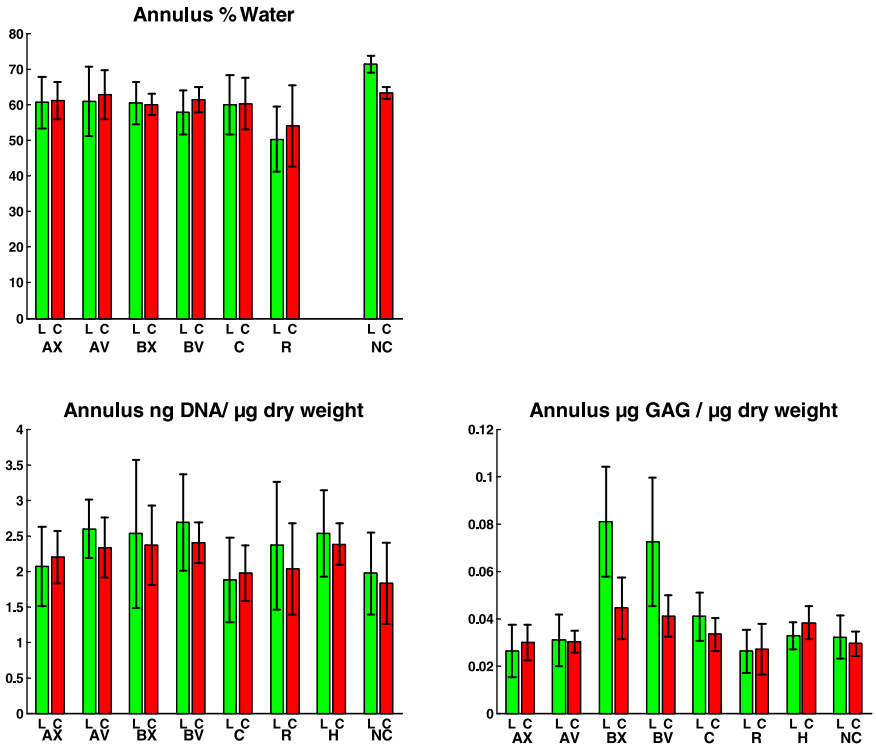

Fig. 4. Annulus hydration (mean and standard deviation [SD]; dry weight as a percentage of wet weight), annulus tissue deoxyribonucleic acid (DNA) content (mean and SD, by Hoechst assay), and glycosaminoglycan (GAG) content (mean and SD by dimethyl methylene blue dye binding method) for the 4 intervention groups—A, B, C, and R—and the sham (H) and control (NC) groups. We made measurements after 5 weeks for all groups. For Groups A and B, AX, AV, BX, and BV identify the group and the side of the disc: X = convex side of the tail; V = concave side of the tail. L = intervention (loaded) disc. C = mean value of adjacent (internal control) discs. Hydration data are not presented for the sham group because of numerous missing values.

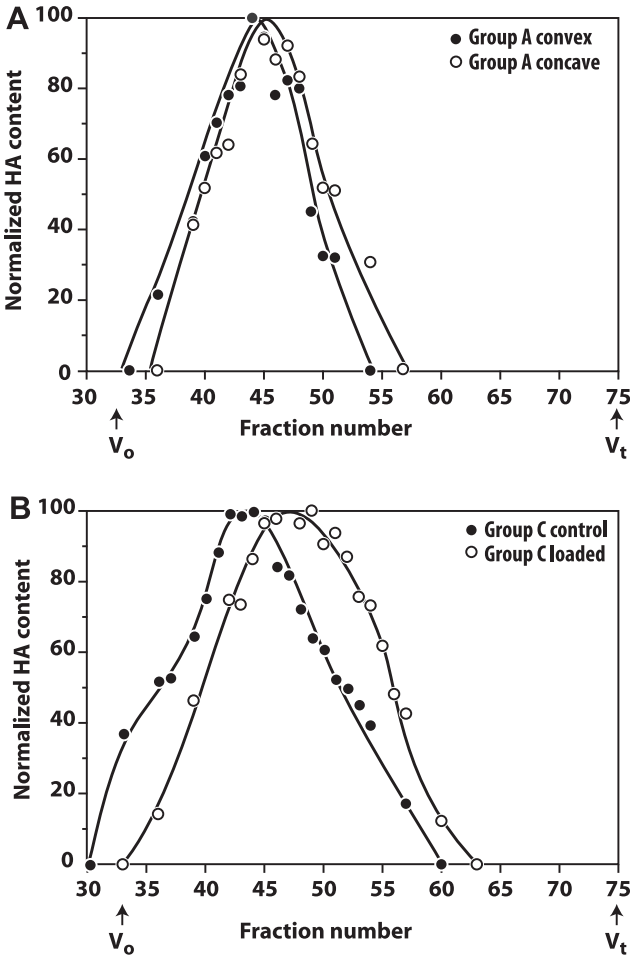

Fig. 5. Hyaluronic acid (HA) size variation in angulated and compressed rat discs. We analyzed pooled extracts from angulated and compressed discs for HA size variation by chromatography through Sephacryl S1000. (A) Comparison of the convex and concave sides of the instrumented (loaded) angulated disc (Group A). There is a small decrease in HA size in the concave side relative to the convex side. (B) Comparison of the instrumented (loaded) and distal control discs of the compression group (Group C). There is a major decrease in HA size in the loaded disc. V_o and V_t = void and total volumes of the column, respectively.

incorporation, there were no significant differences between loaded and control discs and between the concave–convex sides in discs from angulated tails (Fig. 6).

Real-time RT-PCR

We noted the reduced expression of most genes on the concave side of angulated discs in Group A animals at 5 days (convex–concave side differences were significant for collagen 1, TIMP1, TIMP3, and ADAMTS5), but no long-term convex–concave side differences. In Group B, we observed reduced gene expression bilaterally for most genes at 5 days; this was statistically significant for collagen 1, collagen 2, and aggrecan. There was a significant increase in the expression of TIMP3 at 5 weeks in Groups B, C, and R, and in Group R at 5 days. Other degradative enzymes and their inhibitors increased in expression in Group R at both 5 days and 5 weeks, but this was only significant for TIMP3 (Fig. 7).

In analyses of differences between groups, collagen 1 expression was significantly reduced in Groups A and B relative to Group C; TIMP3 was expressed less in Group A than in Group C, aggrecan was expressed less in Group B than in Group C, and both MMP3 and TIMP1 were expressed less in Group A than in Group R.

Discussion

All experimental interventions produced substantial narrowing of the intervertebral disc space of these growing animals over the 5-week period that corresponds to a large proportion of the postnatal growth of the animals. Although the growth curve of rats is not the same as that of humans, this experimental period is considered to be analogous to the human adolescent phase. Despite the substantial narrowing of disc space, measurements of annulus tissue composition after 5 weeks of loading were surprisingly similar at the intervention levels relative to the internal controls, and were symmetrical where angulation had been applied. Lack of statistically significant differences or consistent trends in the results implies that only small effects were present, and that there had been substantial remodeling of the tissue to restore normal composition, hydration, and cellularity.

The changes reported in HA size are consistent with altered disc remodeling in response to the different types of loading, and suggest that compression is associated with HA depolymerization. A decrease in HA size could occur because of a load-induced increase in oxidative stress resulting in free radical production or increase in hyaluronidase expression. However, the observed depolymerization is not extensive, and it is not known whether such a change in HA size could affect disc function.

The synthesis of collagens and aggrecan also indicated a surprising lack of alteration in experimental discs at both 5 days and 5 weeks after loading. Although there were some significant asymmetries of gene expression in angulated groups and some group-wise differences, these did not identify consistent patterns revealing how discs responded to compression, angulation, or reduced mobility. The expected association of loss of disc height with decreased water and GAG content, with reduced synthesis of collagen and GAGs, and with increased expression of genes associated with disc tissue degradation and degeneration was not supported.

The unexpected lack of disc annulus tissue changes in this rat tail model despite substantial disc space loss at the intervention levels may reflect rapid adaptive changes, and also may be associated with differences in the cell population of young rodents compared with human discs [16]. There are limitations in using the rat disc as a model for human discs: notably, the presence of notochordal cells and

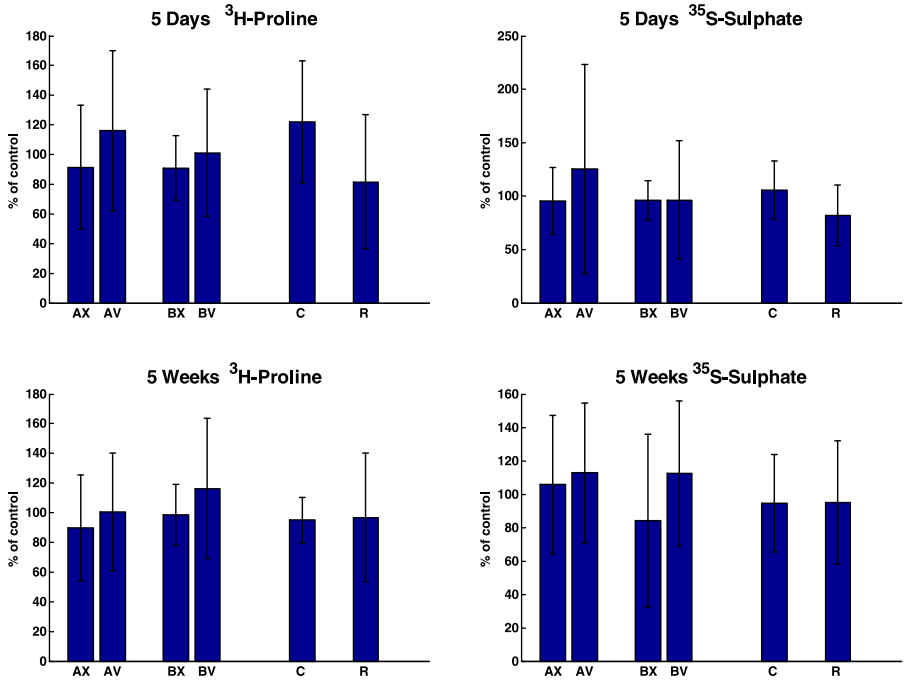

Fig. 6. Incorporation of tritiated proline and of $Na_2{}^{35}SO_4$ after 5 days and 5 weeks in 4 groups of animals (A, B, C, and R). For Groups A and B, X = disc tissue on the convex side of the tail; V = disc tissue on the concave side of the tail. Values for each animal were normalized to the mean value of adjacent (internal control) discs and expressed as a percentage.

the ossified end plate that may alter the nutritional status of the disc, although we studied only annulus tissue here. Because of different disc cell phenotypes, the young rat disc may be able to avoid tissue degenerative changes that occur in adolescent humans and older rats. However, our young rats underwent substantial loss of disc space and wedging, and the intent of the present study was to identify underlying mechanisms. In tails with imposed angulation, the within-animal control discs adjacent to the intervention level were used for comparison and included compensating curves and wedging that could influence asymmetry.

Changes in this rat tail model are induced as the result of an imposed spinal deformity, and the imposed altered mechanical environment included no non-mechanical factors that might be present in human scoliosis. The average amount of force imposed on the tails of Group C animals was typically 1 N at week 1 and increased to 2.2 N at week 5 (to maintain the nominal 0.1-MPa stress on the disc). Thus, it was just less than body weight (125 g) at week 1 and about half body weight (440 g) at week 5.

Disc narrowing [6] and alterations in composition [5] in older animals have been reported in axially loaded (compressed) rat discs, and complete immobilization has been associated with disc degeneration in older rats [5,7] and in fused canine spines [17-19] and articular cartilage [20]. This suggests that reduced mobility, rather than complete immobilization, may be a factor in disc degeneration in human scoliosis, and may explain why expansion thoracoplasty has been associated with disc degeneration in young patients [21].

In humans, altered disc composition was reported in patients with idiopathic scoliosis, as well as those with scoliosis associated with cerebral palsy, and was thought to be the result of the spinal curvature, not the cause of it [22]. Differences in hydration and biosynthetic activity in discs of humans with scoliosis were attributed to an ineffective response to a pathological mechanical environment [23]. In human adolescents with scoliosis, magnetic resonance imaging revealed widespread changes in discs with minimal wedging [24]. Excised tissue had decreased water content and decreased cell density, correlating with increasing severity of curves, and end plate damage and alterations in diffusion were observed earlier than magnetic resonance imaging changes, which suggests that degenerative changes were primarily the result of nutritional factors. End plate calcification has been observed in discs of humans with scoliosis [25] and in a porcine model of induced scoliosis [26], and may contribute to nutritional deficits.

MacLean et al. [7,27] reported altered gene expression in rats older than 12 months. In the annulus, both full immobilization (for 72 hours) and dynamic compression (1 MPa at 0.2 Hz) down-regulated anabolic genes and up-regulated catabolic genes [7], with immobilization apparently contributing the largest effect. Dynamic loading at 0.2 MPa had little effect on gene expression [27]. The compression magnitude in the present study was 0.1 MPa, but was sustained over a 5-week period. Thus, discs in the present study that were subjected to much lesser stress sustained for longer duration and in younger animals (with presumably higher

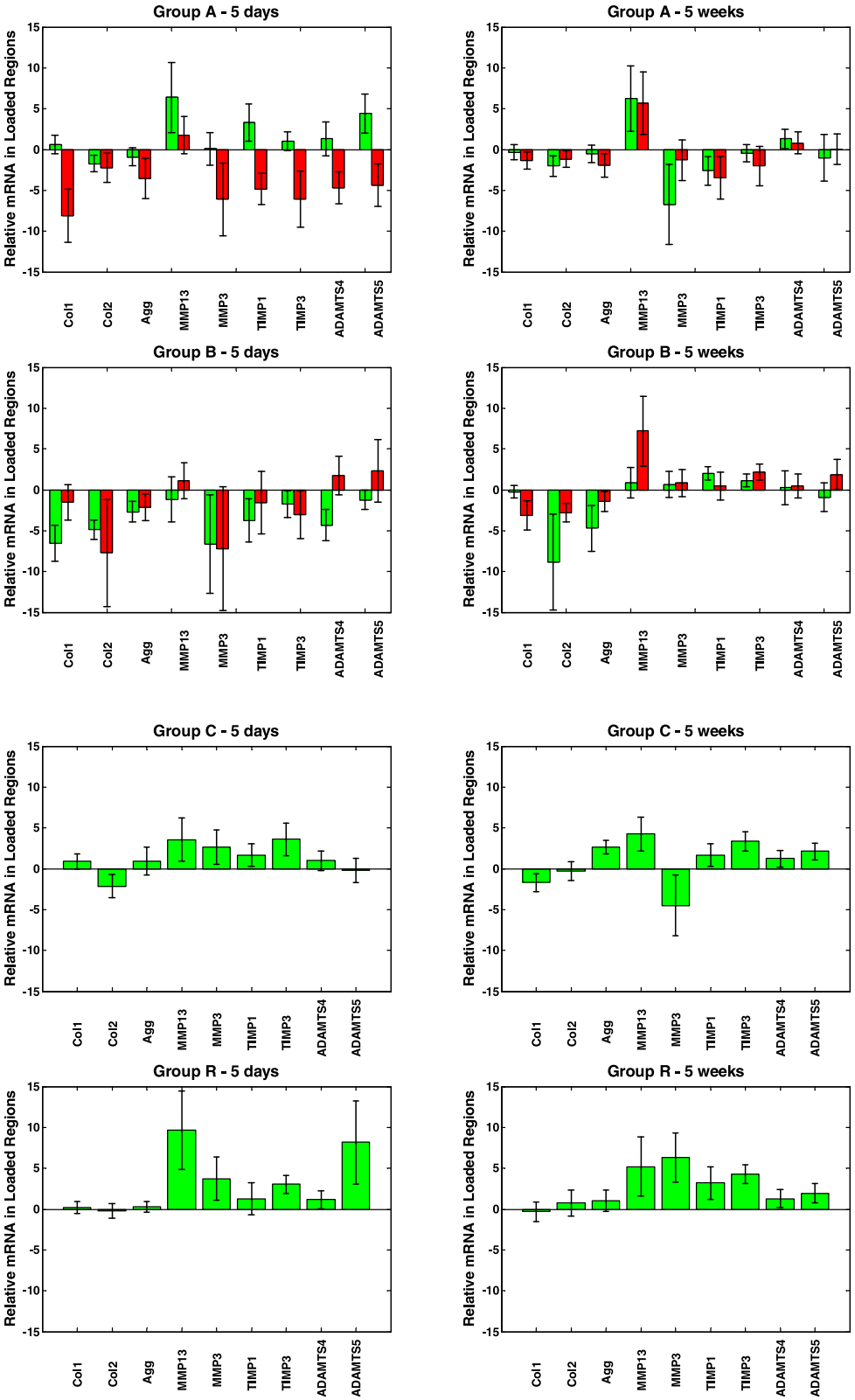

Fig. 7. Gene expression for collagen 1, collagen 2, aggrecan, matrix metalloproteinase 13 (MMP13), matrix metalloproteinase 3 (MMP3), tissue inhibitor metalloproteinase 1 (TIMP1), tissue inhibitor metalloproteinase 3 (TIMP3), ADAMTS4, and ADAMTS5 by real-time reverse-transcription polymerase chain reaction after 5 days and after 5 weeks in Groups A, B, C, and R (mean values and standard deviations). Values are expressed exponentially as fold change, relative to within-animal controls; hence on the vertical axis, 0 = no difference, 1 = doubling of messenger RNA, 2 = 4 times the messenger ribonucleic acid as in control, and so forth. For Groups A and B, the left column corresponds to disc tissue on the convex side of the tail, and the right column corresponds to tissue on the concave side of the tail.

metabolic activity), compared with discs studied by MacLean et al. [7,27] showed no evidence of catabolic response despite their large amount of disc narrowing and the vertebral wedging. Disc narrowing has also been reported in loaded rabbit discs [28], in immobilized rat tail discs [5], and mechanically stressed (overactive) rats [29]. Apoptosis and disc narrowing were reported in compressed skeletally mature mouse tail discs [30], and imposed bending produced cellular changes preferentially on the concave side [31].

This model of altered biomechanics of the immature intervertebral disc was intended to identify which components of the altered biomechanical environment were responsible for structural changes in the discs, especially tissue loss (disc narrowing). Because there were disc changes associated with all experimental interventions and reduced mobility was present in all interventions, reduced mobility could be a major source of disc changes and also a factor in progressive disc deformity in human scoliosis. However, the sparse evidence of compositional, biosynthetic, and differences in gene expression suggests that underlying metabolic processes were highly active in experimental discs, which counters the experimental interventions in this model. The exact relationship of this animal model to intervertebral discs in human scoliosis, in terms of both biomechanical and metabolic differences, remains to be fully explored.

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