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COMBINED GRAFTS FOR REGENERATION OF EXPERIMENTAL ARTICULAR CARTILAGE DEFECTS

341.01. TISSUE ENGINEERING AND CELLULAR CULTURES

Summary of Ph.D. Thesis in Medical Sciences

CHISINAU, 2023

The thesis was developed within the Laboratory of Tissue Engineering and Cell Cultures, PI „Nicolae Testemitanu" State University of Medicine and Pharmacy.

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CONTENT

Introduction

Articular cartilage injuries have a reduced potential for intrinsic regeneration and repair. Since it is a tissue without vascularization, it is mostly nourished only by diffusion from the synovial fluid [1]. At the same time, the structural organization of hyaline articular cartilage and the fixation of chondrocytes in it without the possibility of migration to the site of injury, dramatically hinders the process of cartilage regeneration [2, 3]. Articular cartilage lesions were often asymptomatic, but could be the cause of joint pain, swelling, and articular blockage [4]. It was determined that cartilage defects of various degrees were identified in 60-66% of patients who underwent arthroscopic examination [5, 6]. As a result, the clinician is put in a position to examine thoroughly the patients if they present pain and discomfort at the joint level, the cartilage injury being a burden for both, the patient and the medical system.

Alteration of the biomechanics resulting from an injury sustained at the joint level will lead to the progressive degenerative degradation of the joint. About 12% of all cases of arthrosis were of post-traumatic origin [7]. It was proven that even after surgical stabilization of the joint, the joit degradation will not be stopped due to the molecular and cellular damage that occurred at that level. It was also found, that about 50% of patients after a severe knee injury, developed knee arthrosis up to 20 years, but in case of an intra-articular fracture presence, the risk of arthrosis was up to 75% [7, 8].

It is believed that due to the lack of vascularization, nerves and lymphatic vessels in the structure of articular cartilage, the inflammatory response to any injury it was predominantly chondrocytic, therefore exaggerated, and the early attenuation of this response to an injury could stop the irreversible degradation of chondrocytes and adjacent joint tissues at the molecular level [8]. At the moment there were tested various experimental, non-surgical ways of treating articular cartilage defects, such as utilisation of biologically active compounds such as p188 and rotenone [8], being membrane stabilizers, they inhibit or block p38 protein activated by stress, inhibit the activation of glycogen synthase kinase-3 and the inflammatory process related to IL-6 [9]. Also, has been tested utilisation of proinflammatory cytokine inhibitors such as interleukin-1(IL-1RA) and tumor necrosis factor-α1 (TNF-α1) receptor antagonists. Animal model tests showed that the use of IL-1RA receptor antagonists reduced subchondral bone edema, articular surface fibrillation and chondrocytes necrosis. The antagonist of TNF-α1 receptors, showed a decrease in the expression of the metalloproteinase (MMP) genes MMP-1, MMP-3 and MMP-13, which reduced the synthesis of prostaglandins and increased the synthesis of lubricin [10]. Another group of substances that could be used to stop the process of cartilage degradation after knee trauma, are the agents for cartilaginous matrix protection, among them were the scavengers of reactive oxygen species, inhibitors of nitric oxide, inhibitors of inflammatory cytokines and specific MMP inhibitors [11]. Also, *in vitro* and in animal models, where growth factors were used, promising results have been obtained. The aim was to stimulate the regeneration of damaged cartilage by activating the anabolic process in cartilage [12]. Good results were also obtained with the use of bone morphogenetic protein (BMP) BMP-7 and BMP-2, due to their anabolic and anti-catabolic effects on cartilage [13], among which were listed the stimulation of cartilage regeneration and repair of the articular cartilage, and ensuring of a efficient integration of the newly formed cartilage within the surrounding one [14]. Similar effects were obtained after use of *Insulin-like growth factor-1* in cartilage defects caused by osteoarthritis [15]. Considering that non-surgical methods of cartilage defects treatment were not widespread and their use remained only at the level of

laboratories and research centers, the regeneration of articular cartilage defects has become exclusively the burden of orthopedic surgery and regenerative medicine.

Surgical treatment of articular cartilage defects in big joints largely depends on surgical approach on them. Comparing the accessibility to the hip, knee, ankle and shoulder joints, the treatment of articular cartilage at the hip level is much more complicated to achieve, and cases that would require articular cartilage regeneration at the shoulder and ankle level are much rarer compared to the knee joint. In order to avoid the arthritic degradation of the joint, contemporary treatment tactics tend to surgical repair and the small articular cartilage defects, which in the case of the knee region are smaller than $4cm^2$ [4]. Thus, the aim of articular cartilage injuries treatment always was pain reduction, increasing the amplitude of joint movements, and prevention or delay of arthritic degradation of the joints, with the purpose of delaying or avoiding the performance of total joint arthroplasty [16]. Nowadays, are known many ways of articular cartilage defects treatment, these are various surgical techniques like bone marrow (BM) stimulation [2], allogeneic and autologous osteochondral (OC) tissue [18] transplantation [17], implantation of autologous chondrocytes and mesenchymal stem cells (MSCs) in defects combined or not with a three-dimensional matrix [19]. Unfortunately, the purpose of hyaline cartilaginous tissue obtaining at the end was not achieved [20]. At the same time, the concept of grafts combined with autologous cells that have chondroprogenitor potential for the regeneration of articular cartilage is promising [21]. Was found that the combined grafts obtained for the regeneration of articular cartilage must consist of structures that mimic native articular cartilage and their seeding with cells that have chondroprogenitor potential [13].

The aim of the study is restoration of the experimental cartilage defect in the knee joint using combined osteochondral grafts obtained by tissue engineering techniques.

Research objectives:

1. Development and optimization of methods for isolation and cultivation of cells with chondroprogenitor potential from autologous tissues - hyaline cartilage and bone marrow;
2. The conception of an osteochondral graft for the recovery of articular cartilage defects;
3. Transplantation of grafts combined with cells that have chondroprogenitor potential in experimental defects;
4. Evaluation of regeneration potential of experimental critical articular cartilage defects created at the load-bearing surface of the joint by using combined grafts, compared to the control group.

The scientific novelty of the research. For the first time, several innovative work techniques, processing methods and devices were developed and implemented in the laboratory practice:

1. The innovative technique of bone marrow harvesting for further stem cell isolation, which further was used for *in vivo* tests with autologous cells.
2. Development of a procedure for MSCs culture, that consisted in complete removal of contaminating cells when the MSCs reached a confluence of 40-50%, which allowed obtaining of a high purity MSCs in first passage needed for transplantation.
3. Was significantly reduced the period needed of isolation of a large number of chondrocytes with high-viability.
4. A new method of cellullar isolation using explants was developed;
5. Various types of osteochondral grafts obtained by tissue engineering techniques from allogeneic tissue for combination with autologous cells have been developed and tested;

6. A new device was developed and applied specifically for combining small sized grafts with cells;
7. Establishing for the first time the actual quantity and viability of cells at the time of transplantation;
8. A new histological score was developed for the objective evaluation of the obtained results;
9. Was simplified the way of isolated cells identification by culturing them in overconfluence followed by specific histochemical staining for the investigated tissue.

Theoretical importance of the study. The research results will serve as scientific evidence in the process of obtaining and utilisation of combined grafts, consisting of autologous cells with chondroprogenitor potential - chondrocytes and mesenchymal stem cells from the bone marrow, and a graft obtained by demineralization and decellularization of allogeneic osteochondral tissue.

The applicative value of research. The experience gained in the process of obtaining cells with chondroprogenitor potential, hierarchic biphasic grafts that were tested *in vitro*, but also of combined grafts with their subsequent *in vivo* testing, is used as a basis to perform similar researches in the laboratory, and may also be useful in guiding clinicians in the preparation and transplantation of similar grafts.

Implementation of scientific results. The results of the experimental study are implemented within the Tissue Engineering and Cell Culture Laboratory, in order to carrying out other doctoral theses, in the process of training students, residents and doctors at the Department of anatomy and clinical anatomy, at the Regenerative Medicine course at SUMPh "Nicolae Testemițanu".

1. GENERAL METHODOLOGY

The study on laboratory animals was approved by the Research Ethics Committee of SUMPh "Nicolae Testemițanu", examined at December 14, 2016, with the issuance of favorable decision No. 31 from 14.12.2016.

1.1 General presentation of the study

In the carried out research, I was guided by the „three R" concept, adopted by the International Congress of Biological Standardization (San Antonio, USA, 1979) [33]. As a result, in the research were used 55 rabbits, of 5 ±0.5 months old and 3.51 ±0.49 kg body mass, of whom 30 were females and 25 males. For the research, as cell and tissue donors, served 13 rabbits. The cells which were obtained are MSCs from BM, and chondrocytes isolated from hyaline articular cartilage. From the distal femurs, the osteochondral tissue was collected for preparation of osteochondral demineralized and decellularized (OCDD) grafts. Tissue donor animals were sacrificed in the CO2 chamber according to the standard operating protocol. For the *in vivo* tests, 3 groups by 12 rabbits each were created: the first group consisted of rabbits whose experimental osteochondral defects were treated with OCDD grafts combined with chondrocytes, in the second group the experimental defects were treated with OCDD grafts combined with MSC and the control group consisted of rabbits whose cartilage defects were treated through autologous osteochondral tissue transfer. Were registered 7 cases of failure, of which 2 intraoperative fractures and 5 deaths of unknown cause at different times. The failed cases were replaced with other rabbits. Only one rabbit which failed because of a fracture was used as a tissue donor.

The thesis has a complex traditional structure, and the studies presented within it were carried out in 3 stages, which are presented in table 1.1.

Table 1.1. **Study design.**

Stage No.	The name of the stage
Stage I	Isolation and cultivation of cells with chondrogenic potential and obtaining from allogeneic osteochondral tissue, by tissue engineering techniques, a hierarchically biphasic demineralized and decellularized graft suitable for regeneration of the experimental defects.
Stage II	The experimental study *in vivo*. Transplanation in the critical experimental osteochondral defects of combined grafts obtained from allogeneic OC tissue by demineralisation and decellularization, combined with autologous bone marrow MSCs and autologous chondrocytes.
Stage III	Evaluation of *in vivo* obtained results compared to the control group.

1.2 Preparation and storage of reagents

For carrying out research with cells, were prepared the following solutions and culture media: culture medium for chondrocytes, medium for isolation and cultivation of MSCs, medium for chondrocyte differentiation, medium for MSCs culture, 0.6% collagenase solution, 0.25% trypsin-EDTA solution, medium for cell preservation.

Preparation of reagents for cytotoxicity assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): 0.5% w/v MTT stock solution in Hanks Balanced Salt Solution (HBSS) without phenol red and MTT working solution 0.5% v/v in cell culture medium.

For samples examination under fluorescent microscopy with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) the following reagents were prepared: 4% paraformaldehyde solution, 1mM DAPI stock solution in DMSO; DAPI working solution in Dulbeco phosphate buffered saline (DPBS) whit calcium and magnesium and membrane permeabilization solution.

To perform the enzymatic degradation assay, the following reagents were prepared: the ninhydrin reagent and the 0.01% collagenase solution in phosphate buffered saline (PBS).

The following reagents were prepared for the histological examination: 10% buffered formaldehyde solution, 0.4% Toluidine Blue solution, 0.1M sodium acetate buffer solution; 0.001% and 0.02% Fast Green solutions, 0.1% Safranin O solution and 1% acetic acid solution.

1.3 Anesthesia of laboratory animals

At the level of the hip or back the following drugs were injected Xylla 5 mg/kg (De Adelaar, Holland), diazepex 0.5% – 2 mg/kg (Grindex, Latvia) and ketamine 35 mg/kg (Farmako, Moldova) solutions were injected into the hip or back. For anesthetic prolongation every 30 minutes, half of the initial doses of drugs were added.

Statistical analysis was performed using Microsoft Office Excel version 2020 and SPSS (Statistical Package for the Social Sciences. Version 18.0, Inc. Chicago, IL, USA).

2. ISOLATION, CULTURE AND IDENTIFICATION OF BONE MARROW MESENCHYMAL STEM CELLS.

2.1 Methods

In the experiment were used 9 rabbits. After performing anesthesia and fur removing from the pelvic region, under sterile conditions, using a 5 ml syringe with 1250-2000 U heparin (Balkan Pharmaceuticals, Moldova), BM was collected from the wing of the iliac bone according to a our own technique. In the laboratory, the syringe with the harvested BM was processed under sterile conditions. By centrifugation on concentration gradient HiSep LSM 1077 (HiMedia, India), the nucleated cells were colected and then incubated at 37°C with 5% CO_2 (Smart Cell, Heal Force) in 25 cm^2 cell culture flask, in medium for MSCs isolation and culture. Isolated MSCs were

cultured in two passages. The isolated cells were identified as MSCs by chondrocyte lineage differentiation [25].

2.2 Results

The mean period of BM harvesting from the start of anesthesia lasted in average 36 ±3 minutes, without recording any complications in experimental animals. In average were harvested 3.39 ±1.27 ml of BM. The mean period of cell culture in the first passage to a 70-80% confluence was 7±1 days.

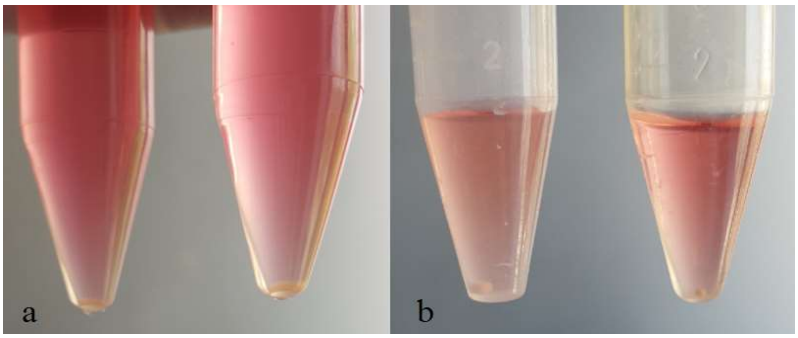

Figure 2.1. **Chondrocytic lineage cell differentiation in 15 ml polypropylene tubes. The appearance of 1x10^6 CSM at the beginning of chondrocyte lineage differentiation (a) and formation of chondrocyte aggregates (b).**

Statistically was determined a strong correlation between the volume of harvested BM and the number of days required to achieve cellular confluence ($p < 0.001$). The higher was the volume of BM, the faster the confluence occurred and vice versa. However, no correlation was determined between the number of obtained cells in the first passage and the volume of harvested BM ($p > 0.5$). This can be explained by the fact that the period needed to achieve the required cell confluence was different, the amount of MSCs differs from case to case, and the level of confluence determined visually is not an objective assessment. As a result, in the first passage, were obtained about 7.47 x10^5 ±6.9 x10^4 cells per flask, and after 5 days of culture in second passage, in 75 cm^2 flasks, up to 80-90% cell confluency were obtained 4.12 x10^6 ±4.64 x10^5 cells per flask. Taking into account the fact that in second passage the period of MSCs culture was the same in all cases, it was determined that the number of obtained cells in the second passage was directly dependent by the number of viable cells obtained in the first passage ($p < 0.001$). As a result, the average period for cell culture during the first 2 passages was 12±1 days, with cell viability being 100% in all cases.

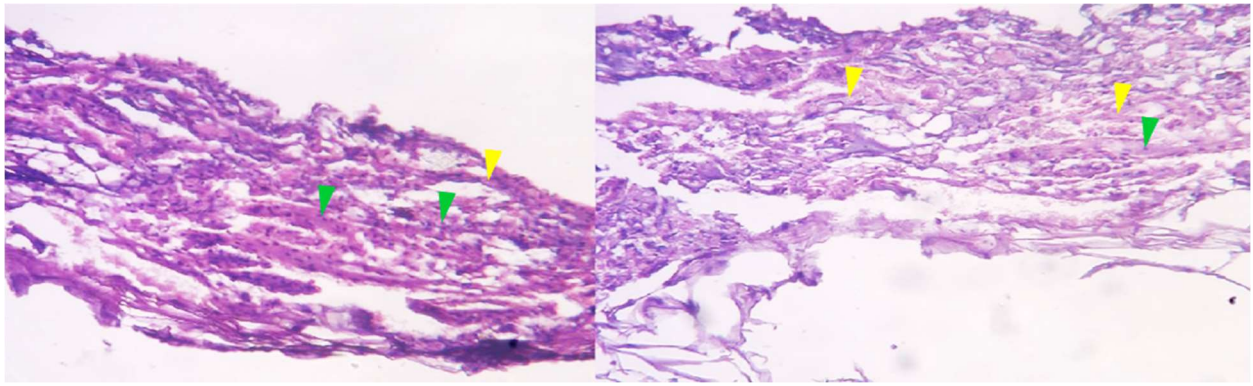

Figure 2.2. **Hematoxylin-Eosin staining of chondrocyte aggregates x60 (MT-2, Olympus). Is determined high density of nuclei (green), and formation of extracellular matrix (yellow).**

In the process of MSCs differentiation in chondrocytes, was determined formation of irregular spherical cellular aggregates which were attached to the bottom of the test tube [25].

Also, during the first days of differentiation, cells could be easily dispersed by pipetting, but after they became egg-like aggregates, it was no longer possible [25]. Even if the cellular aggregates were constituted by the same number of cells, they were of different sizes, between 1.5 and 3 mm in diameter (figure 2.1).

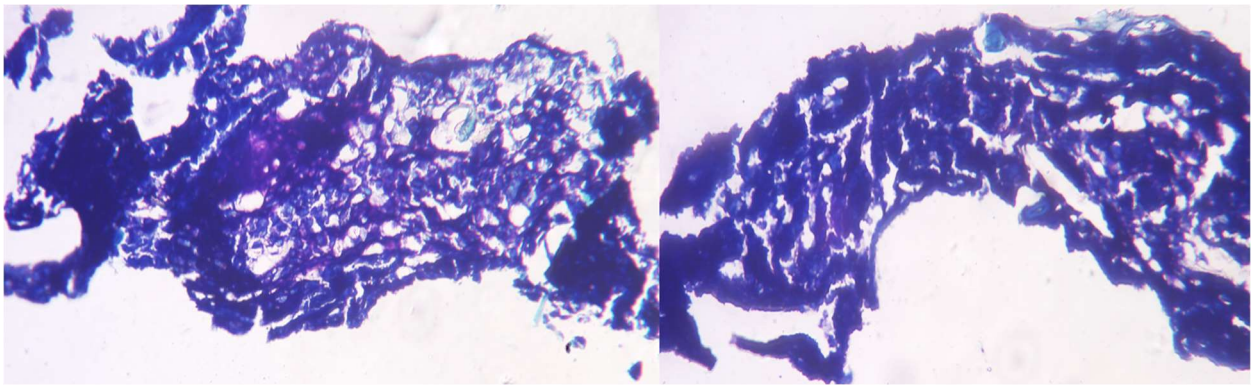

Figure 2.3. **Toluidine Blue and Fast Green staining of formed cellular aggregates x40 (MT-2, Olympus). The presence of a dark blue-violet staining indicates the presence of a high GAG content in the ECM.**

At histological examination with Hematoxylin-Eosin, a rich cellularization of the cellular aggregates was determined, highlighted by a high nuclei density, and formation of extracellular matrix (ECM) (figure 2.2). When staining with Toluidine Blue and Fast Green, the obtained structure was intensely colored in purple and blue, which is specific for glycosaminoglycans (GAG) that are present in the ECM of cartilaginous tissue (figure 2.3). At Safranin O staining, a large number of dark nuclei were determined. The ECM synthesized by the cells, rich in GAG, was stained red, which is specific for cartilaginous tissue (figure 2.4).

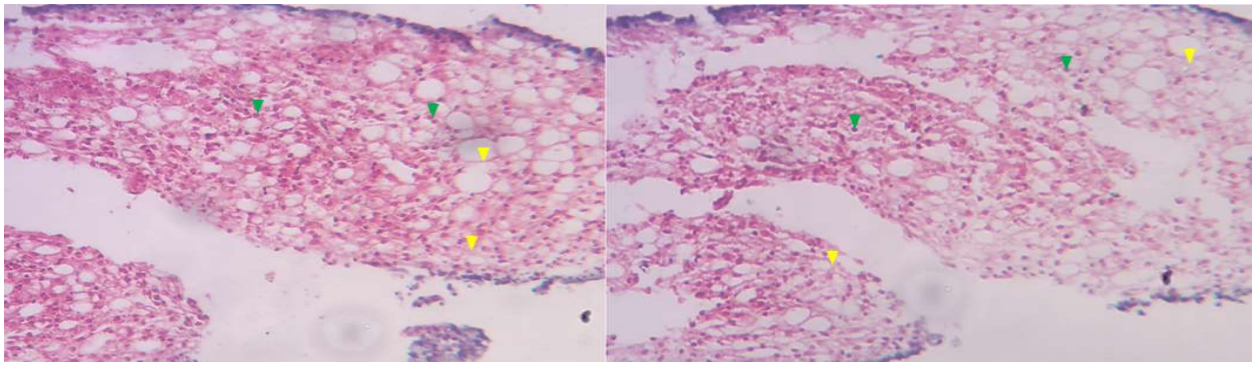

Figure 2.4. **Safranin O staining of the formed aggregates x60 (MT-2, Olympus). ECM stained in red (yellow) due to the presence of GAGs, and a high density of nuclei (green) is determined.**

3. ISOLATION AND CULTURE OF CHONDROCYTES ISOLATED FROM RABBIT ARTICULAR CARTILAGE.

3.1 Methods

Under general anesthesia, in sterile conditions, through a medial parapatellar approach of approximately 1-1.5 cm length, the knee joint was opened [26, 27]. Then, from the non weight-bearing surface of the femoral condyle at the level of the trochlear groove, pieces of articular cartilage were taken and introduced in 15 ml test tube with culture medium for chondrocytes. Under laminar flow hood (LN 090, Nuve), the harvested cartilage pieces were washed with PBS (HiMedia, India) and minced with a scalpel to fragments of 1-2 mm^3, and placed in a sterile 15 ml

tube with 5 ml of 0.25% trypsin-EDTA for predigestion [22]. Cartilage pieces were shaked for 10 min with an orbital shaker (ES-20, Biosan) at 150 rpm, 37°C. Then, the cartilage pieces were resuspended in 2 ml of 0.6% collagenase solution, and 2 groups were formed, each by 9 cases. In group I, the pieces of cartilage with collagenase solution were placed in 15 ml test tubes, and in group II in 25 cm^2 cell culture flasks (Nunc, Sweden). The recipients from both groups were placed in incubator (Heal Force, Start Cell), at 37°C with 5% CO_2, on a rocker shaker (MR-1, Biosan). On the shaker, the test tube was placed in a horizontal position, and the cell culture flask was placed on one of its lateral sides [26]. The recipients from both groups were shaked at 20-25 oscillations/minute. The period of enzymatic digestion in group I was fixed – 360 minutes, and in group II it varied depending on the amount of cells present in the field of view. Thus, after 120 minutes of digestion in cell culture flask, the process was monitored under microscope every 10-20 minutes, with the interruption of the process when the released chondrocytes from the tissue, occupied about 50-60% of field of view, even if the cartilage was not completely digested. The obtained chondrocytes, in each case were placed in a 25 cm^2 cell culture flask (Nunc, Sweden) and incubated at 37°C, 5% CO_2.

The isolated cells were identified as chondrocytes after culture in overconfluence during 16 days, as control in parallel were cultured MSC. The ECM synthesized by the isolated cells was identified as cartilaginous by using specific for GAG stainings – Toluidine Blue with Fast Green and Safranin O.

3.2 Results

As a result of implementation of a new method for chondrocytes isolation, their number increased significantly. The period of exposure to collagenase in group I - control, was fixed – 360 minutes, while the period of exposure in group II - experimental, was in average 140 ±10 minutes. As a result, in the control group were isolated $9.2x10^4$ $±3.1x10^4$ chondrocytes [26], when in the experimental group, through continuous monitoring, were isolated almost 2 times more – $1.6x10^5$ $±3.4x10^4$ cells (p =0.001) (figure 3.1). Hence, the long exposure to collagenase negatively influenced the number of isolated cells in the control group compared to the experimental group (p <0.001) (figure 3.2).

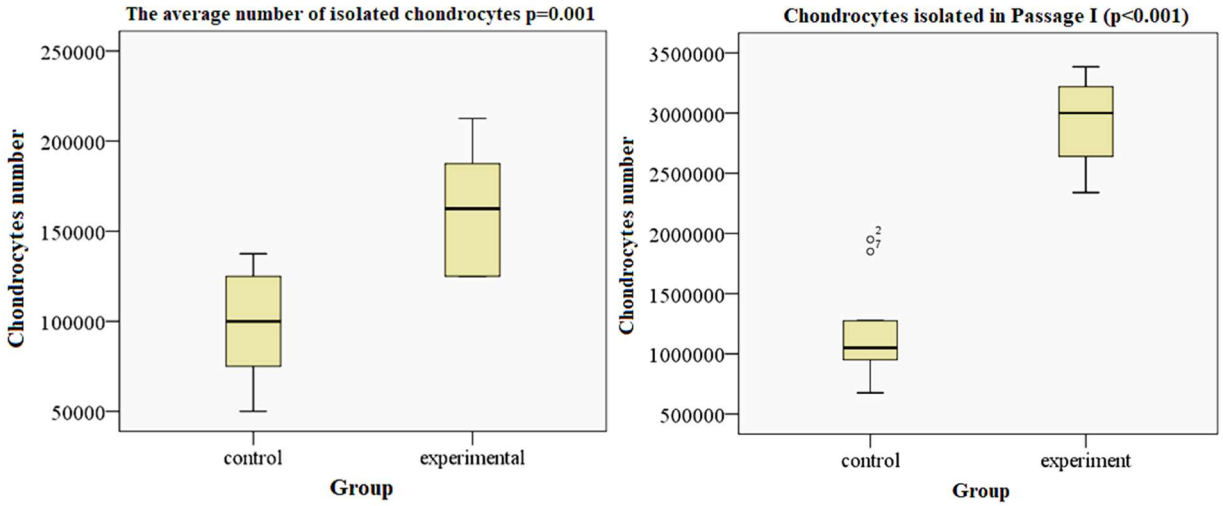

Figure 3.1. **Comparative presentation of isolated chondrocytes number (left), and total number of chondrocytes obtained in the first passage (right).**

When comparing percentage values, after counting cells in the hemocytometer by 0.4% trypan blue exclusion, in the control group was obtained a cell viability rate of 85.36% (95% CI:72.73-97.97). A non-critical difference (p =0.055), but in case if a small number of cells was

isolated, because the other cells were digested by collagenase, any reduction in viability can have negative effects on their cultivation potential [25, 26]. Considering that the number of viable cells in the control group that were cultured in the first passage was $7.5x10^4$ $\pm 2.1x10^4$ cells, and in the experimental group of $1.6x10^5$ $\pm 3.5x10^4$ cells (p=0.001), the period of chondrocytes culture, which includes adhesion to the surface, and confluence up to 70-80%, in both groups was different.

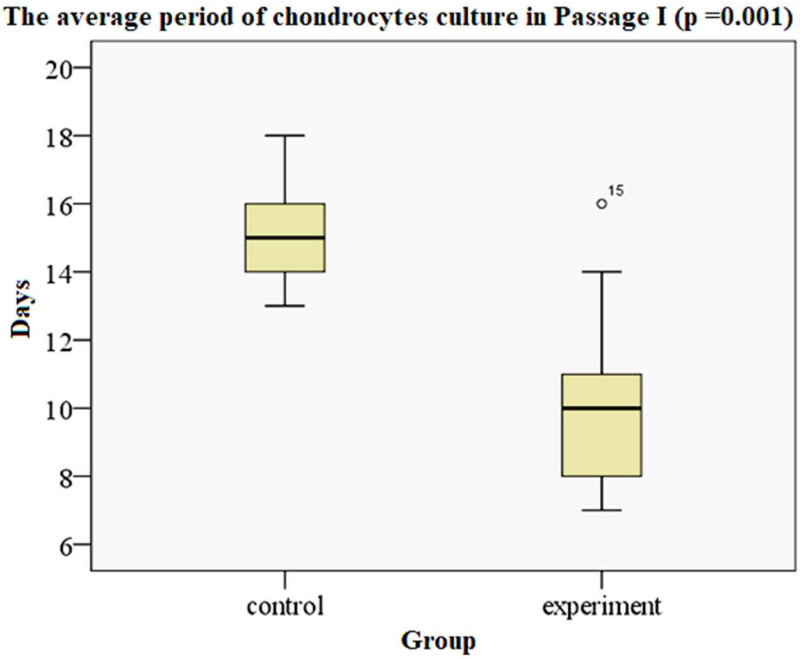

Figure 3.2. **The chondrocytes confluence during first passage.**

In the control group the period of cell culture in the first passage was 17±2 days, and in the experimental group 11±3 days (p =0.001) (figure 3.2). As a result, the number of cells obtained in the first passage in the control group was $1.2x10^6$ $\pm 4.3x10^5$ cells, and in the experimental group $2.92x10^6$ $\pm 3.6x10^5$ cells, with 100% viability in both groups, which represents a significant difference ($p<0.001$).

Implementation of histochemical techniques aimed to stain the ECM secreted by chondrocytes during 16 days of culture in overconfluence, allowed us to stain the matrix in red-orange with Safranin O, while in the control group staining was absent or very weakly expressed. The same was characteristic and for staining with Toluidine Blue and Fast Green, where the ECM was colored violet-blue. This indicates that isolated cells from articular hyaline cartilage secreted cartilage-specific extracellular matrix and are chondrocytes.

4. BIPHASIC GRAFT OBTAINING FROM OSTEOCHONDRAL TISSUE THROUGH TISSUE ENGINEERING TECHNIQUES.

4.1 Methods

The femoral condyles of euthanized rabbits were demineralized in 0.6M HCl (Sigma, USA) solution, that was changed daily until the demineralisation. Then, pieces of osteochondral demineralized tissue (ODC) were defatted, washed, and treated with buffer solutions for pH normalization. After demineralization and washing, 6 experimental groups of OC tissue were created. Depending on used decellularization agents and their doses, were created groups decellularized with various concentrations of sodium dodecyl sulphate (Sigma, Great Britain) - SDS 2%, SDS 1% and SDS 0.5%; and groups decellularized with various concentrations of Triton X-100 (HiMedia, India) - TrX 2%, TrX 1% and TrX 0.5%. The control group was represented by

osteochondral demineralised (OCD) tissue and normal osteochondral tissue (OCN). Decellularization was performed on an shaker with incubation (ES-20, Biosan) at 200 rpm, room temperature, for 24 hours.

In order to select a suitable graft for combination with autologous cells with chondroprogenitor potential, several tests were performed: the DNA quantification in the samples, parallelly with the detection of the nuclei at the histological examination with Hematoxylin-Eosin staining; was evaluated the biocompatibility of the samples by carrying out cell viability tests with MSCs and chondrocytes, using MTT assay, and the assessment of cellular adhesion to the scaffolds; the physico-chemical properties of the samples were analyzed by carrying out the swelling test, determination of elasticity modulus and the enzymatic degradation assay;

4.2 Results

The DNA quantification

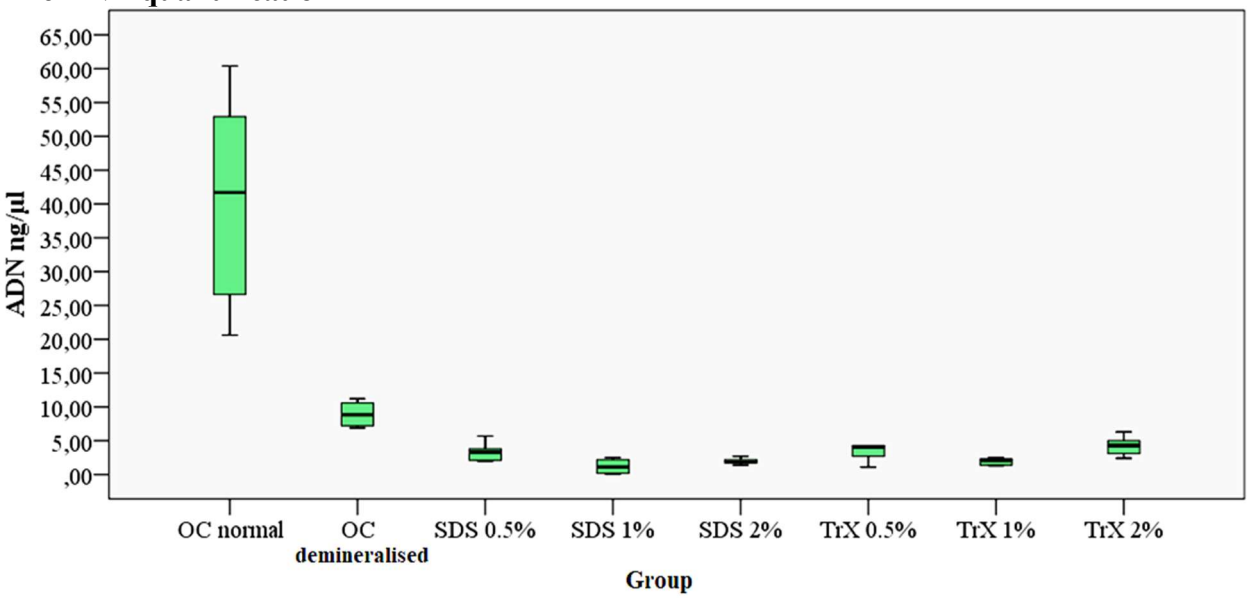

Figure 4.1. **DNA concentration in normal, demineralized, and demineralized-decellularized OC tissue.**

At utilisation of the Thermo Scientific GeneJET genomic DNA purification kit (Thermo Scientific, Lithuania) and the working protocol provided by the manufacturer, was determined that normal OC tissue contain 40.65 ±15.25 ng/µl of DNA [28]. Following the use of 0.6M HCl to demineralize bone from the osteochondral complex, was determined that HCl also contributed to the significant removal of DNA from the tissues, approximately with 78% ($p < 0.001$). Decellularization of demineralized OC tissue with various concentrations of surfactants during 24 hours reduced DNA concentration in the samples to smaller values. Bud, the DNA removal to a content below 5% was achieved with solutions of 1% and 2% SDS, and 1% TrX (figure 4.1).

When comparing the DNA concentration in the control group - OCD tissue, with that of the experimental samples, a significant difference was determined ($p < 0.01$). The difference between the amount of DNA remaining in the control group and in the experimental groups compared to the normal OC tissue is represented in table 4.1. It was determined that the concentration and type of surfactant influence the rate of decellularization of the OC tissue. When comparing the decellularization effectiveness when using the same concentration of surfactants, was determined that concentrations of 0.5% and 1% of SDS and of TrX, does not show major differences ($p > 0.1$), but when these surfactants were used with a concentration of 2%, a significant difference was

obtained in the level of decellularization (p= 0.003), when using 2% TrX the DNA content was much higher table 4.1.

Table 4.1. **Amount of DNA remaining in OC tissues after demineralization and decellularization relative to normal OC tissue.**

Sample type	The amount of DNA (ng/µl) X ±*DS*	p	DNA concentration (%)	95% CI:
OC demineralized	8.93 ±1.93	p <0.001	21.96%	16.97-26.97
SDS 0.5%	3.37 ±1.37	p <0.001	8.29%	4.73-11.82
SDS 1%	1.2 ±1.01	p <0.001	2.95%	0.34-5.55
SDS 2%	1.95 ±0.46	p <0.001	4.79%	3.60-5.98
TrX 0.5%	3.42 ±1.29	p <0.001	8.41%	5.06-11.74
TrX 1%	1.95 ±0.49	p <0.001	4.79%	3.53-6.06
TrX 2%	4.23 ±1.39	p <0.001	10.40%	6.81-14.01

The histological examination

At histological examination, was reconfirmed that the decellularization of the OC tissue depends on the type and concentration of used surfactant. The presence of nuclei or nuclear fragments was determined predominantly in TrX-decellularized OC tissues. Also, a different intensity of specific staining with Toluidine Blue and Fast Green was determined in GAG content in decellularized cartilage. The intensity of staining was higher when the OC demineralized tissue was decellularized with SDS compared to TrX, which denotes a higher GAG concentration in the SDS-treated samples, but much lower compared to the control sample and normal cartilage [28].

MTT cell viability assay

Such results indicate the presence of various growth factors in the tested OC demineralized and OCDD samples, which were favorable to chondrocyte activity, the highest being at 72 hours, with mote than 140% (95% CI: 121.98-165.88), when the chondrocytes comed into contact with the OC demineralized tissue samples (p <0.05), without being treated with surfactants. The lowest cellular viability, of 116.34% (95% CI:114.02-118.66) was of the OC tissue samples thatwere demineralized and then decellularized with 1% TrX. Compared to chondrocytes, the metabolic activity of MSCs was more limited, but good. In the first 24 hours MSCs viability varyed between 90% and 100%, and at 48 and 72 hours between 80-90%, without differences between the groups (p >0.5) (figure 4.2).

The assessment of cellular adhesion to the scaffolds

After culturing MSCs and chondrocytes on OCD and OCDD tissue samples with various concentrations of surfactants, was determined that cellular multiplication was more abundant in the case of chondrocytes at 7th day of culture. At 14th day of cellular culture, the degree of samples population with cells was uniformly abundant with both, chondrocytes and MSCs (figure 4.3).

Scanning Electron Microscopy (SEM)

The SEM showed articular cartilage samples populated with MSCs and chondrocytes. At SEM was determined the presence of structural difference between the samples populated with cells and those which were not. In case of samples populated with cells, the presence of single cells was determined, but also were determined cells that formed films on the surface of cartilaginous tissue section, in some places with thickening. On the unpopulated samples was visualized the ultrastructure of osteochondral tissue, also were identified lacunae in which the chondrocytes were located. During examination was determined that the samples treated with 1% and 2% SDS, the chondrocytes were absent within the lacune.

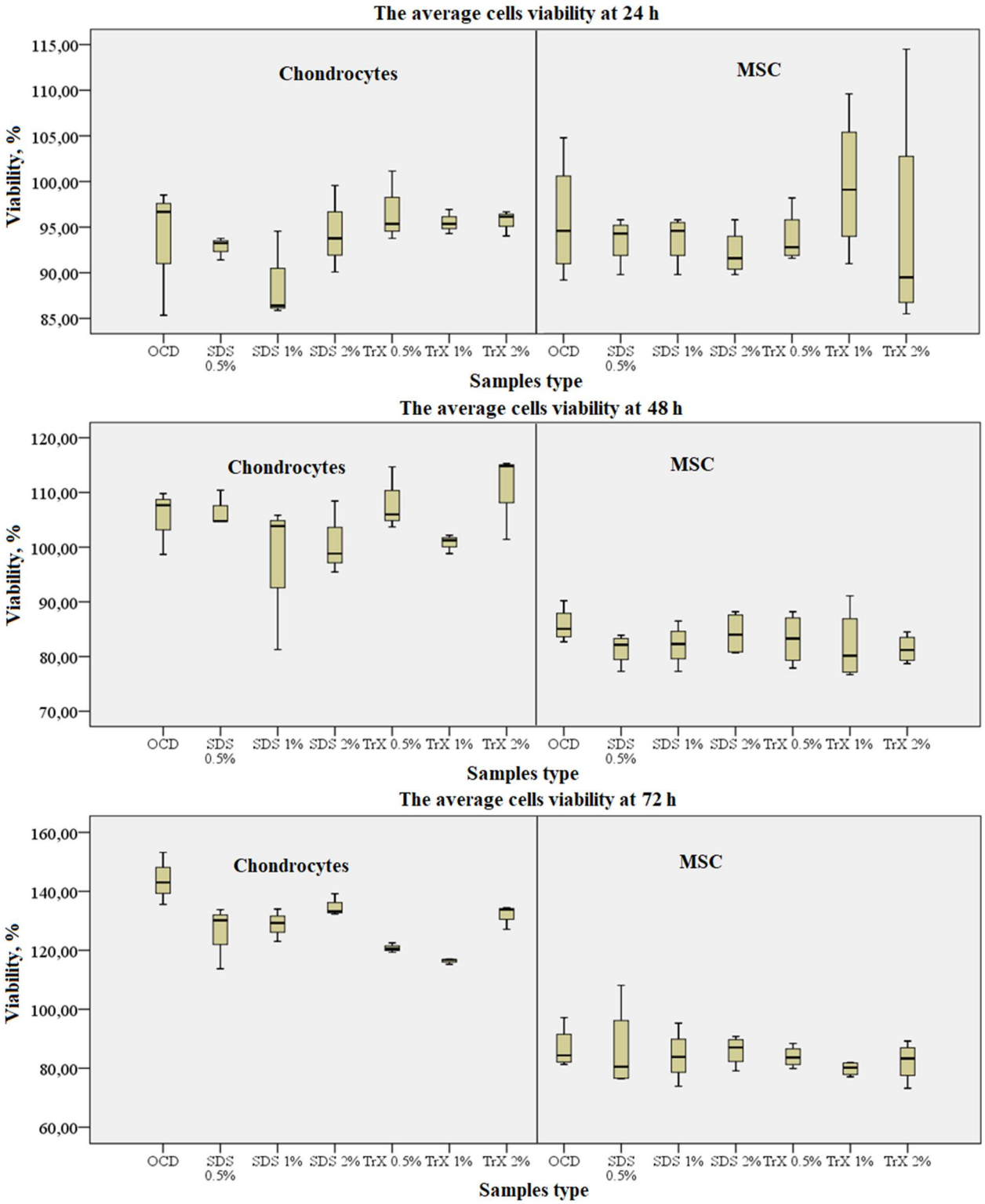

Figure 4.2. **The results of samples cytotoxicity test, performed by MTT assay, with chondrocytes and CSM after 24, 48 and 72 hours.**

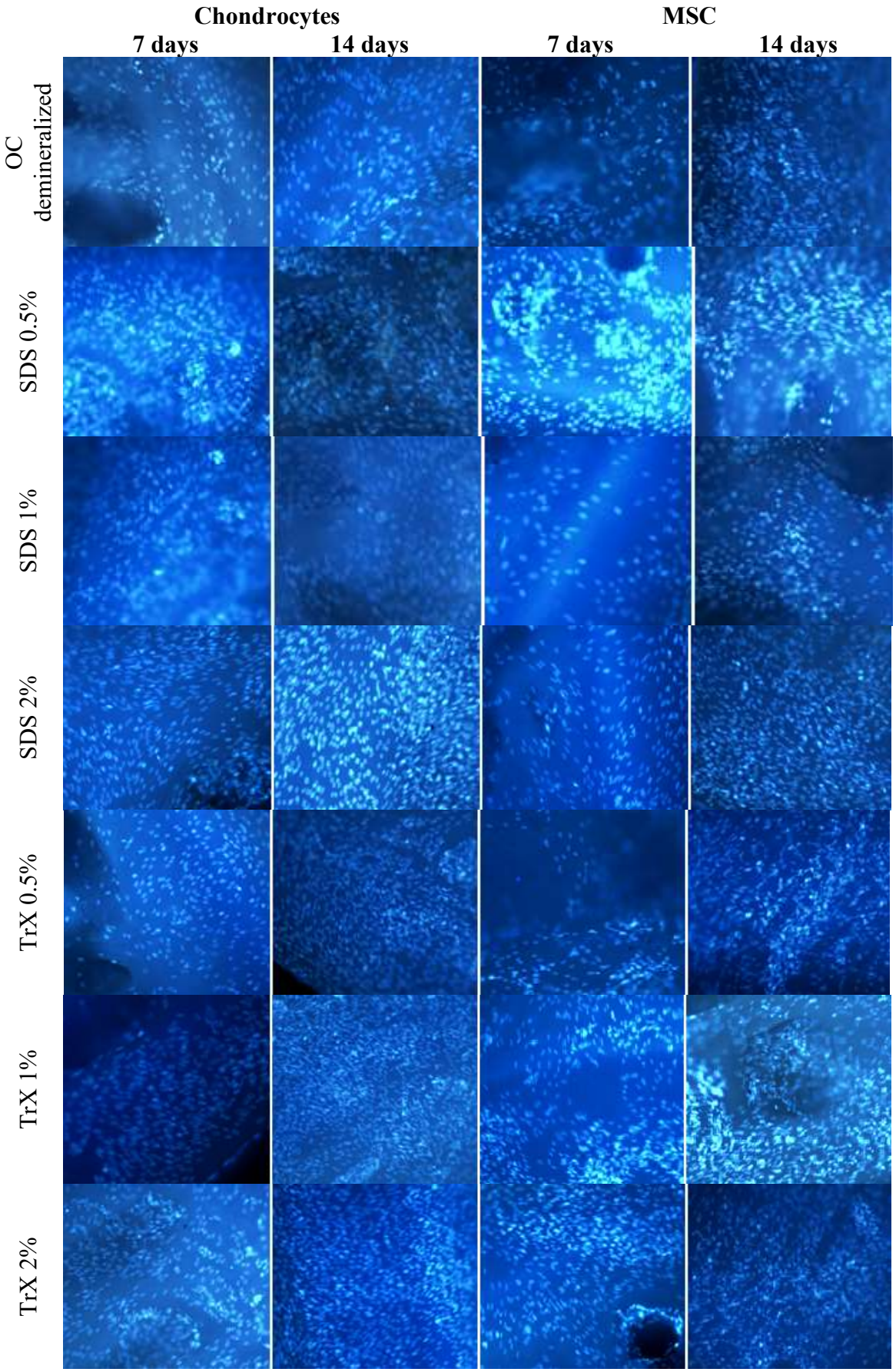

Figure 4.3. **Results of population of osteochondral tissue samples with chondrocytes and CSM, stained with DAPI and visualized by fluorescence microscopy (DM IL, Leica).**

Enzymatic degradation assay

According to the obtained results, there is a statistically significant difference between the research groups in different periods of enzymatic degradation. It was determined that the demineralized OC tissue samples (control) and those decellularized with TrX 0.5% had the fastest speed of enzymatic degradation ($p > 0.5$), reaching the peak of maximum concentration of detected amino acids on the 7^{th} day. The next samples with a high speed of degradation were those decellularized with 1% and 2% TrX ($p > 0.1$), compared to the control group, they did not reached their maximum values until the 14^{th} day of the experiment ($p > 0.1$). It was determined that OCD tissue samples that were decellularized with SDS had a much slower rate of enzymatic degradation compared to control and TrX decellularized samples, this is clearly observed starting with 24 hour period of enzymatic degradation ($p < 0.01$). It also was found that the higher was the concentration of used SDS for decellularization, the lower was the rate of samples enzymatic degradation (figure 4.4). The lowest degradation speed was in the group of OC tissues demineralized and decellularized with 2% SDS ($p < 0.01$).

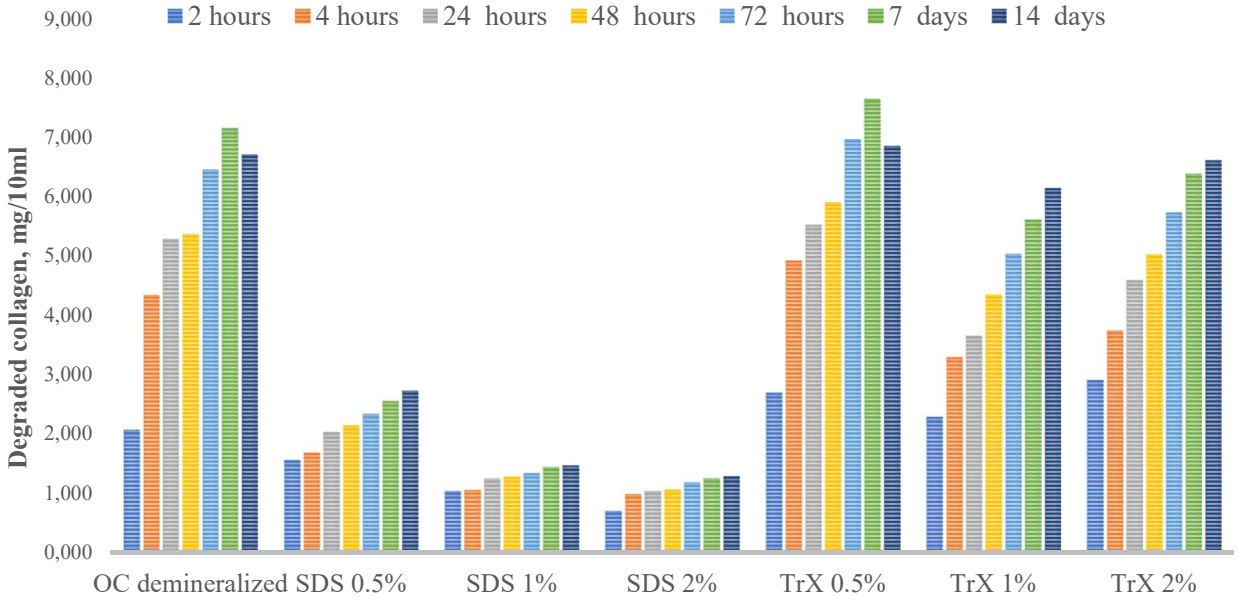

Figure 4.4. **Graphical representation of the results of enzymatic degradation of samples with collagenase.**

The swelling test

The results obtained at determination of swelling capacity of demineralized OC tissue samples and those which were decellularized with SDS and TrX do not show statistically significant results ($p > 0.05$).

The elasticity modulus determination

A statistically significant difference has been obtained when comparing the elasticity modulus of normal OC tissue, OC demineralised and OCDD tissue groups ($p = 0.001$). A significant difference was also determined between the Young's modulus of the demineralised OC tissue group and the group that was decellularized with 0.5% SDS ($p = 0.009$), while in the other cases between the control group (OCD) and the other experimental groups, no significant difference was detected ($p > 0.1$). This can be explained by the inhomogeneity of compared osteochondral tissues structure, as they come from different animals, are characterized by a different degree of tissue demineralization, cartilage thickness and compaction degree of the demineralized bone tissue.

5. IN VIVO TESTING OF COMBINED GRAFTS IN ARTICULAR CARTILAGE REGENERATION.

5.1 Methods

Preparation of grafts for transplantation

From rabbits euthanized in the experiment, distal femurs were removed and stored at -84°C (ULUF 450-2M, Arctiko). To obtain grafts, from the harvested distal femurs all soft tissues were removed, and then demineralized using 0.6 M HCl. With a self made biopsy punch from the bearing surface of each condyle, 31 pieces of osteochondral tissue with a diameter of 3.61 ±0.1mm and a height of 4.33 ±0.11mm and an average volume of 44.05 ±1.15mm^3 were sectioned. The grafts were perforated longitudinally with a 23G gauge syringe needle and degreased with 3% H_2O_2 (Eurofarmaco, Republic of Moldova) for 24 hours and 70% alcohol (Eladum Pharma, Republic of Moldova) for 6 hours on a shaker incubator at 200 rpm. The obtained OCD tissue pieces were washed with distilled water, followed by decellularization in 1% SDS solution for 24 hours. Then, the OCDD and longitudinally perforated grafts were washed again in distilled water for 3 days, changing the water 2-3 times a day. In the laminar flow hood (LN 090, Nuve) grafts were sterilized with 70% alcohol for 2 hours. The sterilized grafts were washed with HBSS (Lonza, Belgium) for 24 hours, the solution was changed 3 times. The next day grafts were desiccated by centrifugation on sterile gauze wicks in 15 ml sterile tubes at 4000 rpm for 20 min (Universal 32R, Hettich Zentrifugen), then frozen at -84°C (ULUF 450-2M, Arctiko) until use (figure 5.1).

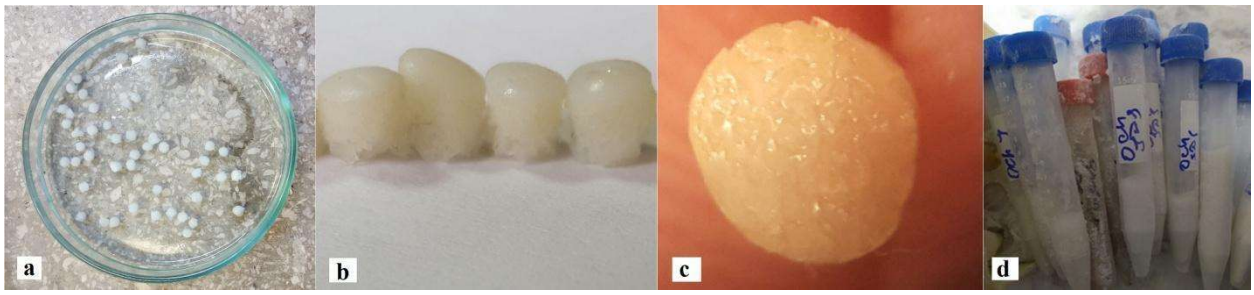

Figure 5.1. **Obtaining and preserving OCDD grafts: (a) graft processing, (b) unperforated OCD tissue, (c) longitudinally perforated OCDD graft, (d) OCDD grafts preserved at -84°C.**

Obtaining cells with chondroprogenitor potential and combining them with OCDD grafts

The cells with chondroprogenitor potential needed to obtain the combined grafts, were obtained according to the methods outlined in the previous chapters. Longitudinally perforated OCDD grafts have been combined with autologous cells - MSCs and chondrocytes. Only cells from the first passage were used for transplantation purposes. MSCs were cultured according to the method described in the previous chapter, in a 25 cm2 cell culture flask using the culture medium composed of DMEM/F-12 Ham (Sigma, UK) and 10% Fetal Bovine Serum (FBS) (Lonza, Belgium) and 1% antibiotic antimycotic solution. On the day when MSCs and chondrocytes reached 80-90% confluence, OCDD grafts were thawed and heated to 37°C in a shaker incubator (ES-20, Biosan) for transplantation, and the cells detached from the cell culture surface of the flask. After centrifugation, the cells were resuspended in one ml cell type-specific nutrition medium. The cellular suspension after pipetting was transferred into 1.5 ml Eppendorf tubes, which were centrifuged at 3500 rpm for 3 min (Combi-Spin FVL-2400N, Boeco). Subsequently, the medium in the tubes was partially extracted, leaving a small volume of 50-60 µl at the bottom. Sterile 15 ml tubes with OCDD grafts and the device for fixation and cellularization of small grafts (DFCSSG) [27, 28] were placed in the laminar airflow hood (LN 090, Nuve). DFCSSG was required for graft cellularization by the gravity method and its use has been

described in previous work [27]. The device was inserted into a stand and the cover removed. The graft with forceps was removed from the tube and inserted into the holder of the device [32]. With a micropipette (LightDrop 10-100 µl, ThermoFisher) cellular suspension from the Eppendorf tube were pipetted into remaining 50-60 µl of cell culture medium, followed by cell suspension transfer onto the OCDD graft placed in the holder of DFCSSG. Then, the cap was applied to the device and inserted into a 15 ml test tube (figure 5.2), which was then placed in the incubator with the lid ajar, at 37°C, 5% CO2 (SmartCell, HealForce). After, transplantation of the combined graft, Eppendorf tubes and DFCSSG containers were flushed with cell-specific nutrition medium, and cells were counted in a hemacytometer excluding dead cells with 0.4% trypan blue solution (Invitrogen, USA) to determine how many cells were actually transplanted [26, 32].

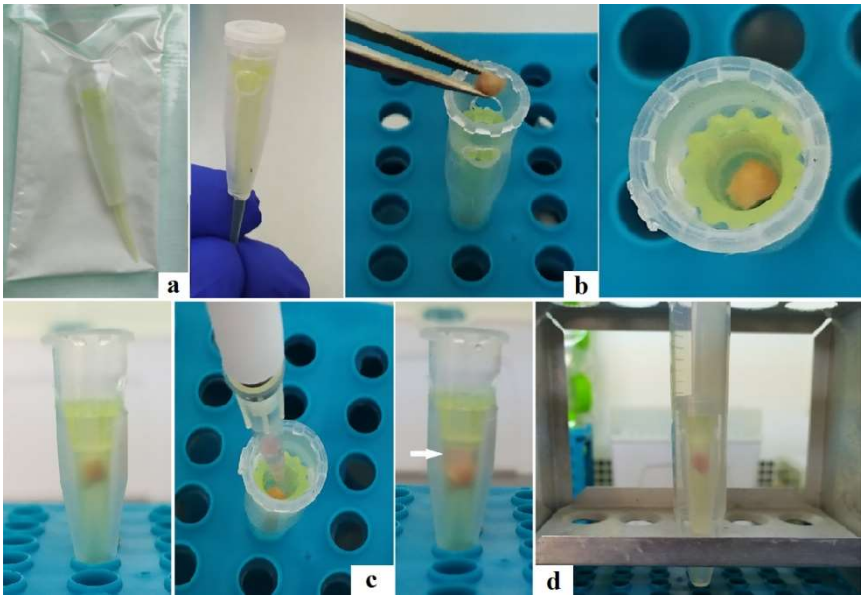

Figure 5.2. **The process of OCDD grafts seeding with cells: (a) sterile DFCSSG, (b) inserting the OCDD graft into the device holder, (c) transferring the cells onto the graft, the white arrow indicate the level of the cell suspension above the graft, (d) inserting the DFCSSG in a 15 ml test tube.**

Transplantation of combined grafts in experimental critical defects

The transplantation of combined grafts consisted of the preparation of grafts and experimental animals for transplantation. After introduction of grafts populated with cells into the incubator, all rabbits were unweighed and anesthetized. Preparation of the operative field at the level of one knee joint, through a medial parapatellar approach, with an incision of 4-5 cm, the knee joint was opened. After haemostasis the patella was dislocated laterally and the knee flexed. Then, on the weight-bearing surface of the medial femoral condyle, consecutivly with 1.5, 2, 3, 3.5 and 3.7 mm diameter drill-bits were performed with caution [27], at low speeds, without opening the medullary canal, a critical defect of 3.7 mm in diameter and 4-4.5 mm in deep [24]. At that point the combined grafts were taken from the incubator and brought to the operating room, where the containers with the grafts combined with MSCs or chondrocytes, were placed on the operating table. The combined graft holders were sectioned at the base with scissors, and the grafts were carefully pushed into the defects with a pin without damaging or squeezing the cells. All grafts were transplanted with their cartilaginous side facing upwards (figure 5.3). After grafts implantation, the dislocated patella was reduced, and with the knee mainteined in extension the wounds were sutured and dressing with 10% povidone-iodine solution (EGIS, Hungary) was applied.

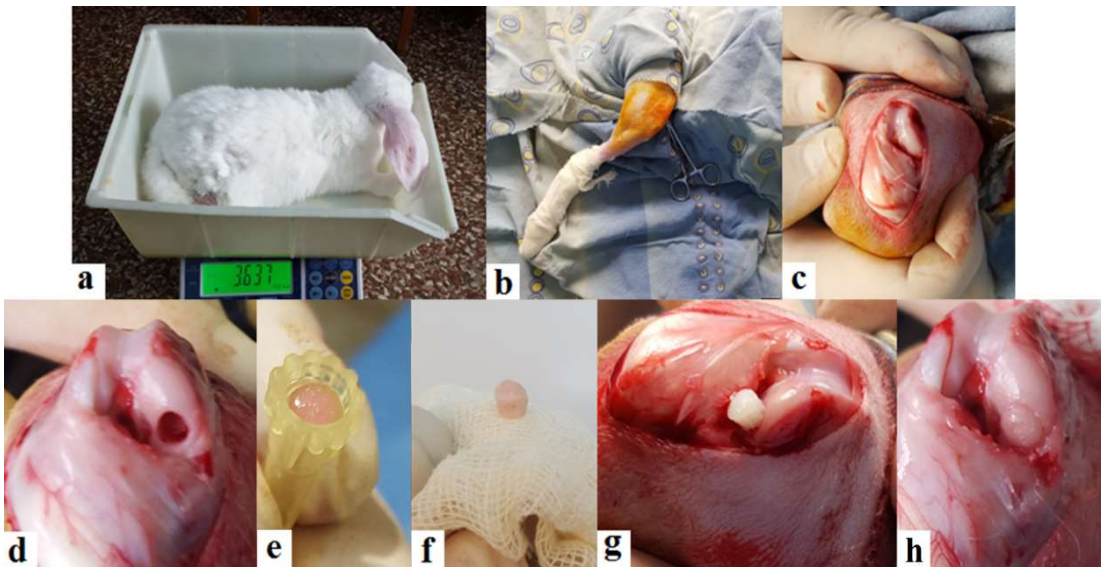

Figure 5.3 **Transplantation of OCDD grafts combined with MSCs and autologous chondrocytes, (a) animal weighing and general anesthesia, (b) operative field preparation, (c) medial parapatellar arthrotomy with lateral patellar dislocation, (d) creation of the experimental defect, (e, f) graft combined with autologous cells, and (g, h) graft implantation in the experimental defect.**

Keeping the knee in extension, a thin splint, wider than the limb and corresponding in length to the distance from the hip to the ankle, was applied to the lateral side of the operated lower limb and fixed to the operated limb with a plaster bandage, leaving a window at the level of the postoperative wound for dressings. The plaster was applied with caution not to compress the soft tissues, only to keep the knee in extension for 10 days.

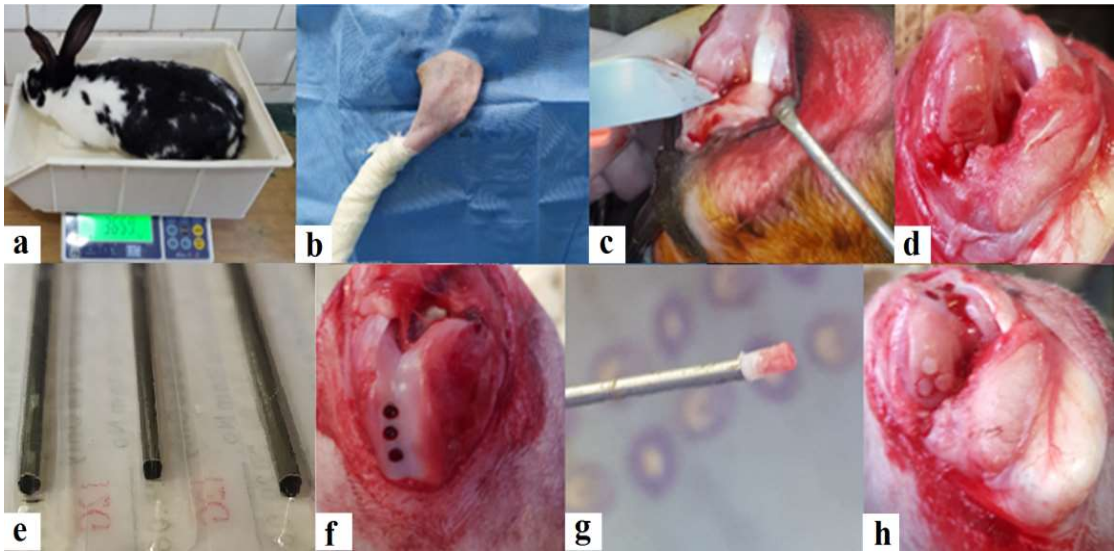

Figure 5.4. **Autologous osteochondral tissue transfer, (a, b) anesthesia of the rabbit and preparation of the operative field, (c, d) creation of the experimental defect, (e, f, g) harvesting of autologous osteochondral grafts from the non-bearing surface of the femoral articular surface and their transplantation.**

Transfer of autologous osteochondral tissue

The method of treatment of critical experimental defects by autologous tissue transfer was selected as a method for treatment of cartilage defects in control groupe because it is a cartilage tissue regeneration technique that use own tissues in the treatment process [16]. The group of rabbits (n=12) to whom autologous osteochondral tissue was transplanted, were prepared for

surgery in the same way as the experimental groups. After arthrotomy, lateral dislocation of the patella and flexion of the knee, with a scalpel the cartilage was removed over an area of 3.7 mm in diameter at the level of weight-bearing surface of the medial femoral condyle. Using a 12G piercing needle with an internal diameter of 1.7 mm, attached to a drill with slow rotations to avoid overheating the tissue, pieces of autologous osteochondral tissue were harvested from the trochlear groove [27], that were implanted into newly created defects at the same height or up to 1 mm higher than the adjacent normal cartilage (figure 5.4).

	Criteria			Scara
1	Cell pattern in regenerated cartilage		Hyaline cartilage	0
			Predominantly hyaline cartilage	1
			Predominantly fibrocartilage	2
			Non-cartilage only	3
2	Cell morphology (initially choose category - a, b, c, or 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 area	1
			<25% of tissue with columns in the radial area (disorganized structure)	2
		(c)	**about 50% round cells with chondrocyte morphology:**	
			> 75% of tissue with columns in the radial area	2
			25-75% of tissue with columns in the radial area	3
			<25% of tissue with columns in the radial area (disorganized structure)	4
		(d)	**mostly spindle cells (fibroblast-like)**	5
3	Formation of chondrocyte clusters		Absent	0
			<25% of cells	1
			25-100% of cells	2
4	Level of cellularisation		Normal cellularity	0
			Mild hypocellularity	1
			Moderate hypocellularity	2
			Severe hypocellularity	3
5	Matrix metachromasia		normal	0
			slightly reduced	1
			moderately reduced	2
			reduced marking	3
			does not colour	4
6	Joint surface		Smooth and intact	0
			Shallow horizontal lamination	1
			Cracks up to 25% to 100% of thickness	2
			Severe disturbances, ruptures, including fibrillations	3
7	Integration of the graft with the surrounding articular cartilage		Continuity and normal integration	0
			Reduced cellularity	1
			Gap or lack of continuity on one side	2
			Gap or lack of continuity on two sides	3
8	Architecture of the whole defect without including edges		normal	0
			1-3 small defects	1
			1-3 large defects	2
			severe destruction	3
9	Thickness of newly formed cartilage		100% of normal adjacent cartilage	0
			50-100% of normal adjacent cartilage	1
			0-50% of normal adjacent cartilage	2
10	Presence of degenerative changes in 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 surface of the adjacent normal cartilage		111-125 %	1
			91-110 %	0
			76-90 %	1
			51-75 %	2
			26-50 %	3
			<25 %	4
12	Formation of the demarcation line		Full	0
			75-99 %	1
			50-74 %	2
			25-49 %	3
			<25 %	4
13	Percentage of new subchondral bone restored		90-100 %	0
			75-89 %	1
			50-74 %	2
			25-49 %	3
			<25 %	4

Histological Unified Regenerated Cartilage Score (HURCS)

After reduction of the dislocated patella, holding the knee in extension the wound was sutured and aseptic dressing was applied. Then, with the knee in extension, knee immobilization was applied for 10 days.

Evaluation of *in vivo* test results performed on rabbits

The operated rabbits were removed from the experiment by euthanasia in the CO_2 chamber, at 6 weeks 3 animals from each group and at 12 weeks the other 9 from each group. Distal femurs were placed in 10% buffered formaldehyde, pH =7.4. After decalcification and embedding in paraffin, samples were sectioned with the microtome by 5 μm in thickness and applied to slides. The samples were stained with Hematoxylin-Eosin, Safranin O and Toluidine Blue with Fast Green.

The objective assessment of results was performed according to the Histological Unified Regenerated Cartilage Score (HURCS), which was compiled by combining three histological scores widely used in the evaluation of regenerated cartilage in animals, authored by Sellers et al. (1997) [27, 29], Wakitani et al. (1994) [30] and O'Driscoll et al. (1986) [31]. HUSRC consists of 13 evaluation criteria [27]. To each criterion was assigned a personal scale and each numerical value in this score has its own specific characteristic. The maximum score of all criteria is 43 points and the minimum - 0 points. The result of the evaluation of the quality of the regenerated tissue depends on the accumulated score, the higher is the quality of the regenerated tissue, the lower is the score and vice versa, the higher is the obtained score, the lower is the quality of the regenerated tissue.

5.2 Results

In order to combine OCDD grafts with cells that have chondroprogenitor potential from hyaline articular cartilage were isolated $1.8x10^5 \pm 5.4x10^4$ chondrocytes with a viability of 96.79% (95% CI:93.02-100.54), and $2.94x10^6 \pm 3.77x10^5$ chondrocytes with a viability of 99.89% (95% CI:99.64-100.13) were obtained after chondrocytes culture in the first passage during 10 ±3 days. Also, from 4 ±1.2 ml of bone marrow after 11±3 days of culture were obtained $1.55x10^6 \pm 3.76x10^5$ MSCs with a viability of 98.03% (95% CI:95.35-100.71). No difference was determined between groups in cell culture duration ($p > 0.3$) and cellular viability ($p > 0.1$). After transplantation of the combined grafts, following washing of the Eppendorf tubes, the 15 ml tubes in which the cells were centrifuged, and the holder of DFCGDM, the actual number of transplanted chondrocytes was determined to be $2.54x10^6 \pm 2.73x10^5$ cells, with a viability of 87.59% (95% CI:84.51-90.67), which represents 86.97% (95% CI:84.09-89.84) of the total chondrocytes obtained by culture. Also, the actual number of transplanted MSCs was $1.37x10^6 \pm 3.57x10^5$ cells, with a viability of 90.91% (95% CI:88.95-92.85), representing 88.32% (95% CI:86.45-90.17) of the total number of obtained MSCs, which is a significant difference compared to the initial number of cells obtained from the first passage ($p < 0.001$).

Table 5.1. **Difference between the number of cells obtained in primary culture and those transplanted with grafts from experimental groups.**

	Total number of obtained cells X ±*DS*	**Actual number of transplanted cells X ±*DS***	**p**
Chondrocytes	$2.94x10^6 \pm 3.77x10^5$	$2.54x10^6 \pm 2.73x10^5$	$p < 0.001$
MSC	$1.55x10^6 \pm 3.76x10^5$	$1.37x10^6 \pm 3.57x10^5$	$p < 0.001$

Therefore, it was determined that total viability of all transplanted cells was 89.25% (95% CI:87.42-91.07), which is a significant statistical difference when compared to cellular viability immediately after cells detachment in primary culture ($p > 0.05$), but with no significant difference

between the rates of viable cells transplanted per group (p >0.5). There was also found no difference between the duration from cell detachment to cell transplantation in the experimental groups (p >0.5). Thus, for the grafts combined with chondrocytes this period was 73 ±12 minutes and for grafts combined with MSC it was 69 ±14 minutes. The difference between the quantity of obtained cells and the transplanted one, and their viability is shown in tables 5.1 and 5.2. Following the utillisation of the DFCSSG, the total transplanted cell rate was 87.64% (95% IÎ:86.04-89.24), which is a very good result.

Table 5.2. **Difference between the viability of obtained cells in primary culture and those transplanted with grafts from the experimental groups.**

	Viability of detached cells	95% CI:	Viability of transplanted cells	95% CI:	p
Chondrocytes	99.89%	99.64-100.13	87.59%	84.51-90.67	p <0.001
MSC	98.03%	95.35-100.71	90.91%	88.95-92.85	p =0.001

The animals removed from the experiment at 6 weeks according to USHCR in all cases had a nearly similar histological score. In the first evaluation the control group scored 26.33 ±1.53, the group treated with OCDD grafts combined with chondrocytes scored 28.33 ±1.53 points (p >0.1) and the group treated with grafts combined with MSCs scored 27.67 ±2.08 points (p >0.2). At second evaluation the control group obtained 28 ±1 points, the group treated with OCDD grafts combined with chondrocytes obtained 30 ±3.61 points (p >0.4) and the group treated with grafts combined with MSC – 28 ±2.65 points (p =1). As a result, no significant difference was determined between the control and experimental groups (figure 5.5).

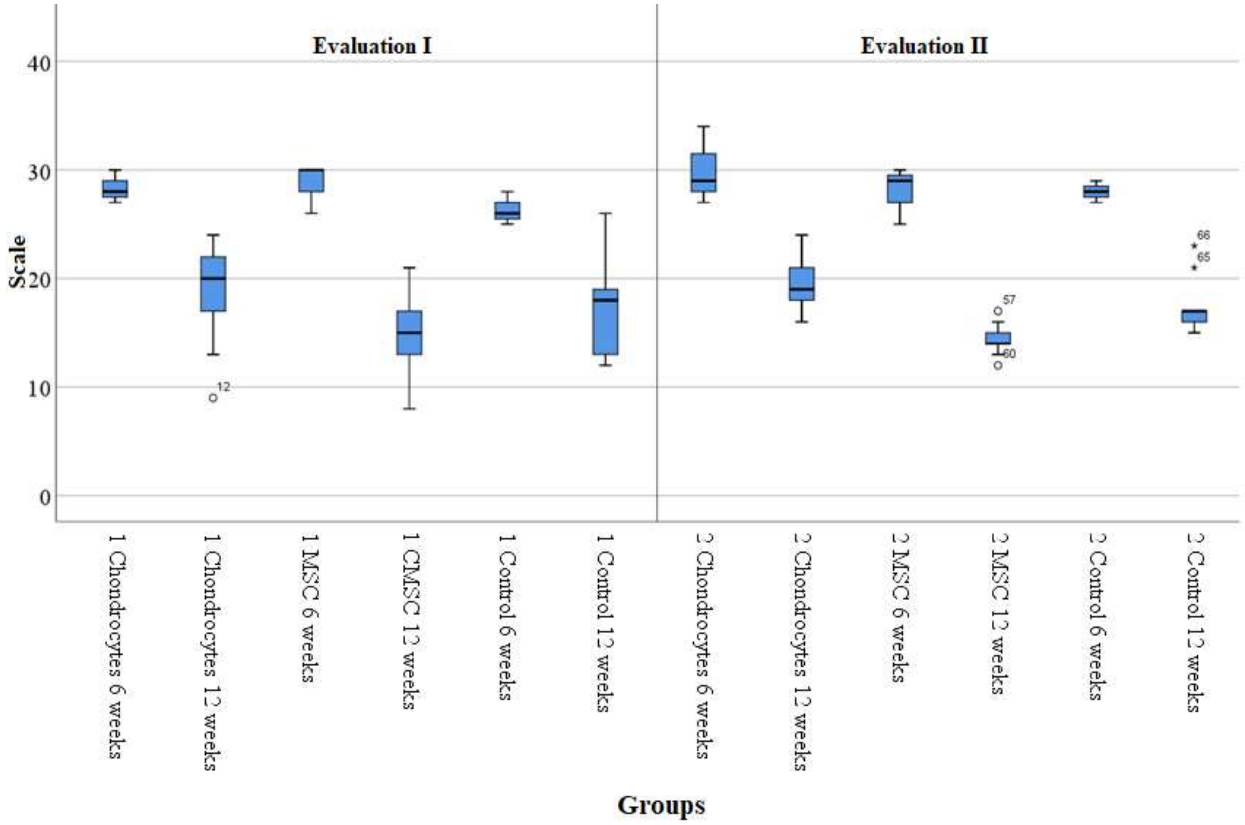

Figure 5.5. **Graphical representation of both evaluation of experimental and control groups, at 6 and 12 weeks postoperatively.**

When analysing the SHUCR results of animals removed from the experiment at 12 weeks, a score of 17.22 ±4.84 points was obtained at the first evaluation for the control group. The group

treated with OCDD grafts combined with chondrocytes obtained 18.68 ±5 points (p >0.5) and the group treated with MSCs – 14.89 ±3.76 points (p >0.2). In the second evaluation the control group scored 17.67 ±2.60 points, the group treated with grafts combined with chondrocytes – 19.22 ±2.44 points (p >0.2) and the group treated with grafts combined with MSCs – 14.33 ±1.5 points (p =0.004).

Table 5.3. **Statistical comparison of UHSRC results given by 2 experts (p).**

Group	Evaluation 1	p_1* compared to control group	Evaluation 2	p_2* compared to the control group	p_1/p_2*
Control 6 weeks	26.33 ±1.53		28 ±1		p =0.189
OCDD+chondrocytes 6 weeks	28.33 ±1.53	p >0.1	30 ±3.61	p >0.4	p =0.502
OCDD+MSC 6 weeks	27.67 ±2.08	p >0.2	28 ±2.65	p =1	p =0.759
Control 12 weeks	17.22 ±4.84		17.67 ±2.60		p =0.811
OCDD+chondrocytes 12 weeks	18.68 ±5	p >0.5	19.22 ±2.44	p >0.2	p =0.768
OCDD+MSC 12 weeks	14.89 ±3.76	p >0.2	14.33±1.5	p =0.004	p =0.745
***p_1** – statistical significance compared to the control group in Evaluation I; ***p_2** – statistical significance compared to the control group in Evaluation II; ***p_1/p_2** – the significance between Evaluation I and Evaluation II.					

As a result, in the first evaluation no significant difference was determined between the control and experimental groups, whereas at the second evaluation a significant difference was obtained between the control group and the group treated with OCDD grafts combined with MSC (figure 5.5), noting that there is no significant difference between the results of both assessments of defects treated with OCDD and CSM grafts (p =0.745), table 5.3.

When comparing the results obtained within the same group, in animals that were removed from the experiment at 6 and 12 weeks, a significant difference between the obtained results was determined (p <0.05). The association degree between the number of cells really transplanted and the quality of articular cartilage regeneration was also assessed. Was determined that the number of chondrocytes or MSCs used in combination with the OCDD graft did not influenced the quality of cartilaginous tissue regeneration (p >0.1). The independent evaluation of histological results by 2 experts are shown in figure 5.5 and table 5.3.

SUMMARY OF OBTAINED RESULTS

Regenerative medicine as a new field of modern medicine that faces many challenges including the regeneration of articular cartilage problem. The basis of regenerative medicine is to obtain and cultivate cells that have the potential to regenerate damaged tissues and organs. The regeneration of articular cartilage is a difficult process because of its anatomical peculiarities and the way it is nourished, the causes of cartilage damage being various, leading ultimately to arthritic degradation of the joint.

In the course of performed study, new methods were developed and implemented in laboratory practice: the collection of BM for isolation of MSC without animal scarification, which gave the possibility to use autologous MSCs in the treatment of articular cartilage lesions; the period of chondrocytes isolation was significantly reduced and the number of isolated cells from rabbit articular cartilage was significantly increased, reducing the risk of important fibroblast degradation of chondrocytes during culture in passages, the used technique can also be implemented without problems in the process of chondrocyte isolation for autologous chondrocyte

transplantation in humans; several methods of obtaining grafts by tissue engineering techniques have been developed and tested; for combining the obtained grafts with autologous cells - MSCs and chondrocytes for transplantation, a special device has been developed with the purpose to fix and populate small grafts with cells; in the realised study, for the first time was determined the actual quantity of transplanted cells and their viability at the time of transplantation; also, in order to objectively evaluate the results of *in vivo* tests, a histological score was developed – the Unified histological score of regenerated cartilage.

In the realised study the process of developing and testing a combined grafts with autologous cells that have chondroprogenitor potential is comprehensive, because it includes *in vitro* graft development and testing as well as *in vivo* animal testing before it can be used in the clinic.

CONCLUSIONS

1. The developed method of collecting bone marrow from the iliac bone, without animal scarification, allowed utilisation of a smaller number of animals in the research, but also, to use autologous MSCs when performing *in vivo* tests.
2. The method of continuous monitoring of the enzymatic digestion process of articular cartilage, increased the number of isolated chondrocytes and reduced the period of isolation process, and development of method that allows cells isolation from the explant using volumetric regulation cycles, allowed the faster isolation of a large number not only of chondrocytes, but also of other cells of mesenchymal origin.
3. In the process of preparing OCDD grafts for combination with chondroprogenitor cells, the use of HCl significantly reduced the DNA content in osteochondral tissue, which allowed during a short exposure of 24 hours, a significant reduction of residual DNA when using 1% SDS solution. Also, the use of SDS in the process of grafts preparation, significantly reduced the speed of grafts enzymatic degradation compared to the control.
4. DFCSSG used in the transplantation of combined grafts significantly increased the populating rate of grafts with cells, but there was found no association between the number of transplanted cells and the quality of the regenerated tissue.
5. The UHSRC allowed objective assessment of regenerated tissue after transplantation of combined grafts. The results of the 6- and 12-weeks evaluation differed in both, experimental and control groups, and when comparing the experimental groups with the control group only after 6 weeks, no difference was determined between the obtained results. After 12 weeks, it was determined that the group in which the defects were treated with grafts combined with MSCs appeared to show better results. However, within 12 weeks complete or near complete filling of the defects with fibrocartilaginous or fibrous tissue was achieved, with an disorganized microscopic appearance and structure, not specific to hyaline articular cartilage.

PRACTICAL RECOMMENDATIONS

1. *In vivo* testing in laboratory animals of biological materials created for the purpose of articular cartilage regeneration, is required to be realised by implantation in critical defects in both weight-bearing and non weight-bearing joint surfaces.
2. Critical defects performed on a weight-bearing articular surface in the rabbit to test the potential for articular cartilage regeneration, should not exceed the minimum critical diameter of

3 mm, because larger defects present a risk of intraoperative fracture of the femoral condyle, significantly reduce the bearing surface of the femoral condyle, but also the regeneration potential through tissue engineering techniques.

3. The assessment of effective decellularisation of a tissue is necessary to be realised through: determination of DNA concentration in ng/mg; identification of cell remnants on histological examination by Haematoxylin-Eosin staining; identification of DNA remnants by fluorescent examination with DAPI; and by appropriate determination of GAG and hydroxyproline concentration.

SELECTIVE BIBLIOGRAPHY

1. VILLALVILLA, A., GÓMEZ, R., LARGO, R., HERRERO-BEAUMONT, G. Lipid transport and metabolism in healthy and osteoarthritic cartilage. Int J Mol Sci. 2013; 14(10), pp. 20793-808. ISSN 1422-0067. Disponibil: DOI: 10.3390/ijms141020793

2. KARUPPAL, R. Current concepts in the articular cartilage repair and regeneration. J Orthop. 2017; 14(2), pp. A1-A3. ISSN 0972-978X. Disponibil: DOI: 10.1016/j.jor.2017.05.001

3. KNUTSEN, G., DROGSET, JO., ENGEBRETSEN, L., GRØNTVEDT, T., LUDVIGSEN, TC., LØKEN, S., SOLHEIM, E., et al. A randomized multicenter trial comparing autologous chondrocyte implantation with microfracture: Long-term follow-up at 14 to 15 years. J Bone Joint Surg Am. 2016; 98(16), pp. 1332-9. ISSN 0375-9229. Disponibil: DOI: 10.2106/JBJS.15.01208

4. JEUKEN, RM., VAN HUGTEN, PPW., ROTH, AK., TIMUR, UT., BOYMANS, TAEJ., VAN RHIJN, LW., BUGBEE, WD., EMANS, PJ. A systematic review of focal cartilage defect treatments in middle-aged versus younger patients. Orthop J Sports Med. 2021; 9(10):23259671211031244. ISSN 2325-9671. Disponibil: DOI: 10.1177/23259671211031244

5. ARØEN, A., LØKEN, S., HEIR, S., ALVIK, E., EKELAND, A., GRANLUND, OG., ENGEBRETSEN, L. Articular cartilage lesions in 993 consecutive knee arthroscopies. Am J Sports Med. 2004; 32(1), pp. 211-5. ISSN 0363-5465. Disponibil: DOI: 10.1177/0363546503259345

6. TETTEH, ES., BAJAJ, S., GHODADRA, NS. Basic science and surgical treatment options for articular cartilage injuries of the knee. J Orthop Sports Phys Ther. 2012; 42(3), pp. 243-53. ISSN 1938-1344. Disponibil: DOI: 10.2519/jospt.2012.3673

7. FELSON, DT. Osteoarthritis as a disease of mechanics. Osteoarthritis Cartilage. 2013; 21(1), pp. 10-5. ISSN 1947-6035. Disponibil: DOI: 10.1016/j.joca.2012.09.012

8. CHUBINSKAYA, S., HAUDENSCHILD, D., GASSER, S., STANNARD, J., KRETTEK, C., BORRELLI, J., JR. Articular cartilage injury and potential remedies. J Orthop Trauma. 2015; 29(12), pp. S47-52. ISSN 0890-5339. Disponibil: DOI: 10.1097/BOT.0000000000000462

9. BAJAJ, S., SHOEMAKER, T., HAKIMIYAN, AA., RAPPOPORT, L., PASCUAL-GARRIDO, C., OEGEMA, TR., et al. Protective effect of P188 in the model of acute trauma to human ankle cartilage: the mechanism of action. J Orthop Trauma. 2010; 24(9), pp. 571-6. ISSN 0890-5339. Disponibil: DOI: 10.1097/BOT.0b013e3181ec4712

10. REESINK, HL., WATTS, AE., MOHAMMED, HO., JAY, GD., NIXON, AJ. Lubricin/proteoglycan 4 increases in both experimental and naturally occurring equine osteoarthritis. Osteoarthritis Cartilage. 2017; 25(1), pp. 128-137. ISSN 1947-6035. Disponibil: DOI: 10.1016/j.joca.2016.07.021

11. MORE, AS., KUMARI, RR., GUPTA, G., LINGARAJU, MC., BALAGANUR, V., PATHAK, NN., et al. Effect of iNOS inhibitor S-methylisothiourea in monosodium iodoacetate-

induced osteoathritic pain: implication for osteoarthritis therapy. Pharmacol Biochem Behav. 2013; 103(4), pp. 764-72. ISSN 0091-3057. Disponibil: DOI: 10.1016/j.pbb.2012.12.013

12. FORTIER, LA., BARKER, JU., STRAUSS, EJ., MCCARREL, TM., COLE, BJ. The role of growth factors in cartilage repair. Clin Orthop Relat Res. 2011; 469(10), pp. 2706-15. ISSN 0009-921X. Disponibil: DOI: 10.1007/s11999-011-1857-3

13. CAO, R., ZHAN, A., CI, Z., WANG, C., SHE, Y., XU, Y., et al. A biomimetic biphasic scaffold consisting of decellularized cartilage and decalcified bone matrixes for osteochondral defect repair. Front Cell Dev Biol. 2021; 9:639006. ISSN 2296-634X. Disponibil: DOI: 10.3389/fcell.2021.639006

14. CHUBINSKAYA, S., HURTIG, M., RUEGER, DC. OP-1/BMP-7 in cartilage repair. Int Orthop. 2007; 31(6), pp. 773-81. ISSN 1432-5195. Disponibil: DOI: 10.1007/s00264-007-0423-9

15. DECKER, RS. Articular cartilage and joint development from embryogenesis to adulthood. Semin Cell Dev Biol. 2017; 62:50-56. ISSN 1084-9521. Disponibil: DOI: 10.1016/j.semcdb.2016.10.005

16. OZTÜRK, A., OZDEMIR, MR., OZKAN, Y. Osteochondral autografting (mosaicplasty) in grade IV cartilage defects in the knee joint: 2- to 7-year results. Int Orthop. 2006; 30(3), pp. 200-4. ISSN 1432-5195. Disponibil: DOI: 10.1007/s00264-005-0068-5

17. GODIN, JA., SANCHEZ, G., CINQUE, ME., CHAHLA, J., KENNEDY, NI., PROVENCHER, MT. Osteochondral allograft transplantation for treatment of medial femoral condyle defect. Arthrosc Tech. 2017; 6(4), pp. e1239-e1244. ISSN 2212-6287. Disponibil: DOI: 10.1016/j.eats.2017.04.010

18. HANGODY, L., VÁSÁRHELYI, G., HANGODY, LR., SÜKÖSD, Z., TIBAY, G., BARTHA, L., BODÓ, G. Autologous osteochondral grafting--technique and long-term results. Injury. 2008; 39(1), pp. S32-9. ISSN 0020-1383. Disponibil: DOI: 10.1016/j.injury.2008.01.041

19. NIEMEYER, P., ALBRECHT, D., ANDEREYA, S., ANGELE, P., ATESCHRANG, A., AURICH, M., et al. Autologous chondrocyte implantation (ACI) for cartilage defects of the knee: A guideline by the working group "Clinical Tissue Regeneration" of the German Society of Orthopaedics and Trauma (DGOU). Knee. 2016; 23(3), pp. 426-35. ISSN 0968-0160. Disponibil: DOI: 10.1016/j.knee.2016.02.001

20. MADEIRA, C., SANTHAGUNAM, A., SALGUEIRO, JB., CABRAL, JM. Advanced cell therapies for articular cartilage regeneration. Trends Biotechnol. 2015; 33(1), pp. 35-42. ISSN 1879-3096. Disponibil: DOI: 10.1016/j.tibtech.2014.11.003

21. KRAEUTLER, MJ., KAENKUMCHORN, T., PASCUAL-GARRIDO, C., WIMMER, MA., CHUBINSKAYA, S. Peculiarities in ankle cartilage. Cartilage. 2017; 8(1), pp. 12-18. ISSN 1947-6035. Disponibil: DOI: 10.1177/1947603516642572

22. BERNINGER, MT., WEXEL, G., RUMMENY, EJ., IMHOFF, AB., ANTON, M., HENNING, TD., VOGT, S. Matrix-assisted autologous chondrocyte transplantation for remodeling and repair of chondral defects in a rabbit model. J Vis Exp. 2013; (75), pp. e4422. ISSN 1940-087X. Disponibil: DOI: 10.3791/4422

23. TAKAHASHI, T., SATO, M., TOYODA, E., MAEHARA, M., TAKIZAWA, D., MARUKI, H., et al. Rabbit xenogeneic transplantation model for evaluating human chondrocyte sheets used in articular cartilage repair. J Tissue Eng Regen Med. 2018; 12(10), pp. 2067-2076. ISSN 1932-7005. Disponibil: DOI: 10.1002/term.2741

24. MENG, X., ZIADLOU, R., GRAD, S., ALINI, M., WEN, C., LAI, Y., et al. Animal models of osteochondral defect for testing biomaterials. Biochem Res Int. 2020; 2020:9659412. ISSN 2090-2255. Disponibil: DOI: 10.1155/2020/9659412

25. **COBZAC, V.,** MOSTOVEI, A., JIAN, M., NACU, V. An efficient procedure of isolation, cultivation and identification of bone marrow mesenchymal stem cells. Mold Med J. 2019; 62(1), pp. 35-41. ISSN 2537-6373. Disponibil: DOI: 10.5281/zenodo.2590011

26. **COBZAC, V.,** VERESTIUC, L., JIAN, M., NACU, V. Chondrocytes isolation from hyaline cartilage by continuous monitoring method. Mold Med J. 2021; 64(6), pp. 13-19. ISSN 2537-6373. Disponibil: https://doi.org/10.52418/moldovan-med-j.64-6.21.03

27. **COBZAC, V.,** JIAN, M., GLOBA, T., NACU, V. Joint cartilage experimental defect regeneration by hierarchic biphasic combined grafts. Mold Med J. 2022; 66(2), pp. 20-29. ISSN 2537-6373. Disponibil: https://doi.org/10.52418/moldovan-med-j.65-2.22.03

28. **COBZAC, V.,** VEREŞTIUC, L., JIAN, M., NACU V. Assessment of ionic and anionic surfactants effect on demineralized osteochondral tissue. In: International Conference on Innovative Research - ICIR EUROINVENT 2019 16–17 Mai 2019, Iasi, Romania. IOP Conference Series: Materials Science and Engineering; 2019; 572:012084. ISSN 1757-899X. Disponibil: DOI: 10.1088/1757-899X/572/1/012084

29. SELLERS, RS., PELUSO, D., MORRIS, EA. The effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the healing of full-thickness defects of articular cartilage. J Bone Joint Surg Am. 1997; 79(10), pp. 1452-63. ISSN 0375-9229. Disponibil: DOI: 10.2106/00004623-199710000-00002

30. WAKITANI, S., GOTO, T., PINEDA, SJ., YOUNG, RG., MANSOUR, JM., CAPLAN, AI., GOLDBERG, VM. Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. J Bone Joint Surg Am. 1994; 76(4), pp. 579-92. ISSN 0375-9229. Disponibil: DOI: 10.2106/00004623-199404000-00013

31. O'DRISCOLL, SW., KEELEY, FW., SALTER, RB. The chondrogenic potential of free autogenous periosteal grafts for biological resurfacing of major full-thickness defects in joint surfaces under the influence of continuous passive motion. An experimental investigation in the rabbit. J Bone Joint Surg Am. 1986; 68(7), pp. 1017-35. ISSN 0375-9229.

32. **COBZAC, V.,** JIAN, M., NACU, V. Cellularization of small sized grafts from biological material using the gravitational modality principle. In: International Conference on Innovative Research - ICIR EUROINVENT 2021, 20–21 Mai 2021, Iasi, Romania. J. Phys.: Conf. Ser. 2021; 1960:012004. ISSN 1742-6596. Disponibil: DOI: 10.1088/1742-6596/1960/1/012004

33. KALTER, SS, HENNESSEN, W. The standardization of animals to improve biomedical research, production, and control. In: Karger Basel S. (ed.) IABS International Congress for Biological Standardization 1979, 16-20 Septembrie 1979, San Antonio, Texas, USA. International Association of Biological Standardization; 1980. 247 p. ISSN 0301-5149

LIST OF PUBLICATIONS

- **Articles in scientific journals abroad:**
  - ✓ **articles in ISI journals, SCOPUS and other international databases**
    1. **Cobzac V.,** Verestiuc L., Jian M. Nacu V. Assesment of ionic and anionic surfactants effect on demineralized osteochondral tissue. In: IOP Conf. Ser.: Mater. Sci. Eng. 2019; 572:012084. Disponibil:10.1088/1757-899X/572/1/012084 (**SCOPUS**).
    2. **Cobzac V.,** Jian M., Nacu V. Cellularization of small sized grafts from biological material using the gravitational modality principle. In: J. Phys.: Conf. Ser. 2021; 1960:012004. Disponibil: 10.1088/1742-6596/1960/1/012004. (**SCOPUS**).

3. **Cobzac V.,** Jian M., Globa T., Nacu V. The cartilaginous tissue regeneration on weight bearing and non-weight bearing surfaces of the knee. In: IFMBE Proceedings. 2022; 87:334–341. Disponibil: https://doi.org/10.1007/978-3-030-92328-0_44. (**SCOPUS**).

- **Articles in accredited national scientific journals:**
  - ✓ **articles in journals of B/B+ category**

4. **Cobzac V.,** Mostovei A., Jian M., Nacu V. An efficient procedure of isolation, cultivation and identification of bone marrow mesenchymal stem cells. *Mold Med Journal*. 2019; 62(1):35-41. Disponibil: 10.5281/zenodo.2590011. (**DOAJ**).
5. **Cobzac V.,** Verestiuc L., Jian M., Nacu V. Chondrocytes isolation from hyaline cartilage by continuous monitoring method. *Mold Med Journal*. 2021; 64(6):13-19. Disponibil: https://doi.org/10.52418/moldovan-med-j.64-6.21.03. (**DOAJ**).
6. **Cobzac V.,** Jian M., Globa T., Nacu V. Joint cartilage experimental defect regeneration by hierarchic biphasic combined grafts. *Mold Med J*. 2022; 66(2):20-29. Disponibil: https://doi.org/10.52418/moldovan-med-j.65-2.22.03. (**DOAJ**).
7. Jian M., **Cobzac V.,** Nacu V. Dispozitive și metode brevetate cu utilizare în ingineria tisulară. *INTELLECTUS*. 2022; 1:94-100. Disponibil: https://doi.org/10.56329/1810-7087.22.1.09.

- **Abstracts/thesis in proceedings of international scientific conferences:**

8. **Cobzac V.,** Verestiuc L., Jian M., Nacu V. Preclinical testing of decellularized and demineralized osteochondral allografts on rabbit model. *The 56th annual meeting of veterinary sciences „Towards a global health”*. Iași, România; 2017. p.79.
9. **Cobzac V.,** Coșciug S., Jian M., Nacu V. Apreciation of cartilage permeability within demineralized and decellularized osteochondrol allogeneic graft with trypan blue. *Матеріали Науково-практичної конференції з миждородною участю. Актуальні питання травматології та остеоситезу.* Чернівці, Україна; 2017. p. 140.
10. **Cobzac V.,** Jian, M., Coșciug S., Nacu V. Comparison of *in vitro* effect of osteochondral demineralised and demineralised-decellularized grafts on cells used in cartilage regeneration. *EATB 26.* Treviso, Italia; 2017. p. 24-25.
11. **Cobzac V.,** Verestiuc L., Jian M., Coșciug S., Nacu V. Preclinical testing of ODDG. Preliminary report. *2nd International Biomedical Congres of Sofia*. Sofia, Bulgaria; 2017. p.74.
12. Nacu V., **Cobzac V.,** Jian M., Verestiuc L. Decellularized-demineralized osteochondral allografts testing on rabbit model. Preliminary report. *TERMIS 2018.* Kyoto, Japonia; 2018. p.78.
13. **Cobzac V.,** Nacu V., Jian M., Coşciug S., Paladi C, Macagonova O. Method of collagen sponge obtaining. *The International Student Innovation and Scientific Research Exhibition „Cadet INOVA ’18”.* Sibiu, Romania; 2018. p. 207.
14. **Cobzac V.,** Nacu V., Croitor G., Coşciug S., Jian M. Grafting for restoring the osteochondral defect. *The International Student Innovation and Scientific Research Exhibition „Cadet INOVA ’18”.* Sibiu, Romania; 2018. p. 208.
15. **Cobzac V.,** Nacu V., Croitor G., Coşciug S., Jian M. Grafting for restoring the osteochondral defect. *Proceedings of the 10th Edition of Euroinvent European Exhibition of Creativity and Innovation*. Iasi, Romania; 2018. p. 191.

16. **Cobzac V.,** Nacu V., Jian M., Coşciug S., Paladi C, Macagonova O. Method of collagen sponge obtaining. *Salonul Internaţional al Cercetării Ştiinţifice, Inovării şi Inventicii, Ediţia XVI*. Cluj-Napoca, Romania; 2018. p. 186-187.
17. **Cobzac V.,** Verestiuc L., Jian M., Nacu V. Assesment of ionic and anionic surfactants effect on demineralized osteochondral tissue. *The 5th International Conference on Innovative Research. ICIR 2019.* Iasi, Romania; 2019. p. 122.
18. **Cobzac V.,** Nacu V., Jian M., Sarev V., Macagonova O. Device for reticulation of collagen sponge with glutaraldehyde vapors. *The International Student Innovation and Scientific Research Exhibition „Cadet INOVA '19".* Sibiu, Romania; 2019. p. 175.
19. **Cobzac V.,** Nacu V., Jian M., Coşciug S., Paladi C., Macagonova O. Method of collagen sponge obtaining. *Proceedings of the 11th Edition of Euroinvent European Exhibition of Creativity ond Innovation.* Iasi, Romania. 2019. p. 195-196.
20. **Cobzac V.,** Nacu V., Jian M., Sarev V., Macagonova O. Device for reticulation of collagen sponge with glutaraldehyde vapors. *Proceedings of the 11th Edition of Euroinvent European Exhibition of Creativity ond Innovation*. Iasi, Romania. 2019. p. 197.
21. **Cobzac V.,** Nacu V., Croitor G., Coşciug S., Jian M. Grafting for restoring the osteochondral defect. *Salonul Internaţional al Cercetării Ştiinţifice, Inovării şi Inventicii, Ediţia XVII.* Cluj-Napoca, Romania. 2019. p. 213-214.
22. **Cobzac V.,** Jian M., Mostovei A., Nacu V. Bone augmentation with natural and synthetic materials. *International Chemical Engineering and Material Symposium, SICHEM 2020.* Bucharest, Romania; 2020. p. 114.
23. **Cobzac V.**, Nacu V., Glavan A., Jian M., Sedaia E., Ababii P. Small-sized graft fixation and cellularization device. *The International Student Innovation and Scientific Research Exhibition „Cadet INOVA '20".* Sibiu, Romania; 2020. p. 233.
24. **Cobzac V.**, Nacu V., Glavan A., Jian M., Sedaia E., Ababii P. Small-sized graft fixation and cellularization device. *Proceedings of the 13th Edition of Euroinvent European Exhibition of Creativity and Innovation*. Iasi, Romania; 2021. p. 194.
25. Jian M., Nacu V., **Cobzac V.,** Branişte T. Method of isolation of cell cultures. *Salonul Internațional de Invenții și Inovații „Traian Vuia", ediția a VIII-a.* Timișoara, România; 2022. p. 51.
26. **Cobzac V.,** Jian M., Nacu V. The efficiency of device for fixation and cellularization of small sized grafts. *International Conference on Innovative Research, ICIR 2021.* Iași, Romania; 2021. p. 79.
27. **Cobzac V.,** Verestiuc L., Jian M., Nacu V. Selection of an appropriate cross-linking method of collagen sponges for articular cartilaginous tissue engineering. *Virtual International Scientific Conference on "Applications of Chemistry in Nanosciences and Biomaterials Engineering – NanoBioMat 2021".* Bucharest, Romania; 2021. p. 40.
28. Jian M., Nacu V., **Cobzac V.,** Branişte T. Method of isolation of cell cultures. *Proceedings of the 14th Edition of Euroinvent European Exhibition of Creativity and Innovation*. Iasi, Romania; 2022. p. 171.

- **Abstracts/thesis in proceedings of national scientific conferences:**

29. **Cobzac V.,** Jian M., Madan V., Croitor Gh., Nacu V. Collagen/Chitosan hybrid sponge as a scafold for cell culture. *Maedica, Ediție specială: Al VIII-lea congres național de ortopedie și traumatologie cu participare internațională.* Chișinău; 2016. 4(61), p. 47.

30. **Cobzac V.,** Jian M., Madan V., Croitor Gh., Nacu V. Method of chondrocytes isolation from hyaline cartilaj. *Maedica, Ediție specială: Al VIII-lea congres național de ortopedie și traumatologie cu participare internațională.* Chișinău; 2016. 4(61), p. 47-48.
31. **Cobzac V.,** Jian M., Croitor Gh., Nacu V. Cultivarea de condrocite autologe pentru terapia celulara. *Maedica, Ediție specială, Conferința Stiintifică „Nicolae Anestiadi - Nume etern al Chirurgiei Basarabene".* Chisinău; 2016. 3(60), p. 10.
32. **Cobzac V.,** Jian M., Croitor Gh., Nacu V. Suport celular pentru ingineria tisulară. *Maedica, Ediție specială, Conferința Stiințifică „Nicolae Anestiadi - Nume etern al Chirurgiei Basarabene".* Chișinău; 2016. 3(60), p. 9.
33. **Cobzac V.,** Nacu V., Jian M., Paladi C., Sarev V. Dispozitiv pentru reticularea spongiei de colagen cu vapori de glutaraldehidă. *Catalog oficial Expoziţia Internaţională Specializată „Infoinvent 2017", Ediţia a XV-a*. Chișinău; 2017. p. 144.
34. **Cobzac V.,** Nacu V., Jian M., Coşciug S., Paladi C., Macagonova O. Metodă de obţinere a spongiei de colagen. *Catalog oficial Expoziţia Internaţională Specializată „Infoinvent 2017", Ediţia a XV-a*. Chișinău; 2017. p. 145.
35. **Cobzac V**., Nacu V., Croitor G., Coşciug S., Jian M. Grefă pentru restabilirea defectului osteocondral. *Catalog oficial Expoziţia Internaţională Specializată „Infoinvent 2017", Ediţia a XV-a.* Chișinău; 2017. p. 145-146.
36. Jian M., **Cobzac V.,** Popescu V., Nacu V. Verestiuc L. Obtaining of a suitable osteochondral graft for articular cartilage engeneering. *The 7th International medical congress for students and young doctors, MedEspera2018.* Chișinău; 2018. p. 202.
37. **Cobzac V**., Nacu V., Glavan A., Jian M, Sedaia E., Ababii P. Dispozitiv pentru fixarea și celularizarea grefelor de dimensiuni mici. *Catalog oficial Expoziţia Internaţională Specializată „Infoinvent 2019" Ediţia a XVI-a*. Chișinău; 2019. p. 119.
38. **Cobzac V.,** Verestiuc L., Jian M., Nacu V. Tissue engineered grafts in articular cartilage defects treatment. *Congresul consactrat anoversării a 75-a de la fondarea USMF „N. Testemițanu"*.Chișinău; 2020. p. 500.
39. **Cobzac V.,** Jian M., Globa T., Nacu V. The cartilaginous tissue regeneration on weight bearing and non-weight bearing surfaces of the knee. *The 5th International conference on Nanotechnologies and Biomedical Engineering. ICNBME-2021.* Chișinău; 2021. p. 89.

- **Patents, registration certificates, materials at invention fairs:**

40. **Cobzac V.**, Nacu V., Jian M., Sarev V., Macagonova O. Dispozitiv pentru reticularea spongiei de colagen cu vapori de glutaraldehidă. Brevet de invenţie MD 1137 Y. BOPI nr.4/2017, 2017.04.30.
41. **Cobzac V.**, Nacu V., Jian M., Coşciug S., Paladi C., Macagonova O. Metodă de obţinere a spongiei de colagen. Brevet de invenţie MD 1169 Y. BOPI nr.7/2017, 2017.07.31.
42. **Cobzac V.**, Nacu V., Croitor G., Coşciug S., Jian M. Grefă pentru restabilirea defectului osteocondral. Brevet de invenţie MD 1177 Y. BOPI nr.8/2017, 2017.08.31.
43. **Cobzac V.**, Nacu V., Glavan A., Jian M., Sedaia E., Ababii P. Dispozitiv pentru fixarea şi celularizarea grefelor de dimensiuni mici. Brevet de invenţie MD 1332 Y. BOPI nr.4/2019, 2019.04.30.
44. **Cobzac V.**, Nacu V., Jian M. Dispozitiv pentru separarea pastei osoase demineralizate. Brevet de invenţie MD 1582 Y. BOPI nr.12/2021, 2021.12.31.
45. Jian M., Nacu V., **Cobzac V.**, Branişte F. Metodă de izolare a culturilor celulare. Brevet de invenţie MD 1584 Y. BOPI nr.12/2021, 2021.12.31.

- **Participation with communications in scientific fora:**
  - ✓ **international**

46. **Cobzac V.,** Coșciug A., Jian M., Nacu V. Appreciation of cartilage permeability within demineralised and decellularized osteochondral allogeneic graft with trypan blue. *Науково-практична конференція з міжнародною участю. Актуальні проблеми травматології та остеосинтезу.* Чернівці, Україна, 27 квітня 2017.
47. Nacu V., Țîmbălari T., **Cobzac V.,** Coșciug S., Cociug A. Tissue and cell transplantation in the Republic of Moldova. *Науково-практична конференція з міжнародною участю. Актуальні проблеми травматології та остеосинтезу.* Чернівці, Україна, 27 квітня 2017.
48. **Cobzac V.** Preclinical testing of decellularized and demineralized osteochondral allografts on rabbit model. Preliminary report. *Simpozionul „Medicină Regenerativă – știință practică și intrdisciplinaritate".* Iasi, Romania, 18 octombrie 2017.
49. **Cobzac V.** Preclinical testing of ODDG. Preliminary report. *2nd international biomedical congress of Sofia*. Sofia, Bulgaria, 17-19 November 2017.
50. **Cobzac V.,** Jian M., Coșciug S., Nacu V. Comparison of *in vitro* effect of osteochondral demineralised and demineralised-decellularized grafts on cells used in cartilage regeneration. *EATB 26*. Treviso, Italia, 17- 22 octombrie 2017.
51. **Cobzac V.,** Vereștiuc L., Jian M., Nacu V. Assesment of ionic and anionic surfactants effect on demineralized osteochondral tissue. *The 5th edition of International Conference on Innovative Research, ICIR 2019.* Iași, Romania, 16-18 May 2019.
52. **Cobzac V.** Combined grafts in the treatment of osteochondral defects. *SPEAKER în cadrul conferinței CONFESTESIS, ediția a II-a*. Iași, România, 24-26 octombrie 2019.
53. **Cobzac V.,** Jian M., Mostovei A., Nacu V. Bone augmentation with natural and synthetic materials. *International Chemical Engineering and Material Symposium, SICHEM 2020.* Bucharest, Romania, 17-18 September 2020.
54. **Cobzac V.,** Jian M., Nacu V. The efficiency of device for fixation and cellularization of small sized grafts. *The 7th edition of International Conference on Innovative Research, ICIR 2021.* Iași, Romania, 20-21 May 2021.
55. **Cobzac V.,** Verestiuc L., Jian M., Nacu V. Selection of an appropriate cross-linking method of collagen sponges for articular cartilaginous tissue engineering. *Virtual International Scientific Conference on "Applications of Chemistry in Nanosciences and Biomaterials Engineering – NanoBioMat 2021".* Bucharest, Romania, 25-26 June 2021.

  - ✓ **national**

56. **Cobzac V.,** Jian M., Globa T., Nacu V. The cartilaginous tissue regeneration on weight bearing and non-weight bearing surfaces of the knee. *The 5th International conference on Nanotechnologies and Biomedical Engineering. ICNBME-2021.* Chișinău, 3-5 November 2021.
57. Jian M., **Cobzac V.,** Popescu V., Verestiuc L. Obtaining of a suitable osteochondral graft for articular cartilage engeneering. *The 7th International medical congress for students and young doctors, MedEspera2018.* Chișinău, 3-5 May 2018.
58. **Cobzac V.,** Vereştiuc L., Jian M., Coşciug S., Nacu V. Matrixuri tridimensionale în ingineria tisulară. *Zilele Universităţii şi Conferinţa ştiinţifică anuală, consacrate aniversării a 90-a de la naşterea ilustrului medic şi savant Nicolae Testemiţanu.* Chișinău, 16-20 octombrie 2017.

59. **Cobzac V.** Grefe tridimensionale în restabilirea cartilajului articular. *Zilele Universităţii şi Conferinţa ştiinţifică anuală.* Chișinău, 15-19 octombrie 2018.
60. **Cobzac V.,** Vereștiuc L., Jian M., Nacu V. Grefe ob-ținute prin inginerie tisulară în tratamentul defectelor de cartilaj articular. *Congresul consacrat aniversării a 75-a de la fondarea Universităţii de Stat de Medicină şi Farmacie „Nicolae Testemiţanu" din Republica Moldova*. Chișinău, 21-23 octombrie 2020.
61. **Cobzac V.** Regenerarea defectelor de cartilaj articular. *Conferinţa ştiinţifică anuală consacrată aniversării a 77-a de la fondarea Universităţii de Stat de Medicină şi Farmacie „Nicolae Testemiţanu" din Republica Moldova.* Chișinău, 18-21 octombrie 2022.

- **Participation with posters in scientific fora:**
  ✓ **international**
62. Nacu V., **Cobzac V.,** Jian M., Verestiuc L. Decellularized-demineralized osteochondral allografts testing on rabbit model. Preliminary report. *The 5th TERMIS World Congress*. Kyoto, Japan, 5-7 September 2018.

- **Research projects on the thesis topic:**
  ✓ **national**
63. Project within the state program „Regeneration by cell therapy of affected tissues and organs", period 2014-2015, the project „ Testing the possibilities of using stem cells to restore the hyaline cartilage of diarthrodial joints in degenerative dystrophic conditions", project number 14.519.04.01A, with funding from the state budget - project executor.
64. Project within the state program „Regeneration by cell therapy of affected tissues and organs", period 2016-2017, the project „ The clinical application of cellular therapy in restoring the hyaline cartilage of diarthrodial joints in degenerative dystrophic conditions", project number 16.00354.80.01A, with funding from the state budget - project executor.

- **Research internships abroad:**
65. "Eugen Ionescu" scholarship for doctoral research within the Center for Training and Research in Tissue Engineering, Artificial Organs and Regenerative Medicine of UMPh "Grigore T. Popa" from Iași, România, April 24 - July 23, 2017.
66. "Eugen Ionescu" scholarship for doctoral research (renewal) within the Center for Training and Research in Tissue Engineering, Artificial Organs and Regenerative Medicine of UMPh "Grigore T. Popa" from Iași, România, March 19 – June 19, 2018.
67. World Federation of Scientists (WFS) Scholarship, June 1, 2019 - May 31, 2020.
68. Training visit in the Leibniz Research Laboratories for Biotechnology and Artificial Organs (LEBAO) at the Hannover Medical School (MHH), Germany, organised in the framework of the Horizon2020 project "NanoMedTwin", 1 October 2021 - 31 March 2022.

COBZAC VITALIE

COMBINED GRAFTS FOR REGENERATION OF EXPERIMENTAL ARTICULAR CARTILAGE DEFECTS

341.01. TISSUE ENGINEERING AND CELLULAR CULTURES

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