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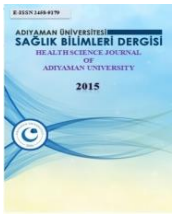

Araştırma/Research

Comparison of Antioxidant mRNA expression levels of advanced Protruded and Extruded Nucleus Pulposus in Degenerative Lumbar Disc Herniations using the RT-PCR method

Şeyho Cem Yücetaş[1], Necati Üçler[1]
Adıyaman Üniversitesi Beyin Cerrahi AD. Adıyaman. Turkey

Abstract

Purpose: The aim of the present study is to compare the antioxidant levels of nucleus pulposus in advanced protruded and extruded lumbar disc hernias (LDH) with a steady lumbar posterior longitudinal ligament with the Real Time-Polymerase Chain Reaction (RT-PCR) method.

Method: This study was conducted with the comparison of the disc sections of a total of 40 patients who underwent an operation due to advanced protruded (n=20) and extruded (n=20) LDH with the TRI-Reagent® and Ribonucleic acid (RNA) isolation and the RT-PCR method between January 2013 and May 2016. The study included patients diagnosed with lumbar disc herniation who were applied with microdiscectomy surgery. The antioxidant levels of the disc material, which caused compression because it was highly protruded or extruded, were detected using the RT-PCR method, and the expression levels of the genes were determined.

Results: The antioxidant levels of the disc materials of a total of 40 patients, who underwent an operation due to protruded and extruded disc hernia, were determined with the RT-PCR method. The patients were found 25 females with a mean age of 36.4 years and 15 males with a mean age of 39.26 years. Of the total patients, 20 had MacNab grade 2 (protruded) and 20 had MacNab grade 3 (extruded) disc hernias. The expressions of protruded LDH materials were found to be lower than those of extruded LDH materials.

Conclusion: Although the expression levels of the significant antioxidant molecules of TGF-β, FGF-β, IGF-1, NGF, MMP-3, and GAPDH mRNA, in patients with extruded LDH were significantly higher compared to those with protruded LDH, no distinctive features of these levels could be determined in terms of discogenic pain and postoperative clinical improvement.

Key Words: Lumbar microdiscectomy; extruded disc; RT-PCR; Gene Expression.

Yazışmadan Sorumlu Yazar

Şeyho Cem Yücetaş

Adıyaman Üniversitesi Beyin Cerrahi AD. Adıyaman. Turkey
Tel : +90 05056563744

Email: seyhocem@hotmail.com

Doi: 10.30569.adiyamansaglik. 486946

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RT-PCR yöntemi kullanılarak Dejeneratif Lomber Disk Herniasyonunda İleri Düzey Protrüde ve Ekstrüde Nükleus Pulposusun Antioksidan mRNA ekspresyon düzeylerinin Karşılaştırılması

ÖZ

Amaç. Bu çalışmanın amacı, Real Time-Polimeraz Zincir Reaksiyonu (RT-PCR) yöntemi ile lumbar posterior longitudinal ligamentte ileri düzey protrude ve ekstrüde lomber disk hernilerinde (LDH) nükleus pulposusun antioksidan düzeylerini karşılaştırmaktır.

Metot: Bu çalışma, Ocak 2013 ile Mayıs 2016 arasında izolasyon ve RT-PCR yöntemi ile ileri düzey protrüde (n = 20) ve ekstrüde (n = 20) LDH ile TRI-Reagent® ve Ribonükleik asit (RNA) ile ameliyat edilen toplam 40 hastanın disk bölümlerinin karşılaştırılması ile yapıldı. Çalışmaya, mikrodisektomi cerrahisi uygulanan lomber disk herniasyonu tanısı alan hastalar dahil edildi. Sıkışmaya neden olan disk materyalinin antioksidan seviyeleri, RT-PCR yöntemi kullanılarak tespit edildi ve genlerin ekspresyon seviyeleri belirlendi.

Bulgular: Protrude ve ekstrüde disk hernisi nedeniyle ameliyat edilen toplam 40 hastanın disk materyallerinin antioksidan düzeyleri RT-PCR yöntemi ile belirlendi. Çalışmadaki 25 kadın hastanın yaş ortalaması 36.4 ve 15 erkek hastanın yaş ortalaması 39.26 olduğu belirlendi. Toplamda hastaların 20'sinde MacNab grade 2 (protruded) ve 20'sinde MacNab grade 3 (ekstrüde) disk fıtığı vardı. Ekspresyon sonucunda protrüde LDH materyallerinin ekstrüde LDH materyallerinden daha düşük olduğu bulundu.

Sonuç: Ekstrüde LDH'si olan hastalarda TGF-β, FGF-β, IGF-1, NGF, MMP-3 ve GAPDH mRNA'nın anlamlı antioksidan moleküllerinin ekspresyon seviyeleri, protrüde LDH hastalara göre anlamlı olarak daha yüksek bulunmasına rağmen, bu düzeylerin diskojenik ağrı ve postoperative klinik iyileşme açısından ayırt edici özellik olarak belirlenemedi.

Anahtar Kelimeler: Lomber mikrodiskektomi; ekstrüde disk; RT-PCR; Gen İfadesi.

Introduction

Lumbar disc herniation (LDH) is a problem that frequently affects the spinal cord, particularly in young and middle-aged patients. Symptomatic LDH affects 1-3% of the general population and only 15-20% of these cases require an operation (1). In addition, the need for surgery is evaluated if no significant improvement can be achieved over 6 months with conservative treatment in patients with severe or typical symptoms (2,3).

Microdiscectomy (MD) is an alternative to conventional and more aggressive open approaches for the treatment of LDH. This method offers many important advantages such as less damage to the surrounding tissue, less blood loss, shorter duration of surgery, and more rapid postoperative recovery time (4,5). Therefore, it has become a preferred standard method in suitable patients compared to open surgery.

Intervertebral degenerative disc (IDD) is a multifactorial disorder. Etiological factors include aging, smoking, infection, abnormal mechanical stress, diabetes, obesity, trauma, and genetic predisposition (6–10). However, many antioxidant levels have been shown to increase during the formation of degenerative lumbar disc herniations (9–11).

There are many methods for determining the change in antioxidant levels in IDD. One of these methods is the detection of antioxidants by the RT-PCR method(12,13). In the present study, it was aimed to measure the Transforming growth factor- β (TGF-β), Fibroblast growth factor-β (FGF-β), Insulin-like growth factor-1 (IGF-1), Nerve growth factor (NGF), Matrix metalloproteinase-3 (MMP-3), and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) levels in the excised disc materials of the patients who underwent microdiscectomy due to protruded and extruded disc herniation.

Materials and Methods

Patient selection

The disc materials of 40 patients who underwent an operation with the microdiscectomy method between January 2013 and May 2014 in the Kafkas University Medical Faculty Neurosurgery Clinic were used in this retrospective study. Approval for the study was granted by the Local Ethics Committee and an informed consent form was signed by all patients included in the study.

Inclusion and exclusion criteria

A total of 40 patients over 18 years of age, who underwent an operation due to MacNab'sprotruded (Grade 2) (n=20) and extruded (Grade 3) (n=20) disc herniation according to the Spinal MRI results, were included in the study (**Figure 1**). The patients included were those with a definitive diagnosis made from Spinal Computed Tomography (CT) and/or MRI, of neurological and radiological protruded or extruded herniation and who underwent an operation with the microdiscectomy method.

Patients who had lumbar surgery history, severe spinal stenosis, lumbar fracture, signs of infection or malignancy in the disc space or vertebra corpus were not included in the study. Patients who were diagnosed with diabetes mellitus, hypothyroidism, smoking or multivitamin supplementation were not included in the study because these would affect antioxidant levels in the materials taken from patients.

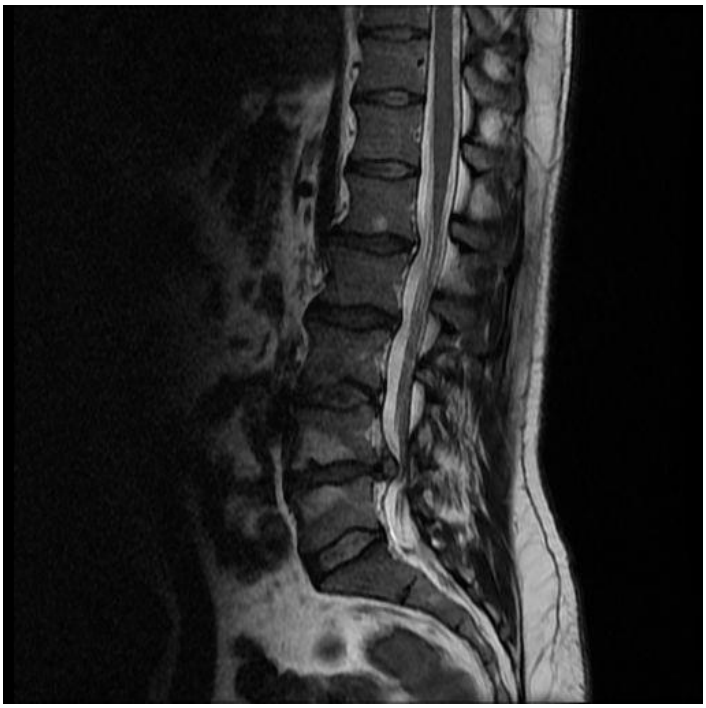


Figure 1 L4-L5 Intervertebral extruded lumbar disc herniation.

Surgical Intervention

Microdiscectomy was performed under epidural or general anesthesia. A 3 cm midline skin incision was made in the prone position and the paravertebral muscles were dissected subperiosteally using monopolar cautery. Using the muscle retractor, medicon speculum-

retractor (medicon, Tuttingen Germany), partial laminectomy, and medial facetectomy (limited to less than one-third of the entire facet joint) were performed under microscopy and the ligamentum flavum was extracted.

The ruptured disc fragment was exposed by gentle retraction of the unilateral sac and the transverse nerve root. The extruded disc part, the intra-circular disc part, and the partial nucleus pulposus were extracted by protecting the end plate.

Total RNA isolation (TRI)

The nucleus pulposus tissue obtained from the discs was dissolvedand then centrifuged at 7000 rpm for 10 minutes. Total RNA isolation was performed after removal of the supernatant and using the TRI reagent (Sigma®) in cell precipitation.

Total RNA isolation with the TRI solution (Tri Reagent)

RNA samples were measured at wavelengths of 260 and 280 nm (nanometers) in the Nano Drop spectrophotometer. During these measurements, the buffer with which the pellets of RNA samples were dissolved was used blindly. The amount of RNA was determined using the values read in the spectrometer in the formulas below.

Production of cDNA from the total RNA with the reverse transcription (RT) reaction

The Fermentas Revert Aid First Strand cDNA synthesis Kit® (catalog number: 1622) was used for the RT reaction. All steps were carried out in accordance with the kit procedure (14).First, 5µg of RNA sample obtained from the RNA isolation was placed in ice in a way to react in the PCR tubes of 0.5, and 1µl oligo dT18 was added and the volume was completed to 12 µl with sterile distilled water. The reaction tube was put into ice after being kept at 70°Cfor5 minutes. A total of 4µl of 5x Reaction buffer, 1µl of Ribolock Ribonuclease inhibitor and 2µl of 10 mMdNTP mixture were added into the reaction tube in the ice, and it was incubated at 37ºC for 5 minutes. At the end of the incubation period, 1µl of M-MuLV reverse transcriptase enzyme was added. The tube with the prepared mixture was kept at 42ºC for 60 minutes. At the end of this period; the reaction tube was incubated for 10 minutes at 70ºC to inhibit the enzyme. It was then stored at -20 ºC until assay.

PCR Reaction Conditions

The cDNA obtained from the source DNA with the RT reaction was used in the PCR reaction. The PCR reaction was generated by half-decayed (F and R) primers: 12.3 µl dH_2O was added on 2.5 µl 10xbuffer, 2.5 µl 25 mM $MgCl_2$, 2 µl 2.5 µM dNTP mixture, 2.5 µl F, 2.5 µl R, 0.5 µl cDNA mold (1:10 diluted), 0.2 µl TaqDNA Polymerase enzyme (5u/µl) in a way that the final volume was25 µl. The DNA fragments processed on the agarose gel were checked on the UVP transilluminator device and the data were recorded with the UV-Photometer gel documentation device (UviTec®).

Statistical Analysis

Analyses of the study data were made using SPSS 25.0®software (IBM Corporation, Armonk, New York, USA).The continuous variables were expressed as mean and standard deviation values and the categorical variables as median values. The SPSS 21 Chi-square and Mann Whitney U tests were used.

Results

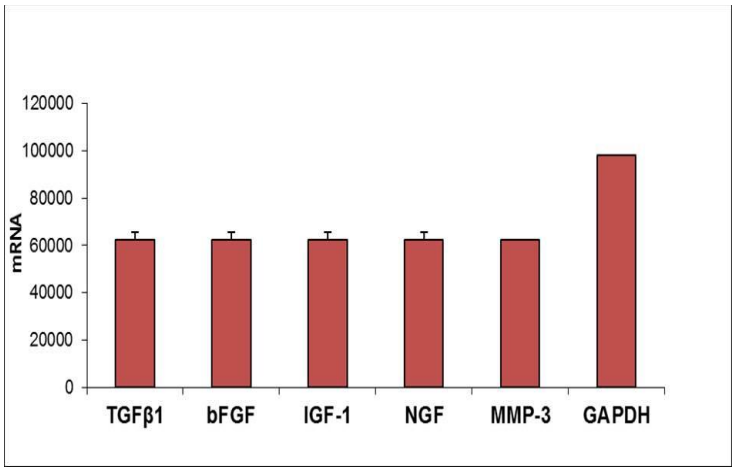
The antioxidant levels of the disc materials of a total of 40 patients, (25 females of mean age 36.4 years and 15 males of mean age 39.26 years) with an age average of 38.4 years, who underwent an operation due to protruded and extruded disc hernia, were evaluated with the RT-PCR method (**Table 1**).

Table 1. Demographics.

Variables	**Protruded (MacNab Grade 2) (n=20)**	**Extruded (MacNab Grade 3) (n=20)**
Gender (F/M)	14/6	11/9
Age (Year)	38.35 ± 7.76	38.45 ± 7.07
Disc location	L4-L5 (100%)	L4-L5 (100%)
Hernia side (right/left)	11/9	12/8
Hernia location (medial/lateral)	4/16	8/12

All the lumbar disc herniations were in the lumbar L4-5 range; 20 patients had highly protruded and 20 patients had extruded disc herniations. The disc material was located in the lateral recess in 16 and in the midline in 4 of the patients with a protruded disc. It was located in the lateral recess in 12 and in the midline in 8 of the patients with extruded disc herniations (**Table 1**).

In lumbar disc herniations, the mRNA levels in the highly protruded and extruded disc materials were found as 60.000 copies/µL for TGF-β, FGF-β, IGF-1, NGF, and MMP-3 and as 100.000 copies/µL for GAPDH (**Graphic 1**).



Graphic 1. Numbers of mRNA copies (copy/µL)

The expression of lumbar protruded genes was determined to be lower than that of the extruded disc materials. The expression was found to be lower in lumbar disc herniations than in those exposed to compression.

Discussion

The lumbar intervertebral disc is a complex structure consisting of collagen, which helps to appropriately disperse the forces applied to the spine, proteoglycans and infrequently ordered fibrochondrocytic cells. Mechanical damage to this structure or prolonged pressure causes the stimulation of the cells, activation of the macrophages and acceleration of the inflammatory processes. As a result, the oxidant / antioxidant equilibrium is impaired, inflammatory molecules accelerate cell destruction, and important clinical outcomes occur

such as clinically long-term low back pain and persistent neurological symptoms. As yet there is no clarification of biological evaluations of whether the pain is only related to the pressure or if the disc material is associated with the antioxidant level. However, in this study, it was thought that the low level of expression leads to an increase in the pressure in proportion to the weakness of the defense system.

The aim of this study was to compare the TGF-β, FGF-β, IGF-1, NGF, MMP-3, and GAPDH mRNA expression levels in patients with protruded and extruded lumbar disc herniation using the RT-PCR methods. From the results of the present study, it was observed that the expression of antioxidant molecules increases in line with the increase in pressure and the severity of disc herniation. However, there is still no clear explanation of the relationship between this condition and discogenic pain.

Lumbar disc herniation must be evaluated radiologically before the operation, and MRI is currently the imaging modality with the best sensitivity and specificity (15,16). The patient's clinical findings, as well as the stage of the disc (Grade), are evaluated in cases where a decision is to be made for LDH surgery.

The staging of herniation with MRI is often assessed according to the Schneiderman's or MacNab scoring systems. According to the Mac Nabclassification, disc degeneration is classified as grade 1: normal, grade 2: protruded, grade 3: extruded, and grade 4: sequestered(17). The patients who undergo surgery are often highly protruded, extruded or sequestered disc herniation patients. In the present study, the spinal MRI images of patients who had been clinically examined were assessed according to the MacNab classification and those with grade 2 and 3disc degenerations were compared.

Previous studies have shown that MMP-3 and MMP-7are elevated in herniated disc material and particularly the high MMP-3 is effective in the resorption of the disc material (18). In addition, it has also been determined that MMP-3 levels increase with the stimulation of the cells in nucleus pulposus by macrophages (19). In the present study, MMP-3 transcription was seen to be significantly increased, which was consistent with the literature.

In a study by Kim et al., it was stated that patients with spinal stenosis had higher TGF-β1, TIMP-1, and TIMP-2 levels in ligamentum flavum tissue than patients with disc herniation, although no significant difference was found between the two groups in terms of

serum levels. Based on this information, it was emphasized that spinal stenosis has a local inflammatory and oxidative effect on the lumbar disc (20). In the present study, TGF-β1 levels in the extruded nucleus pulposus group were significantly higher than those of the protruded group in accordance with the literature. Since this was a retrospective study, serum TGF-β1 levels were not evaluated in the patient groups.

During degeneration, the cells become more rounded in a similar manner to the chondrocytes, whereas in normal conditions, the ring-shaped cells are rather spindle-shaped. Chondrocyte-like cells that frequently form clusters affect the extracellular matrix cycle. Previous studies have emphasized the growth factor in the transformation of TGFβ−1 andTGFβ−2, basic fibroblast growth factor in the herniated disc tissue, and the platelet-derived growth factor (PDGF). In a study by Tolonen et al., it was shown that bFGF was stained immunehistochemically only in damaged intervertebral discs, and no staining was observed in the control group. However, bFGF has been shown to be more highly expressed in chondrocyte-like disc cells, which are more intense than anterior annulus fibrosis, compared to the fibroblast-like disc cells (21). In the present study, the bFGF transcription was positive in accordance with the literature and no significant difference was found between the two groups in terms of bFGF levels. This was thought to be related to the similarity of disc pressures in terms of their localization.

In recent years, it has been emphasized that the NGF levels in symptomatic lumbar herniations increased more compared to findings in previous studies. It has been shown that the elevated NGF developed secondary to annular rupture and was closely associated with discogenic pain(22). In the present study, NGF levels were seen to be higher in patients with extruded discs than in patients with protruded discs. However, no significant difference could be shown between the patients in terms of discogenic pain.

The present study had some important limitations. That it was a single-centered, retrospective study, the low number of patients and the lack of findings related to clinical outcomes (eg, length of hospital stay, clinical improvement, complications, and pain) may be considered as limitations of the study. However, the fact that the patient groups in the present study had quite close characteristics to each other made the comparison more appropriate.

In conclusion, while the expression levels of the significant antioxidant molecules of TGF-β, FGF-β, IGF-1, NGF, MMP-3, and GAPDH mRNA, were significantly higher in patients with extruded LDH compared to those with protruded LDH, no distinctive characteristics could be found for these levels in terms of discogenic pain and postoperative clinic improvement. There is a need for multicenter, prospective studies in this area, which include healthy control groups.

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