

# ***In vivo* Evaluation of a Collagen Scaffold Preconditioned with Adipose-derived Mesenchymal Stem Cells Used for Bone Regeneration**

## **A histological study**

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*The use of collagen scaffolds and stem cells for obtaining a tissue-engineering complex has been an important concept in promoting repair and regeneration of the bone tissue. Such units represent important steps in the development of an ideal scaffold-cell complex that would sustain new bone apposition. The aim of our study was to perform a histologic evaluation of the healing of critical-sized bone defects, using a biologic collagen scaffold with adipose-derived mesenchymal stem cells, in comparison to negative controls created in the adjacent bone. We used 16 Wistar rats and according to the study design 2 calvarial bone defects were created in each animal, one was filled with collagen seeded with adipose-derived stem cells and the other one was considered negative control. During the following month, at weekly intervals, the animals were euthanized and the specimens from bone defects were histologically evaluated. The results showed that these scaffolds were highly biocompatible as only moderate inflammation no rejection reactions were observed. Furthermore, the first signs of osseous healing appeared after two weeks accompanied by angiogenesis. Collagen scaffolds seeded with adipose-derived mesenchymal stem cells can be considered a promising treatment option in bone regeneration of large defects.*

**Keywords:** collagen scaffold, mesenchymal stem cells, osteogenic differentiation, bone regeneration

Bone grafts have been used in order to stimulate new bone formation after fractures, trauma, infections and other diseases, around joint implants and systems used to maintain the bone alignment (plates and screws); their role was to promote the healing process and to assist bone regeneration. The most important aspects used to evaluate the efficiency of a bone autograft, allograft or substitutes are represented by their osteogenic, osteoinductive and osteoconductive properties. The first is based on the osteoblasts and mesenchymal stem cells, which mediate bone formation. The second refers to a scaffold or matrix that promotes the cell growth on the bone surface, while the third represents the stimulation of mesenchymal stem cells to differentiate into pre-osteoblasts and to start bone formation. [1,2] The autograft is considered to be the gold standard bone graft; it contains both osteogenic cells and an osteoconductive mineralized extracellular matrix, which acts as a support on which these cells can grow and proliferate. Its disadvantage is related to the need to be transplanted from the iliac crest, proximal tibia or distal femur, which limits their use and raise the risk of complications as pain, scarring or blood loss. [3] The use of bone allografts is another option but they lack the osteogenic capacity and have a high risk of infection or immune rejection reactions [4]. Bone tissue engineering techniques developed bone grafts substitutes aiming to obtain improved properties by incorporating growth factors and bone progenitor cells into a matrix that reproduces the

bone environment. The components of the extracellular matrix were a starting point in developing scaffolds based on natural materials and therefore, collagen scaffolds were widely used for culturing a large variety of stem cells for tissue engineering applications. [5,6] The most known and studied is type II collagen, which has a molecular mass of 1461.64 g/mol and a molecular formula  $C_{65}H_{102}N_{18}O_{21}$ . (fig. 1) Type I collagen fibril is always found as a group of triple-helical chains twisted together to form a collagen aggregate of 290 nm long and 1.5 nm diameter (fig. 2).

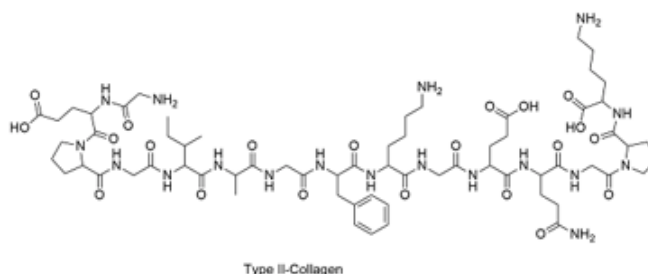


Fig.1. Chemical structure of type II collagen molecule [7]

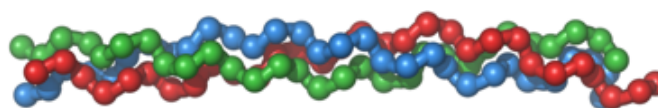


Fig.2. Representation of type I collagen fibril [8]

Designing bone scaffolds requires a thorough understanding of bone biology and physiology; bone can

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be considered a material composed of an extracellular matrix, osteogenic cells, growth factors and hydroxyapatite, which has a complex vascular system [9-11]. The cells represent up to 10% of its volume and include osteoprogenitor cells with mesenchymal origin (osteoblasts and osteocytes) and bone resorbing cells (osteoclasts) with a hematopoietic origin [12]. The extracellular matrix is composed of collagenous and non-collagenous proteins, including glycoproteins and proteoglycans. The collagens represent 90% of the extracellular matrix proteins, mainly collagen type I (97%) and reduced quantities of types III, V, XI and XIII. The non-collagenous proteins form only 10% of the bone mass. [13] The glycoproteins are represented by alkaline phosphatase, bone sialoprotein, osteopontin and osteocalcin (which modulate the mineralization process) and osteonectin (determines the diameter of collagen fibrils) [14,15]. The proteoglycans are up to 10% of the non-collagenous proteins and offer resistance to compressive forces; they also have the capacity to bond to growth factors that can be stored in bone extracellular matrix for future use [7]. The combination of collagen scaffolds with mesenchymal stem cells to enhance bone regeneration was based on the potential of these cells to differentiate into a specific cellular type in response to a certain signal and on their need to rely on an extracellular matrix in order to survive and develop.

Up to date, a variety of collagen scaffolds for bone regeneration have been studied, but there is still a lack of in vivo experiments to validate the utility of these biomaterials. Therefore, we designed a histological study applied to an experimental animal model, in order to evaluate the healing process of critical-sized calvarial bone defects, using a biologic collagen scaffold seeded with adipose-derived mesenchymal stem cells, in comparison to negative controls created in the adjacent bone.

## Experimental part

### *Material and methods*

This experimental study began after obtaining the approval from the Ethics Committee of our university, based on decision Nr. 137/10.11.2016. We used 16 male Wistar rats weighting between 550-600 grams. The animals were kept in the animal facility prior to the experiment, with unrestricted access to water and food. Based on the study protocol, in each animal two bone defects located in the parietal bones were made, the ones located on the right side being the study lesion and the left ones being used as negative controls. In this way we could obtain a sufficient number of defects by using a relatively small number of animals. The method used in our experiment developed based on the following phases: prelevation of adipose tissue, isolation and cultivation of the autologous stem cells, seeding of the cells on the collagen scaffold and subsequent implantation in the bone defect, prelevation of specimens for histological examination.

### *Harvesting of the adipose-derived stem cells*

After anesthesia, fragments of 1 cm<sup>3</sup> of well vascularized subcutaneous tissue from the dorsal aspect of the interscapular region were prelevated. These were introduced into sterile culture medium, using 100 mL solution containing 50 mL DMEM (Dulbecco Modified Eagle Medium) + 10% FBS (Fetal Bovine Serum) + 2% antibiotic/antifungal substance and stored in this environment for 30 min. The donor sites were closed with sutures and the animals received an intramuscular analgesic substance for pain control. The tissue specimens were sliced into 1

mm<sup>3</sup> fragments and placed in collagenase type I solution, filtered to eliminate the detritus and centrifuged for 5 min at 1000 rpm. Then, the fragments were placed in ammonium chloride for removing red blood cells, centrifuged for 5 min at 1000 rpm and placed into flasks at 37°C in an atmosphere with 5% CO<sub>2</sub>.

### *Collagen scaffold engineering*

Fresh bovine pericardial sacs were cleaned in sterile saline solution and the tissue strips obtained were rinsed in double-distilled water at 4°C for 12 h. In this way decellularization by inducing a hypotonic shock was obtained. The tissue fragments were washed and treated for 6 days at 22°C with 0.25% sodium-deoxycholate, 0.1% ethylenediamine-tetraacetic acid (EDTA), 0.02% sodium azide and hydrochloric acid buffered at a pH of 7.8. The fragments were rinsed with double-distilled water and introduced in 70% ethanol for cleaning out detergents. In order to remove nucleic acids, the specimens were treated with deoxyribonuclease/ribonuclease mixture with a concentration of 360 mU/mL at 37°C for 24 h. The strips were rinsed in double-distilled water and incubated in ultrapure elastase 10U/mL, hydrochloric acid buffered at pH 7.8 and 0.02% calcium chloride at pH 8 for 6 days at 37°C using mild agitation. After 3 days the elastase was replaced with a new solution. Scaffolds were rinsed with 70% ethanol and kept in a solution of sterile saline with 0.02% sodium azide at 4°C. This method was described by Tedder et al. [16]

### *The seeding of collagen matrix with stem cells*

The tissue strips were cut into circular fragments of 5 mm diameter and introduced in sterile recipients containing 700 µL nutritive solution with 500.000 cells. After 24 h the scaffolds were turned on the other side and immersed in 700 µL fresh solution containing 700.000 cells. On the third day the membranes were implanted in the bone defects.

### *Surgical protocol for calvarial bone defects*

The animals were anesthetized with a mixture of ketamine 80mg/kg and xylazine 10 mg/kg and kept in these conditions during the entire procedure. The implantation sites were shaved and betadine was used as local antiseptic. The sites were further isolated using sterile drapes. With an aseptic technique and sterile instruments, a cranial incision was made along the midline, in an anterior-posterior direction. The subcutaneous tissue and periosteum were removed in order to expose the calvaria. With a sterile round trephine bur of 4.5 mm diameter two full-thickness defects were made in the parietal bones. In order to avoid overheating the bur was often cooled with saline solution. During the procedure, we were careful not to induce lesions to dura mater, which is known as an important condition for the re-ossification of the defect. In each case the right side defect was filled with a collagen scaffold seeded with stem cells and the left side defect was used as negative control. The periosteum and skin were repositioned and closed with sutures and for one week the animals were monitored regarding wound healing, clinical signs of infection and food intake. After 1, 2, 3 and 4 weeks the animals were euthanized by intraperitoneal administration of ½ doses of ketamine and xylazine previously used for anesthesia followed by intracardiac injection with 0.1 mL of T61 (embutramide 200mg, chlorhidric tetracaine 5 mg, mebezonium iodide 50 mg). The calvarial defects with surrounding tissue were harvested and sent for histologic examination.



### Histologic protocol

The histologic protocol was based on: fixation in 10% formaldehyde solution for 3 days, decalcification in EDTA 14% for 2 weeks, dehydration in increasing concentrations of ethanol and embedded in paraffin. After sectioning into 5 microns thick slices, the specimens were stained with hematoxylin and eosin and examined under a microscope (Olympus BX50, Olympus Japan) connected to a CCD camera. Each specimen was evaluated by an experienced specialist.

### Results and discussions

The animals remained healthy throughout the experimental period, showing no signs of toxicity or adverse reactions. The results obtained after histological examination are presented in figures 3-10.

The presence of collagen fibers and stem-like cells can be regarded as early signs of healing of the bone defects. With no proof regarding the type of the new cells that were formed, we could not confirm the apposition of new osseous tissue.

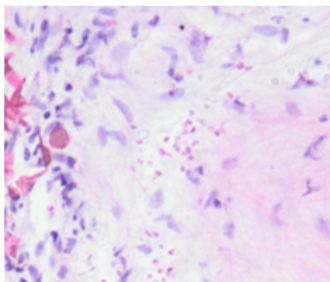


Fig.3. In the control group, after 1 week, the histological evaluation showed the presence of granulation tissue in various evolute stages and the bone defect partially surrounded by adult osseous tissue. This is bordered by adipose cells and loose connective tissue showing moderate inflammation. (H&E stain)

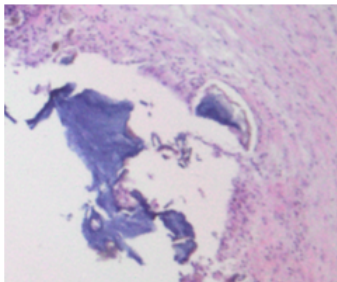


Fig. 5. The histologic examination of the control sites performed at 2 weeks showed little differences compared to the previous week. Oriented connective fibers were present at the periphery of the defect. (H&E stain)

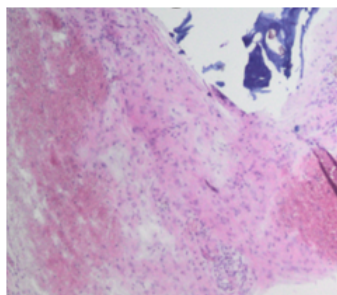


Fig.7. Control sites at 3 weeks evidenced the first signs of healing, due to the presence of collagen fibers and fibroblasts, with minimal inflammation. (H&E stain)

The reconstruction of large bone defects, occurring usually after traumatic injuries or surgical excisions is unable to heal spontaneously, especially in older patients. The need to improve the treatment options of these cases led to the development of new biomaterials resembling bone structure, represented by biologic scaffolds seeded with stem cells. The adipose-derived stem cells were considered to be a good candidate for autologous transplantation, given their properties and large availability. One of the basic conditions for bone tissue regeneration is the use of a scaffold with a good tissue biocompatibility and suitable biodegradable profile, which would provide the framework for cell growth, differentiation and attachment [17,18].

Collagen is a major component of the extracellular matrix which has been extensively used for constructive remodeling, in order to enhance cell growth and differentiation. Its widespread use in clinical applications is due to the bioinductive, mechanical and degradable characteristics. Collagen scaffolds can sustain physio-

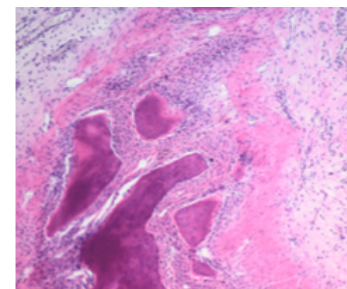
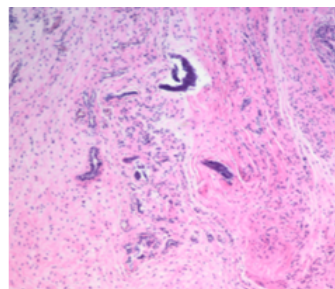


Fig. 4 A After 1 week, the defects filled with collagen scaffold and stem cells were partially bordered by adult bone tissue and a heterogeneous granulation tissue in different evolute stages was also noted. B. The pericardial scaffold showed the characteristic aspect of an inflammatory reaction, due to the presence of microcytes and histiocytes. The lack of necrosis confirms the absence of rejection process. On the periphery numerous cells with stem-like morphology were noticed (stem cells). (H&E stain)

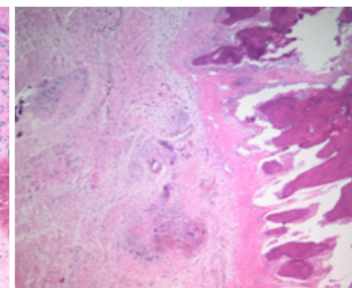
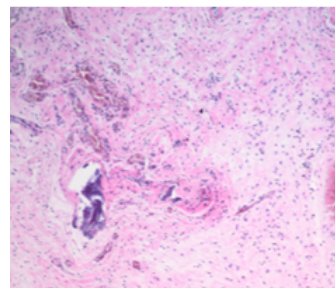


Fig. 6 A. After 2 weeks, the defects filled with collagen scaffold and stem cells were surrounded by adult osseous tissue. There is a minimal inflammatory reaction in the implant zone without signs of infection or rejection. A numerous population of mesenchymal cells can be observed. B. Between the implant and the bone tissue, young fibrous tissue with myxoid morphology is present. (H&E stain)

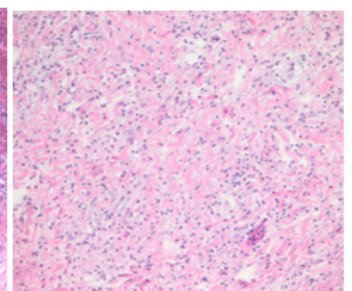
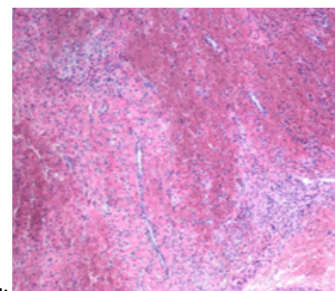


Fig. 8 A. In the study group, at 3 weeks, stem-like cells were observed at the periphery of the defect. Fibrous myxoid tissue is present at the interface implant-bone tissue. B. Fibrous tissue with signs of angiogenesis. (H&E stain)

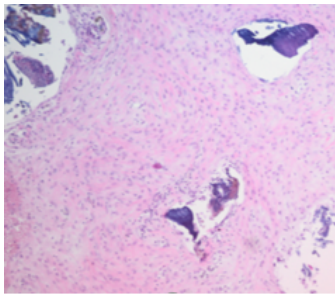


Fig. 9. In the fourth week, the control sites showed the continuation of the healing process: the presence of collagen fibers and small blood vessels, with a persistent minimal inflammatory reaction. (H&E stain)

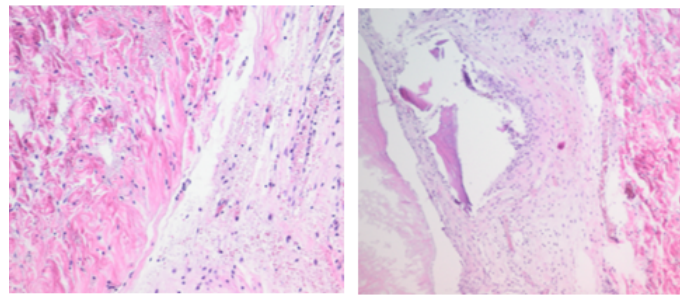


Fig.10 A. In the study group, at four weeks we noticed the presence of an oriented fibrous tissue with blood vessels and stem cells. B. The collagen matrix is still present with signs of minimal resorption, which shows its proper morphologic integration.

logical processes during healing, offering a platform for the development of a functional tissue with vascular ingrowth, which is an absolutely necessary for the survival of the scaffold implanted cells [19]. For bone tissue engineering, the bone marrow derived stem cells were extensively used due to their multi-potency, being considered the most rich and accessible source of human stem cells. However, the harvest of these cells is an invasive procedure and their potential and life span can decrease in older patients. [20,21] On the other hand, adipose tissue had shown to contain an important population of multipotent cells, known as adipose-derived stem cells. These can be easily extracted, differentiate into an osteogenic lineage or can be genetically modified. In addition, it was pointed out that any improvement of scaffolds design must be based on the understanding of the behavior and the response of the cells cultures on these biomaterials [22,23]. In an animal model, Calabrese et al [24] evaluated the capacity of a collagen scaffold seeded with adipose-derived stem cells to induce ectopic bone formation after subcutaneous implantation in rats. Their *in vivo* study, completed with the *ex vivo* histological evaluation, demonstrated that this combination was able to recruit host cells and further stimulate them to osteo- and angiogenesis, thus inducing spontaneous bone augmentation. They also showed that the addition of stem cells to the scaffold prior to implantation improved significantly the mineralization and vascularization of the new bone tissue. Based on these observations, we used the same method in our study, embedding the collagen scaffolds into cell culture before placing them inside the calvarial bone defects.

Murphy et al [25] considered that a limited local inflammation could promote healing and new vascularization, but chronic inflammatory reactions, adverse immune response or their combination could compromise the implant. In our study, the histological examination performed after 2 weeks showed the first signs of healing, accompanied by reduced inflammation and angiogenesis, which is in accordance to previously published data. Under ideal conditions, biodegradation eliminates the need for a second surgical intervention, as the material disappears simultaneously with the formation of the new tissue, resulting in the repair and regeneration of the lost bone tissue.

Studies in mice models suggested that mesenchymal stem cells promote the neovascularization process by stimulating the growth of endothelial cells that will form the inner layer of blood vessels, which was recognized as an important feature in promoting tissue regeneration. The results of our study confirm the observations of Cacciafesta et al [26] who denied spontaneously healing of bone defects over 5 mm diameter in adult rats. Histological data

showed the presence of a new connective tissue preceding osseous apposition at the periphery of the defects. Sun et al [27] used an animal model to compare four types of collagen scaffolds seeded with mesenchymal stem cells and concluded that all materials had a good cytocompatibility and promoted the osteogenic differentiation of rat mesenchymal stem cells. The best results were observed when a scaffold of collagen and hydroxyapatite was used, which was considered to be a promising substitute material in bone tissue engineering.

The future of bone healing and regeneration will certainly be influenced by the advances in tissue engineering techniques; the use of a combination of scaffolds and stem cells might solve the present treatment limitations. Therefore, the development of such constructions will offer promising substitutes for autologous bone grafts, considered for a long period of time the gold standard of large bone defects repair.

## Conclusions

Collagen scaffolds seeded with mesenchymal stem cells proved to sustain the regeneration and revascularization of bone defects, being suitable for plane lesions. The lack of a reject reaction indicates the high biocompatibility of the complex used in our study, which gives us confidence to use the material for further studies on soft tissues.

The development of a collagen scaffold that meets all desired properties, such as biocompatibility, structure stability, conductivity and osteoinductivity to obtain optimal bone regeneration is still a great challenge. These shortcomings can be addressed with the development of bioprinting technology, biomimetic mineralization and gene therapy, for a successful clinical application of collagen scaffolds for bone regeneration.

## References

1. AMINI, A.R., LAURENCIN, C.T., NUKAVARAPU, S.P., Crit.Rev.Biomed.Eng. **40**, nr.2, 2018. doi:10.1615/CritRevBiomedEng.v40i5.10.
2. WILLIAMS, D.F. Biomaterials. **29**, nr.20, 2008, p. 2941
3. POLO-CORALES, L., LATORRE-ESTEVEZ, M., RAMIREZ-VICK, J.E., J Nanosci Nanotechnol. **14**, nr.1, 2014, p.15
4. GRAHAM, S., LEONIDOU, A., ASLAM-PERVEZ, N., HAMZA, A., PANTELADIS, P., HELIOTIS, M. et al., Expert Opin Biol Ther. **10**, 2010, p. 885
5. CATELAS, I., SESEN, W.U., WU, B.M., DUNN, J.C., HELGERSON, S., TAWIL, B., Tissue engineering. **12**, 2006, p.2385
6. CHAN, B.P., HUI, T.Y., YEUNG, C.W., LI, J., MO, J., CHAN, G.C., Biomaterials. **28**, 2007, p. 4652
7. SHOULDERS, M.D., RAINES, R.T., Annu Rev Biochem. **78**, 2009, p. 929
8. \*\*\* <https://commons.wikimedia.org/w/index.php?curid=379964>

9. MINARDI, S., CORRADETTI, B., TARABALLI, F., SANDRI, M., VAN EPS, J., CABRERA, F.J. et al., *Biomaterials*. **62**, 2015, p. 128
10. SUN, F., KANG, H.G., RYU, S.C., KIM, J.E., PARK, E.Y., HWANG, D.Y. et al., *J Biomed Nanotechnol.* **9**, nr.11, 2013, p. 1914
11. STOICA, A.M., STETIU, A.A., BURUIAN, M., STETIU, M., MONEA, M., *J. Optoelectron. Adv. M.* **18**, nr.5-6, 2016, p.562
12. SUNDELACRUZ, S., KAPLAN, D.L., *Semin Cell Dev Biol.* **20**, nr.6, 2009, p.646
13. ARAVAMUDHAN, A., RAMOS, D.M., NIP, J., HARMON, M.D., JAMES, R., DENG, M. et al., *J Biomed Nanotechnol.* **9**, nr.4, 2013, p.719
14. CALABRESE, G., GIUFFRIDA, R., FORTE, S., FABBI, C., FIGALLO, E., SALVATORELLI, L. et al., *Scientific Reports*. 2017, doi:0.1038/41598-017-07672-0.
15. CAZAN, I., SUSANU, S., BOISTEANU, O., TATARCIUC, D., COSTAN, V.V., COSTULEANU, M., *Rev Chim.(Bucharest)*, **69**, no.4, 2018, p.906
16. TEDDER, M.E., LIAO, J., WEED, B., STABLER, C., ZHANG, H., SIMIONESCU, A. et al., *Tissue Eng Part A.* **15**, nr.6, 2009, p.1257
17. NING, L., MALMSTROM, H., REN, Y-DE, *J of Oral Implantol.* 2015, doi:10.1563/AAID-JOI-D-12-00298.
18. NEMTOI, A., DANILA, V., DRAGAN, E., PASCA, S., NEMTOI, A., CONSTANTIN, M. et al., *Rev Chim. (Bucharest)* **68**, no.4, 2017, p.693
19. CHAN, E.C., KUO, S.M., KONG, A.M., MORRISON, W.A., DUSTING, G.J., MITCHELL, G.M. et al., *PLoS ONE.* **11**, nr.2, 2016, :e0149799.
20. SUCIU, B.A., HALMACIU, I., BUD, V., COPOTOIU, C., FODOR, D.R.P., TRIMBITAS, C., VUNVULEA, V., MOLNAR, C., BRINZANIUC, K., *Mat. Plast.* **54**, no.3, 2017, p.520
21. De WITTE, T.M., FRATILA-APACHITEL, L.E., ZADPOOR, A.A., PEPPAS, N.A., *Regenerative Biomaterials.* **9**, nr.5, 2018, p. 197
22. XU, C., SU, P., CHEN, X., MENG, Y., YU, W., XIANG, A.P. et al., *Biomaterials.* **32**, nr.4, 2011, p. 1051
23. SUCIU, B.A., HALMACIU, I., BUD, V., COPOTOIU, C., FODOR, D., TRAMBITAS, C., GODJA, D., VUNVULEA, V., MOLNAR, C., BRINZANIUC, K., *Mat. Plast.* **54**, no.4, 2017, p.626
24. CALABRESE, G., FORTE, S., GULINO, R., CEFALI, F., FIGALLO, E., SALVATORELLI, L. et al., *Front Physiol.* 2017. 8:50, doi:10.3389/fphys.2017.00050.
25. MURPHY, C.M., O'BRIEN, F.J., LITTLE, D.G., SCHINDELER, A., *European Cells and Materials.* **26**, 2013, p.120
26. CACCIAFESTA, V., DALSTRA, M., BOSCH, C., *Tissue Engineering and Regenerative Medicine.* **23**, nr.6, 2001, p.733
27. SUN, X., SU, W., MA, X., ZHANG, H., SUN, Z., LI, X., *Regenerative Biomaterials.* **5**, nr.2, 2018, p.93

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Manuscript received: 3.10.2018