THE EFFECTS OF SPACE FLIGHT ON THE COMPOSITION OF THE INTERVERTEBRAL DISC

Jerry A. Maynard, Ph.D.
Department of Orthopaedic Surgery
University of Iowa

ABSTRACT

The lumbar annuli of rats flown on a COSMOS space flight were compared with those of three control groups and a ground antigravity tail suspension model. The wet and dry weights of the space flight annuli were significantly smaller than the three control groups. In addition, the collagen-to-proteoglycan ratio was significantly greater in the flight group due to a proportional increase in collagen and a decrease in proteoglycan. Finally, it appears microgravity may have altered the nature of the proteoglycan population as more proteoglycans leached from the annuli of flight animals than control animals when immersed in water.

INTRODUCTION

Intervertebral discs consist of three separate structures, the nucleus pulposus (NP), the annulus fibrosus (AF) and the cartilaginous endplates (EP). Each consists primarily of collagen, proteoglycans (PG) and water, but the extent of collagen and PG varies markedly among the three structures and is related to the function of each. The EP is hyaline cartilage and is key in absorbing axial loads and in facilitating the diffusion mechanisms necessary for nutrition of the NP and AF[1,10,15]. The AF is primarily composed of types I and II collagen embedded in a PG gel and the NP consists of a high concentration of PG and type II collagen. The NP attracts water and exerts a swelling pressure enabling it to support an applied load while the collagenous AF resists this expansion and enables the disc to sustain the rotation and shear forces associated with movement[7]. Under normal circumstances, the balance between swelling pressure and axial loading determines tissue hydration which relates to the functional and health status of the disc. Temporary changes in environmental conditions, e.g., weightlessness, may alter the composition of the disc which in turn may affect the state of hydration and therefore the ability of the disc to withstand axial loading upon return to normal loading forces.

The effects of weightlessness on the disc have not been well defined. Tyrrell et al.[16] reported fluid loss together with disc deformation as being primarily responsible for changes of nearly 1% in human height between morning and night. Thorton et al.[17] attributed fluid imbibition as being the most likely cause for the 5 cm increase in height associated with spaceflight. In order to investigate the problem further, in 1988 our laboratory was invited to participate in a joint NASA-COSMOS Biomedical Life Science Conference in Moscow to plan studies for upcoming COSMOS spaceflight missions. At this conference, it was arranged for us to receive intervertebral discs from animals to be flown on the COSMOS 2044 space flight September 29 to October 12, 1989. Ten male Czechoslovakian Wistar rats were flown on this 14-day flight. We received discs from five of these animals and their appropriate counter controls in order to determine the effects of weightlessness on the collagen, PG and water content of the AF.

MATERIALS AND METHODS

Mission and Animals.

Ten male Czechoslovakian Wistar rats were flown on the COSMOS 2044 Space Flight for 14 days. Intervertebral discs L3-4 to L6-S1 from rats numbered 6-10 were used in our studies. The Vostok vehicle was launched from the Plesetsk facility in the USSR at 9:30 a.m. on September 29, 1989. The biosatellite made 224 orbits at an inclination of 82.3° with an apogee of 294 km and a perigee of 216 km. During reentry, the animals experienced forces of 3-4G, and on landing the impact reached a maximum of 30G for a few milliseconds. During the flight, ambient temperatures were 23-26.5°C during days 1-11 and on days 12 and 13 for unknown reasons the temperature rose to a peak of 29.4°C. On day 14, the temperature returned to previous levels. The lighting regimen was controlled at 16 hours light to 8 hours dark commencing at 8 a.m. Control animals were represented by five animals from each of the following groups: basal (B), killed at the beginning of the flight; vivarium (V), housed in similar colony cages during the flight time period; and synchronous (S), exposed to simulated flight conditions. In addition, five tail-suspended (T) animals were compared with the flight (F) and control groups. Dietary and water intake were similar for all groups throughout the study.

Tissue Collection.

At landing, the F rats were transported to the dissection site approximately 30 minutes away by helicopter. The rats were killed by decapitation 3-11 hours after touchdown. The lower spinal segments were harvested, packed

in dry ice and immediately stored at -70°C. Following recovery of the F tissues, the control tissues were similarly harvested in Moscow and shipped to our laboratories in dry ice. Upon arrival to our laboratories, the L4-5 and L5-6 discs were isolated for biochemical studies and the L3-4 and L6-S1 discs from two animals in each group were used for ultrastructure studies. The L3-4 and L6-S1 disc from a third animal in each group was used for light microscopy and the remaining L3-4 and L6-S1 discs from the other two animals stored as backup tissue. The NP was reamed out from each disc with a 27 gauge syringe needle and all studies were conducted on the AF and the connecting EP.

Weight of Annuli.

The initial wet weights of the L4-5 and L5-6 annuli were recorded at the time of dissection (Time 0). Each annulus was then immersed in water for two hours and weighed at 30-minute intervals until a stable weight was reached. Each sample was then lyophilized and the dry weight determined. The hydrating medium was saved to determine the amount of PG leaching from the tissue into the imbibing media.

Light Microscopy.

The L3-4 and L6-S1 discs were thawed while being fixed in a solution containing 1.5% glutaraldehyde (0.05M cacodylate buffer), 1% buffered formalin and 0.5% ruthenium red for 24-48 hours. After fixation, the discs were embedded in paraffin, sectioned at 6 microns and stained with Safranin-O for PG content.

Electron Microscopy.

The discs for electron microscopy were thawed in 5 ml of cold (4°C) 2.5% glutaraldehyde (0.1M cacodylate buffer, pH 7.4) for 30 minutes. Each disc was then divided into anterior, transitional (the area immediately adjacent to the NP) and posterior segments. Small pieces consisting of several laminae were then stripped from each segment, processed with ruthenium red for collagen-proteoglycan relationships and embedded in Spurr embedding resin. Cross sections were examined at 20,000 magnification and longitudinal sections at 60,000 magnification for each sample using a Hitachi H-7000 electron microscope.

Biochemistry.

The L4-5 annuli from each animal were finely minced, lyophilized and digested with papain at 60°C for 24 hours. Aliquots of each digest containing 150-160 μg of dry tissue were hydrolyzed in 6N HCl at 130°C for four hours. After drying under vacuum, the hydrolyzates were dissolved in 1 ml of water, microfuged and filtered through 45 nm filters. Hydroxyproline was then determined with the use of 0.1 ml of the filtered hydrolyzates and 1 ml sample volume according to Woessner[21].

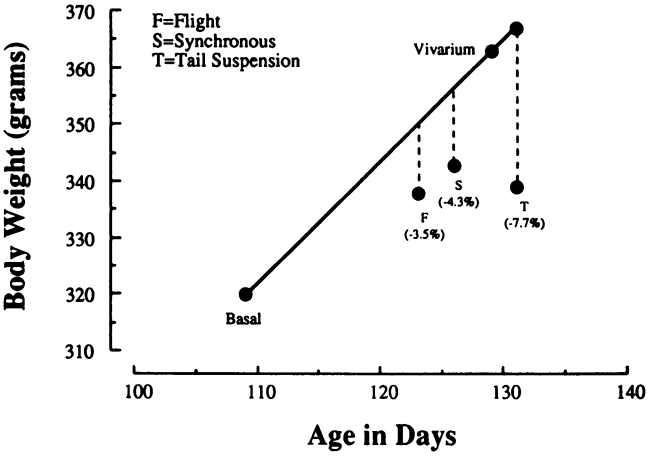

Figure 1.
Body weight-age comparisons of animals involved in COSMOS 2044. If a linear growth rate is assumed between the basal and vivarium groups, F animals are 3.5% below expected values, S controls 4.3% and T animals 7.7%.

RESULTS

Body Weights.

Due to the demands on the dissection team, the five groups of animals were killed on a staggered schedule. Hence, the B group, sacrificed at the start of the flight, constituted the youngest group and the T group, the oldest group. Assuming a normal linear rate of growth between the B and V groups between 109 and 129 days, the F group was 3.5% less, the S group 4.3% less and the T group 7.7% than predicted by the group curve (Fig. 1). The weights of the F and T groups were not significantly different from the weight of the S group ($p>0.05$).

Weight of Annuli.

For each animal, we determined the weights of the L4-5 and L5-6 annuli. The weights of the two annuli were not significantly different; therefore, we used their mean value (L4-6, Table 1) to compare groups. The wet and dry weights of the annuli from the three control groups were not significantly different from each other although the annuli from the youngest group (B) were consistently the heaviest. The annuli from the flight group were 20% to 25% smaller than the annuli from the three control groups ($p<0.03$), and also significantly smaller than the T group ($p<0.002$), Table 1.

Water Content.

The water contents of the annuli were similar among all animals and across all groups. At the time of dissection (Time 0), water represented 52% to 57% of the weight of

Table 1: Wet and Dry Weights of Annuli of Cosmos 2044 Rats

GROUPS		*BASAL*	*VIVARIUM*	*SYNCHRON*	*FLIGHT*	*SUSPENDED*
Age of Animals (days)		109	129	127	123	131
Weight of Animals (g)		320±5	363±5	343±17	338±5	339±21
Wet Weight of Annuli (mg)						
	L4-L5	12.2±0.5	10.3±2.1	10.3±3.1	7.7±1.1	10.3±1.7
	L5-L6	12.8+3.8	9.8±3.7	10.6±1.3	8.2±2.0	13.6±3.1
	L4-L6	**12.5±2.7**	**10.1±2.9**	**10.5±2.2**	**7.9±1.6***	**12.0±2.9****
Stable Time	L4-L5	21.1±2.4	18.7±4.4	16.5±2.9	15.0±1.5	15.1±1.8
	L5-L6	22.0±4.5	18.2±7.8	19.5±3.4	16.5±3.7	20.5±4.4
	L4-L6	**21.5±3.4**	**18.4±6.0**	**18.0±3.4**	**15.8+2.8**	**17.8±4.5**
Dry Weight of Annuli (mg)						
	L4-L5	6.0±1.5	5.0±1.0	4.7±1.3	3.6±0.5	4.5±0.9
	L5-L6	5.5±1.7	4.5±17.7	4.4±0.3	3.6±0.9	6.3±1.5
	L4-L6	**5.8±1.5**	**4.8±1.3**	**4.5±0.9**	**3.6±0.7***	5.4±1.5**

All Data = mean ± standard deviation
*Flight group significantly smaller than all control groups $p<.03$.
**Suspended group significantly greater than flight group $p<.002$.

the annuli. When immersed in water, the annuli imbibed water to a constant level representing 70% to 77% of the weight. No significant differences occurred among the groups. It therefore appears that weightlessness had a negative effect on the size of the annulus, but it did not impair the ability of the tissue to imbibe water, expressed as a percentage of the total weight, and to swell.

Light Microscopy.

No discernible difference in the light microscopic features of the IV discs as revealed by the Safranin-O reaction for PG was detectable among the five groups. In all specimens there was an intense Safranin-O reaction centrally in the NP and the adjacent transitional zones of AF. Near the periphery, the Safranin-O reaction decreased markedly, with the stain being confined to the pericellular areas (Fig. 2).

Electron Microscopy.

Collagen fibril diameters: Mean collagen fibril diameters were determined at the anterior, transitional and posterior regions of the L3-4 discs. One hundred fibrils in each location were measured from each specimen used for electron microscopy, and the fibrils were grouped using 20 nanometer increments ranging from 30 to 270 nanometers. In the anterior and transitional locations, the greatest percentage of fibrils were in the 70-90 nm (80) category in all groups except for the tail suspension group transitional area in which a slightly higher percentage of fibrils were found in the 50-70 (60) nm class. In the posterior regions, the greatest percentage of fibrils were in the 50-70 (60) nm class in all groups except for the vivarium group in which the peak percentage was found in the 90-110 (100) nm class (Fig. 3). Fibrils in the anterior locations exhibited the greatest overall range in diameters (30- 270 nm). If the frequency distributions are divided into small (<110 nm) and large (>110 nm) components, the anterior regions contain 50% to 70% small fibrils, the posterior area 75% to 90% small fibrils and the transitional area (except for the V group) 90% to 100% small fibrils (Table 2).

Table 2: Percent Collagen Fibrils Smaller than 100 nm in Anterior, Transitional and Posterior Regions of Lumbar Discs L3-4

	Anterior	*Transitional*	*Posterior*
	L3-4	L3-4	L3-4
Basal (B)	57	93	86
Vivarium (V)	68	65	82
Synchronous (S)	51	98	83
Flight (F)	67	99	75
Tail Suspension (T)	59	100	89

Collagen-proteoglycan relationships: Ruthenium red staining delineated electron dense PG particles in regular

periodic array along the collagen fibrils in all groups. The anterior region exhibited the largest fibrils and fewer particles compared to the transitional and posterior areas. Anteriorly the basal group appeared to have the greatest concentration of granules and the flight group the poorest. In the transitional and posterior regions, there appeared to be an increased density of granules accompanied by a decrease in collagen fibril size (Fig. 4). These areas also exhibited the most intense Safranin-O staining (Fig. 2).

Biochemistry.

Proteoglycans: It has been reported that PG leach from swollen annuli when placed in an imbibing media[18]. To determine the degree of "leaching out" of the annular PG during two hours of imbibition, we lyophilized the hydrating medium of each sample, redissolved it in 1 ml of water and determined the amount of hexuronate by the

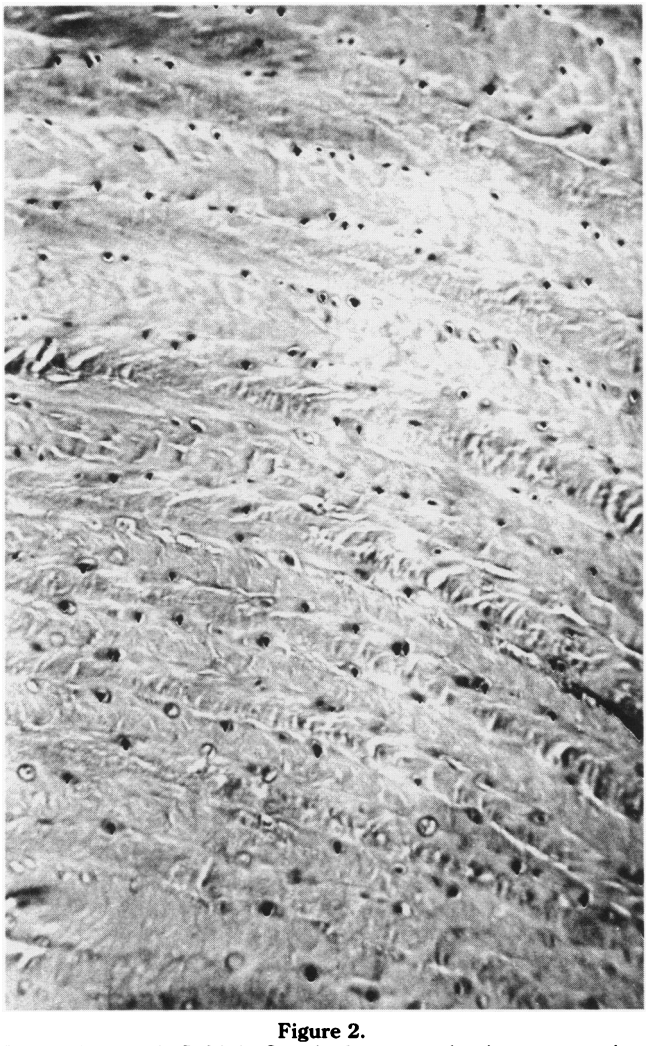

Figure 2.
Light microscopic Safrinin-O stain demonstrating intense reaction (red) near nucleus pulposus (NP) with decreasing intensity toward periphery (green), indicating a decreasing PG gradient from the NP to periphery. X90

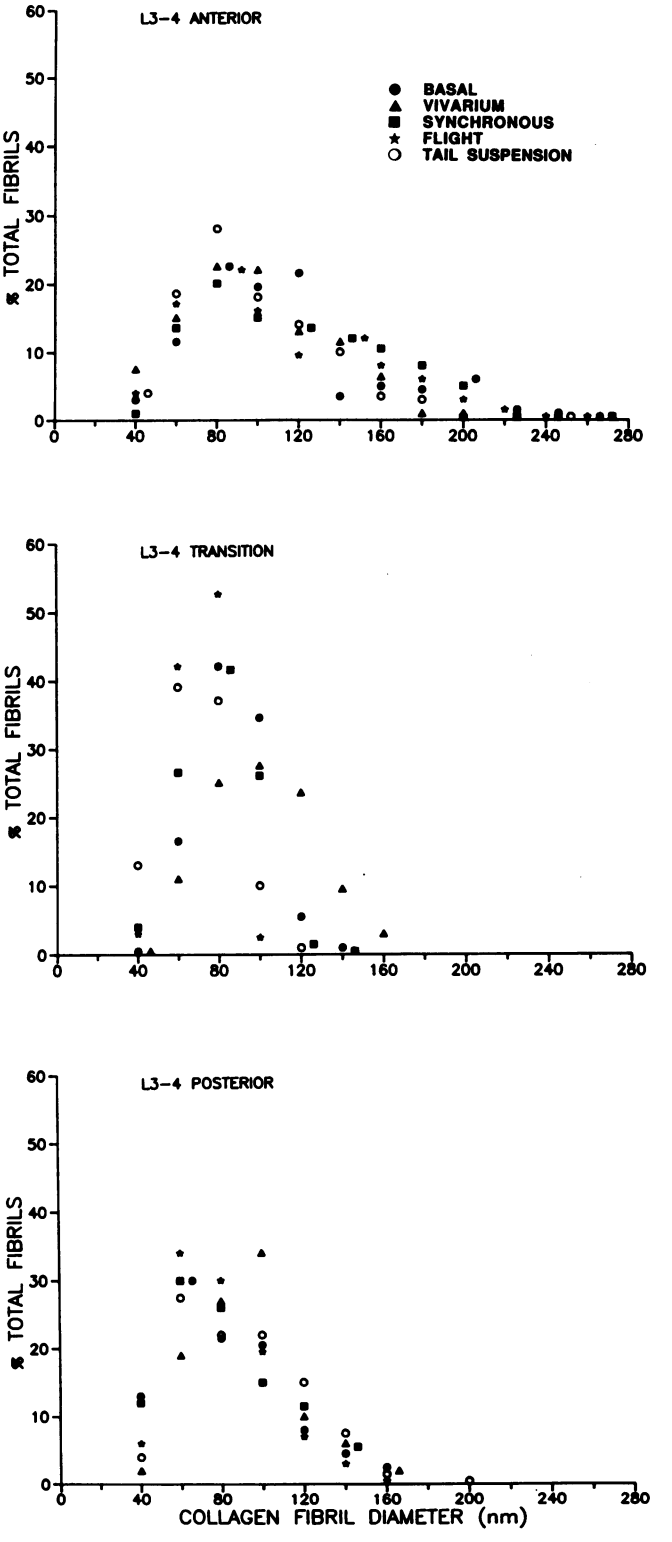

Figure 3.
Collagen fibril diameter frequency distributions in anterior, transitional and posterior regions of the L3-4 discs. Fibrils in the anterior regions exhibit the greatest range in diameters (30-270 nm) whereas transitional fibrils exhibit the smallest diameter range (30-170 nm).

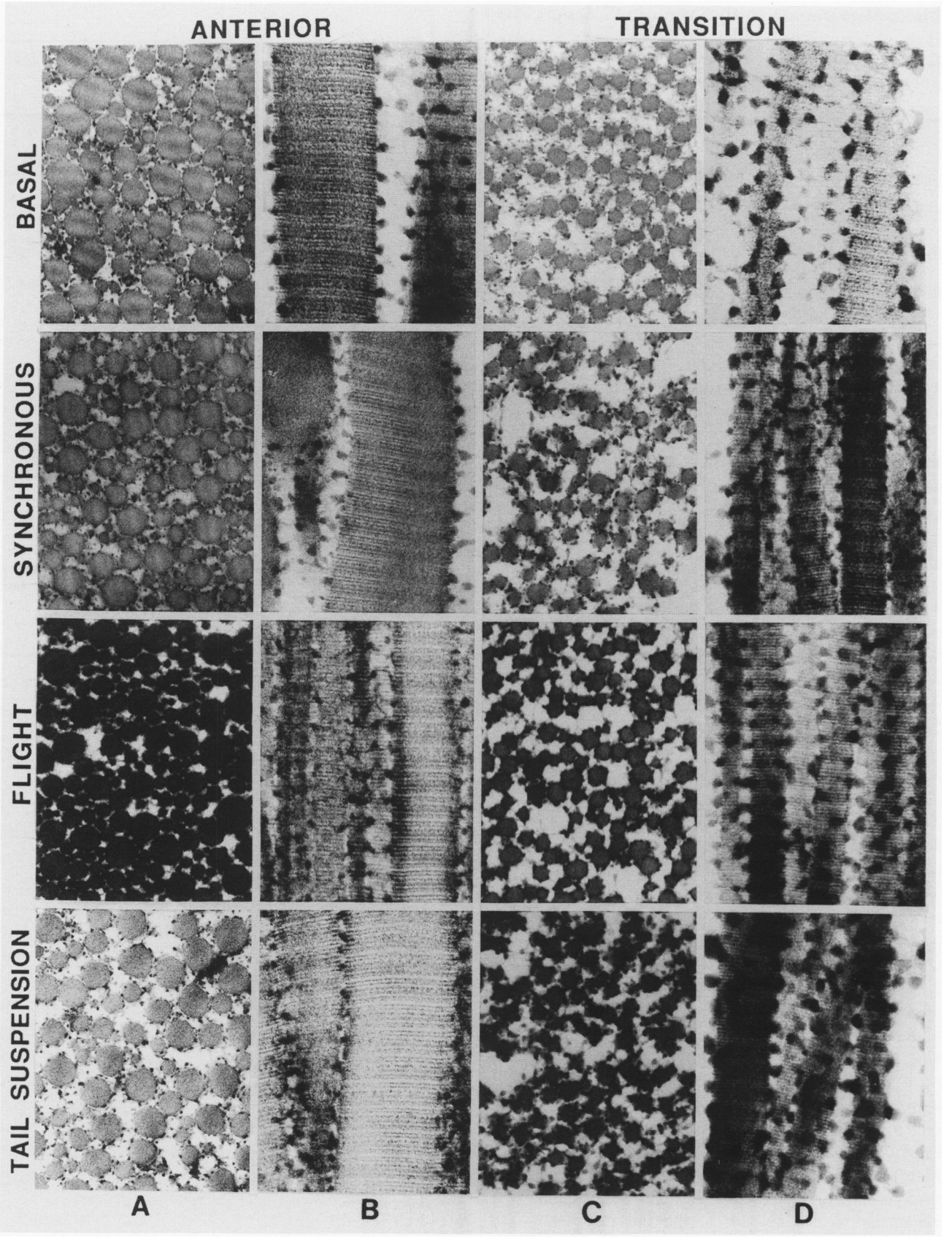

Figure 4.
Ruthenium red-stained electron-micrographs demonstrating fibril sizes and collagen-PG relationships in anterior and transit and areas from B, S, F and T groups. Transitional and posterior regions demonstrated an increased PG density and decreased fibril size. Cross-sections X 40,000, longitudinal sections X 90,000.

Table 3: Hexuronate in the Annuli of Cosmos 2044

GROUP	*TISSUE*	*MEDIUM*	*TOTAL*	*TISSUE*	*MEDIUM*
		(% Dry Tissue)		*(% of Total)*	
Basal (B)	(1.53 ± 0.17)	0.15 ± 0.07	1.68 ± 0.23	91.0 ± 2.7	9.0 ± 2.7
Vivarium (V)	1.53 ± 0.29	0.19 ± 0.03	1.72 ± 0.29	88.9 ± 2.4	11.1 ± 2.4
Synchronous (S)	1.94 ± 0.32	0.11 ± 0.05	2.05 ± 0.32	94.6 ± 2.7	5.4 ± 2.7
Controls (B+V+S)	1.67 ± 0.32	0.15 ± 0.06	1.82 ± 0.32	91.5 ± 0.32	8.5 ± 3.4
Flight (F)	1.35 ± 0.33	0.32 ± 0.14	1.67 ± 0.35	80.8 ± 8.2	19.2 ± 8.2*
Suspended (T)	1.35 ± 0.26	0.20 ± 0.10	1.55 ± 0.35	87.8 ± 3.3	12.2 ± 3.3**

All Data = means ± standard deviations
*Flight group significantly greater than control gorups combined (B+V+S), $p<.001$.
**Tail suspension group significantly greater than control groups combined (B+V+S), $p<.05$.

Blumenkrantz[2] method. The amount of hexuronate remaining in the tissue was determined by the same method after papain digestion of the tissue. The total amount of hexuronate originally present in the annulus was calculated from the amount of hexuronate in the imbibing media plus the amount in the tissue.

The annuli of the B and V groups contained nearly identical amounts of total PG while the annuli of the S group contained nearly 20% more hexuronate. This difference, however, was not statistically significant due to the small sample size. There was also no significant difference between the total hexuronate in the annuli of the F and T groups compared to the control groups individually or when the three control groups (B-V-S) were combined (Table 3).

The amount of hexuronate leaching out from the annuli of the control groups varied from 5.4% in the S group to 11.1% in the V group. The annuli of the F group released 19.2% of their hexuronate into the hydrating media. This was significantly greater ($P<.001$) than the amount released by the 15 animals in the combined groups (B-V-S). The amount of PG released by the annuli of the T group (12.2%) was also significantly greater ($p<.05$) than the amount released by the combined (B-V-S) control groups (Table 3).

Collagen: The mean hydroxyproline content of the three control groups (B-V-S) was 5.91 ± 0.80% of the dry weight with no significant difference between groups. The annuli of the F group were significantly more ($p<.02$) collagenous than the controls with hydroxyproline representing 6.93 ± 0.59% of the dry weight. The amount of hydroxyproline in the annuli of the suspended animals was very similar to the amount in the control tissues and significantly less ($p<.04$) than that of the F group (Table 4).

Table 4: Hydroxyproline in the Annuli of COSMOS 2044 Animals

	HYDROXYPROLINE (% Dry Tissue Wt)
Basal (B)	5.71 ± 0.35
Vivarium (V)	5.36 ± 0.45
Synchronous (S)	6.64 ± 0.91
Controls (B+V+S)	5.91 ± 0.80
Flight (F)	6.93 ± 0.59*
Suspended (T)	5.88 ± 0.71**

*Flight group significantly greater than control groups combined, $p<.02$
**Suspended group sig nificantly less than flight group $p<.04$

Collagen-proteoglycan ratio: Based on the assumption that hydroxyproline represents 14% of the collagen molecule and hexuronate 20% of the weight of PG, mean collagen-PG ratios were calculated for each group and for the three control groups (B-V-S) combined. The mean collagen-PG ratio of the F group (7.71) was significantly greater than the three control groups considered individually ($p<.04$) or combined ($p<.001$). The T group collagen-PG ratio (6.28) was significantly greater than the S group ($p<.05$) and the combined control groups ($p<.04$). No significant differences were found between any of the control groups nor between the F and T groups (Table 5).

Table 5: t-Test-p-Values Comparing Mean Collagen-PG Ratio Between Groups

GROUP	*MEAN + S.D.*	*B*	*V*	*S*	*F*	*T*
Basal (B)	(5.40 ± 0.47)		p<.69	p<.39	p<.02	p<.07
Vivarium (V)	(5.20 ± 1.44)			p<.69	p<.04	p<.19
Synchronous (S)	(4.97 ± 0.91)				p<.02	p<.05
Controls (B+V+S)	**(5.19 ± 0.96)**				**p<.001**	**p<.04**
Flight (F)	(7.71 ± 1.77)					p<.14
Suspended (T)	(6.28 ± 0.84)					

DISCUSSION

The 14-day spaceflight adversely affected the size of the annulus fibrosus. The wet and dry weights of the annuli from flight animals were significantly smaller than the three control groups and the tail suspended animals. The water content of the tissue expressed as percent of tissue weight was, however, the same in the flight and control groups. The smaller annuli in the flight group could result from a loss of tissue or from a failure to increase the size of the annulus through normal growth. We have no evidence to support either an increase in resorption or a decreased synthesis. The only applicable data, the weights of the annuli, are misleading because their decrease with age is probably related to the closure of the growth portion of the endplate and not to an actual loss of the annulus fibrosus. In previous studies of bone exposed to weightlessness, the reduction in bone has been related to a suppressed growth rate more so than to an increase in resorption[3,8,9,17,19,20,22]. It can be suggested that weightlessness might reduce the contact area between disc and blood supply from the vertebral body, thereby negatively affecting the nutrition of the disc and the metabolic activity of the cells as has been reported to occur in the fused sections of dog spines[6]. It is also plausible that weightlessness might induce hormonal changes that in turn might affect the metabolism of the disc. Further investigations will be necessary to clarify such possibilities.

The water content of the annuli of control and experimental rats at the time of dissection was between 52 and 57% of the weight of the tissue. This is considerably lower than found in 15 samples of human annuli of individuals between the ages of 4 and 76 years where water represents 70% ± 3% of the weight of the tissue[5]. The difference in the water content of rats and human annuli is most probably related to the amount of PG present in the two tissues, the human annulus containing 2.5 times more PG than the rat's annulus[5]. When the rat's annuli were excised from vertebral bodies placed in water for two hours, their water content increased to 70% to 77% of the weight of tissue while 8.5% of the PG leached out from the annuli of the control animals. Our data are consistent with the imbibition studies of human annuli of Urban and Maroudas[18] who demonstrated that 500 μm thick annular slices immersed in 0.015M NaCl for 70 minutes increase their water content by nearly 30% while loosing 15% of their PG to the hydration medium. Under similar conditions 45% of the PG leached out from the corresponding NP. The relatively small loss of PG from the annuli and the greater loss from the corresponding NP during a short imbibition time are related to differences in the structures of the PG and the collagen framework in the two tissues. The NP consists of randomly dispersed collagen fibrils of small diameter without any organized complex structure immersed in a highly hydrated gel of PG, 75% of which are in the monomeric form and easily extracted under associative conditions[14]. In the annulus, PG surrounds the collagen fibrils and nearly 50% of the PG is in the aggregated form which are effectively extracted only with a dissociative medium[14]. The significant increase in the amount of hexuronate leaching out from the annuli of the flight and tail suspended groups in comparison to the three control groups would seem to suggest that the experimental conditions had caused a change in the PG population. Weightlessness and tail suspension deprive the cells of the stimuli related to cyclic application of compressive forces and, like immobilization, might result in a reduced proportion of PG aggregates and an increased amount of PG extractable by water or low salt concentrations[11]. It seems reasonable to suggest that any PG abnormality caused by weightlessness or tail suspension might be entirely reversible, as it is in short-term joint immobilization, through the formation of a normal PG population induced by the return to normal gravitational conditions. In spite of the difference in the amount of PG extracted by immersion in water, ultrastructure examination of the tissues did not reveal an obvious difference between groups in the collagen-PG relationships demonstrated by ruthenium red staining.

If collagen fibrils are to have high tensile strength, they need to be large in order to maximize the possibility of intermolecular cross-links, but if the collagen network is to return to its original form after compression, the network must have sufficient collagen-ground substance interactions to inhibit nonrecoverable creep. This latter property can best be met with small fibrils[12,13]. Hence, tissues like the disc are comprised of a wide variety of collagen fibril sizes to meet both requirements. Our data indicate that within any single disc the largest fibrils are in the anterior region and a greater proportion of small fibrils is in the transitional and posterior regions. This suggests that in the rat, the anterior area might be submitted to higher tensile forces, whereas the transition and posterior regions sustain greater compressive forces. Interestingly, at the L3-L4 disc, only the tail suspension model demonstrated a larger mean fibril diameter in the posterior region compared to the transitional region. This raises the question as to whether altered spinal forces resulting from tail suspension contributed to an increase in posterior region collagen fibril size.

The annulus contains seven types of collagen (I, II, III V, VI, IX, XI), although types I and II represent more than 80% of the collagen and form the fibrous framework of the annulus[4]. Collagen represents 42% of the dry weight of the annuli of the three control groups. The smaller annuli of the flight group contain 49.5% collagen and therefore are significantly ($p<.02$) more collagenous than their normal counterparts. The slightly reduced PG content and the increased collagen content result in a mean collagen-PG ratio in the flight group significantly greater than each of the control groups individually ($p<.04$) and all control groups combined ($p<.001$). The collagen-PG ratio of the tail suspension model is also significantly greater than the ratio found in the controls ($p=.04$, Table 5).

If we consider the weight as well as the composition of the annuli, the biomechanical demands placed upon the annulus by nearly identical body weights (340 g) are sustained in the control groups by 455 μg PG and 2110 μg collagen and in the flight group by only 300 μg PG and 1781 μg collagen. This represents 34% less PG and 15% less collagen to sustain identical body weights upon return to 1 g and might affect the tensile stress of the disc, associated with the fibrous component, and/or the compression strength regulated by the ground substance (Fig. 5). Each annulus of suspended animals contains 418 μg PG and 2267 μg collagen, representing 8% less PG but 7% more collagen compared to the controls.

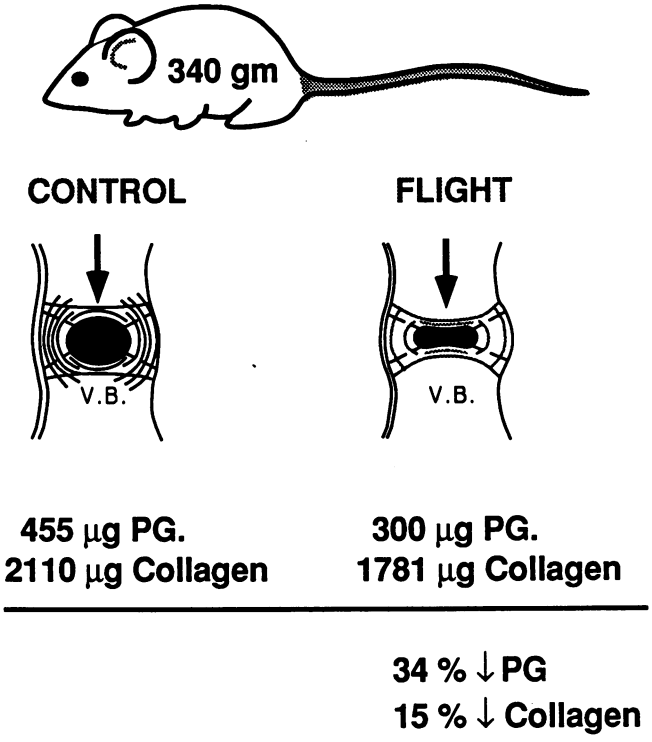

Figure 5.
Schematic diagram illustrating changes in flight animal intervertebral discs compared to control animals.

CONCLUSIONS

Our findings indicate that after 14 days weightlessness the annulus fibrosus is undergoing alterations of its matrix components as indicated by (1) a smaller size, (2) an increased collagen-proteoglycan ratio, (3) an increase in amount of proteoglycan leaching out of the tissue and (4) a proportional increase in collagen content. The tail suspension model does not produce a reduction in the size of the annulus, but the changes in the matrix of the suspended animals may indicate a similar but slower ongoing process that may eventually produce findings similar to those of the flight animals. Future research involving the annulus fibrosus should focus on whether prolonged weightlessness may predispose the disc to injury, whether the observed changes are reversible, and, if so, the time period required to restore the disc to its normal stature.

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