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Joint cartilage experimental defect regeneration by hierarchic biphasic combined grafts

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Abstract

Background: The existing surgical techniques used to regenerate articular cartilage fail. Utilisation of hierarchical, biphasic structures obtained from osteochondral tissue, through demineralisation, decellularization, longitudinal perforation and combination with chondroprogenitor cells, presents a high potential in cartilage defects regeneration.
Material and methods: The research was performed on 36 rabbits, separated equally in two experimental and one control group. In the experimental groups, the experimental osteochondral defects of 4-4.5 mm in depth, were performed with a 3.7 drill bit at the level of weight bearing surface of the medial femoral condyle. In the 1st group the defects were treated with grafts combined with autologous chondrocytes, and in the 2nd group with grafts combined with autologous mesenchymal stem cells. In the control group, cartilaginous defects were treated by transferring the osteochondral plugs taken from the trochlear groove. The rabbits were removed from the experiment at 6 and 12 weeks. The results were evaluated by Unified Histological Score of Regenerated Cartilage (UHSRC).
Results: At 6 weeks, according to UHSRC, the 1st group had 28.33±1.53 points, the 2nd group –27.67±2.08 points and the control group –26.33±1.53 points ($p>0.1$; $p>0.2$). At 12 weeks the 1st group had 18.68±5 points, the 2nd group –14.89±3.76 points and the control group –17.22 ±4.84 points ($p>0.5$; $p>0.2$).
Conclusions: According to UHSRC, the experimental groups don't show a significant difference compared to the control group at 6 and 12 weeks, also the quality of regenerated cartilage is poor.
Key words: cartilage, regeneration, biphasic hierarchic graft, chondroprogenitor cells, unified histological score.

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Introduction

Articular cartilage is a tissue with a very poor regenerative capacity, and in the case of large defects this is impossible [1-4]. The surgical treatment techniques used today mostly lead to the formation of fibrous or fibrocartilaginous tissue within the defect, with very low mechanical properties [2, 3, 5-7]. Combining of grafts obtained from synthetic or biological structures with various types of cells, seems to be an effective way to regenerate organs and tissues. There are several ways described in the literature to regenerate tissues and organs by combining three-dimensional matrices with various cells. Human amniotic membrane combined with bone marrow mesenchymal stem cells is used in the treatment of rabbit skin defects [8], combining of synthetic trachea obtained from polycaprolactone by 3D printing with bone marrow mesenchymal stem cells (BM-MSC), chondrocytes induced from pluripotent stem cells and human bronchial epithelial cells, to obtain a functional organ [9]. It is also known that biphasic blood vessels can be synthesized from polycaprolactone and type I collagen combined with smooth muscle cells and endothelial cells [10]. The combination of different three-dimensional monophasic structures obtained from collagen type I, I/III or atelocollagen type I with cells was also used to restore articular cartilage [7, 11].

As a result, the treatment methods with high perspective for articular cartilage regeneration, seem to be those that use multiphasic grafts combined with cells that have chondroprogenitor potential [3, 5, 12-15]. Utilization of hybrid structures, bi- or triphasic, obtained by mixing collagen with chitosan [5], with hyaluronic acid with or without chondroitin sulfate [12, 13, 16], with silk threads [17], and other non collagenic structures agarose [18], sodium alginate, gelatin [19], polycaprolactone [20] and others, are widely used in obtaining grafts that can be combined with mesenchymal stem cells (MSC) or chondrocytes with the aim of regenerating articular cartilage. The most of those three-dimensional matrices used in combination with chondroprogenitor cells are of synthetic origin, which tend to correspond to the specific properties of cartilage in order to be used in combination with MSC or chondrocytes [21, 22]. Elaboration of the graft that was used in the ex-

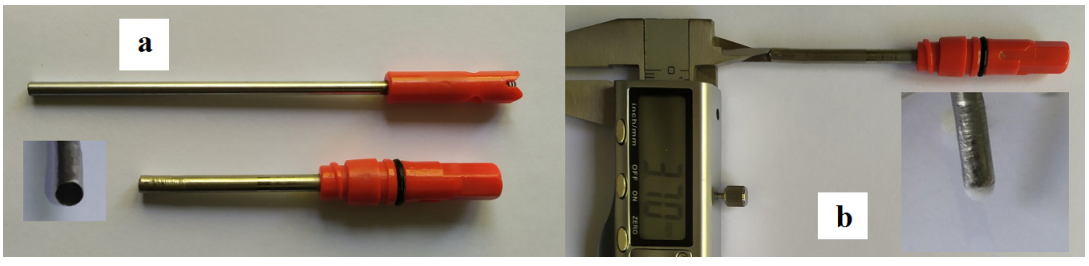

Fig. 1. The selfmade circular knife which was used to make circular sections of the grafts: (a) the device consists of circular knife and the trocar to push out the graft, (b) presentation of knife internal diameter and the device with a sectioned graft

periment from osteochondral tissue through demineralization, decellularization and longitudinal perforation [4], shows a high potential for utilization in combination with MSCs or chondrocytes for articular cartilage regeneration at the level of weight-bearing regions of the femoral condyle by creating favorable conditions that will protect the graft from deterioration after transplantation [6]. Since the graft is obtained of tissue with a biphasic, hierarchical structure [23], and the ability of cellular penetration and proliferation through it is ensured by its high degree of perforation, the use of such a graft seems to be reasonable. Also, the lack of immune response to decellularized bone and cartilage reduces the possibility of a graft rejection reaction, and its combination with autologous cells such as chondrocytes and bone marrow MSC could ensure the tissue remodeling process [24].

Material and methods

The *in vivo* experimental researches were carried out on 36 domestic rabbits with an average age of 4.5 ±0.5 months. The animals were divided into three groups, by 12 rabbits per group as follows:

- Group I – rabbits treated with osteochondral demineralised decellularized (OCDD) grafts combined with chondrocytes, consists of 4 males and 8 females, average weighing 3.52 ±0.42 kg;
- Group II – rabbits treated with OCDD grafts combined with MSC, consists of 7 males and 5 females, average weight 3.37 ±0.57 kg;
- Control group – rabbits treated with autologous osteochondral grafts, consists of 7 males and 5 females, average weight 3.52 ±0.62 kg.

There was no significant difference between the age, body mass and gender of the rabbits in the created experimental groups ($p > 0.2$).

Preparation of the hierarchic biphasic grafts

During the experiment 6 additional rabbits were sacrificed, from which were collected the distal femurs needed for grafts preparation. The collected distal femurs were stored in ultra-freezer (ULUF 450-2M, Arctiko) at -84°C until use. After removing the soft tissues from the distal femurs, they were demineralized in 0.6 M HCl [3, 4]. Then with a self-made circular knife (fig. 1) from the weight-bearing surface of each condyle, pieces of osteochondral tissues were sectioned with the diameter of 3.61 ±0.1mm and the height of 4.33 ±0.11mm, with the average volume of 44.05 ±1.15mm^3. The grafts were longitudinally perforated with a 23G syringe needle more than 120 times, degreased with 3% H_2O_2 (Eurofarmaco, the Republic of Moldova) for 24 hours and and with 70% alcohol (Eladum Pharma, the Republic of Moldova) for 6 hours by shaking at 200 rpm in a shaker-incubator (ES-20, Biosan).

The grafts were washed with distilled water by shaking 3 hours, the water was changed every hour, and decellularized in 1% sodium dodecyl sulphate (SDS) during 24 hours also by shaking at room temperature. After decellularization the grafts were washed with distilled water during 3 days, changing the water 2-3 times a day. In the laminar flow hood (LN 090, Nuve) the grafts were washed with 70% alcohol (Eladum Pharma, the Republic of Moldova) a few minutes. The graft sterilization was performed also with 70% alcohol in a laminar flow hood during 2 hours. Then, the sterilized grafts were washed with HBSS (Lonza, Belgium) for 24 h, changing the solution 3 times. The next day by 2-3 small strips of sterile gauze were inserted into the sterile 15 ml tubes, and the grafts by one were placed on the sterile gauze from the tubes. In order to dry the grafts, the test tubes were centrifuged at 4000 rpm for 20 minutes (Universal 32R, Hettich Zentrifugen), and then stored at -84°C (ULUF 450-2M, Arctiko) until use (fig. 2).

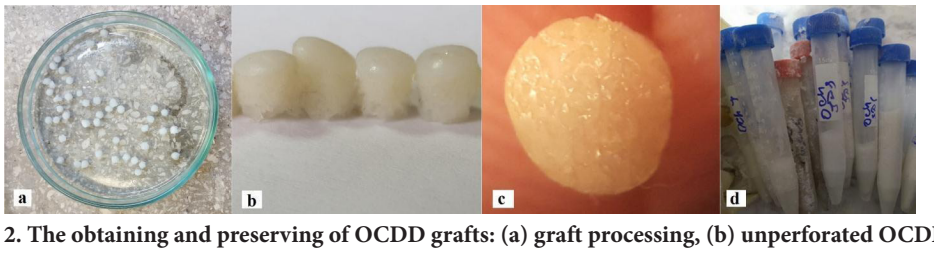

Fig. 2. The obtaining and preserving of OCDD grafts: (a) graft processing, (b) unperforated OCDD grafts, (c) longitudinally perforated OCDD graft, (d) OCDD grafts preserved at –84°C

The isolation of cells with chondroprogenitor potential

The necessary cells with chondroprogenitor potential to obtain the combined grafts were isolated according to the methods presented in previous publications carried out in the laboratory. MSCs were isolated by culture from rabbit bone marrow, which was collected into a heparinized syringe by aspiration under general anesthesia from the iliac bone [1, 25]. There were aspirated 4 ±1.2 ml of bone marrow. After centrifugation with concentration gradient HiSep 1077 (HiMedia, India), the mononuclear cell layer was extracted and washed in other 15 ml tube with HBSS (HiMedia, India) and cell culture media composed of DMEM/F-12 Ham (Sigma, UK) and FBS (Lonza, Belgium) and 1% antibiotic antimycotic solution (HiMedia, India). Then the cells were transferred to a 25 cm^2 cell culture flask (Nunc, Denmark) with 5 ml of cell culture media and incubated at 37°C, 5% CO_2, changing the media every 2-3 days until a 80-90% confluence.

Chondrocytes were isolated by enzymatic digestion of articular cartilage taken from one of knes under general anesthesia. The harvested cartilage pieces were digested with 0.6% collagenase using the continuous monitoring method [2], the process being stopped when the chondrocytes from the digested cartilage occupied the entire field of view. After washing, the chondrocytes were counted and seeded in 25cm^2 cell culture flasks (Nunc, Denmark) at a density of 7100 ±2100 cells/cm^2, in 5 ml of cell culture medium composed of DMEM (Sigma, UK) with SFB (Lonza, Belgium) and 1% antibiotic antimycotic solution (HiMedia, India). The cell culture media was changed every 2-3 days until a 80-90% confluence.

The combined grafts obtaining

OCDD grafts were combined with rabbits autologous cells and transplanted on the day when MSCs and chondrocytes reached 80-90% confluence. Initially, OCDD grafts were thawed and heated to 37°C in a thermostat, followed by cells detachment from the cell culture surface of the flask by trypsinization [3]. After trypsin inactivation with cell culture medium specific for each type of cells, the contents of the flasks were placed in 15 ml tubes and centrifuged at 1000 rpm for 10 minutes at room temperature (Universal 32R, Hettich Zentrifugen).

After centrifugation, the cell culture media with inactivated trypsin was removed, and by 1 ml of fresh cell culture media specific to the cells type was poured into the tubes. After pipetting, the cellular suspension was transferred to 1.5 ml Eppendorf tubes, which were centrifuged at 3500 rpm for 3 minutes (Combi-Spin FVL-2400N, Boeco) (fig. 3). After centrifugation from the Eppendorf tubes, the medium was extracted, except for 50-60 µl. Meanwhile the 15 ml tubes with the OCDD grafts and the sterile devices for fixation and cellularization of small sized grafts (DFCSSG) [3, 6] were placed in the hood (LN 090, Nuve). The used device was necessary for cellularization of grafts by the gravitational method, and its utilisation has been described in previous work [6]. The device was placed into

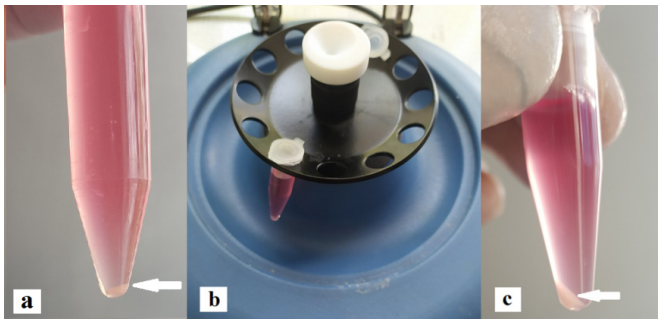

Fig. 3. Cells preparation to be combined with OCDD grafts: (a) cellular pellet in 15 ml tube after first centrifugation, (b) centrifugation of Eppendorf tube with cellular suspension in microcentrifuge, and (c) cells pellet at the bottom of Eppendorf tube, the pellet is indicated with white arrow

a stand, the cap from the tube with the graft was removed and with a tweezer the graft was extracted from the tube and inserted into the container of the fixation device. With a micropipette (LightDrop 10-100 µl, Thermo Fisher) the cellular pellets from the Eppendorf tubes were pipetted into the remaining 50-60 µl, and the cellular suspensions were poured onto the OCDD grafts which were fixed in the container of DFCSSG. Then, the lid was applied on the device and it was placed in a 15 ml tube [6], with the slightly opened cap and the divice was placed in incubator (SmartCell, Heal Force) at 37°C, 5% CO_2 humid environment.

Transplantation of combined grafts

After introduction of the combined grafts in the incubator, the rabbits were weighed and anesthetized. After preparing the operating field at the level of the unoperated knee joints, through a medial parapatellar approach of approximately 4-5 cm the knee joints were opened. After hemostasis, the patella was laterally dislocated and the knee flexed. Then, on the weight-bearing articular surface of the medial femoral condyle, with drill-bits of 1.5, 2, 3, 3.5 and 3.7 mm diameter, consecutively, with caution, at low speed and without opening the medullary canal, the defects of 3.7 mm in diameter and 4-4.5 mm in depth were created. Then the combined grafts were taken to the operating room. The containers with the combined grafts were placed on the operating table respecting all sterility principles. Intraoperatively, the base of the support with one of the combined grafts was sectioned with scissors and the graft was slowly pushed out into the defect with a K-wire, without damaging it or squeezing out the cells from the graft. All grafts were transplanted with its cartilaginous part facing up (fig. 4). After graft implantation, the dislocated patella was reduced and with the knee in extension, the wound was closed and aseptic dressing was applied. Maintaining the knees in extension, at the lateral side of the operated lower limbs thin planks were applied, wider than the limb and with the length corresponding to the distance from the hip to the ankle, which were fixed to the operated limbs with a cast from the pelvis to the ankle, holding a window at the level of the postoperative wound for dressings (fig. 6).

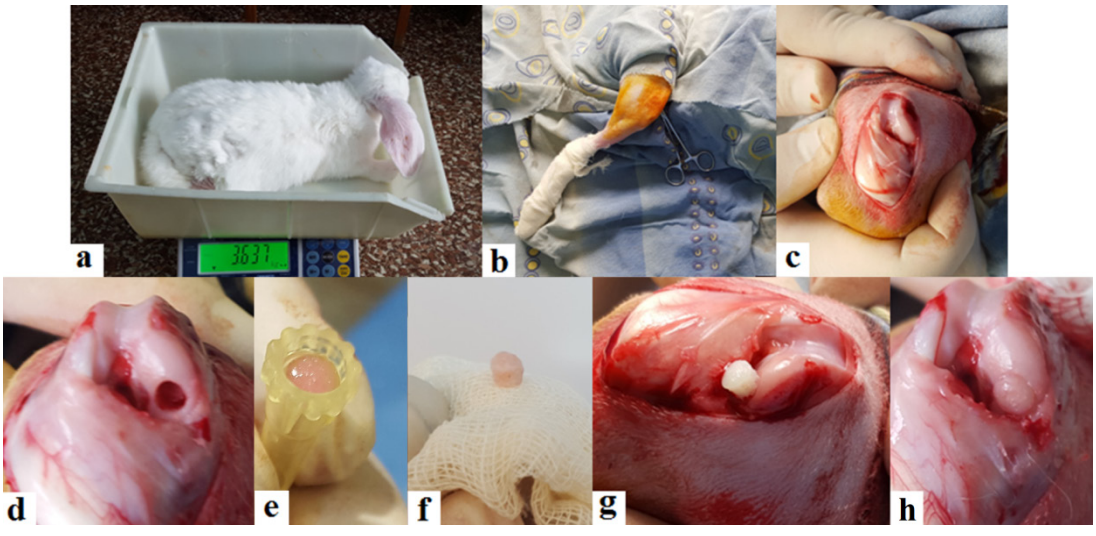

Fig. 4. Transplantation of the OCDD grafts combined with MSCs autologous chondrocytes, (a) animal weighing and general anesthesia performing, (b) operating field preparation, (c) medial parapatellar arthrotomy with lateral dislocation of patella, (d) creatig of the experimental defect, (e, f) aspect of the graft populated with autologous cells and (g, h) graft implantation into the experimental defect

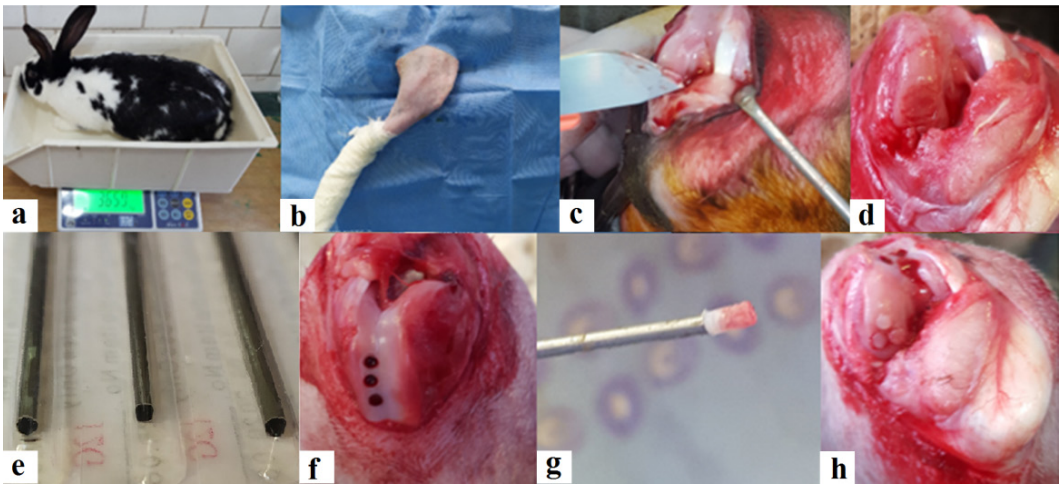

Fig. 5. Transplantation of autologous osteochondral tissue: (a, b) general anesthesia of the animal and preparation of the operating field, (c, d) creating the experimental cartilage defect, (e, f, g) harvesting of the autologous osteochondral tissue grafts from the non weight-bearing surface of the femoral trochlear groove with 12G needle and (h) their transplantation.

Transplantation of autologous osteochondral tissue

To the rabbits from the control group was performed transplantation of autologous osteochondral tissue (AOCT). The rabbits were prepared for surgery in the same way as the rabbits from the experimental groups. Through the same approach, after arthrotomy, lateral dislocation of the patella and knee flexion, with a scalpel, a cartilaginous defect was made on the articular weight-bearing surface of the medial femoral condyle of 3.7 mm in diameter, at the same level where were transplanted the combined grafts. In the created cartilage defect with a small diameter drill-bit, 3 holes of approximately 1.7 mm in diameter were made, in the form of a triangle, with a depth of 4-4.5 mm. With a 12 G piercing needle, that has internal diameter of 1.7 mm, at low speed, to avoid overheating the osteochondral tissue, from the trochlear groove were taken pieces of autologous osteochondral tissue and implanted in the drilled holes at the same height or with almost 1 mm higher than the adjacent normal car-

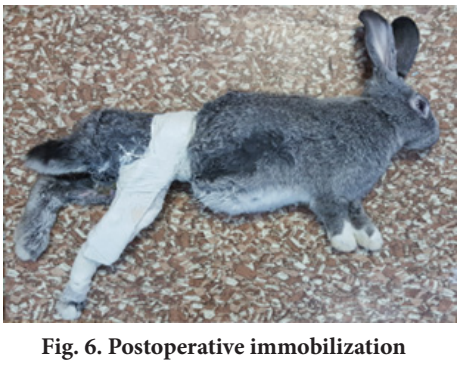

Fig. 6. Postoperative immobilization of the operated knees in rabbits

tilage (fig. 5). After reducing the dislocation of the patella and keeping the knee in extension, the wound was sutured and aseptic dressing was applied, followed by knee immobilization as described above (fig. 6).

Postoperative care

The cast immobilization of the operated knees was removed after 10 days. Postoperatively the rabbits received antibiotics for another 2-3 days depending on the case. Dressings were performed every 1-2 days, with monitoring of the general condition of the animals. All animals were kept in clean conditions, fed and watered daily.

The operated rabbits were removed from the experiment after 6 weeks – by 3 rabbits from each group, and after 12 weeks the rest, and the operated distal femurs were sampled. After removing of all soft tissues, the distal femurs were placed in 10% buffered formaldehyde, pH=7.4. For the microscopic examination slides of 5 µm were sectioned with microtome and fixed on the coverslip. Histochemical staining was performed with Hematoxylin-Eosin (H-E), Safranin O and Toluidine Blue with Fast Green. The evaluation of the results was performed according to the Unified Histological Score of Regenerated Cartilage (UHSRC) [3], which was composed by combining 3 scores widely used in the evaluation of regenerated articular cartilage in animals, whose authors are Sellers R. (1997), Wakitani S. (1994) and O'Driscoll S. (1986) [3, 26]. Each of the scores proposed by the authors contain many similar evaluation criteria, which evaluate the general morphology of the regenerated tissue and make difference between hyaline cartilage, fibrocartilage and fibrous tissue. Those scores also evaluate the degree of defect filling, thickness of the regenerated cartilage and the presence of lesions in it, the metachromasia of the regenerated tissue and the degree of integration of the regenerated tissue with the healthy one. Each score also contains specific individual criteria, such as the cellularity level of the regenerated cartilage in the O'Driscoll score, or the cellular pattern in the regenerated tissue present in the Wakitani score, and formation of chondrocytes clusters in the Sellers and O'Driscoll scores. At the same time, the Sellers score includes such criteria like evaluation of the subchondral bone condition and formation of the demarcation line between the normal and the calcified cartilage in the repaired osteochondral unit, which is very important, because the subchondral bone was also involved in the process of *in vivo* testing of combined biphasic hierarchical grafts [23] for articular cartilage regeneration. The UHSRC is presented in Table 1, and consists of evaluation of 13 criteria, the values assigned to each criteria on the scale are in descending order, in other words, the higher is the quality of the regenerated tissue, the lower will be the evaluation score, and vice versa, the higher is the evaluation score, the lower is the quality of the regenerated tissue, the value of the lowest quality being 43 points.

Table 1. Unified histological score of regenerated cartilage (Sellers, Wakitani and O'Driscoll)

	Criteria			Scale
1	Cellular pattern in regenerated cartilage	Hyaline cartilage		0
		Predominantly hyaline cartilage		1
		Predominantly fibrocartilage		2
		Only non-cartilaginous structure		3
2	Cellular morphology (category – a, b, c, d)	(a)	**Normal**	0
		(b)	**Mostly round cells with chondrocytic morphology**	
			> 75% of tissue with columns in the radial zone	0
			25-75% of tissue with columns in the radial zone	1
			<25% of tissue with columns in the radial zone (disorganized structure)	2
		(c)	**About 50% round cells with chondrocytic morphology**	
			> 75% from tissue with columns in the radial zone	2
			25-75% from tissue with columns in the radial zone	3
			<25% of tissue with columns in the radial zone (disorganized structure)	4
		(d)	**Mostly spindle-shaped cells (fibroblast-like)**	5
3	Chondrocytes clusters formation	Absent		0
		<25% of cells		1
		25-100% of cells		2
4	The cellularization level	Normal cellularity		0
		Mild hypocellularity		1
		Moderate hypocellularity		2
		Severe hypocellularity		3
5	Matrix metachromasia	Normal		0
		Slightly reduced		1
		Moderately reduced		2
		Severely reduced		3
		Staining is absent		4
6	The articular surface aspect	Smooth and intact		0
		Superficial horizontal lamination		1
		Cracks from 25% to 100% of the thickness		2
		Severe disorders, ruptures, including fibrillation		3
7	Integration of the graft with the surrounding articular cartilage	Normal continuity and integration at the border		0
		Reduced cellularity at the border		1
		Gap or lack of continuity on one side		2
		Gap or lack of continuity on both sides		3
8	The architecture of entire defect without including edges	Normal		0
		1-3 small defects		1
		1-3 large defects		2
		severe destruction		3
9	The thickness of the newly formed cartilage	100% of the neighboring normal cartilage		0
		50-100% of the neighboring normal cartilage		1
		0-50% of the neighboring normal cartilage		2

10	The presence of degenerative changes in the adjacent cartilage	Normal cellularity, no clusters, normal staining	0
		Normal cellularity, few clusters, moderate staining	1
		Moderate cellularity, moderate number of clusters, moderate staining	2
		Severe hypocellularity, without staining	3
11	Filling of the defect in relation to the adjacent normal cartilage surface	111-125 %	1
		91-110 %	0
		76-90 %	1
		51-75 %	2
		26-50 %	3
		<25 %	4
12	Formation of the demarcation line	Complete	0
		75-99 %	1
		50-74 %	2
		25-49 %	3
		<25 %	4
13	The percentage of newly restored subchondral bone	90-100 %	0
		75-89 %	1
		50-74 %	2
		25-49 %	3
		<25 %	4

Results

In order to combine OCDD grafts with cells that have chondroprogenitor potential, $1.64x10^5 \pm 7x10^4$ chondrocytes were isolated from hyaline articular cartilage with a viability of 96.79%, from which in the first passage during 10±3 days of culture were obtained $2.94x10^6 \pm 3.77x10^5$ chondrocytes with a viability of 99.89%. Also, from 4 ±1.2 ml of bone marrow collected from the rabbits, during 11±3 days were obtained $1.55x10^6 \pm 3.76x10^5$ MSC with a viability of 98%.

As a result of the implementation of the UHSRC, the animals removed from the experiment at 6 weeks in all cases had an almost similar histological score. The group of rabbits whose experimental defects were treated with OCDD grafts combined with chondrocytes had a score of 28.33±1.53 points, the group treated with grafts combined with MSC obtained a score of 27.67±2.08 points and the control group had a score of 26.33±1.53 points. As a result, there is no significant difference between the control group and the experimental groups in which tissue defects were treated with OCDD grafts combined with chondrocytes ($p > 0.1$) and in that treated with grafts combined with MSC ($p > 0.2$) (fig. 7).

At the animals removed from the experiment at 12 weeks after surgery, according to the UHSRC were determined the following results: the experimental group in which the experimental defects were treated with OCDD grafts combined with chondrocytes had a score of 18.68 ±5 points, the experimental group treated with OCDD grafts combined with MSC had a score of 14.89 ±3.76 points and the control group had a score of 17.22 ±4.84 points. According to obtained results, it was determined that there was no significant difference between the control group

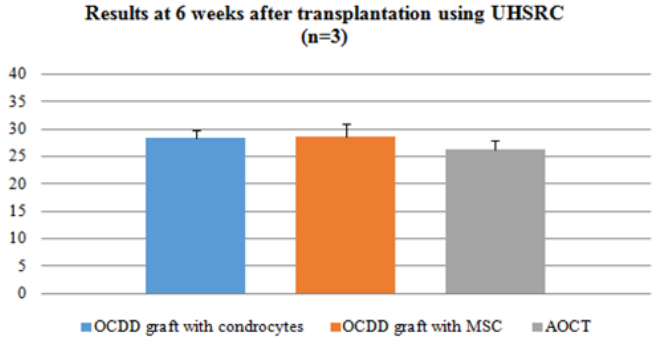

Fig. 7. Comparative results of experimentally created defects and treated with hierarchical biphasic grafts combined with chondrocytes and BM-MSC at 6 weeks after transplantation

and the group in which the cartilaginous defects were treated with OCDD grafts combined with chondrocytes ($p > 0.5$) and the group in which the defects were treated with OCDD grafts combined with MSC ($p > 0.2$) (fig. 8).

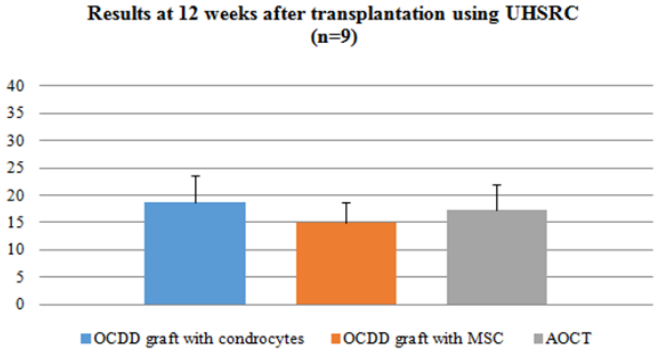

Fig. 8. Comparative results of regenerated defects at 12 weeks after surgery

At comparison of the results obtained within the same group of animals that were removed from the experiment at 6 and 12 weeks after surgery, a significant difference is determined between the obtained results, which are much better in the case of animals that were removed from the experiment later, the data are shown in table 2.

Table 2. The comparison of UHSRC obtained by groups depending on the evaluation term of the regenerated defects

	UHSRC at 6 weeks (n=3)	UHSRC at 12 weeks (n=9)	t-test
OCDD grafts combined with chondrocytes	28.33±1.53	18.68±5	p=0.009
OCDD grafts combined with BM-MSC	27.67±2.08	14.89±3.76	p<0.001
Transplantation of AOCT	26.33±1.53	17.22±4.84	p=0.004

During the histological examination of the regenerated articular cartilage after transplantation of grafts that

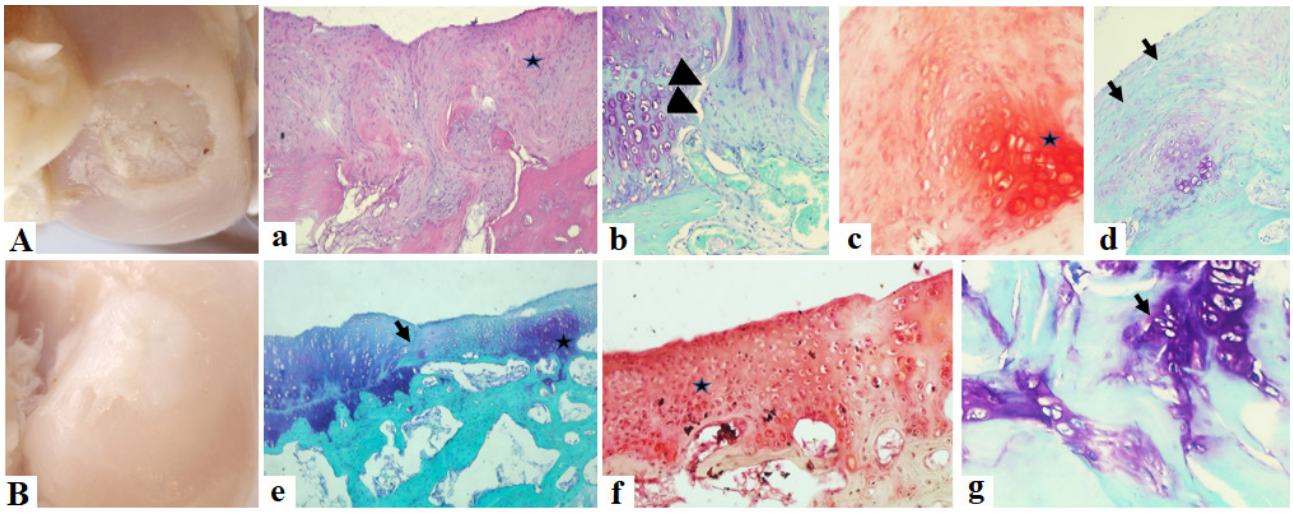

Fig. 9. Regeneration of articular cartilage using grafts combined with chondrocytes at 6 (A) and 12 weeks (B) after transplantation: (a, d) moderate cellularised fibrocartilage (× 40), H-E and Toluidine Blue with Fast Green; (b) regenerated cartilage cracks (× 100); (c) uneven GAGs distribution (x100), Safranin O; (e, g) chondrocytic clusters delimited by fibrous tissue (× 40, × 100), Toluidine Blue with Fast Green and H-E; (f) GAGs homogeneous content (× 40), Safranin O

were combined with chondrocytes (fig. 9), at 6 weeks after transplantation was determined that the defects were filled with fibrocartilage with moderate cellularity unevenly distributed. It was also determined that the deep areas of the neo-cartilage were populated by chondrocytes, arranged in isogenous groups, with signs of cellular hypertrophy and preservation of their integrity, and the superficial part of the regenerated cartilage was represented by fibroblast-like cells surrounded by a predominantly fibrous matrix. The collagen fibers were determined as being thinned and fragmented, and the concentration of glycosaminoglycans (GAG) was gradually reduced from the deep areas to the periphery. In the place of contact with the native cartilage, long and deep cracks were observed. At 12 weeks after transplantation, the quality of regenerated articular cartilage was significantly better, it was smoother, and its thickness was thinner compared to the native cartilage. In the deep and middle areas newly formed hyaline cartilage was identified, and fibrous cartilage was frequently detected in the superficial areas. The cells in the regenerated cartilage were unevenly distributed, being predominantly of the chondrocytic type, organized in isogenic groups. At the border with the native cartilage chondrocytic clusters were identified, which were delimited by masses of fibrous tissue. The collagen fibers were highly disorganized, and the content of GAGs was more homogeneous, being identified in all areas of the newly formed cartilage.

In the case of experimental defects regenerated by using grafts combined with MSC, no big difference was observed compared to grafts combined with chondrocytes from the

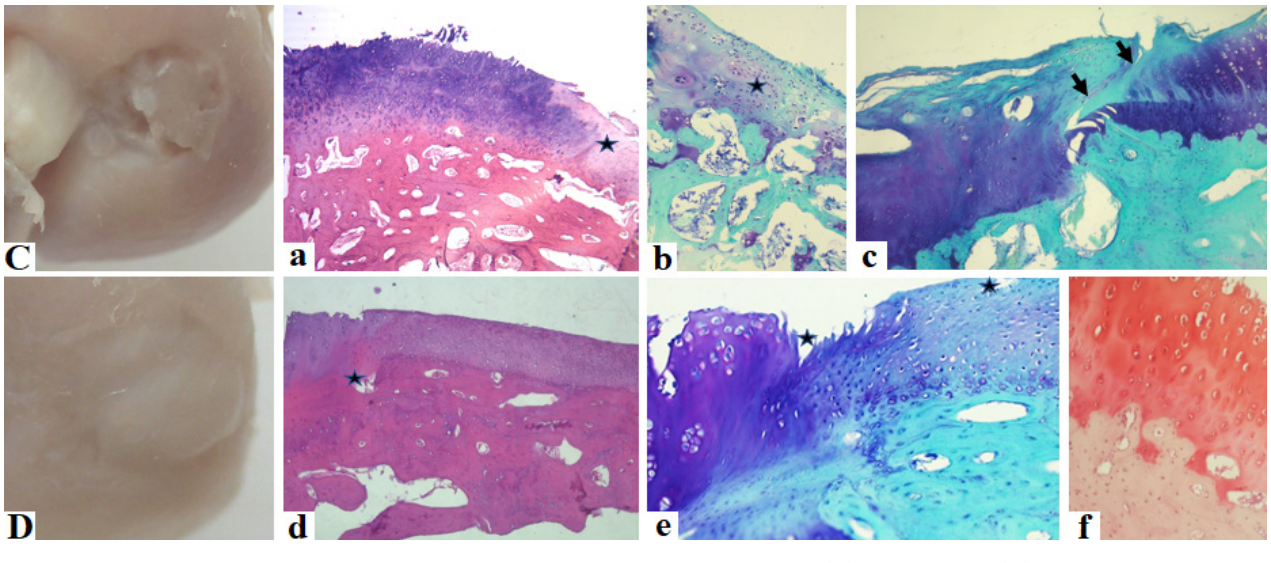

Fig. 10. Regeneration of articular cartilage using grafts combined with MSC at 6 (C) and 12 weeks (D) after transplantation: (a) moderate cellularised fibrocartilage (× 40), H-E; (b, f) GAGs homogeneous content with cells in upper part of the cartilage (× 40, × 100), Toluidine Blue with Fast Green and Safranin O; (c, e) regenerated cartilage cracks and fibrillation (× 40); (d) chondrocytic clusters delimited by fibrous tissue (× 40), H-E

same periods. At 6 weeks was determined that the presence of chondrocytes and chondroblasts was predominantly in the superficial and middle areas of the regenerated cartilage. While at 12 weeks was determined that the thickness of the regenerated cartilage is similar to that of the native one, the concentration of GAGs being slightly lower, and the demarcation line was better visualized (fig. 10).

In the case of cartilage defects regeneration by autologous osteochondral tissue transfer, at 6 weeks, in all cases, were identified areas where the defects were not completely filled with regeneration tissue, but also the areas with the lack of adherence to native articular cartilage. There are cases when the autologous transplant was located in the same plane as the surface of native articular cartilage, or even slightly protruding into the joint cavity. The cartilage within the autologous grafts underwent partial or in some cases total lytic changes, with partially or totally destroyed chondrocytes. In the contact areas of the native cartilage with the transplanted tissue, were observed structural changes like hypercellularity with uneven cellular distribution and their tendency to form large clusters of chondrocytes and extracellular matrix with intense metachromasia. At 12 weeks, the defects areas were incompletely filled, the recovery of the defect was achieved only at the level of bone tissue, with its non-homogeneous filling. Also, at the periphery of the transplanted tissue, immature cartilaginous tissue was observed, on some places with signs of maturity, and in the center of the transplant area a fibrillar matrix with fibroblast-like cells was present, with the content of GAGs being inhomogeneous (fig. 11).

Discussion

The process of obtaining cells for articular cartilage regeneration with combined grafts is an essential one. It is necessary to mention that there is an obvious difference between the number of MSC and chondrocytes transplanted within the experiment ($p<0.001$). This significant difference between the groups is due to the fact that MSC are larger than chondrocytes, for this reason, the amount of transplanted MSCs is almost twice lower comparing to chondrocytes. However, in each transplantation, one cell culture flask with 80-90% cells confluence was used. As a result, if the amount of transplanted cells during the experiment is reported to have a defect of 1 cm^3, then it turns out that for the treatment of a defect of 1 cm^3 will be necessary to transplant approximately $6.67x10^7 \pm 8.55x10^6$ chondrocytes or $3.51x10^7 \pm 8.53x10^6$ MSC.

During the performed research, the experimental defects should have been only of articular cartilage, without involving the subchondral bone, according to the main objective of the research, which is the regeneration of the articular cartilage, but not of the subchondral bone. This was not done because it was practically impossible to prepare and fix a decellularized cartilaginos graft combined with cells, which after transplantation would be constantly subjected to mechanical stress forces, because the articular cartilage has a very weak regeneration potential, even being combined with cells that have chondroprogenitor potential, it has poor possibility to adhere at the adjacent bone and cartilage. At the same time, the subchondral bone in the region with degenerated cartilage is important for the nutrition of the deep layers of the cartilage and the transfer of mechanical stimuli from the cartilage to the rest of the bone [27], as a result, the bone within the biphasic graft being demineralized ensures a rapid regeneration of the subchondral bone due to its osteoinductive and osteoconductive properties [15, 23], but also a more stable fixation of the graft. Because the demineralized, decellularized and longitudinally perforated grafts have much lower mechanical strength than normal osteochondral tissue [4],

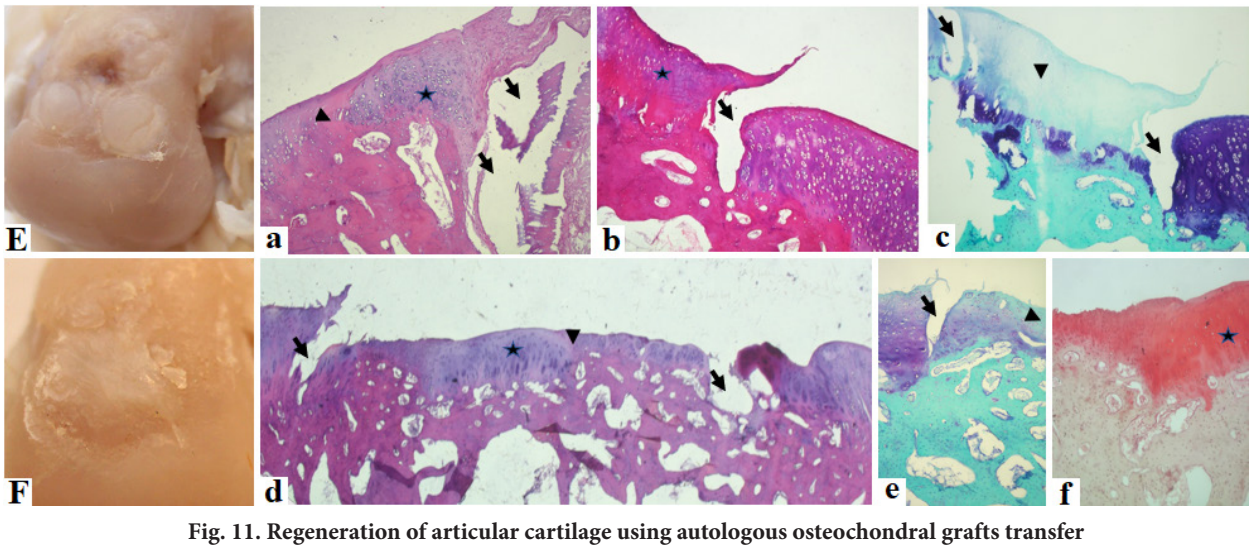

Fig. 11. Regeneration of articular cartilage using autologous osteochondral grafts transfer at 6 (C) and 12 weeks (D) after implantation: (a, b, c, d, e) lack of regeneration between the transferred tissue and the native cartilage with signs of partial or total osteo- and chondrolysis (× 40), H-E and Toluidine Blue with Fast Green; (a, d) hypercellularity at the level of transferred tissue and lack of cells at the border between graft and native cartilage; (e, f) inhomogeneous GAGs content (× 40), Toluidine Blue with Fast Green and Safranin O

in order to ensure joint rest for the process of osteogenesis and chondrogenesis, plaster immobilization was applied for a period of 10 days.

Transplantation of biphasic osteochondral, allogeneic, demineralized, decellularized, longitudinally perforated grafts and combined with autologous cells with chondroprogenitor potential to regenerate the articular cartilage, can be qualified as articular cartilage regeneration through tissue engineering techniques. The tissue engineering technique that uses three-dimensional matrices combined with autologous chondrocytes is called Matrix-assisted Autologous Chondrocyte Implantation (MACI) [2, 3], and the technique that uses three-dimensional matrices populated with MSC is called Matrix Autologous Stem-cells Implantation (MASI) [14]. Thus, in the performed research, were compared two tissue engineering techniques of articular cartilage regeneration with the autologous osteochondral tissue transfer through the mosaicplasty technique [28]. Given that the UHSRC at 12 weeks in the groups that used grafts combined with chondrocytes and grafts combined with MSC are different (fig. 8), the best score being in the case of grafts combined with MSC – 14.89±3.76 points, comparatively to those combined with chondrocytes with a score of 18.68 ±5 points, from a statistical point of view, there is no significant difference between these 2 groups ($p = 0.08$). The research that compares the MACI and MASI techniques, using chondrocytes isolated from the articular cartilage and MSC isolated from the articular synovium, shows slightly better results when MASI technique was performed [14].

Considering that the UHSRC obtained in the experimental groups do not show a significant difference compared to the control group at 6 and 12 weeks, and the histological picture is far from ideal, the obtained cartilage results are far from good.

Conclusions

There was no big difference at 6 and 12 weeks between results of the grafts combined with chondrocytes and those combined with MSC, even though the number of transplanted chondrocytes was almost twice bigger ($p > 0.8$; $p = 0.08$), and the control group did not show better results.

Utilisation of UHSRC allowed a thorough evaluation of the obtained results. Thus, a weak regeneration degree of the experimental cartilage defects was achieved in both cases: using grafts combined with chondrocytes and MSC, and the control group.

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VC – conducted literature review, obtained raw data, created the unified histological score of regenerated cartilage and wrote the manuscript; MJ – interpreted the data and drafted the manuscript; TG - created the objective evaluation score of histological samples; VN – conceptualized the idea, designed the research and monitored the experiment. All the authors approved the final version of the manuscript.

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Ethics approval and consent to participate
The research project was approved by Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (Protocol No 31, 14.12.2016).

Conflict of interests
No competing interests were disclosed.