

[Original Article]

Differential Carbonyl Stress Expression in the Intervertebral Disc between Singular- and Persistent- Mechanical Injuries

Itaru Hibino^{1,2}, Simon Tang²

Abstract

Low back pain is a significant public health problem worldwide. Intervertebral disc degeneration is most significant known risk factor for low back pain. Yet the mechanisms of degeneration remain relatively unknown. Carbonyl stress and oxidation have been implicated in cartilage and fibrocartilage degeneration. Here we investigate the role of oxidative stress and carbonyl production in the intervertebral disc after mechanical injury using an *in vitro* organ model of the mouse functional spine unit. We use a single- stab insult to model mild injury, and the three- stab insult to model severe trauma. Our results indicate that mild injury increases the carbonyl response that may be required for tissue repair, while severe trauma tempers this response and rapidly accelerates degeneration.

Keywords: Low back pain, Intervertebral disc degeneration, Mechanical injury, Carbonyl stress

Introduction

Low back pain is a significant public health problem worldwide [5, 13, 19]. Although there are a number of causes for low back pain, degeneration of the intervertebral discs (IVDs) is the most significant known cause of low back pain [3, 8, 12]. The degenerative cascade of the IVD includes changes that affect the structural,

mechanical, and inflammatory behavior of the disc. Multiple interdependent factors have been implicated in disc degeneration including reduced nutrient supply, hereditary factors, altered mechanical loading, age, up-regulated levels of pro-inflammatory cytokines and associated catabolic enzymes, and cellular and extra-cellular biochemical changes [2, 8, 10–13, 18, 21]. The occurrence of one or more of

1 Department of Physical Therapy, Faculty of Rehabilitation Sciences, Nagoya Gakuin University, Nagoya, Aichi, JAPAN

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2 Department of Orthopaedic Surgery, Washington University in St. Louis, St. Louis, MO

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Correspondence to: Itaru Hibino

E-mail: hibino@ngu.ac.jp

these factors initiates and propagates the IVD degeneration process.

The biochemical changes in the IVD include post-translational oxidative changes in the extracellular matrix of the IVD. In aging, the generation of free radicals outpaces the cellular scavenging mechanism resulting in a net increase of tissue oxidative stress [2, 7, 16]. Furthermore, increased inflammation during injury and pathology can exacerbate this oxidative burst [4, 6, 15]. Oxidative stress has been implicated in IVD failure [18], interfering with protein folding, increasing susceptibility to MMPs degradation, and impairing mechanical properties. Given the paramount importance of oxidative stress, it is thus critical that models investigating disc degeneration account for and properly account for the role of oxidative stress.

Investigations of the IVD using human tissues pose additional challenges because of the scarcity of normal, healthy human tissues. Furthermore, cadaveric or aged tissues are not suitable for disease initiation studies because of typical degree of advanced degeneration. Thus many disease initiation studies of disc degeneration are conducted in animal models. Animal models of IVDs degeneration can be classified as either experimentally induced, either by mechanical overloading, injury, or other insults to the disc or spontaneous, which include animals genetically altered or specially bred to develop degenerative disc disease [3, 8, 10, 12–13, 21]. In the injury model, the use of needle puncture has gained popularity mainly because of its reproducibility

and the short time required to produce the desired degenerative discs [1]. Studies by our group and others have shown that the needle puncture model recapitulates the degenerative and inflammatory cascade of pathological IVDs, suggesting that this in vitro injury approach provides the structural and molecular characteristics of disc degeneration. Yet the effect of this needle injury on IVD oxidation remains unknown. We hypothesized that the injurious response induced by a needle would induce elevated oxidation from the cells that would subsequently increase the net di-carbonyl presence in the tissue. The objective of this study is to examine and compare the oxidative response of the IVD after a single- and repeated- stab injury using a model of organ culture, with the long term goal of improving the understanding of the mechanisms contributing to IVD degeneration and low back pain.

Materials and Methods

Disc harvesting and organ culture

The experimental protocols were approved by the university committee on the use and care of animals at the Washington University in St. Louis. We used an in vitro model of IVDs ($n = 28$) organ culture of BALB/c mice [1]. The animals were euthanized and 3 IVDs of the tail, containing an intact vertebrae-disc-vertebrae structure, from each animal were dissected and removed under sterile conditions. The IVDs were rinsed in saline and cleaned of soft tissues using dissecting scissors prior to organ culture.

The IVDs were randomly assigned to three groups: control and stab (mechanically injured using a 27-gauge hypodermic needle). The samples of both groups were cultured in 2mL of DMEM containing 20% fetal bovine serum and 1% penicillin-streptomycin and maintained in organ culture in 12-well cell culture plates. The complete medium was refreshed every 48 hours for both groups.

Needle puncture model

The samples of stab group were further divided to two groups: single- and three-stabs ($n = 8/\text{group}$). The samples of single-stab group were stabbed on day 0. The samples of three-stab group were stabbed on day 0, 5 and 10. The samples of all groups were maintained up to 15 days. Although we have previously utilized the single-stab model, we also use a three- stab model [20] to induce severe degeneration and inflammation.

Histological analysis

After the culture period, the structure of IVDs was fixed in 10% neutral buffered formalin, for 48 hours and decalcified in Imunocal (StatLab Medical Products, USA) for 5 days, paraffin-embedded, and sectioned to 5 μm thickness with a microtome. The sections were observed at intermediate time points using Safranin-O/Fast Green staining and polarizing microscopy and were scored according to the classification system for grading the histological changes which is from total score 0 (normal disc) to 5 (severe degeneration). The histological

grading by preceding studies [8, 13, 17], with some modifications (Table 1) was performed separately on annulus fibrosus (AF), nucleus pulposus (NP), and the AF and NP border (Border), and the scores from these regions were combined to provide a composite score.

Oxidized protein concentrations

The proximal and posterior vertebral bodies of the sample were removed from each sample at the growth plate using a dissection microscope, and the homogenized using a tissue pulverizer. The concentration of oxidized proteins in isolated IVDs was quantified using 2, 4-dinitrophenylhydrazine (DNPH) spectrophotometric assay [18]. 200 μL of sample solution was added to 100 μL of 10 mM DNPH solution, mixed, and incubated for 60 min in the dark at room temperature. After the addition of 0.6 mL of trichloroacetic acid (20% w/v) the mixture was vortexed. The mixture was incubated on ice for 10 min before centrifugation at 1,000g for 10 min. The supernatant was discarded without disturbing the pellet that was washed three times with 1.5 mL of ethanol/ethyl acetate (1/1; v/v) to remove free DNPH reagent. The sample pellet was resuspended in 0.5 mL of 6 M guanidine-HCl before absorbance measurement at 370 nm, using 6 M guanidine-HCl as blank solution, and then normalized to protein concentration determined by Bradford's method using serialized BSA as standard.

Comparisons between groups were made between groups using ANOVA with Tukey's posthoc comparisons. Significance was

Table 1 : Definition of Histological Grading Scale

I. Annulus fibrosus
Grade:
0. Normal, pattern of fibrocartilage lamellae (U-shaped in the posterior aspect and slightly convex in the anterior aspect) without ruptured fibers and without any bending / kinks / wavy patterns within the annulus
1. Minor disruptions (<10%) and no rupturing of annulus fibers
2. Somewhat (<25%) ruptured fibers or bending / kinks / wave pattern in annulus
3. Moderate (<50%) ruptured fibers or bending / kinks / wave pattern in annulus
4. Severe (<75%) ruptured fibers or bending / kinks / wave patterns pattern in annulus
5. Extreme (>75%) rupturing of the annulus fibers and significant bending / kinks / wave patterns, no distinguishable U-shaped lamellae
II. Border between the annulus fibrosus and nucleus pulposus
Grade:
0. Nomal (clear distinction between AF and NP)
1. Minimally interrupted (<10%)
2. Somewhat interrupted (<25%) border
3. Moderate (<75%) interruption
4. Severe (<75%) interruption
5. No distinguishable boundary between AF and NP tissue
III. Nucleus Pulposus
Grade:
0. Normal un-fragmented appearance, normal proteoglycan (red) staining, no contraction of the nucleus.
1. Minimal (<10%) contraction of the extracellular matrix, no significant proteoglycan staining
2. Somewhat (<30%) contraction of the extracellular matrix, fragmentation of the matrix, and decreased proteoglycan staining
3. Moderate (<50%) contraction of the extracellular matrix, fragmentation of the matrix, and decreased proteoglycan staining
4. Severe (<75%) contraction of the extracellular matrix, fragmentation of the matrix, and decreased proteoglycan staining
5. Complete disorganization and loss of nucleus matrix, severely decreased proteoglycan staining

Histological grading scale based on 3 categories of degenerative changes with scores rating from a normal disc with 0 points (0 points in each category) to a severely degenerated disc with 15 points (5 points in each category).

considered to be $p < 0.05$.

Results

After 15 days of culture, the control IVDs remained viable and structurally intact. The single-stab injury caused a loss of structure in the NP and reduced Safranin-O uptake, while the three-stab resulted in complete loss of proteoglycans and the NP structure, suggesting that severe granulated fibrosus of these tissues (Figure 1). The modified Masuda's grading system confirms that the discs are categorically degenerated in the single-stab treatment, and

highly degenerated in the three-stab group (Table 2). The normalized DNHP assay reveals significantly higher levels of reactive carbonyls in both the single- ($p < 0.01$) and three- ($p < 0.05$) stab groups. Normalized to the controls, the single-stab differentially upregulated carbonyl production while the three-stab resulted in a reduction (Figure 2).

Discussion

Here we examined the effects of single- and three-stabs injury on the structure and oxidation of the mouse IVDs in culture. Our

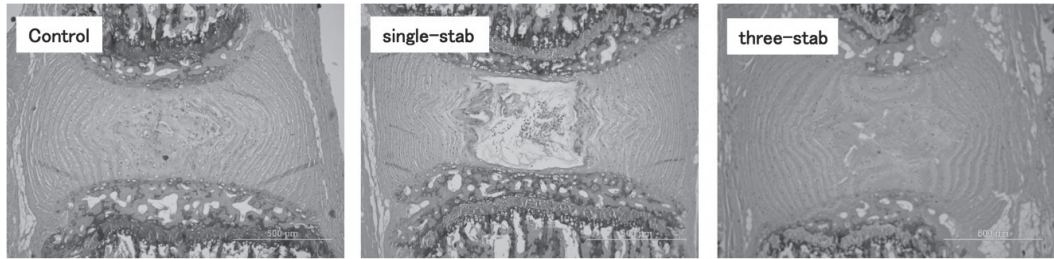


Figure 1 : Histological assessment by Safranin-O (Fast Green Counter stain) reveals that the single- stab group has an increase disorganized nucleus pulposus, and disruption of the annular-nucleus interface. In contrast, the three- stab group shows a complete ablation of the nucleus, yet the annulus cells remain viable.

Table 2 : The histological grading after the culture

Group	I AF	II Border	III NP	composite
Control	1	2	2	5
single-stab	2	2	3	7
three-stab	3	5	5	13

The quantitative assessment of the degeneration of the control, single-, and three- stab groups show that the three-stab group is significantly more degenerated than the control and the single-stab group.

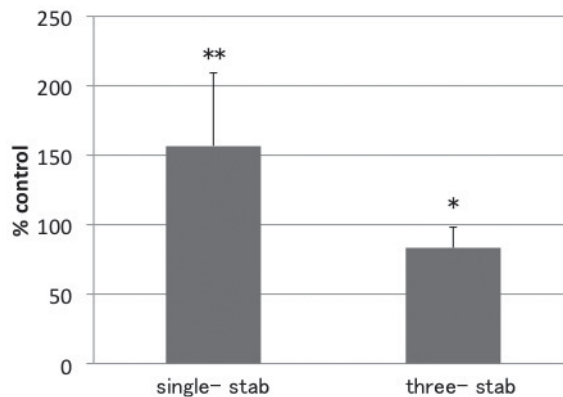


Figure 2 : The 2, 4-dinitrophenylhydrazine shows significantly higher carbonyl production by the single- stab group (* ; $p < 0.01$) while the three-stab group results in significantly lower carbonyl (* ; $p < 0.05$), suggesting this cellular response mechanism is impaired.

results show that single-stab IVDs causes mild structural changes, and the IVD cells adapt by expressing elevated levels of carbonyls. Since carbonyls can stimulate the damage repair [4, 6, 15] by cells and are part of the immune and

inflammatory response in cells, the elevated expressions of the DNHP assay in the single-stab group may be part of the insult-mediated reparative response of the IVD cells. Since the resulting degeneration is relatively mild,

especially since the nucleus pulposus and the annular-nucleo border appear intact, albeit disorganized, the IVD under a single stab insult may eventually repair itself if given sufficient time. It is noteworthy however, that this organ culture system preserves only the IVD cells (annulus fibrosus, nucleus pulposus, and end-plate), the macrophage and the endothelial progenitors that may be required for full regeneration are missing from this system. Nevertheless, the increased carbonyl response suggests at least that the IVD cells are responsive to its environment and may be capable of generating recruitment signals for regeneration.

In contrast, the three-stab IVDs exhibit total depletion of the NP as well as an inability to produce carbonyls, suggesting that this injury is catastrophic and the cells cannot repair itself from this magnitude of injury. In place of the NP are granulated tissues that are completely impervious to the Safranin-O stain, demonstrating that this fibrotic tissue is devoid of glycosaminoglycans. This phenotype is reminiscent of the loss of HIF-alpha, a transcription factor necessary for the homeostasis of nucleus pulposus cells [14]. In this phenotype the HIF-alpha results in the inability of NP cells to undergo anaerobic glycolysis, forcing the NP cells to undergo oxidative phosphorylation. Our observations are consistent with this phenotype: the triple-stab results in a loss of carbonyl production, and results in a loss of the nucleus pulposus despite maintaining the disc height. In our previous works, depletion of the GAGs results

in loss of the water content and declined viscoelastic behavior [9, 11] that would increase its susceptibility to mechanical damage. Taken together, the extreme trauma induced by triple-stab reduces the oxidative response required for regeneration, and increases further propensity for damage.

It is noteworthy that in this experimental model, we have isolated the functional spine unit in culture. This model maintains the viability and homeostasis of just the IVD structure and cells, and thus other cells such as the macrophage that may be required for the intact inflammatory and repair response. However, this approach also allows us to precisely isolate and stimulate responses only in the intervertebral disc, which allow us to understand response solely attributable to the disc. It will be important to investigate the mechanisms here in vivo to ensure translatability. An improved understanding of mouse injury models to the IVD will enable us to conduct more mechanistic investigations relating to structure, oxidation, and downstream inflammatory factors. It may be possible to develop therapies targeting oxidation in the future.

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References

- [1] Abraham AC, Liu JW, Tang SY. (2016) Longitudinal changes in the structure and inflammatory response of the intervertebral disc due to stab injury in a murine organ culture model. *J Orthop Res.* 34: 1431–1438
- [2] Alexeyev MF. (2009) Is there more to aging than mitochondrial DNA and reactive oxygen species? *FEBS J.* 276: 5768–5787
- [3] Alini M, Eisenstein SM, Ito K, Little C, Kettler AA, Masuda K, Melrose J, Ralphs J, Stokes I, Wilke HJ. (2008) Are animal models useful for studying human disc disorders/degeneration? *Eur Spine J.* 17: 2–19
- [4] Cathcart MK. (2004) Regulation of Superoxide Anion Production by NADPH Oxidase in Monocytes/Macrophages: Contributions to Atherosclerosis. *Arterioscler Thromb Vasc Biol.* 24: 23–28
- [5] Dagenais S, Caro J, Haldeman S. (2008) A systematic review of low back pain cost of illness studies in the United States and internationally. *Spine J.* 8: 8–20
- [6] De la Fuente M, Miquel J. (2009) An update of the oxidation-inflammation theory of aging: the involvement of the immune system in oxi-inflamm-aging. *Curr Pharm Des.* 15: 3003–3026
- [7] Haigis MC, Yankner BA. (2010) The Aging Stress Response. *Molecular Cell.* 40: 333–344
- [8] Issy AC, Castania Y, Salmon CEG, Nogueira-Barbosa MH, Del Bel E, Defino HLA. (2013) Experimental model of intervertebral disc degeneration by needle puncture in Wistar rats. *Braz J Med and Biol Res.* 46: 235–244
- [9] Jazini E, Sharan AD, Morse LJ, Dyke JP, Aronowitz EA, Chen LKH, Tang SY. (2012) Alterations in T2 Relaxation Magnetic Resonance Imaging of the Ovine Intervertebral Disc Due to Nonenzymatic Glycation. *Spine.* 37: E209–215
- [10] Korecki CL, Costi JJ, Iatridis JC. (2008) Needle puncture injury affects intervertebral disc mechanics and biology in an organ culture model. *Spine.* 33: 235–241
- [11] Liu JW, Abraham AC, Tang SY. (2015) The high-throughput phenotyping of the viscoelastic behavior of whole mouse intervertebral discs using a novel method of dynamic mechanical testing. *J Biomech.* 48: 2189–2194
- [12] Markova DZ, Kepler CK, Addya S, Murray HB, Vaccaro AR, Shapiro IM, Anderson DG, Albert TJ, Risbud MV. (2013) An organ culture system to model early degenerative changes of the intervertebral disc II: profiling global gene expression changes. *Arthritis Research & Therapy.* 15: R121
- [13] Masuda K, Aota Y, Muehleman C, Imai Y, Okuma M, Thonar EJ, Andersson GB, An HS. (2005) A Novel Rabbit Model of Mild, Reproducible Disc Degeneration by an Annulus Needle Puncture: Correlation Between the Degree of Disc Injury and Radiological and Histological Appearances of Disc Degeneration. *Spine.* 30: 5–14
- [14] Merceron C, Mangiavini L, Robling A, Wilson TL, Giaccia AJ, Shapiro IM, Schipani E, Risbud M. (2014) Loss of HIF-1 α in the Notochord Results in Cell Death and Complete Disappearance of the Nucleus Pulposus. *Plos ONE.* 9: e110768
- [15] Preynat-Seauve O, Coudurier S, Favier A, Marche PN, Villers C. (2003) Oxidative stress impairs intracellular events involved in antigen processing and presentation to T cells. *Cell Stress Chaperones.* 8: 162–171
- [16] Roberts CK, Sindhu KK. (2009) Oxidative

- stress and metabolic syndrome. *Life Sciences*. 84: 705–712
- [17] Rutges JPHJ, Duit RA, Bekkers JEJ, Oner FC, Castelein RM, Dhert WJA, Creemers LB. (2013) A validated new histological classification for intervertebral disc degeneration. *Osteoarthr Cartil*. 21: 2039–2047
- [18] Scharf B, Clement CC, Yodmuang S, Urbanska AM, Suadican SO, Aphkhazava D, Thi MM, Perino G, Hardin JA, Cobelli N, Vunjak-Novakovic G, Santambrogio L. (2013) Age-Related Carbonylation of Fibrocartilage Structural Proteins Drives Tissue Degenerative Modification. *Chemistry & Biology*. 20: 922–934
- [19] Suka M, Yoshida K. (2009) The national burden of musculoskeletal pain in Japan: Projections to the year 2055. *Clin J Pain*. 25: 313–319
- [20] Ulrich JA, Liebenberg EC, Thuillier DU, Lotz JC. (2007) Repeated Disc Injury Causes Persistent Inflammation. *Spine*. 32: 2812–2819
- [21] Zhang H, Yang S, Wang L, Park P, Marca FL, Hollister SJ, Lin CY. (2011) Time course investigation of intervertebral disc degeneration produced by needle-stab injury of the rat caudal spine: laboratory investigation. *J Neurosurg Spine*. 15: 404–413

〔原著〕

機械的損傷の程度の違いがカルボニルストレス発現に及ぼす影響

日比野 至^{1,2}, サイモン タング²

要 旨

腰痛は世界中で重大な公衆衛生上の問題である。椎間板変性は、腰痛の最も重要な既知の危険因子であるが、その変性メカニズムについては明らかではない。カルボニルストレスおよび酸化は、軟骨および線維軟骨の変性に関与している。そこで、マウス機能脊柱ユニットの培養モデルを用いて、針挿入による機械的損傷後の椎間板における酸化ストレスおよびカルボニル産生の役割を調べた。軽度の損傷をモデル化するために針挿入を1回、重度の損傷をモデル化するために針挿入を3回行った。軽度の損傷モデルは、組織修復に必要とされるカルボニル応答を増加させるが、重度の損傷モデルはこの応答を緩和し、変性を急速に加速することを示した。

キーワード：腰痛，椎間板，機械的損傷，カルボニルストレス

1 名古屋学院大学リハビリテーション学部理学療法学科

2 セントルイス・ワシントン大学