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Biochemical Structure and Functions of the Lumbar Disc after an Autologous Chondrocyte Transplantation (ACT) Evaluated by Studies of Two Operated Cases of Repeated Disc Herniation

Abaaslam F[1,2], Marszałek P[2], Głowacki M[1], Włuka A[1], Sierzputowski T[2] and Czernicki Z[1,2*]

[1]*M. Mossakowski Institute of Experimental and Clinical Medicine, Polish Academy of Sciences, Warsaw, Poland*

[2]*Department of Neurosurgery, 2nd Faculty of Medicine, Medical University of Warsaw, Poland*

Abstract

Transplantation of autologous chondrocytes obtained from cellular culture constitutes one of the methods of the biological treatment of the degenerative disc disease (DDD). The study covered a group of 50 patients operated on for single-level lumbar discopathy in whom material for autologous chondrocyte culture was sampled from a removed fragment of an intervertebral disc. The chondrocytes obtained from the culture were next implanted into the pre-operated space with the use of the percutaneous method, the implantation being followed by a radiological and clinical evaluation. In two cases a repeated prolapse of a nucleus pulposus fragment was reported after an earlier transplantation. The patients were operated on again. The material obtained from the recurred nucleus pulposus hernia allowed for a biological evaluation of the effect of the transplantation on the structure of the intervertebral disc with a light and confocal microscope. The examination showed good integration of the transplanted cells with the cellular matrix and their correct production activity in the form of the generation of typical matrix components – type I, II, III, IX collagen, proteoglycans and aggrecan as well as type IV collagen, atypical for the matrix, the presence of which is a subject of further research works.

Keywords: Lumbar disc; Chondrocytes; Disc herniation; Spine

Introduction

The degenerative spine disease affects a very large part of the population. According to sources available, 70 to 80% of people report pain-related complaints in different parts of the spine, the lumbar segment being most common. Over the past two decades a number of biological methods of intervertebral disc treatment have been developed. The most promising of them seems to be the cellular therapy based on a percutaneous transplantation of autologous chondrocytes into the intervertebral disc previously operated on with the purpose of stimulating production by the matrix, increasing the population of chondrocytes as well as restoring the disturbed catabolism-anabolism balance. The chondrocytes transplantation was carried out by commonly used approach in vertebroplasty procedure [1].

It should be emphasized that the research carried out so far has been primarily based on animal material. By contrast, our research involved a human population consisting of 50 patients operated on for one-level discopathy who underwent implantation of autologous chondrocytes into the operated space. Two cases of a repeated intervertebral disc herniation after cellular transplantation were registered. This created a unique possibility of a biological evaluation of the intervertebral disc after a performed implantation with the use of both a light and a confocal microscope. The use of labelled antibodies allowed assessing the regeneration of individual intercellular matrix components as well as the vitality of the transplanted chondrocytes. Direct evidence was thus obtained of the integration of the transplanted cells with the intracellular matrix which seems to confirm the effectiveness of this method of treatment in the management of degenerative processes affecting the intervertebral disc.

The purpose of the study was to show directly the biochemical and structural results of autologous derived chondrocytes transplantation (ADCT) in DDD treatment of two patients who were operated again after cells transplantation.

Material

The study group included 50 patients, 26 women and 24 men, aged 18 to 40, who underwent single-level L4-L5 or L5-S1 discectomy. Clinical examination of the patients before the operation revealed acute radicular syndrome within the lumbar segment (ODI and VAS scales).

MRI scan showed unilevel lumbar herniation with no significant concomitant clinical and laboratory adverse conditions. Biopsy material for histological evaluation of the operated intervertebral disc and for cellular culture was sampled from fragments of the removed nuclei pulposi. The cultured autologous chondrocytes were implanted percutaneously into the space previously operated on. The effects of the implantation were evaluated radiologically (MRI) and clinically (VAS and ODI). In two cases a repeated prolapse of the intervertebral disc was reported and the patients were again operated on. The obtained biopsy material from the thus removed hernia provided material for the biological evaluation of the nucleus pulposus after cellular implantation.

All patients were informed that information concerning the case would be used for publication.

Case I: a 34 years old female patient, operated on in 2011 for L5-S1 discopathy revealed on MRI, with an acute left-side radicular syndrome, who underwent implantation of autologous chondrocytes obtained from culture after four weeks (26.5 million cells of 98% vitality). The 12-month follow-up showed a good clinical effect (VAS 0-1, ODI 0-20%) in the form of withdrawal of the pain syndrome and restoration

***Corresponding author:** Zbigniew Czernicki, Mossakowski Medical Research Centre, Polish Academy of Sciences, Department of Neurosurgery, 2nd Faculty of Medicine, Medical University of Warsaw, Poland, Tel: +48 22 552 00 00; E-mail: neurochirurgia@bielanski.med.pl

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of full physical function. In 2013, after 18 months from the first surgery, the patient was again hospitalized as MRI confirmed discopathy on the same level, with acute radicular syndrome symptoms. The patient was again operated on. The then removed fragment of the nucleus pulposus (biopsy – 3118.06 mg) was subjected to biological analysis.

Case II: a 28 years old female patient, operated on in 2012 for MRI confirmed L5-S1 discopathy, with an acute right-side acute radicular syndrome, underwent implantation of autologous chondrocytes (27.3 m of 99% vitality) obtained from culture. Pain symptoms subsided over the following 11 months (VAS 0-1, ODI 0-20%). In the 12th month after the implantation pain of the initial intensity returned and MRI showed a repeated prolapse of the L5-S1 intervertebral disc. The patient was again operated on and the removed fragment of the nucleus pulposus (biopsy specimen of 2900.17 mg) was subjected to a biological analysis.

Method

The material obtained from the removed fragments of the nucleus pulposus (biopsy) during both the first and the second surgical procedure was examined with both a light and a confocal microscope. Immediately after the procedures the material was frozen and subsequently prepared for a microscopic examination (slicing and staining). The quantity of type I, II, III, IV, IX collagen, aggrecan, proteoglycans as well as the number of chondrocytes in the biopsy material before and after the implantation were analyzed. The biopsy material examined in a light microscope was stained with hematoxylin and eosin.

The biopsy material for examination in a confocal microscope was placed on glass slides. After drying the preparations were fixed in a freshly prepared 4% paraphormaldehyde in phosphate buffered saline (PBS) for 15 minutes and rinsed three times in PBS without Ca^{2+} and Mg^{2+}. Where the immunocytochemical analysis concerned internuclear antigens, the preparations were subjected to the process of membrane permeabilization with 1% Triton X-100 in PBS for 15 minutes at room temperature. Next, the preparations were incubated for 60 minutes with a blocking mixture consisting of 5% albumin in PBS. After 60 minutes the blocking mixture was removed and monoclonal antibody diluted in the blocking mixture was applied. Preparations labeled without incubation with a monclonal antibody constituted the controls. A list of the antibodies used is given in Table 1.

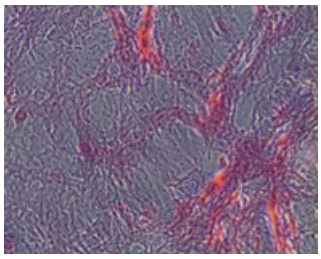
Incubation with antibodies was conducted throughout the night in a wet chamber at 4°C. After the incubation the preparations were rinsed three times for 5 minutes in PBS. Next, fluorochrome-bound Alexa Fluor 488 and 546 polyclonal antibodies (goat anti-rabbit) in a 1:500 dilution was applied for 30 minutes and the preparations were placed in a dark room.

Cellular nuclei were stained with 5 μM solution of Hoechst 33258 dye in PBS for 30 minutes at room temperature. Next, the preparations were rinsed three times for 5 minutes in PBS and sealed with the use of a medium for fixing fluorescence labeled cells (Dako Cytomation Fluorescent Mounting Medium, DakoCytomation Glostrup, Denmark). The confocal Zeiss LSM 510 microscope (Carl Zeiss) equipped with helium-neon and argon lasers was used to analyze the preparations. Pictures were taken in Zeiss LSM 510 v.3.2.

Antigen	Monoclonal /Polyclonal	Origin	Subclass	Dilution	Catalogue No.
Type I collagen	M	rabbit	_	1:20	AB745**
Type II collagen	M	rabbit	_	1:20	AB761**
Type IV collagen	M	rabbit	_	1:100	ab6586*
Proteoglycan	M	rabbit	_	1:20	AB5320**
Aggrecan	P	rabbit	IgG	1:100	ab118610*
Type III collagen	P	rabbit	_	1:20	AB747**
Type IX collagen	P	rabbit	IgG	1:200	Ab134568*

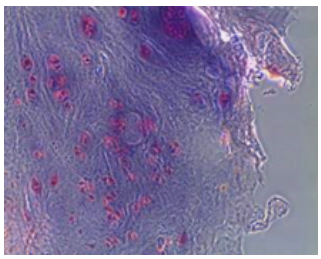
*Abcam
** EMD Millipore

Table 1: List of antibodies used.



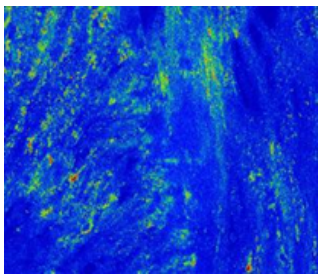
Picture before implantation reveals slightly disorderly tissue structure and single chondrocytes.

Figure 1: A light microscope picture of culture material (Case 1).



Picture shows normal tissue structure, numerous chondrocytes.

Figure 2: A light microscope picture of herniated disc material (Case 1).



A small quantity of type IV collagen.

Figure 3: A rainbow image of type IV collagen in culture material (Case I) - Material obtained during first surgery (before implantation).

Results

Case I

a) Light microscope pictures of the starting material prior to the implantation, used for culture (Figure 1), and the herniated disc material after the implantation (Figure 2). Hematoxylin and eosin staining.

b). Confocal microscope pictures of the starting material before implantation used for cellular culture and the herniated disc material after implantation showing chondrocytes and intercellular matrix components – type IV (Figures 3 and 4), IX collagen (Figures 5 and 6).

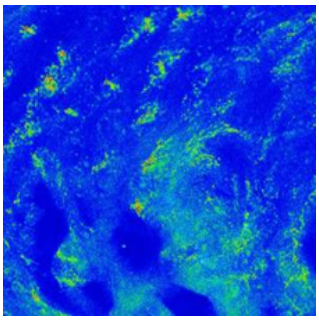
Case II

a). Light microscope pictures of the starting material before implantation used for culture (Figure 7) and herniated disc material after implantation (Figure 8). Hematoxylin and eosin staining.

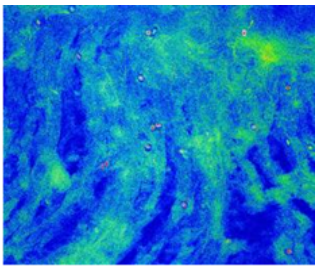
b). Confocal microscope pictures of starting material before implantation used for cellular culture and herniated disc material after implantation showing chondrocytes and intercellular matrix components – type IV, IX collagen.

Discussion

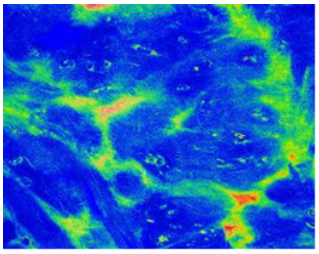
The biological methods of the treatment of the degenerated nucleus pulposus applied different types of cells which were expected to transform into chondrocytes or cells similar in their functions of the production of typical components characteristic of a healthy intracellular matrix. The aim of research was thus to structurally recreate the nucleus pulposus with all its functions as well as to restore balance to the distorted metabolic processes of the degenerated nucleus pulposus.



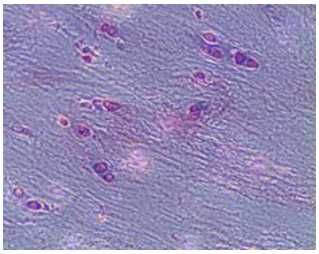
An increased quantity of type IV collagen visible in green, yellow and red.
Figure 4: A rainbow image of type IV collagen in herniated disc material (Case I) - Material obtained during second surgery (after implantation).



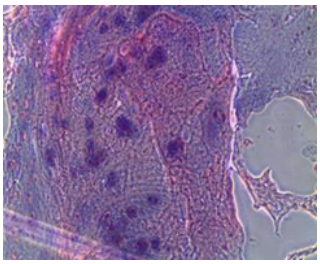
Type IX collagen within cell nuclei in red.
Figure 5: A rainbow image of type IX collagen in culture material (Case I) - Material obtained during first surgery (before implantation).



An increased number of cells and type IX collagen.
Figure 6: A rainbow image of type IX collagen in herniated disc material (Case I) - Material obtained during second surgery (after implantation).



The picture before implantation shows a slightly disorderly tissue structure and single chondrocytes.
Figure 7: A light microscope picture of culture material (Case II).



The picture shows normal tissue structure, numerous chondrocytes.
Figure 8: A light microscope picture of herniated disc material (Case II).

The cells most commonly used in research were chondrocytes (previously widely used in orthopedics), mesenchymal cells and adipose cells.

It should be emphasized that a prevailing majority of these studies concerned animal materials and solely in a few studies the authors attempted to assess the effectiveness of an autologous chondrocyte transplantation however the assessment of the transplantation effect could only be indirect, primarily through radiological (MRI) and clinical examinations.

The integrity of transplanted cells and their productive properties after the transplantation were examined by many authors, both *in vivo* and *in vitro,* on animal material [2-5]. Reports concerning human material are still few and are mostly pilot studies. An interesting study was presented by Coric et al. [6]. Fifteen patients were implanted approximately 20 million immature allogenic chondrocytes which resulted in their good adaptation to the intracellular matrix. The authors stressed also the necessity to perform the implantation prior to the development of advanced degenerative disc processes in the form of damage to the annulus fibrosus and calcification of the end plate. The uniqueness of the study consists in the fact that patients in whom the implantation was performed had not been operated on earlier and the cells inserted into the nucleus pulposus were to complement the loss of cells in result of degenerative changes. Improvement of the quality of discs was shown on MRI (increased signal intensity) as well as on clinical assessment (ODI and VAS).

Orozco et al. studied ten patients while Yoshihawa et al. two reporting a good clinical effect after the application of mesenchymal cells [7,8]. Meisel et al. implanted autologous chrondocytes obtained during discectomy and multiplied through culture [9,10]. The clinical effects were also promising, good integration with intracellular matrix was recorded, with production of matrix components by the implanted cells.

Numerous studies on the properties of chrondocytes, their integration with the intracellular matrix and production of its components were carried out by authors from the Tetlow Centre in Germany, Libera et al. [11-13]. In the *in vitro* environment, the size of the cellular culture and the distribution of cells in the matrix were studied with the use of typical hematoxylin and eosin staining, the number of proteoglycans was estimated with the use of safari-O dye

while picrosirius red served to assess the presence of Type I, II and III collagen with a fluorescence microscope. In addition the presence of vacuoles in the cytoplasm of cells testified to their proper metabolism.

The productivity of the transplanted chondrocytes was also assessed with the analogue of thymidine nucleotide (BrdU), the injection of which into the DNA of a cell allows to identify its products after a performed implantation and thus to differentiate them from the self-repair processes of the cells of the nucleus [3,10].

The reported findings from fairly numerous studies on animal material focusing on cellular culture of material sampled from different sites and effects of its implantation into the intervertebral space also seem promising [3,4,12,14]. Other cells cultured included immature stem cells and cells obtained from adipose tissue enjoying particular interest among researchers recently.

Some authors emphasize their ability to diversity towards the nucleus pulposus cells phenotype under the influence of the components of the nucleus pulposus intercellular matrix environment [14].

However other authors draw attention to the ultimate absence of certain intercellular matrix components produced by the implanted cells. Gorenšek et al. compared two groups of animals with an excised nucleus pulposus, one with implanted autologous chondrocytes and the other without it, to present interesting observations [15,16]. In the group without cellular implantation they found fibrous cartilaginous tissue and granulation tissue forming. In the second group, after the implantation, they found cartilaginous tissue and granulation tissue, with cells irregularly scattered in the matrix, revealing a considerable number of proteoglycans and type II collagen but no elastic fibres. Simultaneously, like healthy disc tissue, the new matrix tissue showed absence of blood supply and maintained a fairly regular annular architecture. This problem was also addressed by other authors.

To complement the deficiency of elastic fibres Arevalo-Silva et al. [5] added the fibroblastic growth factor (FGF) as well as the transforming growth factor ß (TGF-β) to the cellular culture obtaining a good productive effect of implanted chondrocytes also as regards the production of elastic fibres. The authors believe that the substitute cartilaginous tissue produced by the implanted autologous chondrocytes is the best substitute for the healthy matrix of the nucleus pulposus.

In our study we used autologous chondrocytes to multiply them through culture and to subsequently implant them into the previously operated space. The evaluation of the material after the implantation with the use of a confocal microscope provided direct evidence of very good integration of the transplanted chondrocytes with the intercellular matrix and, most importantly, of their typical production of different types of collagen, aggrecans and proteoglycans. The observed regeneration of the nucleus pulposus also proves that the transplanted autologous chondrocytes began to perform their functions in maintaining the anabolism-catabolism balance of the nucleus, contributing to normal metabolic processes in the intercellular matrix.

The two cases of a repeated prolapse of a nucleus fibrous fragment provided a unique opportunity for a direct evaluation of the excellent functioning of the transplanted chondrocytes. Certainly ADCT procedure it is not a method of solving all the problems in DDD, however it seems to be very helpful in the comprehensive treatment of the patient [11,12,13].

Conclusions

The chondrocytes obtained from culture integrate well with the intercellular matrix showing normal productive activity in the form of generation of typical matrix components – type I, II, III, IV, IX collagen, proteoglycans and aggrecan, inhibiting the degenerative processes in the nucleus fibrosus and restoring normal balance in the metabolism of the nucleus fibrosus.

Correct culture of autologous chondrocytes from material obtained from fragments of the nucleus pulposus removed during surgical procedure allows obtaining a large number of valuable chondrocytes to be used in the treatment of the degeneratve disc disease (DDD). However implanted chondrocytes improved the disc matrix, the ADCT procedure was not able to completely prevent the next disc herniation.

Detailed discussion of the results of treatment using ADCT is the subject of another work covering all 50 surgical patients.

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