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Effect of Tail Suspension (or Simulated Weightlessness) on the Lumbar Intervertebral Disc

Study of Proteoglycans and Collagen

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Study Design. An experiment to measure the proteoglycan and collagen content of the lumbar intervertebral discs of rats that had been tail-suspended for up to 4 weeks.

Objectives. To determine the effect of tensile force (or simulated weightlessness) on the intervertebral disc.

Summary of Background Data. During space flight the intervertebral disc experiences low compressive force (because of so-called "weightlessness"), which, in turn, produces, among other things, low hydrostatic pressure acting on the disc cells. Although disc cells respond (*in vitro*) to changes in hydrostatic pressure, it is unclear what effect low levels of hydrostatic pressure have *in vivo* and whether they lead to a degenerative catabolic process. The rat tail-suspension model is appropriate for studying the effects of tensile force on the disc. The disc (especially the anulus) is subjected to tension during various body movements (*e.g.*, bending stretches the posterior anulus, and twisting tensions the whole anulus).

Methods. Thirty-two Sprague–Dawley rats were tail-suspended for either 2 weeks (16 rats) or 4 weeks (16 rats). Sixteen other rats were left unsuspended for 4 weeks; these were used as controls. At the end of 2 or 4 weeks, as appropriate, the rats were killed and their lumbar spines were removed. In each rat the six lumbar discs were bisected and the discs (anulus and nucleus together) were carefully removed. The six lumbar discs from one rat were pooled with the six lumbar discs of a second matching rat (*i.e.*, from the same group) to give one sample. The disc samples were then assessed using enzyme-linked immunosorbent assays.

Results. There was a 35% statistically significant decrease in proteoglycan content going from the control group down to the 4-week group, but no significant differences between the control group and the 2-week group or between the 2-week group and the 4-week group. There were no statistically significant differences between the three groups for collagen I or collagen II.

Conclusions. These findings clearly establish a link between decreased proteoglycan content and tension on the disc, as modeled by the tail-suspended rat. [Key words: collagen, proteoglycan, rat, tail, tension, space flight, weightlessness] **Spine 2002;27:1286–1290**

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It has been reported that astronauts experience back pain (low back pain in particular) during and after space-flight.[6,21] Although the intervertebral disc has not been shown specifically to be the source of the pain in astronauts, it is certainly a source of low back pain in those of us earthbound. During space flight the intervertebral disc experiences low compressive force (because of so-called "weightlessness"), which, in turn, produces, among other things, low hydrostatic pressure acting on the disc cells. Although disc cells respond (*in vitro*) to changes in hydrostatic pressure, it is unclear what effect low levels of hydrostatic pressure have *in vivo* and whether they lead to a degenerative–catabolic process that could generate pain in humans.

There is some literature on the effects of space flight and simulated space flight on the musculoskeletal system.[8,12,20] Space flight can cause a loss of muscle mass,[14] disrupt bone growth, induce bone loss, and result in weakened bone material.[2,19] Space flight has also been shown to produce decreased expression of some muscle and bone-specific genes.[1] Although a short period of space flight (8 days) does not seem to produce any gross irreversible changes to the disc (in terms of disc area and lumbar length),[13] it is not clear what the biochemical effects are with longer missions. Further, little is known about bone remodeling and tissue repair during space flight: What happens if an astronaut suffers a vertebral fracture or disc damage while on a long mission in space? Will weightlessness inhibit repair?

With the above in mind, we decided to carry out an experiment to test the hypothesis that tail-suspending rats for up to 4 weeks causes changes in the proteoglycan and collagen content of the lumbar intervertebral discs. This model has been used many times before in experiments on simulated weightlessness.[3,7] Dehority et al,[3] for example, promote this as a means of simulating the loss of gravitational loading on the skeleton. Maynard[15] used it in studies on the effect of weightlessness on the intervertebral disc. The rat tail-suspension model has two advantages: 1) it is, to date, an accepted animal model to simulate weightlessness (*e.g.*, Garber et al[7]), and 2) it is a good model for studying the effects of tensile force on the disc. The disc (especially the anulus) is subjected to tension during various body movements (*e.g.*, bending stretches the posterior anulus, and twisting tensions the whole anulus). Thus, although the model has been used before, as a means of simulating weightlessness on the intervertebral disc, it is not ideal because it applies tensile

Table 1. Average Results of ELISA for Proteoglycan, Collagen I, and Collagen II for the Three Groups of Rats

	No. of Samples	Collagen I	Collagen II	Proteoglycan
Control	8	0.17 ± 0.05	0.18 ± 0.07	0.098 ± 0.02
2 weeks	8	0.16 ± 0.05	0.16 ± 0.09	0.08 ± 0.02
4 weeks	8	0.15 ± 0.05	0.15 ± 0.03	0.064 ± 0.03

Values are given as μg of epitope/mg of dry tissue weight (mean ± SD).

force (and reduces the hydrostatic pressure inside the disc) rather than just remove gravitational load.

■ Materials and Methods

Animals. After Institutional Animal Care and Use Committee approval had been obtained, 32 female Sprague–Dawley rats (~250 g in weight and approximately 3–3.5 months old) were tail-suspended for either 2 weeks (16 rats) or 4 weeks (16 rats). Sixteen other rats were left unsuspended for 4 weeks; these were used as controls. We only used control rats at the 4-week time point in order to limit the number of rats, and because we also considered our main comparisons to be between 4 weeks suspended and 2 weeks suspended, and between 4 weeks suspended against 4 weeks control. At the end of 2 or 4 weeks, as appropriate, the rats were killed and their lumbar spines were removed. In each rat the six lumbar discs were bisected and the discs (anulus and nucleus together) were carefully removed. The six lumbar discs from one rat were pooled with the six lumbar discs of a second matching rat (*i.e.*, from the same group) to give one sample. We pooled the material for two reasons: 1) to reduce variation, and 2) to obtain a good amount of material for analysis. Thus, each number shown in *Results* (Table 1) represents pooled tissue from 12 lumbar discs harvested from two rats (*i.e.*, N = 8 for statistical comparisons). The pooled discs were then assessed using enzyme-linked immunosorbent assays (ELISA).

Hind Limb Suspension. The experimental rats were placed in modified individual mesh-bottom rabbit cages in hind limb suspension. Each rat was suspended with tape wound around the length of the tail, so that the hind limbs could not contact the cage floor (Figure 1). The maximum angle of the body of the rat to the horizontal was 40° head-down. The tape attached to the tail was connected to a pulley linked to a metal arm attached to the center of the top of the cage. Rats suspended in this way had 360° rotational mobility with full access to the corners of the cage, and to food and water. A radiograph was taken of a suspended rat to check that the lumbar spine was in the correct alignment (*e.g.*, no hyperlordosis). The rats were checked twice daily to ensure that they were hanging correctly and that they had adequate food and water. Observation of the rats revealed that the suspended rats moved about their cages just as readily as the nonsuspended rats. In three rats we attached a spring balance (range 0–500 g) between the swinging arm on the top of the cage and the tail of the rat. This allowed us to observe the tensile force being applied down the length of the tail and through the lumbar spine as the rat moved about the cage. This value ranged from about 150 g (when the rat was stationary) to 450 g (when the rat propelled itself along using its front paws). Assuming that the rat moved about for a third of the day, and was still (or sleeping) for the other two thirds, then the average pull on the rat's tail was 250 g (2/3 × 150 + 1/3 × 450 = 250 g) over a 24-hour period.

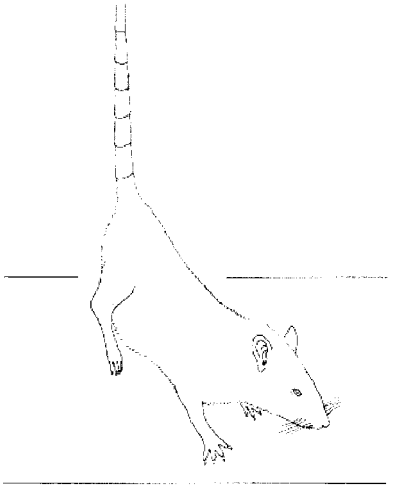

Figure 1. The tail-suspended rat. The rat's hind limbs are off the cage floor. The rat is suspended by a pulley system that allows it access to the corners of the cage and to food and water.

ELISA. For the ELISA the disc material was stored at −80 C before extracting the proteoglycans and collagen. The tissues were added to 0.05 mol/L sodium acetate buffer (pH 6.0) containing 4.0 mol/L guanidinium chloride and the protease inhibitors (0.01 mol/L ethylenediaminetetraacetate, 1.0 mmol/L phenylmethylsulfonyl fluoride, and 1.0 mmol/L benzamidine [15 vol times the dry tissue weight]) and stirred for 48 hours at 4 C. The solution was centrifuged at 15,000*g* at 4 C for 30 minutes. The supernate and the nonsolubilized material (pellet) were separated. The supernatant contained the proteoglycan while the pellet contained the collagen. The supernatant was dialyzed against 1000 volumes of sterile water overnight and lyophilized to dryness. The pellet was washed extensively with sterile water before continuing with the collagen extraction.

Direct ELISA was carried out as described by Rennard et al.[17] All samples were assayed in triplicate. For the proteoglycans, standard curves were established by adding increasing concentrations of a proteoglycan monomer D1 preparation from bovine nasal cartilage (provided by Dr. Tyl Hewitt, National Institutes of Health, Bethesda, MD) dissolved in 0.02 mol/L carbonate buffer (pH 9.2, containing 0.02% sodium azide) to each well in high-protein binding microtiter plates. The source for the antiproteoglycan antibody, MAB 2005, was Chemicon International (Temecula, CA).

For the samples 100 μL of diluted extracted proteoglycan preparation (between 1:1 and 1:100,000 dilution) was added to each well in the microtiter plate and incubated overnight at 33 C. The plates were washed with phosphate-buffered saline containing 0.5% Tween 20 (PBS-T) and unreacted sites were blocked with 2% bovine serum albumin followed by incubation at room temperature for 1 hour. After being washed with PBS-T diluted antiserum (1:2000) was added to each well in 100-μL aliquots. The reaction mixture was incubated for 2.5 hours at room temperature. The plates were washed with PBS-T, incubated for 1.5 hours at room temperature with biotinylated anti-MAb, washed again, and incubated for 1 hour with avidin-conjugated alkaline phosphatase (Vector Laboratories, Inc., Burlingame, CA). Color was developed with the

substrate *p*-nitrophenyl phosphate for 30 minutes before the reaction was stopped with 3 N NaOH. The color was quantified by measuring the absorption difference at 405 nm/630 nm and using an El_x 800-microplate reader (Bio-Tek Instruments, Winooska, VT).

Collagen was extracted from the pellets by adding 10 mL of 0.2 mol/L NaCl and 0.5 mol/L acetic acid containing 1 mg/mL of pepsin (Sigma Chemical). The suspension was stirred for 72 hours at 4 C. The pH was then adjusted to 8.0 with 5 mol/L NaOH. After standing overnight, the suspension was centrifuged for 30 minutes at 15,000*g*. The supernate, which contained the collagen, was removed, dialyzed against sterile water, and lyophilized.

Standard curves for Type I and Type II collagens (Sigma Chemical) were established by serially diluting a stock solution (100 μg/mL stock) in 0.02 mol/L carbonate buffer, pH 9.2, containing 0.02% sodium azide, and adding 100 μL to each well in the microtiter plate. The plates were incubated overnight at 33 C. The plates were washed with PBS-T, and unreacted sites were blocked with 0.2% bovine serum albumin in PBS-T, followed by incubation at room temperature for 1 hour. After washing with PBS-T, either rabbit anti-collagen I or rabbit anti-collagen II 1:200 dilution (Accurate Chemical and Scientific, Westbury, NY) was added to each well in 100-μL aliquots, followed by incubation at room temperature for 2.5 hours. The remaining assay was carried out as described for the proteoglycan ELISA.

To quantitate the results, linear regression plots were made for each standard and each sample. In all cases the linear regression plots of the samples were extrapolated from corresponding values at the same absorbance as the standards. The concentrations were expressed at micrograms epitope per milligram of extracted proteoglycan or collagen.

Statistics. An analysis of variance (ANOVA) was used to compare the average proteoglycan, collagen I, and collagen II contents between the three groups: control, 2 weeks, and 4 weeks. Similarly, an ANOVA was used to compare the ratio of proteoglycan to collagen I, and proteoglycan to collagen II between the three groups: control, 2 weeks, and 4 weeks. A linear contrast was used to test whether there was a significant trend in the results for proteoglycan, collagen I, and collagen II. Pairwise comparisons between each of the three groups for each outcome, proteoglycan, collagen I, and collagen II, was performed only if the overall test was statistically significant at a Type I error of 0.05.

■ Results

The rats tolerated the hind limb suspension quite well. None broke free. We had previously worked out the correct methodology for hanging the rats.[7] There were no areas of pressure ischemia on the tail. The body weights of the rats were stable over the 4-week period.

The three comparative groups were the control group (the 16 rats that were not suspended) plus the two experimental groups (the rats that were suspended for either 2 weeks [16 rats] or 4 weeks [16 rats]). The average results are shown in Table 1. For proteoglycan there was a statistically significant difference between the average values of proteoglycan between the three groups ($P = 0.035$). In addition, there was a statistically significant declining trend from the control group, to the 2-week group, to the 4-week group ($P = 0.01$). There were statistically significant pairwise differences between the control group and the 4-week group ($P = 0.01$) but not between the control group and the 2-week group ($P = 0.16$), or between the 2-week group and the 4-week group ($P = 0.20$). One particularly notable result from Table 1 is the 35% statistically significant decrease in proteoglycan content between the control group and the 4-week group (0.098 for control with 0.064 for 4 weeks).

There were no statistically significant differences between the three groups for collagen I ($P = 0.62$), collagen II ($P = 0.58$), the ratio of proteoglycan to collagen I ($P = 0.34$), and the ratio of proteoglycan to collagen II ($P = 0.27$).

Histologic staining (hematoxylin and eosin and safranin-O) revealed no abnormal difference in the cellular and tissue organization of the discs between the control group and the two experimental groups.

■ Discussion

The main results of this experiment are as follows: 1) there was a significant declining trend in proteoglycan content going from the control group, down to the 2-week group, down to the 4-week group; 2) there was a significant pairwise difference between the control group and the 4-week group for proteoglycan; and 3) there were no significant changes seen in the results for collagen I and collagen II. These findings clearly establish a link between decreased proteoglycan content and tension on the disc, as modeled by the tail-suspended rat. However, it should not be forgotten that the model also produces decreased hydrostatic pressure inside the disc and that the rats were inverted for 2 or 4 weeks, and they may have had changes in blood supply to their intervertebral discs, which could that have influenced our results.

The proteoglycan decrease of approximately 35% (Table 1) is quite remarkable for an experiment lasting only 4 weeks. What could be causing these effects on proteoglycan?

1. It could be that the disc cells are responding to increased tension and/or lower values of hydrostatic pressure inside the disc, and so producing less proteoglycans. It has already been shown in an *in vitro* experiment that the disc cells from dogs respond to lower values of hydrostatic pressure by producing less proteoglycans.[11] In another *in vitro* experiment using human disc cells, Handa et al[9] suggested that low pressure caused decreased proteoglycan synthesis and increased MMP-3 production (MMP-3 can degrade proteoglycan).
2. The nutritional supply to the disc could be impaired because of the lack of normal diurnal changes in compressive load on the disc. There are low levels of compressive load acting on the disc during overnight bed rest and high levels acting on the disc during daily

activities; these pressure changes cause fluid to flow into and out of the disc. The fluids that flow into the disc carry nutrients that are necessary to the disc cells, and the fluids that flow out carry waste products.

The fibrous framework of the intervertebral disc is built from fibrils of collagen I and collagen II.[5,18] These two collagen types are distributed radially in opposing concentration gradients. It is considered that Type II is in the inner anulus and in the nucleus pulposus, and Type I is most concentrated in the exterior of the anulus fibrosus, where it provides the tough lamellar sheets that are anchored into the bone of adjacent endplates. It is interesting that both collagen I content and collagen II content were reduced (although not significantly) in the tail-suspended groups. These changes in both collagens may suggest that the tensile forces mediated the biologic response throughout the disc instead of having a predominant effect on the anulus or the nucleus.

Although we did not directly measure the tensile force across the disc in our model, we can make an estimate based on the following. Using a digital caliper we measured the cross-sectional area of a rat lumbar disc. This gave the area of the anulus as approximately 0.1 cm^2. We assumed that the average tensile force exerted down the length of the rat's tail is 250 g (*Materials and Methods*) and that 100 g is carried by the anulus. This assumption is based on the fact that the muscles and the ligaments will bear the greatest portion of the tensile force (although this balance will change somewhat when the rat is sleeping or extremely active). Thus, the "estimated" average tensile stress exerted on the anulus during a diurnal cycle is approximately 0.1 MPa ($100 \times 9.81 \times 10{,}000$)/($1000 \times 0.1$ 1,000,000). This figure of 0.1 MPa is well below the measured failure stress of the anulus fibrosus as measured in humans (1–3 MPa).[4] However, it should be noted that the disc cells may respond more to peak stress or frequency of stress change, rather than average stress.

In our model we exerted a tensile force on the whole disc for a period of up to 4 weeks. Tensile forces are exerted on the disc during many movements. When we bend forward the posterior anulus sees stretch and tension. When we rotate our upper body the anulus twists and deforms to exert tensile stresses on the anular fibers. When we compress the disc it bulges and the anular fibers go into tension. The results from our experiment show that prolonged tension brings significant decreases in proteoglycan content and decreases (although not significant) in collagen I and collagen II content.

Some of the other results that are not significant are still worth noting (although with some circumspection). There were declining linear trends in collagen I and collagen II going from the control group, down to the 2-week group, down to the 4-week group. There were declining linear trends in both the ratio of proteoglycan to collagen I, and proteoglycan to collagen II going from the control group, down to the 2-week group, down to the 4-week group. If we had increased the number of rats (>16) or if we had continued the experiment for a longer period of time, then these trends may well have become statistically significant.

How relevant is our tail-suspension model to space travel and microgravity and the effect on the lumbar spine? Although this model has been used before in studies on the lumbar discs,[15] it suffers from a lack of true simulation: it pulls on the spine. However, in the model's defense it does remove (or reduce considerably) the compressive load on the disc, and that simulates space travel. Pedrini-Mille et al[16] and Maynard[15] report on an analysis of lumbar anuli from five rats that were in space flight for 14 days. They measured collagen using hydroxyproline content. As compared with controls, they found total collagen to be less in the flight group, but the collagen-to-proteoglycan ratio was found to be more in the flight group. These results are similar to our results; we found the collagen I and the collagen II to be less in the tail-suspended groups, and we also found the proteoglycan-to-collagen I ratio and the proteoglycan-to-collagen II ratio to be less in our 2-week and 4-week groups. These similarities in results tend to support the tail-suspended rat as a good model to simulate rats in space flight (with respect to the lumbar disc).

Astronauts (and cosmonauts) are known to get back pain, and although this may be an eventual consequence of a proteoglycan decrease in the disc, there are other possible causes. 1) If the disc is unloaded for a period of time, it will swell and remain swollen as the hydrophilic side chains of the proteoglycans retain the fluid. There will be little or no diurnal changes in fluid transport during space travel, and this will have implications for disc nutrition. 2) The prolonged tensile loading (particularly peak loads) could produce collagen damage and discogenic or other related pain. 3) The prolonged increase in disc height could cause discogenic pain, as the nerve fibers that penetrate the outer anulus feel mechanical strain. 4) Similarly, the apophyseal joint capsules will be stretched, and being well innervated could be a source of pain. Could it be that short-term (and reversible) back pain is due to disc height increases, whereas long-term unloading of the disc produces proteoglycan decreases and irreversible changes to the disc?

The question arises, in general, for those of us who are earthbound: Do body postures that produce tension in the disc cause long-term changes in proteoglycan content in particular? Bending, a frequently assumed body posture, produces tension in the posterior anulus, and it has already been shown that the posterior anulus in degenerated discs is more undersulfated as compared with other parts of the anulus.[10] Do these facts piece together a picture of repeated bending, especially in flexion, producing tension in the posterior anulus, causing a gradual undersulfation of the posterior anulus? Ebara et al[4] in a study of the tensile properties of anulus fibrosus found that the posterolateral regions of the anulus failed at

much lower values of stress as compared with the anterior regions of the anulus.

Similarly for astronauts, what could be the long-term consequences on the intervertebral disc of a long space flight? Clearly, a decrease in proteoglycans (with their hydrophilic side chains) could cause the disc to lose its ability to draw in its nutritional requirements and thus degenerate more quickly. Only prolonged space flights will reveal the true consequences of long periods of weightlessness and whether any unwelcome consequences are reversible or not. Both for bone cells and disc cells, understanding the effect of weightlessness lies in determining the mechanism by which cells respond to mechanical stimuli.

■ Key Points

- Three groups of rats were tail-suspended for up to 4 weeks to determine the effect of tensile force (or simulated weightlessness) on the lumbar intervertebral disc.
- There was a statistically significant decrease in proteoglycan content going from the control group down to the 4-week group.
- There were no statistically significant differences in collagen I or collagen II.

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