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# EXTRACELLULAR ACTIN AS A FACTOR IN THE DEVELOPMENT OF DEGENERATIVE PROCESSES OF INTERVERTEBRAL DISC

**Sudakov N.P.[1,2,3]*, Klimenkov I.V.[2,4], Byvaltsev V.A.[1,5,6], Nikiforov S.B.[1], Goldberg O.A.[1], Kalinin A.A.[1,5,6], Bardonova L.A.[1,5], Belykh E.G.[1]**

[1] FSBSI Irkutsk Scientific Center of Surgery and Traumatology, Irkutsk, Russia
[2] FSFEI HE Irkutsk State University, Irkutsk, Russia
[3] FSBIS Irkutsk Research Center, Siberian Branch, Russian Academy of Science, Irkutsk, Russia
[4] FSBIS Limnological Institute, Siberian Branch of the Russian Academy of Sciences, Irkutsk, Russia
[5] FSFEI HE Irkutsk State Medical University, Irkutsk, Russia
[6] Non-governmental Health Care Institution Road Clinical Hospital at Irkutsk-Passenger station JSC "Russian Railways", Neurosurgery Center, Irkutsk, Russia

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***Abstract***

*Intervertebral disc is the largest avascular organ in human body, which consists of the nucleus pulposus surrounded by annulus fibrosus at the sides and cartilaginous endplates. Degenerative changes in intervertebral discs are a common pathology and one of the main causes of disability among the population. Inflammatory process is one of the key mechanisms of intervertebral disc degeneration. It is assumed that the degradation products of intervertebral disc cells may be directly involved in the initiation and formation of the inflammatory process. Many of the cellular decay products referring to danger-associated molecular patterns are capable of inducing an inflammatory response, one of which is the actin. Present article describes the possible pathogenetic role of extracellular actin in the necrotic zones of nucleus pulposus in intervertebral disc degeneration.*

*Studies were performed using intervertebral disc fragments obtained during diskectomy and fusion procedure. Fragments of disc tissue were fixed with 4% paraformaldehyde for histochemical analysis. Specimens were stained with fluorescent dye for cell nuclei and phalloidin-fluorescein isothiocyanate for actin microfilaments. In situ analysis of apoptosis was performed by terminal deoxynucleotidyl transferase dUTP nick end labeling assay.*

*Laser confocal microscopy showed that necrotic zones of nucleus pulposus of intervertebral disc were infiltrated by neutrophils and isolated lymphocytes. Terminal deoxynucleotidyl transferase dUTP nick end labeling stated the presence of few cells (15%) with DNA fragmentation in the nucleus, confirming the activation of programmed death mechanisms. The analysis of preparations stained with phalloidin-fluorescein isothiocyanate staining identified large clusters of extracellular filamentous actin. Presence of lymphocytes in the studied areas of necrosis indicated the development of an antigen-specific immune response to the decay products of the nucleus pulposus structures of intervertebral disc, and the participation of extracellular actin as one of possible autoantigens.*

*Thus, it can be assumed that extracellular actin, which is normally cleaved and disintegrated in other tissues, can be potentially involved in the pathogenesis of intervertebral disc degeneration as an autoantigen and a new danger-associated molecular pattern.*

***Keywords:*** *danger-associated molecular patterns, degeneration, extracellular actin, intervertebral disc, laser confocal microscopy, nucleus pulposus, programmed cell death.*

## Introduction

Intervertebral disc is the largest avascular organ in human body. It consists of the nucleus pulposus surrounded by annulus fibrosus at the sides and cartilaginous endplates on the top and bottom. Degenerative changes in intervertebral discs are a common pathology and one of the main causes of disability among people in developed countries [*Resnick D et al., 2009*]. The need to develop effective methods of early diagnosis and treatment determines the importance of a detailed study of individual elements

***Address for Correspondence:***
Sudakov Nikolay Petrovich
Irkutsk Scientific Center of Surgery and Traumatology
1 Bortsov Revolutsii Street, Irkutsk 664003, Russia
Tel.: (3952) 290-339, (89832411369)
E-mail: npsudakov@rambler.ru

in the pathogenesis of this disease.

It is known that inflammatory process is one of the key mechanisms of intervertebral disc degeneration [*Hadjipavlou A et al., 2008; Weber K et al., 2015*]. The immune response generated toward extracellular matrix components such as collagen types I, II, and V and aggrecan has been previously demonstrated [*Capossela S et al., 2014*]. The role of extracellular matrix components in the development of the immune response is being actively investigated. Studies suggest that programmed cell death of chondrocytes plays an important role in the development of degenerative changes in the intervertebral disc matrix [*Kuhn K et al., 2004*]. Mechanical stress is one of the initiators of programmed cell death [*Chen C et al., 2001; Dudli S et al., 2014*]. It is established that chondrocytes are characterized by different variants of programmed cell death: apoptosis, chondroptosis and necrosis [*Zamli Z, Sharif M, 2011*]. It is proved that apoptosis of chondrocytes has its unique characteristics: limited access of phagocytes in the intervertebral disc tissue predetermines the lack of clearance of apoptotic bodies, which subsequently disintegrate and release their contents into the extracellular space [*Krysko D et al., 2006; Polzer K et al., 2007*]. It is assumed that the degradation products of intervertebral disc cells may be directly involved in the initiation and formation of the inflammatory process. It is known that many of the cellular decay products referred to danger-associated molecular patterns are capable of inducing an inflammatory response [*Krysko D et al., 2012; Kaczmarek A et al., 2013*]. One of such danger-associated molecular patterns is the actin, the most common intracellular protein responsible for cell shape and motility [*Ahrens S et al., 2012*]. Extracellular actin may be involved in various disease processes as an inducer of the autoimmune process [*De Scheerder I et al., 1985*]. Probably, deposits of extracellular actin are the initiators and intensifiers of immune response to extracellular matrix components.

Present report objectively shows the presence of extracellular actin deposits in the intervertebral disc tissue of patients with degenerative processes of this structure and discusses its possible involvement in the autoimmunity aspects of intervertebral disc degeneration.

## Material and methods

Intervertebral disc material for the studies was obtained during planned spinal operations. Specimens from 5 patients aged 26-57 years with signs of intervertebral disc degeneration at $L_{IV}$-$L_V$ level were studied. Tissue samples were dissected for analysis from the annulus fibrosus and nucleus pulposus regions. Study protocol was approved by the Ethics Committee of Irkutsk Scientific Center of Surgery and Traumatology (No 10, 31.08.2015), and also the biological material of intervertebral disc of patients, who have signed an informed consent was used.

*Morphological studies:* Intervertebral disc samples $L_{IV}$-$L_V$ were fixed in buffered 10% formalin, and processed in a vacuum tissue processor VIP-E150F (Sakura, Japan). Staining with hematoxylin and eosin was performed in a slide stainer machine DRS-601A (Sakura, Japan). Cover Tech glass coverslips (Microm, Germany) were used on slides. Microscopic examination was carried out with a digital microscopic video system Quantimet 550IW (Leica, England and Olympus, Japan).

*Confocal laser microscopy:* Fragments of the intervertebral disc tissue from $L_{IV}$-$L_V$ were immediately fixed in 4% paraformaldehyde (Sigma-Aldrich, USA). The cell nuclei were stained with fluorescence dye (Sigma-Aldrich, USA); actin microfilaments were stained with phalloidin-fluorescein isothiocyanate (Sigma-Aldrich, USA). The analysis of programmed cell death was performed by detection of fragmented DNA by terminal deoxynucleotidyl transferase dUTP nick end labeling assay Click-iT® TUNEL Alexa Fluor® Imaging Assay (Thermo Fisher Scientific, USA), according to the recommendations of the manufacturer. The slides were imaged under LSM-710 laser confocal microscope (Carl Zeiss, Germany), and three-dimensional reconstructions were generated using Imaris Bitplane 7.2.3 software (Bitplane AG, Switzerland).

## Results and discussion

Patients underwent surgery for spinal canal decompression, removal of $L_{IV}$-$L_V$ disc herniation and transforaminal cage implantation at $L_{IV}$-$L_V$ from posterior approach. Obtained intervertebral disc fragments, as well as pieces of the posterior longitudinal ligament were immediately fixed and trans-

ported to the laboratory for investigation.

Histological analysis by light microscopy of the nucleus pulposus specimens stained with hematoxylin and eosin revealed extensive areas of tissue necrosis. These areas were characterized by dystrophic changes and coagulative necrosis of chondrocytes, destruction of the extracellular matrix (Fig. 1 a-c). Whereby, features of active productive inflammation, such as proliferation of capillaries and fibroblasts, were found in the posterior longitudinal ligament, located in the area close to disc herniation (Fig. 1d).

Laser confocal microscopy of the necrotic zones of nucleus pulposus cells allowed visualizing the infiltration by neutrophils and isolated lymphocytes (Fig. 2). Terminal deoxynucleotidyl transferase dUTP nick end labeling method demonstrated the presence of numerous cells (15%) with DNA fragmentation inside their nuclei, indicating the activation of programmed cell death mechanisms (Fig. 2a). Phalloidin-fluorescein isothiocyanate staining was able to identify cells with clearly visible actin cytoskeletons, such as neutrophils, lymphocytes and chondrocytes. Large clus-

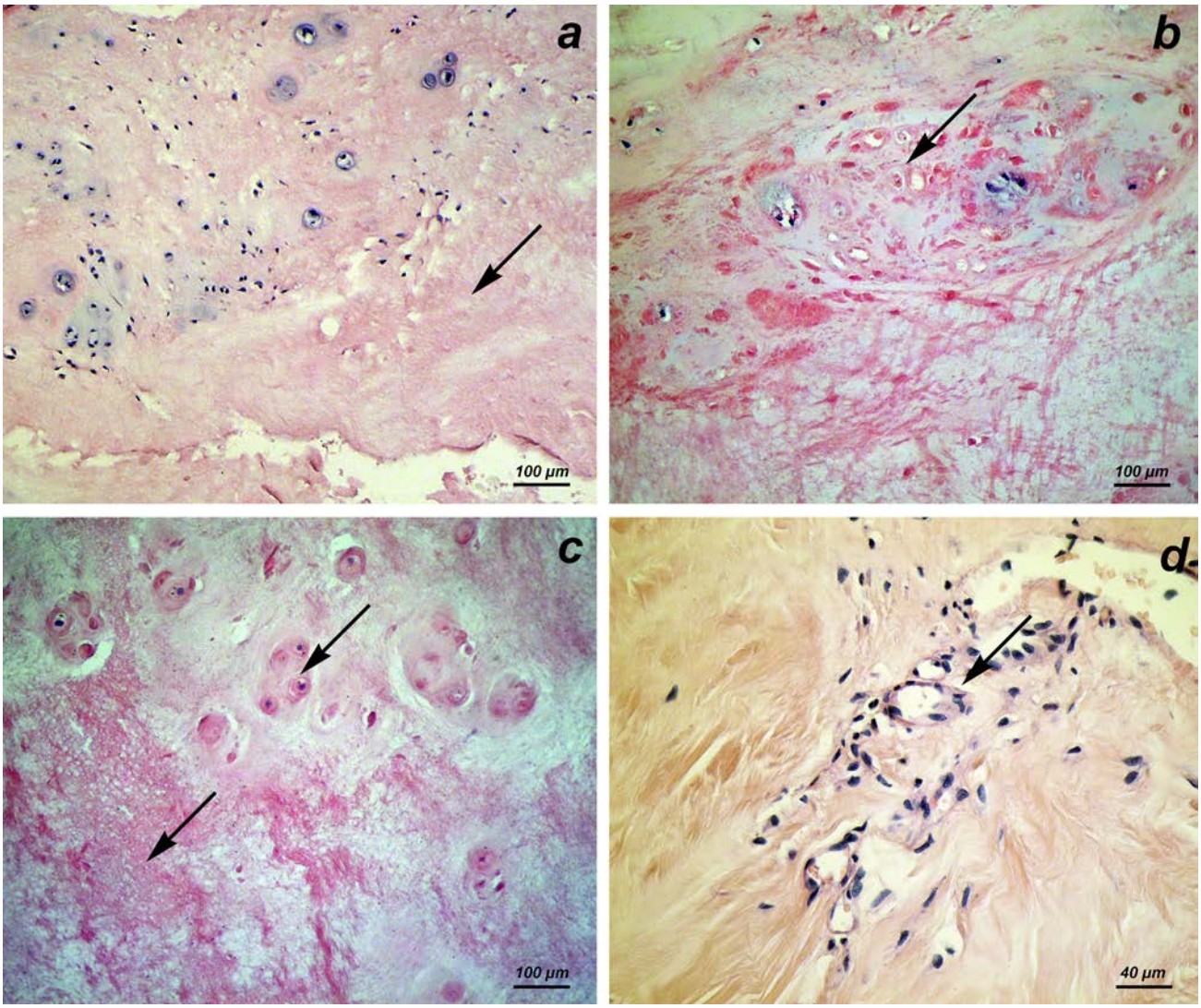


***Figure 1.*** *Light microscopy of intervertebral disc tissues stained with hematoxylin-eosin.* ***(a)*** *Tissue necrosis of nucleus pulposus;* ***(b)*** *Degenerative changes and coagulative necrosis of chondrocytes;* ***(c)*** *Isolated groups of chondrocytes with dystrophic changes, destruction of the extracellular matrix;* ***(d)*** *Posterior longitudinal ligament with proliferation of capillaries, fibroblasts – productive inflammation. Scale bars:* ***(a, b, c)*** *– 100 µm, (**d**) – 40 µm.*

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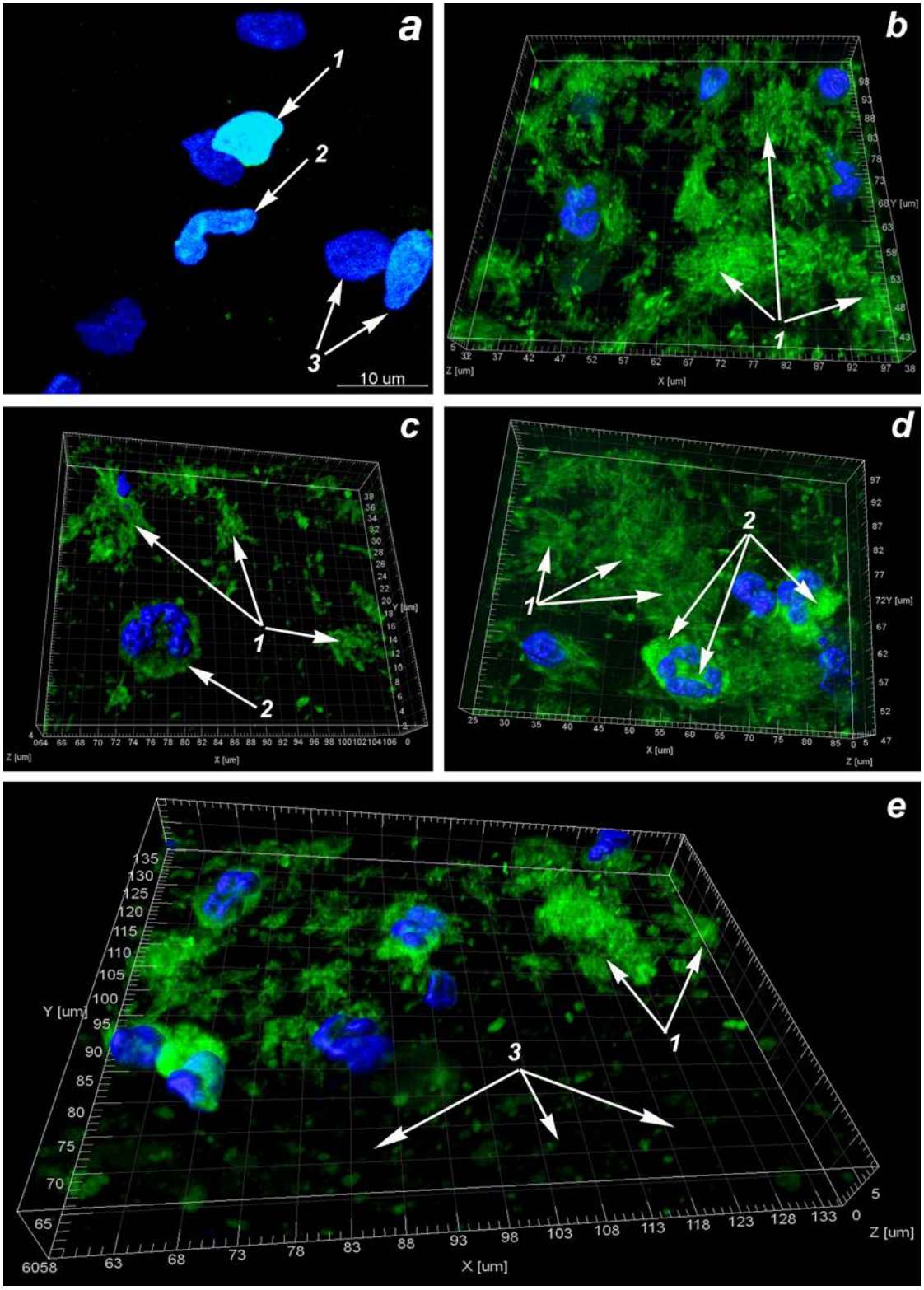


***Figure 2.*** *Cytochemical features of degenerative changes in the necrotic zone of nucleus pulposus.* ***(a)*** *Terminal deoxynucleotidyl transferase dUTP nick end labeling of the positive cell (1), located in the immediate vicinity of the neutrophil (2) and terminal deoxynucleotidyl transferase dUTP nick end labeling of the negative cell (3). Scale bars: 10 µm.* ***(b, c, and d)*** *– intervertebral disc preparations obtained from the same patient show extracellular (1) and intracellular (2) actin microfilaments.* ***(e)*** *Necrotic zone with cells and compartments of extracellular actin (1), bordered with unchanged nucleus pulposus tissue (3).*

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ters of filamentous actin, exceeding the volume of the space occupied by the actin cytoskeleton in these cells, were revealed in the extracellular space of necrotic zones in three patients (Fig. 2 b-e).

In this regard it can be assumed that these actin aggregates could be formed because of actin polymerization in the extracellular matrix. Actin's ability to self-assemble into microfilaments in the extracellular medium was previously confirmed by *in vivo* studies [*Haddad J et al., 1990*]. Obtained results also raise an interesting question about the nature of the nucleation centers for the identified actin aggregates. It has been shown that actin can bind to fibronectin *in vitro*, which is a major extracellular matrix component [*Keski-Oja J et al., 1980*]. The amount of fibronectin in nucleus pulposus has been shown to increase with progression of intervertebral disc degeneration [*Oegema T et al., 2000*]. Perhaps, in this case, fibronectin could serve as a nucleation center. In the studied specimens, tufts of filamentous actin in the extracellular matrix were arranged predominantly radially, concentrating at one or more aggregation centers. There were also single large bundles of parallel-oriented actin microfilaments. It can be assumed that such formations are generated upon the release of actin from the dying cells [*Krysko D et al., 2012; Kaczmarek A et al., 2013*]. The release of actin from the intact cells is also possible [*Smalheiser N, 1996*].

Currently, the mechanism by which the actin can be released from a cell and displayed on the cell's plasma membrane remains unknown, because actin does not contain a signal peptide or transmembrane domain in its structure [*Dudani A, Ganz P, 1996*]. It is known that extracellular actin molecules can be detected on the cell surface [*Moroianu J et al., 1993; Miles L et al., 2006*] and interact with plasminogen and plasmin [*Dudani A, Ganz P, 1996*]. The amount of actin detected on the cell surface was shown to be grown with increased functional activity and stress [*Bachvaroff R et al., 1980*]. With this in mind, it is possible that actin can be released by the intervertebral disc cells under extreme mechanical and hyponutrition conditions, which are one of the major causes of intervertebral disc degeneration.

It is also demonstrated that actin can be released to the systemic circulation [*Erukhimov J et al., 2000*]. The presence of actin in the urine after ischemia related to kidney transplant was previously reported [*Kwon O et al., 2003*]. There are actin scavenger systems in blood plasma that include vitamin D-binding protein (Gc-globin) and gelsolin [*Janmey P, Lind S, 1987*]. Insufficiency of this system functioning leads to the excessive formation of F-actin, providing various negative effects on the cells [*Lee W, Galbraith R, 1992*].

Neutrophils, which probably can be involved in the utilization of the degradation products of dead cells, including extracellular actin, were detected in the areas of necrotic tissue containing extracellular actin deposits. In addition, activated neutrophils may have a negative effect on viable chondrocytes adjacent to the areas of necrosis, in particular, due to the production of reactive oxygen species. This may facilitate the spread of the degenerative process in intervertebral disc tissue. The presence of lymphocytes in the analyzed zones of necrosis implied the development of an antigen-specific immune response toward the nucleus pulposus components. The identification of cytotoxic T-lymphocytes and dendritic cell receptors, namely DNGR-1 (also known as CLEC9A) specific to F-actin, on the surface of immune cells is an important fact confirming the possibility of the involvement of extracellular actin in the autoimmune process [*Ahrens S et al., 2012*]. It has also been shown that antibodies against actin are formed in a number of pathological processes, such as autoimmune hepatitis, celiac disease, idiopathic nephrotic syndrome, heart transplant rejection, hemolytic anemia and herpes dermatitis [*Felder K et al., 2010; Schirru E et al., 2013; Schotte H et al., 2016*].

Overall, the findings combined to the data of discussed studies objectively confirm the participation of extracellular actin as an autoantigen in the inflammatory process, contributing to degenerative changes in the nucleus pulposus of intervertebral disc in spondylarthrosis pathogenesis. Studying the role of extracellular actin in the intervertebral disc pathology may lead to the development of new technologies for the prophylaxis and treatment of disc degeneration.

The clusters of extracellular actin were revealed by using confocal laser microscopy in the necrosis zones of nucleus pulposus in patients with spondylarthrosis. The role of extracellular actin in the development of degenerative processes of intervertebral disc is being discussed, perhaps as an active

autoantigen, initiating and promoting further immune response toward the extracellular matrix components. Future advanced interdisciplinary studies in this area are needed for the development of new diagnostic tests of spondylarthrosis early manifestations or more complex clinical cases related to the processes of spinal canal stenosis.

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