Molecular Biological Effects of Weightlessness and Hypergravity on Intervertebral Disc Degeneration

Di Wu; Chao Zheng; Ji Wu; Rongrong Huang; Xuanyu Chen; Tong Zhang; Lili Zhang

INTRODUCTION: The rate of intervertebral disc degeneration (IVDD) is influenced by environmental factors. Extracellular matrix (ECM) destruction and apoptosis of intervertebral disc cells are major characteristics of IVDD. ECM degradation is closely linked to up-regulation of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMP). This study aimed to elucidate the molecular biological changes during IVDD under conditions of weightlessness and hypergravity.

METHODS: A total of 120 rabbits were divided randomly into four groups: control group, weightlessness group, hypergravity group, and mixed (hypergravity + weightlessness) group. Tail-suspension was used to simulate a weightless environment, and an animal centrifuge (+7 G_z three times for 60 s) was used to mimic hypergravity conditions. After exposure to the above conditions for 30, 60, and 90 d, respectively, 10 rabbits were selected from each group for immunohistochemical determination of MMP-1, MMP-3, and TIMP-1 expression. TUNEL staining was also carried out to detect apoptotic cells in each group at each time point.

RESULTS: MMP-1, MMP-3, and TIMP-1 were rarely expressed in the control group, but were positively expressed in the other three groups. The strongest expression was in the mixed group at every time point, followed by the hypergravity group, and then the weightlessness group. Cell apoptosis index followed a similar trend to MMPs and TIMP-1 expression.

DISCUSSION: The results suggested that weightlessness and hypergravity may both aggravate IVDD over time, with hypergravity having a particularly marked effect.

KEYWORDS: Intervertebral disc degeneration, matrix metalloproteinase, tissue inhibitor of matrix metalloproteinase-1, cell apoptosis, weightlessness, hypergravity.

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Degenerative spinal disease may require pilots to stop training or be grounded, and may reduce noncombat activities. Intervertebral disc degeneration (IVDD) is the most common form of degenerative spinal disease, resulting in low-back pain.[7] Intervertebral discs are highly sensitive to mechanical stress and have been shown to undergo morphological and functional changes in response to changes in gravity. The rate of IVDD is approximately quadrupled in astronauts following spaceflight because of exposure to specific environmental conditions.[3]

IVDD is characterized by extracellular matrix (ECM) degradation and apoptosis of intervertebral disc cells. Intervertebral discs have a complex structure in which the nucleus pulposus (NP) is encapsulated by the endplates and the annulus fibrosus (AF). Discs usually degenerate both morphologically and biochemically from early childhood, especially in the NP. The ECM in intervertebral discs is normally in a state of equilibrium in terms of synthesis and metabolism. However, changes in the matrix component affect the disc through loss of intrinsic elasticity and reduced water content of the NP, affecting disc structure and causing IVDD. A reduction in intervertebral disc cells, mainly caused by cell apoptosis, is an important factor in IVDD. Excessive mechanical load is widely believed to be one of the most important initiators of disc cell apoptosis.[4]

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Matrix metalloproteinases (MMPs) are important components of the ECM and indispensable factors responsible for its degradation. Previous studies showed that MMP-1 and MMP-3 were significantly increased in degenerative intervertebral discs, and that the degree of degeneration was positively correlated with the levels of MMP-1 and MMP-3. Tissue inhibitor of MMP-1 (TIMP-1) is expressed at very low levels under normal physiological conditions. However, TIMP-1 levels increase when discs are subjected to excessive loading. TIMP-1 initially inhibits the effects of the load-induced MMPs, but cannot counteract continued increases in MMPs as a result of long-term loading, resulting in ECM and disc degeneration.[4]

Few studies to date have focused on the molecular biological changes occurring during IVDD under conditions of weightlessness and hypergravity.[3] The only way of achieving true weightlessness and hypergravity is by using rockets, space crafts, or in space labs such as on the International Space Station. However, performing experiments under these conditions is limited by the high costs and limited number of missions. We tested the effects of weightlessness and hypergravity on IVDD in rabbits using tail-suspension to simulate weightlessness and an animal centrifuge to mimic hypergravity conditions. We observed the effects of these conditions on expression levels of MMP-1, MMP-3, and TIMP-1, and on the apoptotic index (AI) in NP cells and cartilage endplate cells after short- (30 d), middle- (60 d), and long-term (90 d) exposure.

METHODS

Animals

A total of 120 skeletally mature male New Zealand white rabbits (mean weight, 3 kg; mean age, 7–8 mo) were divided randomly into four groups: control group, weightlessness group, hypergravity group, and mixed (hypergravity + weightlessness) group. Animals lost/died during the experiment were replaced to maintain the numbers required for statistical analysis. All experimental rabbits were fed with standard animal feed, with the regular addition of carrots and cabbage. The room temperature was maintained at 20–23°C, the photoperiod was 12 h light:12 h dark, the relative humidity was controlled at 40–80%, and the environmental noise was $<$ 60 dB. All experiments were performed in accordance with the guidelines of the Animal Care and Use Committee, the Air Force General Hospital of the Chinese People's Liberation Army, Beijing, China.

Equipment

Weightlessness was simulated noninvasively using a tail-suspension model.[4] Experimental animals were suspended by the tail with both hind legs off the ground and no weight bearing on the fore legs, using a soft cushion to protect the abdomen. The trunk angle was kept at 40–60° to ensure that the spine did not bear any load. Each rabbit was permitted to move freely using its forelimbs to access food and water.

A high-speed animal centrifuge (YL-001, Apos Optoelectronic Corp., Changchun, China) with a square metal mesh box at the end was used to simulate hypergravity conditions. The rotation radius of the centrifuge was 1.6 m. The experimental animals were fixed in the metal box and the centrifuge was operated at a maximum speed of +20 G_z (accuracy $\pm$ 0.1 G). This generated a sine curve of G_z and exposed the experimental animals to the specified hypergravity value (+7 G_z) in a parabolic form.

Procedures

Rabbits in the weightlessness group were maintained in the tail-suspension position for 5 d followed by 2 d of rest, continued for the duration of the experiment. Based on the gravity experienced by air force pilots during routine flights and previous hypergravity experiments in mice,[5] rabbits in the hypergravity group were exposed to +7 G_z three times for 60 s with 10 min intervals, repeated every other day. The mixed group was subjected to both weightlessness and hypergravity as above. Ten rabbits randomly selected from each group were sacrificed by air embolism after 30, 60, and 90 d, respectively. The whole lumbar vertebrae were removed and fixed with formalin. The L7/S1 intervertebral disc was chosen for analysis because this suffered the largest load and the most obvious change. The specimens were embedded in paraffin and sectioned for immunohistochemical staining of MMP-1, MMP-3, and TIMP-1, and for TUNEL staining to detect apoptotic cells. Experiments were conducted at the Air Force Institute of Aviation Medicine, Beijing, China.

Each sample was sectioned at 5 μm thickness and stained with hematoxylin–eosin for morphological assessment and determination of MMP-1, MMP-3, and TIMP-1 expression. The sections embedded in paraffin were deparaffinized and rehydrated and then microwaved in an antigen unmasking solution (0.01 mol $\cdot$ L^{-1} sodium citrate) for 10 min each. Next, endogenous peroxidase was inactivated by incubation with 3% H_2O_2 for 10 min. Then the sections were incubated with the primary antibody (anti-MMP-1, 1:100, anti-MMP-3, 1:100, and anti-TIMP-1, 1:200) for 30 min at 37°C. Finally, the sections were incubated with an appropriate secondary antibody (rat-antirabbit serum, Dako, Glostrup, Denmark). The chromogenic reaction was developed by incubation with 0.4 ml diaminobenzidine (DAB) and the sections counterstained with hematoxylin. The slides were observed under an optical microscope (BX51, Olympus Inc., Tokyo, Japan) equipped with an automated image-acquisition system (DP70, Olympus Inc.). Positively-stained cells were counted in three randomly selected high-power visual fields under a light microscope ($\times$400). The positive-expression rate was obtained by the formula: positive expression rate = number of positive cells / total number of cells $\times$ 100%.

The terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) method is a useful technique for measuring apoptotic cell content. After deparaffinizing and rehydrating, each section was incubated with the 20 μg/ml proteinase K free from DNase for 30 min at 37°C and then washed with PBS three times. According to the manufacturer's instructions, the NP and cartilage endplate cells were developed with

Streptavidin-HRP solution (Beyotime Institute of Biotechnology, Shanghai, China) and DAB, finally counterstained with hematoxylin. Apoptotic cells were counted in three randomly selected visual fields under an optical microscope and the AI was obtained using the formula: AI = number of apoptotic cells / total number of cells × 100%.

Statistical Analysis

All statistical analyses were performed with IBM SPSS, version 20.0 (IBM Corp., Armonk, NY). One-way analysis of variance was used to determine the significance of all variables among the four groups. Group differences were determined by least-significant difference post hoc tests. A P-value < 0.05 was considered statistically significant.

RESULTS

Positive expression of MMPs was indicated by brown–yellow or brown particles in the cytoplasm and blue-stained nuclei (**Fig. 1A–H**) [color figure available online]. MMP-1 and MMP-3 showed similar trends (**Table I**). There were few positive cells in the control group at all time periods (Fig. 1A, E), and there was no significant difference between the time points (MMP-1, $F_{(2,29)} = 1.41$, $P = 0.26$; MMP-3, $F_{(2,29)} = 1.11$, $P = 0.34$). Positive expression rates in the weightlessness, hypergravity, and mixed groups increased gradually with prolonged exposure time, with significant differences between time points in each group ($P < 0.001$). The strongest expression was in the mixed group at all time points (Fig. 1D, H), followed by the hypergravity group (Fig. 1C, G), and the weightlessness group (Fig. 1B, F).

Positive expression of TIMP-1 was indicated by brown granules in the cytoplasm (Fig. 1I–L). TIMP-1 showed a similar expression trend to the MMPs (Table I). There were few TIMP-1 positive cells in the control group at all time points (Fig. 1I). TIMP-1 expression was strongest in the mixed group, followed by the hypergravity and weightlessness groups (Fig. 1J–L). TIMP-1 expression in the control and mixed groups increased at 30 d, increased further at 60 d, and then weakened slightly.

The same second antibody (rat-antirabbit serum) was used to detect MMPs and TIMP and both showed positive expression in the cytoplasm, making it impossible to observe them directly on the same slide. We therefore compared their positive expression rates. Positive expression of MMP-1, MMP-3, and TIMP-1 was most significant in the mixed group (Table I). The ratios of MMP-1/TIMP-1 and MMP-3/TIMP-1 were similar at 30 and 60 d, but both increased at 90 d (Fig. 1M, N).

TUNEL staining indicated apoptotic cells in the NP and cartilage endplate regions by brown or dark brown particles, surrounded by nonapoptotic cells with blue-stained nuclei (**Fig. 2A–H**) [color figure available online]. The AI of intervertebral disc cells in the control group remained similar throughout the experiment (AI of nucleus pulposus cells, $F_{(2,29)} = 0.88$, $P = 0.43$; AI of cartilage endplate cells, $F_{(2,29)} = 2.27$, $P = 0.12$), while the AIs in the weightlessness, hypergravity, and mixed groups increased gradually ($P < 0.001$). AI was highest in the mixed group at all time points, followed by the hypergravity group, the weightlessness group, and the control group (Fig. 2I). The AI trends corresponded with the changes in MMP-1, MMP-3, and TIMP-1 expression (**Table II**).

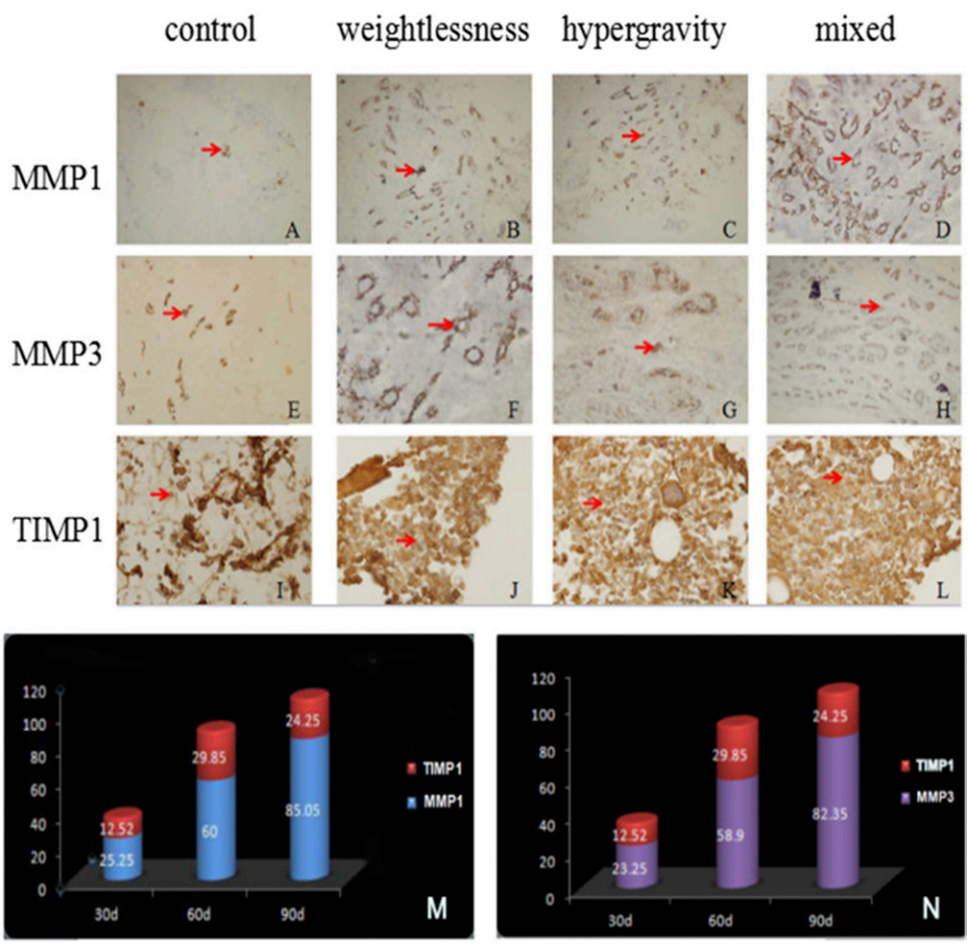

Fig. 1. A–L. Positive expression of MMP-1, MMP-3, and TIMP-1 observed by optical microscopy (×400) in each group after exposure for 90 d. Positive cells indicated by brown–yellow or brown particles in the cytoplasm and blue-stained nuclei (gray arrow in print/red arrow online). M–N. Ratios of MMP-1/TIMP-1 and MMP-3/TIMP-1 in the mixed group at each time point. [Color figure available online.]

Table I. Mean Positive Expression Rate (%) of MMP-1, MMP-3, and TIMP-1 in Groups During the Exposure Time; Standard Deviations Are Indicated in Parentheses.

GROUPS	30 DAYS	60 DAYS	90 DAYS	*F*-VALUE	*P*-VALUE
Control					
MMP-1	5.50 (0.84)	6.40 (1.05)	5.70 (1.33)	1.41	0.26
MMP-3	5.90 (1.37)	5.40 (0.96)	6.30 (1.63)	1.11	0.34
TIMP-1	2.70 (0.94)	2.80 (1.13)	2.00 (0.94)	1.85	0.17
Weightlessness					
MMP-1	11.6 (2.01)	34.6 (2.11)	57.9 (3.17)	862	< 0.001
MMP-3	12.0 (1.56)	35.2 (1.93)	58.7 (2.11)	1448	< 0.001
TIMP-1	6.40 (1.42)	16.4 (1.77)	26.5 (1.08)	475	< 0.001
Hypergravity					
MMP-1	20.3 (1.88)	50.8 (1.81)	72.1 (2.99)	1283	< 0.001
MMP-3	19.6 (1.26)	50.5 (1.84)	71.8 (2.61)	1746	< 0.001
TIMP-1	9.70 (1.49)	25.2 (1.75)	28.0 (1.49)	387	< 0.001
Mixed					
MMP-1	25.4 (2.45)	60.1 (2.13)	85.5 (4.06)	1008	< 0.001
MMP-3	24.3 (2.16)	61.2 (1.87)	84.8 (3.32)	1448	< 0.001
TIMP-1	12.3 (2.00)	29.3 (2.26)	22.3 (1.16)	209	< 0.001
F-value					
MMP-1	218.94	1519	1306		
MMP-3	249.87	2034	1892		
TIMP-1	75.2	435.25	1032.04		
P-value					
MMP-1	< 0.001	< 0.001	< 0.001		
MMP-3	< 0.001	< 0.001	< 0.001		
TIMP-1	< 0.001	< 0.001	< 0.001		

DISCUSSION

Based on the authors' review of the literature, this may be the first published study to focus on the effects of simulated weightlessness and hypergravity on IVDD. The tail-suspension model has been recognized as a reliable method for simulating weightlessness or microgravity.[2] We modified the method for rabbits, which have short tails, by suspending the tail and both hind legs and using a soft cushion to protect the abdomen. This technique maximized survival and thus allowed long-term observation of the experimental animals. We set the hypergravity conditions as +7 G_z based on the daily training load of pilots and the physiological capacity of the experimental rabbits.[5] The animals appeared to tolerate this exposure, with no signs of standing instability, decreased mobility, syncope, or death.

1. MMPs participate in both the synthesis and degradation of the ECM, thereby regulating intervertebral disc dynamics. MMP-1, also known as collagenase 1, is a collagenase that mainly degrades collagen I. Given that collagen I is the most abundant component of the matrix, MMP-1 plays a pivotal role in matrix degradation. MMP-3 is another matrix-degrading enzyme in the ECM, and is able to degrade laminin, proteoglycans, gelatin, collagen, and fibronectin directly, and to activate other MMPs that accelerate the degradation of the ECM. Table I illustrated that MMP-1 and MMP-3 contents gradually increased with prolonged exposure time in the process of IVDD; the reasons as follows:
   1) The capillaries began to appear in the degenerative intervertebral disc, with a large number of macrophages being involved in the ECM, which can stimulate and activate MMPs.
   2) MMPs activated the TNF-α expression in the process of ECM degradation. TNF-α, as an inflammatory factor, can not only accelerate the degeneration of intervertebral discs, but also stimulate the nerve root, which may cause low-back pain.
   3) The increased levels of IL-1β might be induced by MMPs, and IL-1β caused the imbalance of homeostasis in the ECM, resulting in the obstacles of synthesis and metabolism.

Taken together, MMPs may be a target leading the process of IVDD.

In the present experiments, TIMP secretion was increased in the short-term, stimulated by up-regulation of MMPs in response to simulated weightlessness and/or hypergravity. TIMP-1 is a glycoprotein with a relative molecular weight of 28 kDa that can stimulate cell proliferation and inhibit apoptosis. TIMP-1 binds the zinc ions in the corresponding MMPs noncovalently in a 1:1 ratio, thus inactivating the MMPs responsible for degrading the intervertebral discs. At 90 d, TIMP-1 expression in the mixed group was no longer increased compared with that at 60 d (22.3 ± 1.16 vs. 29.3 ± 2.26), which was also verified by the results of MMP/TIMP (Fig. 1M, N). Therefore, under the conditions of weightlessness and hypergravity, MMP-1 and MMP-3 contents gradually increased with prolonged exposure. TIMP-1 also increased initially as a result of feedback regulation, followed by eventual decompensation resulting in IVDD.

Apoptosis is an autonomic and well-organized process leading to cell death. It is genetically controlled and is required to maintain stability of the internal environment and physiological function. Apoptosis in intervertebral discs can be induced by many factors, including excessive loading, abnormal

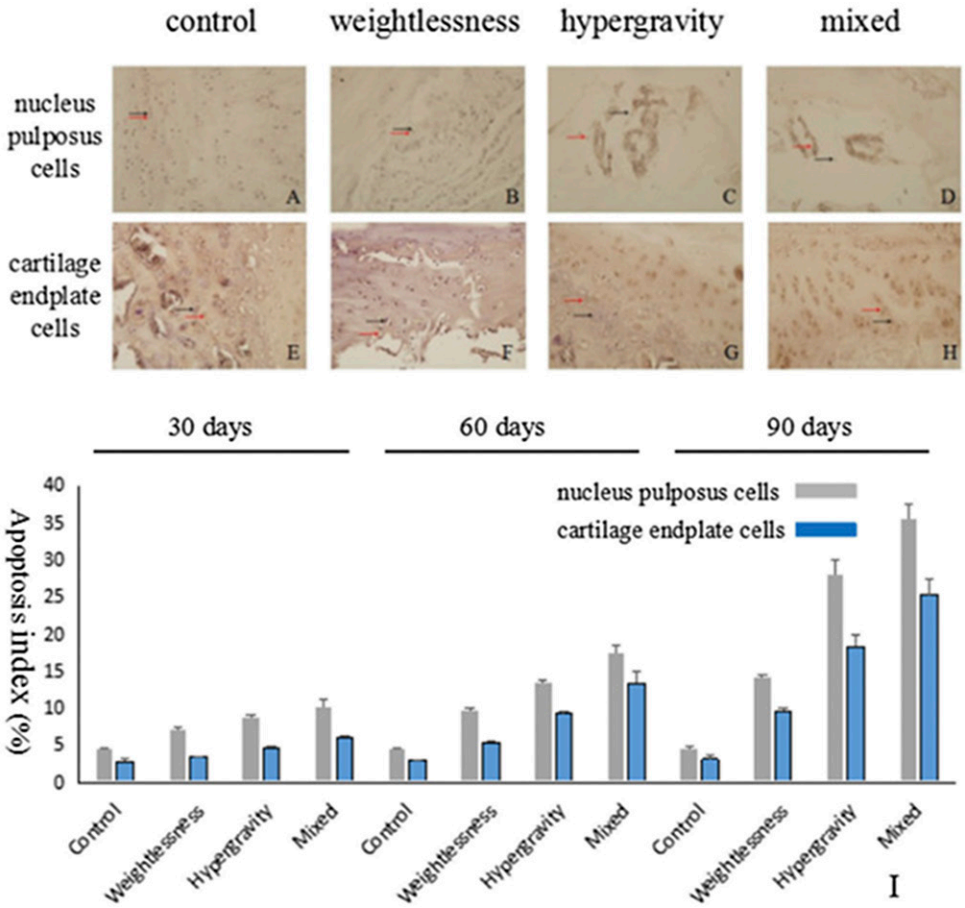

Fig. 2. A–H. Apoptotic index (AI) of nucleus pulposus and cartilage endplate cells observed by optical microscopy (×400) in each group after exposure for 90 d. Apoptotic cells indicated by brown or dark brown particles (gray arrow in print/red arrow online) surrounded by nonapoptotic cells with blue-stained nuclei (black arrow). I. AI in each group at each time point. [Color figure available online.]

stress, the microenvironment of the intervertebral disc, and cytokines. Apoptosis can lead to tissue destruction and to diseases such as IVDD.[1] TUNEL staining in the current study indicated that the AI was generally consistent in the NP and cartilage endplate cells, with a relatively low AI in the intervertebral disc under normal circumstances. However, the AI increased dramatically under conditions of simulated weightlessness or hypergravity, with the combination of weightlessness and hypergravity having the greatest impact on intervertebral disc cell apoptosis. Apoptosis also increased and the viable cell number accordingly decreased with prolonged exposure to weightlessness and hypergravity.

The specific mechanisms whereby weightlessness and hypergravity cause the molecular biological changes in degenerative intervertebral discs remain unclear. Excessive load or uneven mechanical distribution under conditions of weightlessness and/or hypergravity may affect the biomechanical features of the disc, with several possible consequences. Calcification of the cartilage endplate and aging and disappearance of the AF might block the nutritional pathway to the disc, thus reducing nutrients and increasing metabolic products, and further damaging the nutritional supply to the intervertebral disc.[6] A reduction in blood vessels surrounding the disc and increased pressure of the cartilage endplate may decrease the permeability of the disc, further hindering oxygen and nutrient supplies and changing the microenvironment of the disc, e.g., through accumulation of lactic acid, reduced pH, and reduced oxygen partial pressure.[6] These mechanisms may result in a gradual increase in the number of apoptotic cells and activation of MMPs and TIMP, further exacerbating IVDD.

In conclusion, intervertebral disc cell degeneration is affected by simulated weightlessness and hypergravity. Exposure to these conditions may aggravate IVDD by increasing

Table II. Mean Apoptosis Index (%) Of Nucleus Pulposus Cells and Cartilage Endplate Cells in Groups During the Exposure Time; Standard Deviations Are Indicated in Parentheses.

GROUPS		30 DAYS	60 DAYS	90 DAYS	*F*-VALUE	*P*-VALUE
Control	Nucleus Pulposus Cells	4.42 (0.13)	4.57 (0.06)	4.41 (0.44)	0.88	0.43
	Cartilage Endplate Cells	2.83 (0.48)	2.95 (0.07)	3.10 (0.06)	2.27	0.12
Weightlessness	Nucleus Pulposus Cells	7.09 (0.31)	9.60 (0.32)	14.2 (0.23)	1526.61	< 0.001
	Cartilage Endplate Cells	3.41 (0.15)	5.39 (0.21)	9.60 (0.42)	1253.73	< 0.001
Hypergraity	Nucleus Pulposus Cells	8.80 (0.21)	13.35 (0.49)	27.94 (1.69)	952.73	< 0.001
	Cartilage Endplate Cells	4.67 (0.20)	9.25 (0.39)	18.29 (1.66)	439.60	< 0.001
Mixed	Nucleus Pulposus Cells	10.12 (0.98)	17.45 (1.10)	35.62 (1.99)	840.01	< 0.001
	Cartilage Endplate Cells	6.05 (0.29)	13.33 (1.74)	25.28 (2.09)	376.50	< 0.001
F-value	Nucleus Pulposus Cells	216.87	767.41	1089.02		
	Cartilage Endplate Cells	220.38	232.14	516.34		
P-value	Nucleus Pulposus Cells	< 0.001	< 0.001	< 0.001		
	Cartilage Endplate Cells	< 0.001	< 0.001	< 0.001		

MMP-1, MMP-3, TIMP-1, and AI. Hypergravity together with weightlessness has a particularly strong effect on IVDD, while the effect of hypergravity alone is greater than that of weightlessness alone. Further studies are needed to clarify the pathological mechanisms of disc cell apoptosis and ECM degradation under conditions of simulated weightlessness and hypergravity.

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