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Regeneration of intervertebral disc by way using of allogeneic chondrocytes cultivated *in vitro* in the light of concept for restabilization of the spine (experimental investigation)

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The intervertebral disc (ID) has low reparative capacity. In this regard, researcher aimed at improving the capacity of the reparative structures are carried out. A variety of growth factors, mesenchymal stem cells, autologous and allogenic chondrocytes, etc are used for this purposes. Objective: to study the structure of the traumatized intervertebral disc after transplantation of allogenic chondrocytes in combination with spinal restabilization using method of dynamic spine neutralization of vertebral motion unit. Methods for optimizing ID regeneration in the rat tail vertebral segment we selected high-density culture of chondrocytes obtained from the limb bud 7–10 day old rat embryos in which cells retain cartilaginous phenotype and genotype was confirmed by electron microscopy. Performed four series of experiments: I — intact animals, II — ID injury (control), III — traumatic ID injury with dynamic neutralization (method G. Dubois et al, 1999), IV — ID injury with dynamic neutralization and transplantation of chondrocytes. One month the operation the animals were euthanized, and a comparative morphological analysis with morphometry was carried out. Results: it found that uncontrolled movements in the injured vertebral motion unit lead to destructive changes in the ID. In terms of restabilization and dynamic neutralization of the vertebral motor segment ID has been replaced by connective tissue, its height reduced. Chondrocyte culture is different from the fibrous ring and nucleus pulposus cells. However, with the introduction of chondrocytes in the injured ID and maintaining the controlled mobility of the vertebral motion unit the proliferation of hyaline cartilage in its peripheral parts has been noted. Conclusions: combination of allogeneic chondrocytes transplantation into disk and dynamic neutralization leads to the formation of hyaline cartilage territories and maintains the height of the injured ID. Key words: intervertebral disc, allogenic chondrocytes transplantation, regeneration.

Межпозвонковый диск (МПД) обладает низкими репаративными возможностями. В связи с этим проводятся исследования, направленные на повышение репаративного потенциала структур МПД. Для этого используют различные факторы роста, мезенхимальные стволовые клетки, ауто- и аллогенные хондроциты и др. Цель: изучить структуру травмированного МПД крыс после трансплантации аллогенных хондроцитов в комбинации с рестабилизацией и использованием метода динамической нейтрализации позвоночного двигательного сегмента (ПДС). Методы: для оптимизации регенерации МПД в хвостовом отделе позвоночника крыс выбрана культура хондроцитов высокой плотности, полученная из зачатков конечностей 7–10-дневных крысиных эмбрионов, в которой клетки сохраняли хрящевой фенотип и генотип, что подтверждено с помощью электронной микроскопии. Выполнено четыре серии экспериментов: I — интактные животные, II — повреждение МПД (контроль), III — повреждение МПД с динамической нейтрализацией (метод G. Dubois et al., 1999), IV — повреждение МПД, динамическая нейтрализация и трансплантация аллогенных хондроцитов. Через 1 мес. после операции животных выводили из эксперимента, проводили сравнительный морфологический анализ с морфометрией. Результаты: установлено, что неконтролируемые движения в травмированном ПДС приводят к прогрессированию деструктивных изменений в МПД. В условиях рестабилизации и динамической нейтрализации позвоночного двигательного сегмента МПД был замещен соединительной тканью, его высота снижена. Культура хондроцитов отличалась от клеток фиброзного кольца и студенистого ядра. Однако при введении хондроцитов в травмированный МПД и сохранении контролируемой подвижности ПДС отмечено разрастание гиалиновой хрящевой ткани в его периферических отделах. Выводы: сочетание трансплантации в диск аллогенных хондроцитов и динамической нейтрализации приводит к образованию территорий гиалиновой хрящевой ткани и сохранению высоты травмированных МПД. Ключевые слова: межпозвонковый диск, аллогенные хондроциты, трансплантация, регенерация.

Key words: intervertebral disk, allogeneic chondrocytes, transplantation, regeneration

Introduction

Intervertebral disk is one of the most important structures in performing spinal functions. One primary cause of low back pain is the degeneration of the intervertebral disc resulting in the compression of the spinal nerves and adjacent vertebrae [1]. Exact causes of degeneration are unknown, but it is thought that natural aging, and both biological and genetic factors may play a significant role in the degenerative process [2]. Peculiarities of morphological changes of intervertebral discs with ageing and pathological disorders are well studied [3] while the problems of disc regeneration need additional investigation. It is well known, that the intervertebral disc is an avascular tissue that has limited capacity for regeneration.

During the last few years for the purpose of regeneration modulation a sensitivity of intervertebral disk tissue to different growth-stimulating factors has been studied. The sensitivity of intervertebral disk tissues to insulin-like growth factor-1, epidermal growth factor, fibroblast growth factor and transforming growth factor-beta has been found [4, 5].

Cell therapy is an appealing approach to regenerate the intervertebral disc [6]. Bone marrow derived mesenchymal stem cells (MSCs), adipose tissue derived stem cells, chondrocytes have been injected into the damaged disc of animals and largely demonstrated regenerative potential [7–9]. Promising outcomes have moreover been achieved with human disc cell or mesenchymal stem cell transplantations [10–12] and with the delivery of cartilage cells [13].

We want to discuss a possibility of using chondrocytes growing in high-density tissue culture as a biological replacing material transplanting into the injured annulus fibrosus and nucleus pulposus.

The latest concepts about restabilization and dynamic spine neutralization [14] point at the importance of creating specific conditions for the spinal functioning. In this case we think it is advisable to use cartilage cells transplantation under condition of dynamic spine neutralization on the way to restabilization.

Purpose of investigation. To study the structure of the traumatized intervertebral disc after transplantation of allogenic chondrocytes and in combination with spinal restabilization using method of dynamic spine neutralization.

Material and methods

Cell cultures

Cell suspension were prepared from the limb buds of Wistar rats embrions (7–10 days) as was described for chicken embryos by Cs. Hadchazy [15]. Limb buds were removed, rinsed in calcium- and magnesium free phosphate-buffered saline (CMF-PBS), dissociated with 0.25 % trypsin (1:250, Difco, Detroit, Mich., U.S.A.) — 0.15 % EDTA-disodium CMF-PBS for 50 min at 37 °C.

Then cells were centrifugated and resuspended in 5 ml of medium. Limb bud cells were filtered through two layers of No. 20 Nitex and counted using the hemacytometer. Five 10 μl drops of cells (2x106) were inoculated onto coverslips and this was placed on the bottom of plastic dish (35 mm). After 2 h. in a 37 °C incubator for cell attachment, the dishes were flooded with 2 ml of cultural medium Ham's F12 containing 10 % fetal calf serum, antibiotics (50 U penicillin and 50 μg streptomycin per ml) and L-ascorbic acid (50 μg/ml). The medium was changed every other day. The cultures were maintained at 37 °C in an air atmosphere with 5 % CO_2.

Experiments on animals

Operation procedure. The experiments were carried out with adult Wistar rats of either sex weighing (from 260 before 290 g) in 4 groups of 5 each. Intervertebral discs of rats were destructed in the area of the tail spinal segment (from the 5^{th} to the 10^{th}) by injection needle, diameter of 1 mm. The lateral part of the annulus fibrosus and nucleus pulposus was incised transversely about 2 mm long. Rats were operated using thiopentalum narcosis.

Experimental groups:

I series — intact animals;

II series — (control) traumatic disc injury;

III series — (experiment) traumatic disc injury + dynamic neutralization. From the longitudinal dissection the vertebral bodies were denuded and united by 8-shared suturing (Fig. 1). For this purpose a monofilamental (Dexon II, T-19, Guanamid of Greet Britain LTD. Gosport, Hampshire) thread was used. The skin was sutured with nodulous suturing;

IV series — (experiment) traumatic disc injury + transplantation of chondrocytes + dynamic neutralization.

Transplantation of cells. After 14 days' growth in tissue culture the cells were transplanted into freshly traumatized intervertebral disc. The final volume

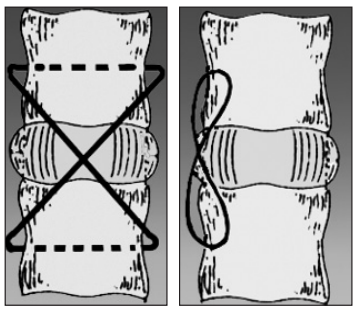

Fig. 1. Scheme of performing dynamic neutralization of the injured disc

of transplantation of the cells suspension was 100 µl, with a total content cells 2,0x107.

The animals were stabled, three per cage, and fed with a standard diet under standard environmental conditions (strictly according to the law of Ministry of Health of the USSR № 724, 13.11.1984 and European law on animal experiments D.L. 116/92). The animals were euthanasied by at 30 days post-operatively. The study was approved by the bioethics committee of SI «Sytenko Institute of Spine and Joint Pathology National Academy of Medical Science of Ukraine» (protocol № 85, 07.02.2011).

Histology

Decalcified specimens were embedded in celloidin and cut in 6–8 µm sections for histological investigation. The sections were stained with hematoxylin & eosin, van-gieson and alzian blue and were examined by light microscopy.

Transmission electron microscope analysis

Aggregates of cells (after 14 days' growth in tissue culture) fixed in 0,1 mmol/l cacodylate-buffered with 2.5 % glutaraldehyde at pH 7.3–7.4 at 40 °C for 2 h. After washing same buffer the specimens were fixed in 1.0 % O_sO_4 0.1 mmol/l cacodylate-buffer (pH 7.2–7.4) at room temperature for 2 h. The samples dehydrated in acetone and embedded in epon-araldit. Sections were treated with uranylacetate and lead citrate and examined in transmission electron microscope (EMV-100 BR, Ukraine).

Ocular measurement

Measurements of discal height have been made in central and peripheral zones on the histological preparations (central part of section) using eyepiece micrometer (MOV-1–16x, LOMO) with a magnification of 108.

A stage micrometer was used to calibrate the object micrometer and a length in µm calculated for each variable. The results have been analyzed statistically with using computer program «Excel» (Microsoft). Statistical significance was $P < 0.05$.

Results and discussion

Cell cultures

In high-density culture was dominated cells a rounded shape (Fig. 2, a). At the electron microscopic study determined chondroblasts. Into cytoplasma presented a well-developed Golgi apparatus and granular endoplasmatic reticulum (Fig. 2, b).

I series

The intervertebral disc consists of a centrally located nucleus pulposus, surrounded by an annulus fibrosus and cartilaginous endplate cranially and caudally at the junction to the vertebral bodies.

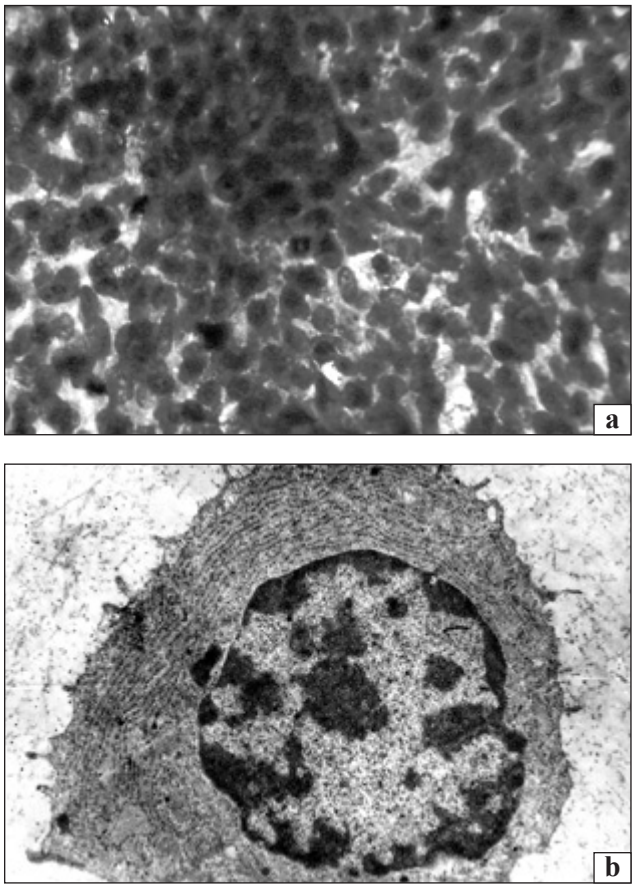

Fig 2. Culture of high-density: a) azur-eozin. Magn. 200; b) hondroblast with well-developed Goldgy apparatus and granulas endoplasmatic reticulum, contrast by osmium. EMV-100 BR. Magn. 12 000

The annulus fibrosus is characterized by differences in the organization of cells and matrix in the outer and inner regions (Fig. 3).

Fibroblast-like cells are arranged between circumferential coaxial lamellas of the collagen fibers in the outer regions of the annulus. In the annulus fibrosus the bundles of collagen fibres were arranged in layers parallel to each other. Among them the regions with radial orientation of collagen fibres were seen. Along the collagen fibers the fibrochondrocytes.

The cells of nucleus pulposus were not numerous. They were united into small groups with the help of long processes as multinuclear syncytia. Within one group the cells were different by density of nucleus. Large multinuclear cells had acidophilic cytoplasm. The nucleus pulposus consists of loosely organized collagen fibres and proteoglycans.

The data of morphometric investigation were represented in table. In edge parts the height of normal

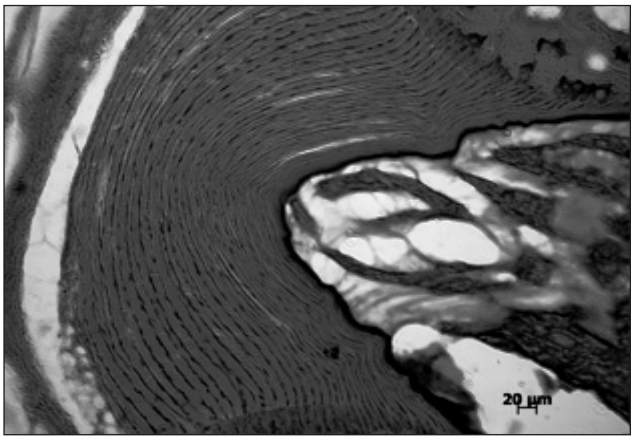

Fig. 3. Intervertebral disc. Nucleus pulposus. Annulus fibrosus. Alzian blue. Magn. 200

intervertebral disc was the same. In the central part was fixed lower indicators.

II series (control)

Intervertebral disc degeneration histologically characterized by a loss of disc height. In morphometric investigation it was stated that the height of traumatized intervertebral discs compared to intact animals decreased in all the points investigated. From the side of the trauma the height of annulus fibrosus is decreased by 9.7 %, and in the region of nucleus pulposus — by 21.4 %, from the opposite side of the annulus fibrosus — by 19 % (table).

In the injured disc the area of the annulus fibrosus was extended, cracks and small fissures were found (Fig. 4). The fragment of annulus fibrosus was without cells and contains basophilic areas of mainly in the edge areas.

The annulus fibrosus was only on the small parts. The nucleus pulposus was destructed. In this area there were extended cavities consisting of multiple clefts within the nucleus substance, areas of amorphous granular material devoid of cells.

III series (experiment) disk injury + dynamic neutralization

The intervertebral disc height (in comparison with the II experimental series) increased by 22.4 % in 1 the area of traumatic injury, 48.6 % in 2 area and 24.4 % in the 3 area. The structure of the annulus fibrosus is disarranged due to loss of lamellae and substitution by connective tissue. Nucleus pulposus is not determined. In this area we discovered the bundles of collagen fibres with high-density fibroblast among them, fissures and small gaps (Fig. 5).

At the edge of the trauma area the destructed parts of annulus fibrosus were replaced by loose fibrous tissue containing a great number of dilated blood vessels. More blood vessels begin to grow into the disc from the outer areas of the annulus fibrosus.

IV series (experiment) traumatized disc + transplantation of allogenic chondrocytes + dynamic neutralization

Introduction of chondrocytes into the traumatized disc and use of dynamic neutralization led to the intervertebral disc height increase comparing to II series in all the investigated points: in the I — by 8.9 %, in the II — by 26.1 %, in the III — by 16.1 %, comparing to III series in all investigated points: in the I — by 33.2 %, in the II — by 87.4 %, in the III — by 44.3 %,

It can be noted that the annulus fibrosus height in the region of trauma was increased and did not practically distinguished from disc height intact animals. In the central parts of annulus fibrosus and in the nucleus pulposus on small sites destructive changes were determined. Collagen lamellas was replaced by connective tissue with high-density fibroblast and areas of chondroid. Linking of cartilage tissue and annulus fibrosus plates was happening without formation of boundaries. Chondrocytes were large, with brightly stained nuclei. At the edges in the

Table

Measurement (µm) of discal height at central and peripheral zones

Types of the treatment	Discal height * (µm)		
	I	II	III
Normal discs (Series I)	1338.14 ± 41.78	1166.67 ± 36.19	1393.66 ± 45.23
Traumatic disc injury (Series II)	1208.57 ± 30.74 P < 0.05	916.57 ± 38.21 P < 0.001	1130.14 ± 33.47 P < 0.01
Traumatic disc injury + dynamic neutralization (Series III)	987.12 ± 27.79 P_1 < 0.001	616.55 ± 45.55 P_1 < 0.001	908.75 ± 52.10 P_1 < 0,001
Traumatic disc injury + transplantation of chondrocytes + dynamic neutralization (Series IV)	1315.14 ± 35.08 P_2 < 0.05 P_3 < 0.001	1155.57 ± 37.69 P_2 < 0,001 P_3 < 0.001	1311.71 ± 34.34 P_2 < 0.001 P_3 < 0.001

Note. * — I, III peripheral zones; II — central zones; P — significant differences between series I and II; P_1 — significant differences between series II and III; P_2 — significant differences between series II and IV; P_3 — significant differences between series III and IV.

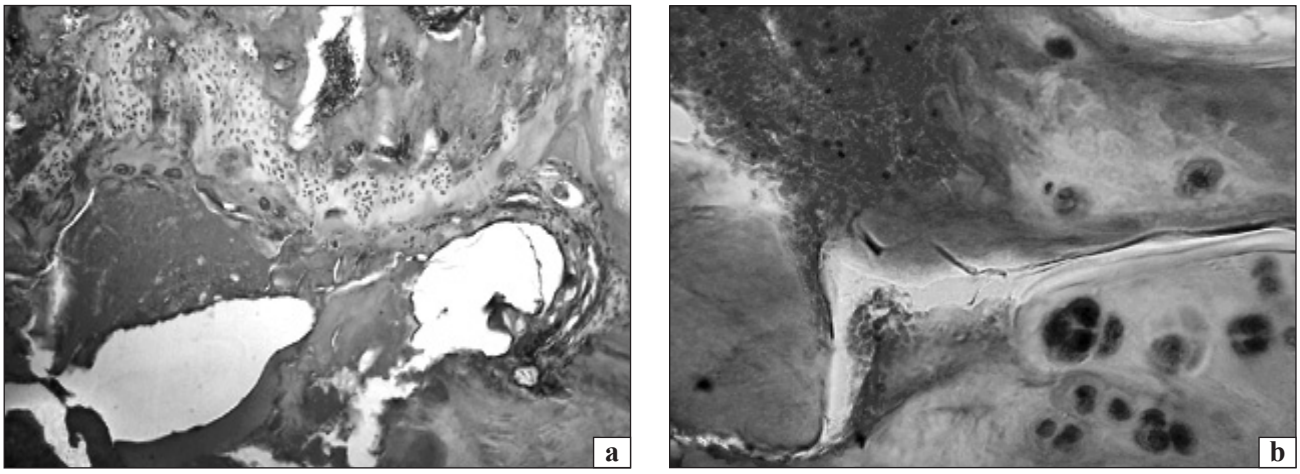

Fig. 4. The insured intervertebral disc: a) annulus fibrosus, crackings, fissures, necrosis of collagen fibres bundles, magn. 80; b) nucleus pulposus, cartilage metaplasia (II series), hematoxyline and eosin. Magn. 200

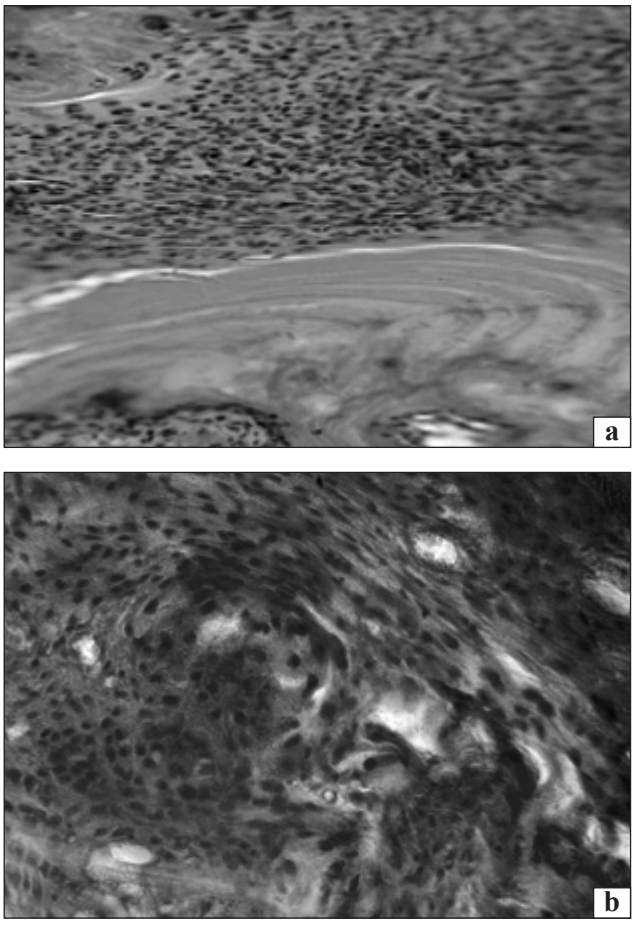

Fig. 5. Fibrous tissue with high-density fibroblasts into annulus fibrosus (a) and chondrocytes into the nucleus pulposus (b). Fissures and gaps. Blood vessels (III series). a) hematoxyline and eosin, magn. 80; b) Alzian blue, magn. 400.

place of annulus fibrosus extended fields of cartilaginous cells could be seen (Fig. 6).

Numerous experimental investigations and clinical observations demonstrate that the reparation of the injured intervertebral disc has limitations. Methods of stimulating anabolic processes, modulation catabolic processes and providing new cells (mesenchymal stem cells, adipose tissue derived stem cells, chondrocytes, fibroblasts) are typically used to repair the degenerative disc [7, 16–18]. In degenerated discs a reduced number of cells have been found that cannot be explained by limited entering into disc of nutrient and ageing. Cells that are transplanted into the intervertebral disc can fulfil several functions, including matrix production, prevention of annulus fibrosus deformation, inflammation control, production of growth factors and prevention of angio- and neurogenesis, regulate local homeostasis and can attract additional cell [18, 19]. Mesenchymal stem cell therapy may be a valid alternative treatment for chronic back pain caused by degenerative disc disease [20].

Eventually, cells have the potential to interact with the resident cell population, [21]. The researchers showed that the trauma of annulus fibrosus have restricted healing. Our experiments also revealed that in the region of the traumatized disc destructive disorders were found in the area of both annulus fibrosus and nucleus pulposus, and the reparation was present as hyperplasia of fibroblast. Decrease of the disc height was observed. Mechanical destabilization of functional three-joint-complex (disc and facet joints) could be one the reasons of such disorders. Non-controlling movements in spine promotes progressing of the destructive changes in the disc.

In a view of the new restabilization concept and dynamic neutralization of spine movement segments (a month later after the operation) height of the intervertebral disc was decreased. The disc was replaced by connective tissue. Destructive changes were not

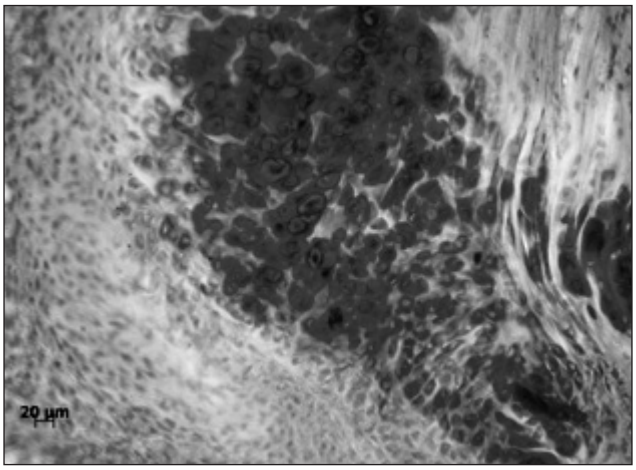

Fig. 6. Chondroid in the central part of the injured disk (IV series). Alzian blue. Magn. 200

found. Possibly, it was caused by the vertebral bodies stabilization with limited level of movements in condition dynamic neutralization, absorbing non-physiological forces (compressions and flexions-extensions) and supressing unwanted movements [14].

Search for possible modulations of the reparative potential of the intervertebral disk tissues is also important due to structural changes in intervertebral discs, that is appear in the 2–3d life decade and increased with ageing. Ageing of nucleus pulposus and annulus fibrosus can lead to the development of degenerative changes, protrusion and prolapse demanding surgical intervention.

To repair degenerative intervertebral discs we chose high-density chondrocytes culture, that is the cells of which have a cartilaginous phenotype and genotype [15]. We was used chondrocytes culture, it was differed from the cells of annulus fibrosus and nucleus pulposus. However, under condition of degenerative disc followed by introduction of chondrocytes into the disc and keeping full vertebral segment mobility we observed the evolution of chondroidal tissue in the peripheral parts of the intervertebral disc. We suggest that such a location of the chondroid related to the transplanted cells. This fact proves the necessity of searching of optimal ways for cells transplantation and what is more important to create conditions retaining these cells in the inner parts of the annulus fibrosus.

Combination of dynamic neutralization method performed with restabilization for the spine as well as transplantation of cartilaginous cells into the disc enables to preserve the interbody gap height on account of the formation of vast cartilaginous tissue territories in the area of nucleus pulposus and annulus fibrosus that we consider important for a biological replacement of the degenerative disc. Thus, the intervertebral disc height can be preserved account of development of chondroid tissue from transplanted chondrocytes when performing restabilization of the vertebral segment with limited volume of movements. That is important for achievement of the dynamic neutralization. We think that application of cells cultures in case to regeneration of the intervertebral disc is promising.

The next stage of the investigations can be a study of possible transplantation of cells of annulus fibrosus and nucleus pulposus which cultured in vitro, for the purpose of biological replacement of intervertebral disc. An important direction is searching ways of combination cells with synthetic biodegrading materials.

Conflict of interest. The authors declare the absence of conflict of interest.

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РЕГЕНЕРАЦИЯ МЕЖПОЗВОНКОВОГО ДИСКА ПУТЕМ ПРИМЕНЕНИЯ КУЛЬТИВИРОВАННЫХ IN VITRO ХОНДРОЦИТОВ В СВЕТЕ КОНЦЕПЦИИ РЕСТАБИЛИЗАЦИИ ПОЗВОНОЧНИКА (ЭКСПЕРИМЕНТАЛЬНОЕ ИССЛЕДОВАНИЕ)

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