Assessment of ionic and anionic surfactants effect on demineralized osteochondral tissue

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Abstract. After demineralization the rabbit distal femoral osteochondral tissues were decellularized, separately with SDS and Triton X-100 for 24, 48 and 72 hours, at concentrations of 2%, 1% and 0.5%, respectively. The greatest DNA removal was achieved with Triton X-100 solutions. Cytotoxicity tests with CSM and chondrocytes have shown good and very good results, but a gradual decrease in cell viability related to the duration of treatment with surfactants compared to the control was registered. The same trend was observed in the cells population test after 7 days, while there was no difference at the 14th day. It was also determined that samples decellularized with SDS have a higher resistance to enzymatic degradation than the control and the decellularized tissue with Triton X-100. The swelling test and elasticity modulus measurements did not show values dependent of the surfactant nature.

1. Introduction
The treatment of articular cartilage injuries is a challenge for regenerative medicine in the field of orthopedics and traumatology [3–7]. Since articular cartilage is an avascular tissue, it is fed only by diffusion from the synovial fluid [8, 9]. However, the structural organization of hyaline articular cartilage and fixation of chondrocytes in it without the possibility of migration to the lesion site, dramatically hinders cartilage regeneration process [4, 6, 7, 9].

Numerous modalities of cartilage defects treatment are known, from surgical bone marrow stimulation techniques [4, 10], transplantation of allogeneic [11, 12] or autologous osteocondral tissue [13–15], implantation in a chondral or osteochondral defect of autologous chondrocytes and mesenchymal stem cells (MSC), attached or not to a three-dimensional matrix [16–19]. Unfortunately, hyaline cartilaginous tissue was not possible to obtain [19, 20]. At the same time, the use in the regeneration of articular cartilage of three-dimensional matrices seeded with cells with chondroprogenitor potential remains a perspective field of research [18, 19, 21].

The three-dimensional matrices used in nowadays as cells support for articular cartilage regeneration, mimics the composition of cartilage extracellular matrix [1]. As a result, utilisation of a three-dimensional matrix, composed of DNA-free cartilage and subcondral bone with perforations for adequate cell invasion and scaffold population, seems to be more reasonable [2].
The actual research papers presents the experimental studies which are related to rabbit demineralized osteochondral tissue (RDOT) what was decellularized with various concentrations of ionic and anionic surfactants.

It is known that how higher is surfactant concentration, the decellularization process is faster, but the decellularized tissue quality is lacking [24, 25], as a result, in this study was analyzed the influence of detergent concentration and the decellularization period on the performances of the obtained scaffolds.

2. Materials and methods
Isolation, culture and utilization of rabbit cells and tissues in research was approved at meeting of ethics committee of Moldovan State University of Medicine and Pharmacy “Nicolae Testemitanu” on 14.12.2016 with No. 31.

2.1. Preparation of osteochondral tissue samples
From 11 freshly sacrifice drab its, femoral con dyles were taken, followed by bone and soft tissues removal excepting the subchondral bone. Osteochondral tissue was subjected to demineralization process with 0.6 MHCl (Sigma, SUA), followed by washing in a large volume of ddH2O and degreasing overnight in 6% H2O2. The next day the samples were washed with ddH2O and kept for 24 hours in PBS, changing the solution 3 times. RDOT was separately decellularized with 2%, 1% and 0.5% sodium dodecylsulphate (SDS) (Sigma, USA) and Triton X-100 (TrX-100) (HiMedia, India) for 24, 48 and 72 hours respectively, with a magnetic stirrer changing the solutions every 24 hours as the case. After decellularization the samples were washed for 24 hours with a large volume of ddH2O and were desiccated by centrifugation at 2700xg for 20 min.

2.2. DNA quantification
DNA extraction was performed with Thermo Scientific GeneJET Genomic DNA Purification Kit (Thermo Scientific, Lithuania).

From each rabbit demineralized decellularized osteochondral tissue sample (RDDOT), normal osteochondral tissue and RDOT, were cut 6 to 9 pieces by 20 mg each, containing bone matrix and cartilage in the same amount. All samples were processed according to the DNA extraction guide provided by the manufacturer. At the end of the extraction process the resulting suspension was scanned with spectrophotometer (Synergy H1, BioTek) at the wavelength of 260 nm.

2.3. The chondrocytes and mesenchymal stem cells obtaining
The MSC and chondrocytes used in the experiment were of rabbit origin, they were isolated with the agreement of the ethics committee from 14.12.2016 No. 31. MSC were obtained from bone marrow extracted from the rabbit iliac bone and processed according to described procedure [23]. The obtained MSC were cultured in 2 passages with DMEM/Ham's F-12 medium (Sigma, UK) supplemented with 10% FBS (Lonza, Belgia) and antibiotic antymycotic solution (HiMedia, India) [33]. The excess of MSC were cryopreserved at -85°C with 10% DMSO (Origen Biomedical, Germany) for later use.

Chondrocytes were isolated from rabbit articular cartilage, which was harvested from the knee joint. The minced cartilage was subjected to enzymatic degradation with 0.6% collagenase (Sigma, UK) [22]. The isolated chondrocytes were grown the first 2 passages in DMEM (Sigma, UK) supplied with 10% FBS (Lonza, Belgia) and antibiotic antymycotic solution (HiMedia, India). The chondrocytes in excess were preserved as MSC.

2.4. MTT cytotoxicity test
To perform the cytotoxicity test with MSC and chondrocytes, pieces of about 6 mm³ were cut from RDOT and RDDOT. They were then sterilized by treating with 70% alcohol for 2 hours and UVC exposure for 30 min followed by washing with sterile ddH2O and HBSS (Sigma, UK). In 48-well tissue culture plates (TPP, Switzerland) was poured by 0.5 ml of suspension with MSC and
chondrocytes at a concentration of $5 \times 10^3$ cells/well. Plaques with MSC and chondrocytes were prepared for cell viability assessment 3 days consecutively after introduction of sterilized samples.

The next day in the wells the samples were introduced by 4 pieces of each type. At 24, 48 and 72 hours from wells, the samples and culture medium was removed and fresh 2.5 mg/ml MTT (Sigma, UK) solution prepared in culture medium characteristic for each cell type, was introduced in wells. The plates were wrapped in aluminium foil and incubated, MSC for 2 hours and chondrocytes for 3 hours. Subsequently, the medium with MTT was replaced with isopropyl alcohol of 99.8% (Stanchem, Poland) and stirred for 10 minutes at 150 rpm (ES-20, Biosan). The results were read with spectrophotometer (Synergy H1, BioTek) at the wavelength of 570 nm.

2.5. Cellular population test

Pieces of each type of osteochondral tissue were prepared with approximately 1 mm thickness. Samples were sterilized by the procedure described above. The day before populate the samples with cells, they were placed in 96-well plates, in specific culture medium for MSC and chondrocytes. The next day, the media was removed from the wells and by $5 \times 10^3$ chondrocytes and MSCs were added. Over one day the samples were transferred to 48-well plates with 1 ml of cells specific culture medium. The culture medium has changed every 2 days. At 7th and 14th day, one of the samples was fixed in 4% paraformaldehyde, followed by DAPI staining (Sigma, UK) and fluorescence microscopy examination.

2.6. In vitro enzymatic degradation studies

The scaffolds degradation was evaluated with the ninhydrin chromogenic assay [31]. The ninhydrin reactive was prepared by dissolving $2.83 \times 10^{-3}$ moles of SnCl$_2$·2H$_2$O in 500 ml of citrate buffer (pH=5) and mixing with 500 ml of 2-methoxyethanol which contained 20 g of ninhydrin.

Freeze-dried RDDOT and RDOT samples ($\approx 25$ mg) were incubated in 15 ml PBS (0.1M, pH=7.4) containing 0.01% collagenase (Sigma, UK), for 2 weeks, at 37ºC. At different periods of time (2, 4, 24, 48, 72 hours, 7days and 2weeks, respectively) 200 µl of solution was extracted from supernatant and replaced with fresh PBS solution, containing the same amount of collagenase (Sigma, UK). The sample solution was mixed with 1 ml ninhydrin reactive and warmed on a water bath for 20 minutes; afterwards, the sample solution was immediately cooled and mixed with 4 ml solution of 2-propanol/distilled water (v:v=1:1) and absorption at 570 nm was registered with a UV-VIS spectroscopy (Schimadzu UV-1700 PharmaSpec, Japonia). The experiments were repeated three times, and the results are expressed as a mean ± standard deviation (SD).

2.7. Swelling properties

Swelling degree of each RDDOT sample and RDOT has been assessed with QIAquickRSpin Columns 50, Ø = 10 mm, attached to a micro syringe. The micro column contains a cellulosic membrane and a cover to avoid water evaporation. The experiments were performed by the immersion of samples in phosphate buffer saline solution (PBS, 0.1 M, pH=7.4, d=1.07g/cm$^3$) at 37ºC, and the retained PBS volume was measured. The swelling degree of samples (SD) has been calculated with the equation (1), where $W_f$ is the final weight (g) of the swollen sample, and $W_i$ is the initial weight of the sample (g). The experiments were repeated three times, and the results are expressed as a mean ± standard deviation (SD).

$$SD(\%) = \frac{W_f - W_i}{W_i} \times 100$$

2.8. Modulus of elasticity

A texture analyzer TA-XT2 Plus (Stable Microsystems, UK) was used for testing the scaffolds elasticity with a cylinder of 12 mm diameter and compression speed of 1 mm/s. The slopes of the stress–strain curves at small deformations were used to calculate an apparent compression modulus.
Stress-relaxation measurements on the free and on the confined samples were made on the TAXT2 instrument, as well. Three lyophilized samples were introduced in PBS pH 7.4 for 4 hours. After that, the height and the areas of the contact surfaces were calculated. An initial fast deformation of 10 % at 1 mm/s was kept constant for 60 s.

3. Results and discussion

3.1. DNA quantification
By demineralisation of the osteochondral tissue with hydrochloric acid significant amount of DNA have been eliminated - about 83% (p˂0.0001). A DNA content below 5% was performed after a treatment with Triton X-100 of 1% and 2% for 48 and 24 hours respectively (p=0.001), stronger decellularization was in RDOT treated with 2% Triton X-100 for 24 hours – 0.97 ng/µl ± 0.4 (Table 1).

Table 1. The results of DNA quantification.

| Sample type  | DNA quantity (ng/µl) |
|--------------|----------------------|
| Normal tissue| 42.33±10.89          |
| RDOT         | 7.13±1.73            |
| SDS 0.5%     | 2.82±0.44            |
| SDS 1%       | 2.52±0.92            |
| SDS 2%       | 2.58±0.40            |
| TrX-100 0.5%| 2.32±0.56            |
| TrX-100 1%   | 1.60±0.52            |
| TrX-100 2%   | 0.97±0.40            |

3.2. MTT cytotoxicity test
Following the MTT cell viability test, high cell viability was determined in dynamics at contact with almost all samples, except for those treated with SDS and Triton X-100 of 1%, at 72 hours of culturing MSC, where cell viability registered values below 80%. Surprising was the extremely high increase in chondrocyte at contact with tested samples, over 100% viability at 48 and 72 hours (see Figure 1), which suggests the presence of various growth factors in RDOT and RDDOT samples that are favorable for chondrocytes. In contrast, in MSC the viability in most cases is 80-90%, see Figure 2.

Figure 1. Chondrocyte viability at contact with tested samples.
3.3. **Cellular population test**

After culturing chondrocytes and MSC on RDOT and RDDOT samples it was determined that at day 7 of cultivation, the cellular multiplication is more abundant in chondrocytes, but at 14th day the samples were almost uniform abundantly populated, see Table 2.

**Table 2. Cellular population test results.**

|          | Chondrocytes |          |          | MSC          |          |
|----------|--------------|----------|----------|--------------|----------|
|          | 7 days       | 14 days  | 7 days   | 14 days      |          |
| RDOT     | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) | ![Image](image5.png) |
| SDS 0.5% | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) | ![Image](image10.png) |
| SDS 1%   | ![Image](image11.png) | ![Image](image12.png) | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) |
| SDS 2%   | ![Image](image16.png) | ![Image](image17.png) | ![Image](image18.png) | ![Image](image19.png) | ![Image](image20.png) |
| TrX-100 0.5% | ![Image](image21.png) | ![Image](image22.png) | ![Image](image23.png) | ![Image](image24.png) | ![Image](image25.png) |
| TrX-100 1%  | ![Image](image26.png) | ![Image](image27.png) | ![Image](image28.png) | ![Image](image29.png) | ![Image](image30.png) |
| TrX-100 2%  | ![Image](image31.png) | ![Image](image32.png) | ![Image](image33.png) | ![Image](image34.png) | ![Image](image35.png) |
3.4. *In vitro enzymatic degradation*

According to the obtained results most rapidly are degraded the RDOT and samples treated with 0.5%TrX-100 (HiMedia, India) reaching the peak of the maximum amino acid concentration at 7th day, followed by samples treated with 1% and 2% TrX-100, which at 14th day of enzymatic degradation did not reached to maximum concentration of amino acids in solution. Samples decellularized with SDS showed slower enzymatic degradation. The amount of free amino acids is indirectly correlated with the increase SDS concentration, the higher was the concentration, the lower were the amount of amino acids; the lowest values have been obtained for samples treated with 2% SDS and the highest in 0.5%SDS. The last one at 14th day presented approximately the same amount of amino acids as samples treated with 0.5% TrX-100 after 2 hours of enzymatic degradation, see Figure 3.

3.5. *Swelling degree*

Samples treated with Triton X-100 have a higher liquid absorption, by comparison to RDOT and those treated with SDS, but no strict correlation was found between samples. However, the samples that have been treated with detergents for 24 hours had a higher degree of swelling compared to those with longer exposure duration, even at a lower concentration of detergents. The highest swelling degree was registered for the samples decellularized with 2% TrX-100, which is 659.72% ±31.82 (Table 3).
Table 3. Swelling degree test results.

| Sample type      | Swelling degree (%) |
|------------------|---------------------|
| RDOT             | 309.55±56.69        |
| SDS 0.5%         | 365.74±83.72        |
| SDS 1%           | 395.24±35.95        |
| SDS 2%           | 448.48±83.98        |
| TrX-100 0.5%     | 453.85±50.44        |
| TrX-100 1%       | 423.02±46.19        |
| TrX-100 2%       | 659.72±31.82        |

Figure 3. Graphic representation of the samples enzymatic degradation.

3.6. Elasticity modulus

After elasticity modulus evaluation, no significant differences were found between RDOT and RDDOT samples.

Table 4. Elasticity modulus appreciation results.

| Sample type      | E(N/m²)       |
|------------------|---------------|
| RDOT             | 175.82±83.72  |
| SDS 0.5%         | 295.63±30.75  |
| SDS 1%           | 252.19±61.99  |
| SDS 2%           | 135.78±63.10  |
| TrX-100 0.5%     | 175.39±114.13 |
| TrX-100 1%       | 295.92±95.44  |
| TrX-100 2%       | 142.62±43.70  |

The samples show different mechanical resistance results; as a result they are incomparable, see Table 4. The cause of incomparable results is different demineralisation degree of subchondral bone, the different cartilage thickness and different compaction degree of the bone.

The osteochondral demineralized, decellularized and perforated graft was designed to combine the transplantation of osteochondral allogeneic or xenogeneic decellularized tissue with the recipient's own cells at the level of cartilage defect, similar to cartilage transplantation [2]. Separate utilisation of
different detergents, in different concentrations and for different time periods, allowed to evaluate their effects on osteochondral tissue. Various combinations of chemicals are described in the literature that being used in decellularization of various tissues and organs. Because articular cartilage is a dense tissue [6-9, 29], it requires a long decellularization period, as a result it was decellularized only after mincing [25-27]. Use of 0.6 M HCl in order to decalciﬁe the subchondral also reduced signiﬁcantly the amount of DNA in tissue for 83% without being minced. According to the literature the HCl utilisation followed by pepsin treatment effectively removes DNA from cartilage with reduction of glycosaminoglycan (GAG) content, preserving the collagen structure [29]. Also, it is reported that Triton X-100 shows a lower loss of GAG in the decellularization of biological tissues comparatively with SDS [24, 30, 32]. Obtained very good results in the cell viability test at chondrocytes utilisation denotes the presence of favorable conditions for their activity. At the same time, cell population test demonstrated that both SDS and Triton X-100 RDDOT ensures a high level of cellular multiplication at 14th day, even if at 7th day the samples were densely populated with chondrocytes.

4. Conclusions
The scaffolds for osteochondral tissue regeneration could be obtained by decellularization of RDOT using SDS or Triton X-100 tensoactives of various concentrations and different periods of treatment. The best results for in vivo testing had the RDDOT treated with 2% Triton-X 100 for 24 hours.

Cytotoxicity tests with MSC and chondrocytes have shown good results and a strong correlation of the cell viability with the duration of treatment was registered. The same trend was observed in the cells population test after 7 days of culturing.

Samples enzymatic degradation differs depending on the type of tensioactif: the SDS-treated samples exhibiting a slower degradation compared to Triton X-100. This can be explained by stiffening the protein molecule of C-terminal portion by binding calcium molecules.

The swelling test and elasticity modulus measurements did not show values dependent of the surfactant nature.

5. References
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