Effect of Lumbar Intralidcal Injection of Tumor Necrosis Factor-alpha and Nerve Growth Factor / Vascular Endothelial Growth Factor on Disc Degeneration, Pain Behavior and Neurovascular Ingrowth in an In-Vivo Rat Model

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Disclosures:

Introduction: Low back pain is a common and debilitating musculoskeletal disorder which is associated with degenerated intervertebral discs (IVDs). Neurovascular ingrowth has been identified in painful IVDs, and it is believed that the irritation of these nerves by the abnormal intradiscal stresses in degenerated IVDs causes pain (1). Neurovascular ingrowth may play a critical role in discogenic pain. The growth of microvascular blood vessels might be facilitated by vascular endothelial growth factor (VEGF), whereas the nerves growth might be promoted by nerve growth factor (NGF) and inhibited by intradiscal pressure and proteoglycans of the extracellular matrix. Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα), is also implicated in discogenic pain as it facilitates the breakdown of proteoglycans and the production of NGF and Substance P. However, the in-vivo effects of NGF and TNFα on neurovascular ingrowth, IVD degeneration and associated pain have not been investigated. Therefore, the objectives of this study were to explore whether NGF and TNFα induce discogenic pain in association with IVD degeneration and neurovascular ingrowth in an in-vivo rat model. We hypothesized that intralidcal injection of NGF and TNFα into the lumbar IVDs will induce progressive discogenic pain together with IVD degeneration, neurovascular ingrowth and elevated expression of pain neuropeptides.

Methods: Thirty healthy, skeletally mature (4-5 months old) Sprague-Dawley rats were used in this study. All experimental procedures were approved and guided by the Institutional Animal Care and Use Committee. The rats were randomly divided into five groups (n=6): 1) naïve, 2) sham surgery, 3) PBS control, 4) TNFα, and 5) NGF/VEGF. The animals were operated under sterile conditions and general anesthesia. After a small midline abdominal incision, the lumbar spine was exposed and the L3-4, L4-5 and L5-6 discs were identified using the pelvic rim as an anatomic landmark. The IVDs were punctured using a 26-gauge needle with a depth of 1.5mm guided by a custom-made stopper. 2.5μL of PBS, TNFα (0.25ng in 2.5ul) or NGF/VEGF (50ng/250ng in 2.5ul) was slowly injected into the 3 disc levels of the rats in the PBS control, TNFα and NGF groups, respectively. The abdominal wound was then closed. No intralidcal injection was performed in the sham surgery group, and there was no surgery in the naïve group. Pain behavior was assessed weekly for up to 6 weeks using mechanical hyperalgesia in the hindpaw with a series of calibrated von Frey filaments (0.6g-26g). Function was also assessed using inclined plane test to determine the maximum inclined angle until the rat was unable to maintain its position (2). The severity of IVD degeneration was examined weekly using radiographic disc height measurements and histology. Six weeks after surgery, the rats were euthanized using CO2 and their lumbar spines were collected, decalcified, embedded in paraffin and sectioned at 5μm. The sections were stained with Hematoxylin and Eosin and Safranin-O/Fast-green/Hematoxylin for morphology and glycosaminoglycan (GAG) content. For statistical analysis, changes in paw withdrawal threshold and disc height with time were compared using repeated measures ANOVA, and the difference in inclined angle between groups was compared using a one-way ANOVA. All statistical tests were performed using SPSS v.20 with p<0.05 being significant.

Results: The average withdrawal threshold (from right and left paws) of the naïve group was maintained throughout the experimental period. The withdrawal threshold transiently decreased after sham surgery (P>0.05), and recovered one week after surgery. The withdrawal threshold decreased immediately after intralidcal injection of PBS, TNFα and NGF/VEGF (P<0.05) (Figure 1). There was a mild recovery of withdrawal threshold in the PBS control group one week after surgery, but the threshold continued to decrease in both TNFα and NGF/VEGF groups (P<0.05). The inclined test evaluated the functional agility of the rats, and the decreased inclined angle showed that the TNFα and NGF/VEGF injected rats had a poor motor control which might be associated with discogenic pain. IVD heights of naïve and sham surgery groups did not show obvious change throughout the experiment. However, there was a continuous decrease over time in disc height after intralidcal injections in PBS control, TNFα and NGF/VEGF groups (P<0.05).

Healthy IVD morphology with organized annulus fibrosus (AF), GAG-rich nucleus pulposus (NP) as well as notochordal NP cells and fibroblast-like annular cells was observed in the sham surgery group (Figure 2). Intralidcal injection of NGF/VEGF and TNFα induced mild to moderate IVD degeneration, including disorganized AF and decreased number of NP cells (Figure 2). There was some variability in histological findings with one of the NGF/VEGF injected disc being highly degenerated with significant decreased GAG, highly disorganized AF and fibrous NP, Along the needle track in the AF of TNFα and NGF/VEGF animals there was prominent structural disruption, loss of GAG staining, and was evidence for irregular (i.e., non-IVD) cells (Figure 3).

Discussion: Intralidcal injection of TNFα or NGF/VEGF induced functional and histological changes representative of discogenic
pain with continuously and significantly reduced paw withdrawal thresholds (i.e. pain), loss of IVD height, degenerated IVDs and decreased performance on the inclined angle test. TNFα and NGF/VEGF are naturally occurring and commonly found in degenerated IVDs, therefore, the findings may provide insight for the pathophysiology of discogenic back pain, inflammation and neurovascular ingrowth. Increased pain-sensitive behaviors were observed in all injected IVDs, which may be associated with IVD degeneration as evidenced by decreased IVD height and histology (Figure 2). The annular puncture might also reduce spinal stability and induce IVD degeneration, and this accelerated degeneration process might be facilitated by intradiscal injection of NGF/VEGF and TNFα which promotes the breakdown of extracellular matrix. Proteoglycans have also been shown to be an inhibitor of nerve growth, and the structural defect and reduced GAG along the needle track at the AF might provide a path for neurovascular ingrowth. High magnification histological assessment suggested invasion of non-IVD cells along the needle track of NGF/VEGF and TNFα groups which may be a sign of neurovascular or macrophage invasion, and further immunohistochemical identification of these cells for nerve fibers and blood vessels and macrophages are warranted. NGF/VEGF and TNFα have also been demonstrated to regulate the production of substance P which is an important neurotransmitter for pain perception (3, 4). Therefore, the nerve endings might be irritated mechanically by the concentrated AF stress and chemically by the upregulation of substance P.

**Significance:** The findings may provide insight for pathophysiology of discogenic back pain and the association between disc degeneration, inflammation and NGF/VEGF. We also present a new discogenic pain model that explores mechanisms for neurovascular ingrowth and may have high significance for screening future treatment modalities and biological methods of tissue repair.

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![Figure 1. The changes of averaged withdrawal threshold in different groups. Significant decreases were observed for after intradiscal injection of PBS, TNFα and NGF/VEGF, however, the changes were more obvious in TNFα and NGF/VEGF groups.](image.png)
Figure 3. Safranin-O/fast-green/hematoxylin staining for NGF/VEGF(a) and TNFα (b) and hematoxylin and eosin staining for NGF/VEGF(c). Arrow marks irregular (i.e., non-IVD) cells along the needle track in the AF.