Induction of intervertebral disc cell apoptosis and degeneration by chronic unpredictable stress

Laboratory investigation

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Object. The purpose of this study was to evaluate the effects of chronic unpredictable stress on the intervertebral discs of rats.

Methods. The cellular events involved in injury- and stress-induced disc degeneration were investigated in male Wistar rats. Disc degeneration and apoptosis were evaluated using microscopic (light and electron) and molecular (immunoblotting and immunohistochemistry) methods. Corticosterone levels were used as markers of stress and measured by radioimmunoassay.

Results. The data gathered in this study showed that chronic unpredictable stress can significantly increase corticosterone levels. Furthermore, biochemical markers of apoptosis (that is, increases in the Bax/Bcl2 ratio and TUNEL reactivity [p < 0.05]) were observed in the stressed animals. Electron and light microscopy also showed disc degeneration and apoptotic cells in the experimental groups.

Conclusions. Taken together, these data demonstrated that chronic stress is most likely to be a risk factor for creating intervertebral disc degeneration and that programmed cell death may be one of the mechanisms of stress-induced disc degeneration.

(http://thejns.org/doi/abs/10.3171/2014.1.SPINE13466)

Key Words • apoptosis • chronic unpredictable stress • degenerative disc • intervertebral disc

Epidemiological data indicate that various risk factors predispose individuals to low-back pain (LBP). The mechanisms of the injury may be confusing, and the studies supporting these findings are variable and conflicting regarding most environmental risks. Furthermore, there are some factors, including emotional circumstances, that heavily influence back disability, yet the cause of back disability is still unknown.37,40

Stress is a state of the body, or it is the mental tension that results from factors that tend to alter an existing equilibrium. Stress is an unavoidable effect of daily life and is an especially complex phenomenon in modern societies. Emotional stress causes biochemical changes in the blood that affect different systems in the body. Stress has been linked to coronary artery disease, immune system suppression, psychosomatic disorders, and various other mental and physical problems (http://encyclopedia2.thefreedictionary.com/Stress). Signs of stress may be cognitive, emotional, physical, or behavioral. Stress is a predictor of low-back disability for persons with previous LBP but not for those without previous LBP.2

Patients with acute stress usually have high levels of catecholamines.2 Stress increases proinflammatory cytokine levels (that is, tumor necrosis factor and interleukins-1α and -1β) in the periphery and brain.22 Elevated levels of inflammatory mediators have been described in pathological intervertebral disc (IVD) tissue, and these levels increase with the severity of the degeneration.39 Therefore, emotional stress may also influence IVD.

Because the effects of chronic stress on disc degener-
Chronic unpredictable stress and intervertebral disc degeneration have not yet been clarified, the present study was designed to investigate the degenerative effects of chronic unpredictable stress (CUS) on the IVDs of rats.

**Methods**

**Experimental Animals**

All experiments were performed on male adult Wistar rats that weighed 300–350 g and were housed 4 per cage on a 12-hour light/dark cycle in a temperature-controlled room (22 ± 1°C). Food and water were available. The experimental procedure was approved by the Animal Experimentation Ethics Committee of the Kerman Neuroscience Research Center. The animals were randomly categorized into 4 groups. Each group included 10 rats, of which 6 were studied with molecular methods and 4 were studied with microscopic methods.

**Study Design**

Group 1 underwent both disc injury (induced degeneration) and CUS (Group 1: degenerated/CUS); Group 2 experienced only CUS (Group 2: CUS); Group 3 was only subjected to disc injury (Group 3: degenerated); and Group 4 was used as a control (Group 4: neither CUS nor disc injury). Both normal and degenerated discs were investigated to study the impact of stress on IVD degeneration. Thus, we also assessed the trends of progression and enhancement of degeneration. Intervertebral disc degeneration was induced in Groups 1 and 3 in the first 3 levels of the rats’ tails according to the method of Han et al. Briefly, the skin corresponding to the tail vertebrae was marked with a permanent marker. Next, the rats were weighed and injected intraperitoneally with 10% chloral hydrate (Sigma Aldrich) at a dose of 3.5 ml/kg body weight. Tail skin was sterilized with iodinated polyvinylpyrrolidone, and the tail vertebral discs were punctured through the skin with a 20-gauge sterile needle that was inserted into the tail in the dorsal-to-ventral direction through the center of the disc until the skin on the opposite side of the tail was ruptured. Extreme care was taken to ensure that the needles were perpendicular with the skin. Palpation of the adjacent vertebrae ensured that the needle was inserted into the centers of the discs. The needle punctured the entire disc and was then rotated 360° and held in position for 30 seconds before extraction. Then, the discs were examined after exposure to CUS. The animals were exposed to CUS by using a modified version of the method recommended by Munhoz et al. and Ortiz et al. Over the next 14 days the procedures listed in Table 1 were repeated; thus, the rats were exposed to CUS over a 28-day period.

On experiment days, 30 minutes after stress induction, blood samples were collected in tubes containing 5% EDTA. Plasma samples were obtained by centrifuging the blood at 2500 rpm for 10 minutes. The samples were frozen immediately at −20°C and stored until the time of the corticosterone assay. Plasma corticosterone levels were measured using a commercial radioimmunoassay kit for rats (DRG International, Inc.). The sensitivity of the assay was 0.25 ng/ml, and the antibody cross-reacted 100% with corticosterone, 0.34% with deoxycorticosterone, and less than 0.10% with other steroids. At the end of the CUS period, all rats were killed, and discs were separated from vertebral endplates under microscopic magnification.

**Immunoblot Analysis**

Discs were homogenized in ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1% sodium dodecyl sulfate, 0.1% sodium deoxycholate, 1% NP-40 with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin, 10 μg/ml aprotinin), and 1 mM sodium orthovanadate. The homogenate was centrifuged at 14,000 rpm for 15 minutes at 4°C. The resulting supernatant was retained as the whole cell fraction. Protein concentrations were measured using the Bradford method (Bio-Rad Laboratories). Equal amounts of protein were resolved electrophoretically on 9% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes (Hybond ECL, GE Healthcare Bio-Sciences Corp.). After blocking overnight at 4°C with 5% nonfat dried milk in Tris-buffered saline with Tween 20 (TBS-T) (blocking buffer, TBS-T, 150 mM NaCl, 20 mM Tris-HCl [pH 7.5], 0.1% Tween 20), the membranes were probed with rabbit monoclonal antibodies for Bax (Δ21: sc-6236) and Bcl-2 (C-2: sc-7382, Santa Cruz Biotechnology, Inc.) at 1:1000 for 3 hours at room temperature. After washing in TBS-T (3 times, 5 minutes each), the blots and a horseradish peroxidase–conjugated secondary antibody (1:15,000, GE Healthcare Bio-Sciences Corp.) were incubated for 60 minutes at room temperature. All the antibodies were diluted in blocking buffer. The antibody-antigen complexes were detected using an ECL system and exposed to Lumi-Film chemiluminescent detection film (Roche). Lab Work software (UVP, LLC) was used to analyze the

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<tr>
<th>Day</th>
<th>Experiment</th>
<th>Duration</th>
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<td>1 (2 p.m.)</td>
<td>restraint</td>
<td>60 min</td>
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<td>2 (9 a.m.)</td>
<td>forced swim</td>
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<td>3 (3 p.m.)</td>
<td>cold isolation</td>
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<td>4 (7 p.m.)</td>
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<td>5 (10 a.m.)</td>
<td>forced swim</td>
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<td>6 (7 p.m.)</td>
<td>water/food deprivation</td>
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<td>7 (2 p.m.)</td>
<td>restraint</td>
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<td>8 (3 p.m.)</td>
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<td>9 (9 a.m.)</td>
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* Animals were exposed to CUS over 28 days (14 × 2 = 28).
expression intensities, and β-actin immunoblotting (the antibody was obtained from Cell Signaling Technology, Inc.; 1:1000) was used as a loading control.

Histopathological Examination

Staining With the TUNEL Method. Tissue sections were dewaxed by heating at 60°C and were then irrigated with xylene and rehydrated using ethanol and deionized water. This process was followed by 15 minutes of incubation in proteinase K solution (10 μg/ml, Boehringer Mannheim) at room temperature. Endogenous peroxidase was inactivated with 2% H2O2. After a short equilibration in terminal deoxynucleotidyl transferase (TdT) buffer, the sections were incubated for 60 minutes with TdT and biotinylated deoxyuridine triphosphate in TdT buffer in a 37°C humidified chamber. The slides were thoroughly washed, and the sections were covered with ExtrAvidin peroxidase. Color was developed by exposing the sections to a 3'3'-diaminobenzidine solution containing H2O2. For negative staining controls, either TdT or ExtrAvidin peroxidase was omitted. The slides were then dehydrated, cleared, and mounted. The numbers of positively stained cells (dark brown) were quantified in 3 noncontiguous fields of the nucleus pulposus for each sample (12 total fields).

Light Microscopy. The IVDs were fixed in 10% formalin, decalcified using 10% standard decalcifying solution (10% EDTA for 48 hours), washed with running tap water, and fixed again in the same fixative. The fixed, decalcified discs were embedded in paraffin and sectioned (5 μm thick). The sections were stained with H & E.

Electron Microscopy. The specimens were fixed sequentially in buffered 2.5% glutaraldehyde (Merck) for 24 hours, decalcified using 10% standard decalcifying solution (10% EDTA for 48 hours), washed in 0.1 M phosphate-buffered saline, and postfixed in OsO4 1% sodium phosphate-buffered saline 0.1 M buffer (pH 7.4) at room temperature for 1 hour. After dehydration in increasing concentrations of ethanol, the specimens were embedded in Epon 812 resin (TAAB Laboratories Equipment Ltd.). Ultrathin sections were cut at 70 nm, stained with lead citrate and uranyl acetate 2%, and viewed with a Philips (EM300) electron microscope.

Statistical Analysis

The results are expressed as the mean ± SEM. The Bax/Bcl2 and β-actin band density values were obtained by band densitometry. These values are expressed as the tested protein/β-actin ratio for each sample. The apoptotic cell values are expressed as the percentage of positively stained cells. Averages were compared across groups with ANOVAs, followed by Newman-Keuls and post hoc Tukey tests. A p value < 0.05 was considered significant.

Results

Corticosterone Assay Findings

The plasma corticosterone levels were 424.50 ± 69.45 ng/ml after CUS and 249.86 ± 42.46 ng/ml in the control group (mean ± SEM); this difference was significant (p < 0.05; Fig. 1).

Biochemical Findings

The mean Bax/Bcl2 ratios were compared across groups (Fig. 2). The Bax/Bcl2 ratio of the degenerated/CUS group (Group 1) was significantly greater than that of the control group (p < 0.01). The Bax/Bcl2 ratios of the CUS and degenerated groups (Groups 2 and 3, respectively) were also elevated compared with that of the control group (p < 0.05). The Bax/Bcl2 increase in the degenerated/CUS group was not significantly different from that of the degenerated group. Thus, cell apoptosis in the discs was significantly increased by CUS, but this process was not intensified in degenerated discs after CUS.

Histopathological Findings

Staining With the TUNEL Method. Apoptotic cells were examined using the TUNEL method (Fig. 3). The degenerated, CUS, and degenerated/CUS groups exhibited positive cell rates of 43%, 52%, and 69%, respectively (Fig. 4). The difference between the degenerated and control groups was significant (p < 0.05). The difference between the CUS and degenerated/CUS groups and the control group was more obvious (p < 0.001), and the difference between the degenerated/CUS and CUS groups was not statistically significant.

Light Microscopy. Light microscopic evaluation of the tail discs revealed normal and well-organized anuli fibrosi and nuclei pulposi in the control group. In the experimental groups, the structures of the IVDs showed some degenerative changes such as the dissociation of multilayered structures, the loss of normal fibrillation of the cartilage, and vascular proliferation. The laminar structure of the anuli fibrosi disappeared, and the fibers were disorganized and fragmented. The cells of the nucleus pulposus were sparse and reduced in number (Fig. 5).

Electron Microscopy. Normal ultrastructure was observed in the cells of the control group on electron microscopic survey. The organelles were intact within the cytoplasm. The nuclear membranes were smooth, and

![Figure 1](image_url)
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Chromatin was dispersed throughout the nuclei. Small and irregular projections on the surfaces of the cells gave these cells a scalloped appearance (Fig. 6A and B). In the CUS group, the shapes of anuli and nuclei of the cells were irregular and exhibited signs of apoptosis. Discontinuities of the plasma membranes were not observed in the bizarrely shaped cells, but the nuclear membranes were disintegrated. The nuclei had irregular shapes, and chromatin margination was evident. The cellular organelles showed some evidence of degeneration, including dilated rough endoplasmic reticulum and Golgi cisterns. The fibers of the anuli fibrosi were not packed densely and were also disorganized. The most noticeable finding in the CUS group was the presence of many bizarrely shaped cells that were not observed in the other groups. Apoptotic bodies were obvious within the cytoplasm (Fig. 6C and D).

Discussion

In this study we found CUS-induced IVD degeneration in rats and confirmed this finding with molecular and structural surveys. Because the molecular and immunohistochemical assays were the main methods of our survey, the results of these assays were presented quantitatively. Because complementary histopathological studies were also performed, the results of these studies were presented descriptively and subjectively.

Intervertebral disc degeneration is caused by abnormalities in collagen, vascular ingrowths, abnormal proteoglycans, local inflammation, and ultimately apoptosis.
Electron photomicrographs showing IVD cells. A and B: Normal ultrastructure in the control group. The nuclear membrane is smooth, and chromatin is dispersed throughout the nucleus. Cytoplasmic organelles are intact. Small and irregular projections on the surface of the cell produce a scalloped appearance. C and D: Nuclear deformation with chromatin condensation in the stressed group. Note the dilated rough endoplasmic reticulum and Golgi cisterns. Original magnification ×11,500 (A), ×15,500 (B and C), and ×19,000 (D).

Apoptosis is an active process that is associated with programmed cell death. In vivo and in vitro studies have indicated that apoptosis has a central function in the degenerative process. The importance of apoptosis in the diverse diseases that are associated with disc degeneration has been extensively reviewed. The Bcl2 gene has antiapoptotic effects and is inversely related to the Bax gene, which promotes apoptosis. Hence, the Bax/Bcl2 expression ratio can be strongly suggestive of cell apoptosis. In our study, the elevated Bax/Bcl2 ratio of the CUS-only group compared with that of the control group indicated the induction of apoptosis. The results of TUNEL staining also confirmed apoptosis in the stress groups. The electron microscopic finding of bizarrely shaped cells that were only observed in the stress groups favors the molecular data. These findings show that stress can somehow induce disc degeneration.

As expected, the Bax/Bcl2 ratios and positive cell percentages were elevated in the degenerated group, but neither the Bax/Bcl2 ratios nor the percentages of apoptotic cells were different between the degenerated/CUS group and the degenerated group. This latter finding indicates that stress did not have significant degenerative effects on discs that were already degenerated. Therefore, it seems that the normal discs were more vulnerable to the effects of chronic stress than the degenerated ones. It is possible that the effects of needle puncture induced enough degeneration that the effects of stress could not be observed. Another presumable explanation for this finding is that some unknown defensive mechanisms may be activated to inhibit further degeneration after its induction, which prevents stress from causing further degeneration.

The mechanism by which stress induced disc degeneration is still unknown. Stress causes biochemical changes in the blood that affect different systems in the body. When the adaptive system is switched on and off efficiently, the body is able to recover from imposed stress. However, when the system is activated repeatedly or the activity is sustained (for example, by chronic or excessive stress), an allostatic load is generated that can lead to disease over long periods of time. In adult males, stress decreases plasma testosterone and fertility. Stress in rats increases corticotropin-releasing hormone and arginine-vasopressin levels in the plasma via the hypothalamic-pituitary-adrenal axis. Elevated corticotropin-releasing hormone increases the risk of osteoporosis, suppresses the immune system, and increases blood sugar through cortisol in humans and corticosterone in rats. Moreover, elevated arginine-vasopressin elevates blood pressure and disturbs the water/salt balance through peripheral vascular resistance. Stress increases serum prolactin in rats. Increases in growth hormone occur after stressful procedures. Acute stress increases human blood catecholamines, which, in turn, causes myofibrillar degeneration, necrosis of heart muscle, and dopaminergic neuron degeneration. Stress can change the structure and function of different brain regions.

Other various effects of stress include exacerbation of liver, skin, gastrointestinal, pulmonary, oncological, otological, and skeletal diseases. Susceptibility to infections, autoimmune disorders, and tumor progression are strongly influenced by the activities of the endocrine and nervous systems in response to stressful stimuli. Chronic stress increases plasma melatonin concentrations in rats, and serum melatonin levels are affected by the disc degeneration process in patients with IVD herniations. Won et al. investigated the effects of hyperglycemia on notochordal cell apoptosis and IVD degeneration in diabetic rats. These authors suggest that diabetes is associated with premature and excessive apoptosis of notochordal cells of the nucleus pulposus, which accelerates the transition from a notochordal to a fibrocartilaginous nucleus pulposus, which in turn leads to early IVD degeneration.

The biochemical changes that accompany IVD degeneration include altered expression of both matrix metalloproteinases and proinflammatory cytokines. Elevated levels of molecular mediators of inflammation have been described in pathological IVD tissue, and these levels increase with increasing grades of degeneration. Such findings have been observed for both interleukin-1 and tumor necrosis factor–α, both of which have established roles in regulating nitric oxide and prostaglandin production, metalloproteinase expression, and apoptosis. Metalloproteinases exert direct and indirect effects through the promotion of neovascularization in the IVD.
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This complex effect acts both in disc matrix degeneration and in the pain generated by contact between the protruding disc and the nerve roots. All of these changes may contribute to the progressive degeneration of the IVD.31

Our data showed that CUS increases plasma corticosterone levels. It seems that the CUS induced IVD programmed cell death and degeneration through corticosterone secretion. However, the actual linkage between stress-induced elevation in corticosterone and disc degeneration is an important issue that needs to be considered. Numerous scientific reports have indicated that stress-induced corticosterone secretion is responsible for the induction of apoptosis in stressful situations in laboratory animals. It has been reported that restraint stress results in significant attenuation of the phosphatidylinositol 3–kinase/Akt signaling pathway and increased apoptosis in rat skeletal muscle.32 Additionally, prolonged exposure to stress-level glucocorticoids causes neuronal cell apoptosis through the activity of caspase-9 and caspase-3.33 Furthermore, chronic isolation stress leads to cytochrome c–mediated activation of caspase-3 and apoptotic cell death in the prefrontal cortex.34 Li and colleagues reported that a single episode of prolonged stress increased the plasma corticosterone level, altered the Bcl-2/Bax ratio, and induced neuronal apoptosis in the rat medial prefrontal cortex.35

The results of our study also showed that chronic stress that is accompanied by corticosterone release promotes the proapoptotic factor Bax and, eventually, IVD apoptosis. Additionally, high circulating levels of catecholamines, matrix metalloproteinases, proinflammatory cytokines, and serum melatonin are linked to both stress and degeneration.12,24 According to our data and the above-mentioned reports, we conclude that there is a probable link between stress and disc degeneration. Stress may influence the discs through all of the above-mentioned mechanisms.

The limitations of this study are as follows: we were unable to evaluate symptomatic IVD degeneration in rats (because we had no access to rats with spontaneously degenerated discs with which to evaluate the effect of stress on the discs), and there are no existing methods to induce occupational stress in animals. Finally, it is difficult to extrapolate these nonprimate results to humans.

Conclusions

Overall, based on our results, stress is most likely to be a risk factor for the degeneration of IVDs, but the mechanism of this effect and its impact on the escalation of IVD degeneration and LBP require further investigation. Future research is also required to clarify the correlation between stress and apoptosis in disc degeneration, to inhibit or eliminate the stress axis and the hormones that are relevant to IVD degenerative processes.

Disclosure

This work was supported by funds received from the Kerman Neuroscience Research Center (KNR/87-31). The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Reihani Kermani. Acquisition of data: Hoboubati, Esmaili-Mahani, Asadi-Shekaari. Analysis and interpretation of data: all authors. Critically revising the article: all authors. Reviewing submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Reihani Kermani. Statistical analysis: Esmaili-Mahani. Administrative/technical/material support: Reihani Kermani. Study supervision: Reihani Kermani.

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