

Tissue engineering of blood vessels using three-dimensional bioprinting of endothelial and smooth muscle progenitor cells

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Phase II report – 2019

Abstract

Although autologous vascular grafts are the gold standard for clinical use, they are not suitable for a large number of patients due to vascular disease, amputations or previous vascular tissue harvesting. Despite the clear clinical needs of vascular substitutes, currently available synthetic grafts have proven to be successful only in large-calibre implant implants; small diameter vascular substitutes (<6 mm) have generally unsatisfactory results, with major negative side effects, such as acute thrombosis, hyperplasia and aneurysm. Therefore, biomimetic reconstruction of blood vessels through tissue engineering remains a difficult task that requires new construction techniques. The project aims to realize bioprinted vascular structures and to investigate the biocompatibility of endothelial and muscle progenitor cells in the presence of collagen and hyaluronic acid hydrogels. The obtained results showed that the cells are viable in the presence of the 3 hydrogels for 21 days. Endothelial and muscle progenitor cells are not subjected to oxidative stress and proliferate better in the presence of hydrogels than when grown in culture plates (2D system). The most intense proliferation was recorded in the hydrogel with 50% hyaluronic acid (H50), both at 7 days and at 21 days, respectively. In vascular constructs the level of VEGF is higher than in 2D system at 7 days. After 21 days of cultivation, the concentration of VEGF in vascular constructs H50 was 0.29 ng/ml, in H25 0.19 ng/ml and in H0 0.21 ng/ml comparable to EPCs cultured in 2D system (0.2 ng/ml). Also, the detection of VEGF in the EPC cultures obtained from AFSC shows that the differentiation towards the endothelial line occurred. The highest amount of nitric oxide was identified in the H50 hydrogel reaching values of 10.7 μ M and 5.65 μ M at 7 days and 21 days respectively. The results show that EPC and SPC express CD44 at 100% and CD49e at 99% for EPC and 94% for SPC. EPC shows increased expression for the CD54 (25%), CD56 (38%), CD105 (95.5%) markers. Also, markers CD73, CD90, and CD146 are expressed by both EPC and SPC in percentages between 70-100%. The hydrogels have a porous structure, forming large pores with a diameter between 40-150 μ M. In the presence of hydrogels, the cells have a fusiform morphology and emit filopods that allow the interaction between cells and with collagen fibres.

Introduction

Cardiovascular diseases are the leading causes of mortality worldwide and require over one million bypass / blood vessel replacements annually in the United States alone [1]. Although autologous vascular grafts are the gold standard for clinical use, they are not suitable for a large number of patients due to vascular disease, amputations or previous vascular tissue harvesting. Despite the clear clinical needs of vascular substitutes, currently available synthetic grafts (for example, Dacron and Teflon) have proven to be successful only in large calibre implants; small diameter vascular substitutes (<6 mm) have generally unsatisfactory results, with major negative side effects, such as acute thrombosis, hyperplasia and aneurysm [2]. Arterial failures are generally attributed to the relatively low blood flow rate in these small vessels, which increases the rate of interactions between cells and molecules in the blood and polymeric implants. Therefore, there are strict requirements regarding the biofunctionality of the small diameter designed grafts. In order to produce grafts with normal vascular functions, at least 2 requirements must be met: (1) there is a confluent endothelium providing a nontrombogenic interface and (2) there is smooth contractile muscle tissue that can withstand haemodynamic stress and local changes in blood pressure that occur through constriction and physiological relaxation of the vessel [3]. Tissue engineering has emerged as a promising alternative approach for viable small diameter arterial grafts. Various strategies including decellularized tissue, synthetic polymeric matrices, self-assembling cell layers and hydrogels have been used to create living vascular structures and replace native blood vessels; these strategies have achieved different levels of success. These approaches generally produce vascular substitutes in the form of a robust tubular matrix containing smooth vascular or fibroblast muscle cells followed by seeding and cultivation of endothelial cells to achieve endothelialisation. This multi-step construction process is slow and requires complicated procedures and long manufacturing periods. In addition, grafting and retention rate of endothelial cells is strongly influenced by seeding mode, surface properties, cell density and culture conditions and, therefore, current methods can hardly be considered reliable for generating an intact functional endothelium. Therefore, biomimetic reconstruction of blood vessels through tissue engineering remains a difficult task that requires new construction techniques [4-7]. An important advantage of 3D bioprinting is the ability to build tissue / organ equivalents with a structure as close as possible to the native one. The precise positioning of cell-loaded bioinks simulates the anatomical features of the target tissue / organ, so that as cells proliferate, migrate and differentiate into a biomimetic construct. In particular, coaxial extrusion technique can use various biomaterials for bioprinting resulting in vessel-like structures [8].

For the successful bioprinting of a functional vessel, an essential factor is the bioink used. It must be biocompatible with the different cell types and promote the functionality of the cells and also allow the direct bioprinting of the vessels. While the hydrogels used to create vessels (fibrin, elastin, collagen, fibroin) generally have poor printability, materials suitable for co-extrusion bioprinting (alginate, gelatine methacryloyl, photopolymerized hyaluronic acid) usually have negative results due to low cellular affinity or poor mechanical properties and the functionality of the tissues is impaired.

The project aims to create vascular structures using collagen and hyaluronic acid hydrogels and endothelial and muscle progenitor cells derived from mesenchymal stem cells isolated from amniotic fluid using 3D bioprinting. Thus, the objectives for 2019 were:

I. "Realization of the bioprinted vascular structure and investigation of the biocompatibility of EPC and SPC in the presence of the used hydrogels". These objectives have the following activities:

I.1 *3D bioprinting of vascular structure and assessment of viability and growth of differentiated progenitor cells;*

I.2 *Research on cell proliferation, oxidative stress and phagocytosis;*

I.3 *Analysis of the secretory profile of EPC and SPC incorporated in the hydrogel.*

II. "Evaluation of the morphology and adhesion capacity of EPC and SPC incorporated in the hydrogel". This objective is accompanied by the following activities:

II.1 *Analysis of the rearrangements of actin filaments and microtubules by immunofluorescence;*

II.2 *Evaluation of cell morphology by scanning / transmission electron microscopy.*

I.1 3D bioprinting of vascular structure and assessment of viability and growth of differentiated progenitor cells.

Computer-aided design (CAD) software (BioCad, RegenHU, Switzerland) was used to create the 3D vascular model. Bioprinting was performed using a needle with a diameter of 0.2 mm, under pressure of 1.2 bar, speed of 1 mm / sec, creating vascular structures with a diameter of 2.5 mm, the thickness and the height of each layer 0.3 mm, followed by polymerization of each layer for 5 seconds (Fig. 1).



Fig. 1. Bioprinted 3D vascular structure using collagen-hyaluronic acid hydrogel and progenitor cells

For bioprinting, 3 hydrogels were used:

- H0, collagen hydrogel containing 2% collagen, RehenHu (Villaz-St-Pierre, Switzerland)
- H25, H0 hydrogel to which 25% hyaluronic acid (MW 1-1.6 MDa, 1%) was added
- H50 H0 hydrogel to which 50% hyaluronic acid (MW 1-1.6 MDa, 1%) was added

Mesenchymal stem cells isolated from amniotic fluid (AFSC) were differentiated into endothelial (EPC) and muscle (SPC) progenitor cells using specific culture media and growth factors.

Endothelial differentiation of AFSC was achieved by cultivation in M200 medium supplemented with 10% FBS (fetal bovine serum), 40 ng / ml vascular endothelial growth factor (VEGF), 20 ng / ml insulin growth factor (IGF-1), 10 ng / ml epidermal growth factor (EGF), 10 ng / ml fibroblast growth factor (bFGF), 100 µg / ml penicillin, 100 µg / ml streptomycin and 50 µg / mL neomycin (Merck, Frankfurter, Germany).

Muscle differentiation was performed in M231 medium supplemented with FGF (2 ng / ml), EGF (0.5 ng / ml), heparin (5 ng / ml), IGF (2 pg / ml) and bovine serum albumin (0.2 pg / ml). The cells were maintained in these environments for 4 weeks and passed to reach the subconfluence. Cell cultures were maintained at 37°C with 5% CO₂ and 21% O₂ in a humidified atmosphere.

Viability assessment was performed using the LDH test (ThermoFischer Scientific, USA). This test measures the amount of LDH (lactate dehydrogenase) in the culture medium. LDH is a cytosolic enzyme and its presence in the culture medium demonstrates that the cells were destroyed and released extracellular LDH. Vascular constructs with EPC and SPC cultured for 21 days in DMEM medium were evaluated to determine cell viability at 7 days and 21 days.

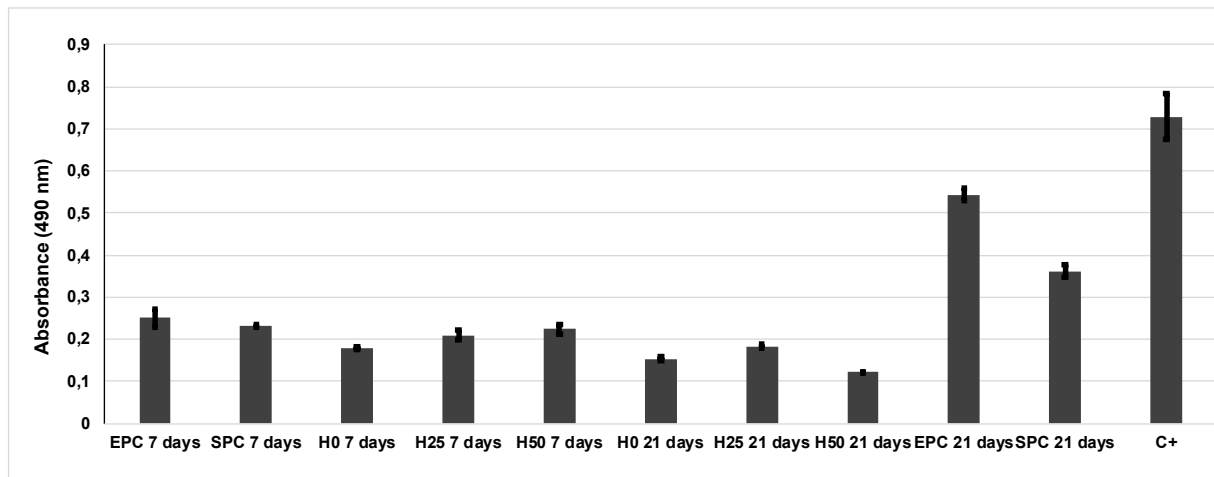


Fig. 2 Assessment of the viability of EPC and SPC in H0, H25 and H50 hydrogels, at 7 and 21 days using LDH assay

The obtained results showed that the cells are viable in the presence of the 3 hydrogels for 21 days. At 21 days EPC and SPC grown in plates without hydrogel (2D system) showed a high level of LDH compared to those in hydrogels, which suggests that collagen-hyaluronic acid hydrogels provide an optimal environment for cell growth (Fig. 2).

I.2 Research on cell proliferation, oxidative stress and phagocytosis

The cellular proliferation of EPC and SPC in hydrogels was investigated using the MTT assay. Based on this quantitative colorimetric method, the proliferation, viability and cytotoxicity of the cell are assessed. The method is based on reducing a yellow MTT tetrazolium salt (3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide) to dark-blue formazan. The reduction achieved by mitochondrial enzymes (especially succinate dehydrogenase) is an indication of cellular / mitochondrial integrity. The insoluble formazan can be solubilized with isopropanol, dimethyl-sulfoxide or other organic solvent. The optical density (DO) of the solubilized formazan is evaluated spectrophotometrically, obtaining a function absorbance-concentration dye-number of metabolically active cells in culture. Vascular constructions grown for 7 days and 21 days, were treated with 15 μ l 12 mM MTT and incubated at 37°C for 4 hours. Subsequently, 100 μ l SDS-HCl solution was added, and energetically pipetted to solubilize formazan crystals, followed by one-hour incubation. Then pipetted for homogenization and remove all the bubbles to not interfere with reading. The reading was performed at the spectrophotometer at 570 nm (TECAN, Männedorf, Switzerland).

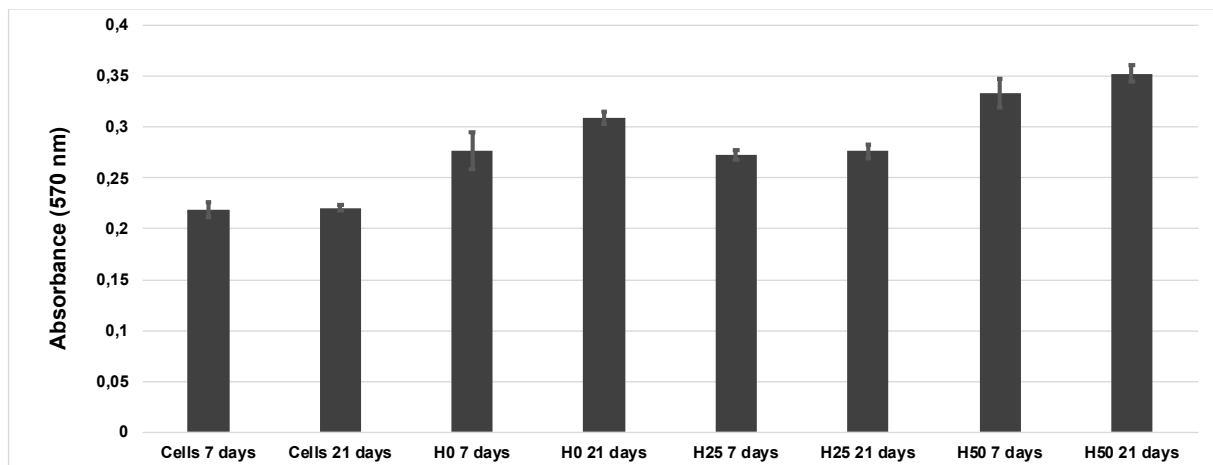


Fig. 3 Evaluation of EPC and SPC proliferation in H0, H25 and H50 hydrogels, at 7days and 21 days using the MTT assay

The results showed that the cells proliferate better in the presence of hydrogels than when grown in culture plates (2D system). In the hydrogel with 50% hyaluronic acid (H50) the most intense proliferation was recorded, both at 7 days and at 21 days. The H0 hydrogel caused an increase in proliferation by 28% at 7 days and 36% at 21 days compared to cells grown in 2D system. The H25 hydrogel allowed cell proliferation for 21 days, resulting in an increase in proliferation by 28% at 7 days and 22% at 21 days compared to cells grown in the 2D system. Hydrogel with 50% H50 hyaluronic acid caused a proliferation of over 57% at 7 days and 59% at 21 days compared with 2D control (Fig. 3).

Oxidative stress assessment was performed using the GHS assay. This kit measures the amount of glutathione, an antioxidant agent. The glutathione produced by the cells is transformed by glutathione S-transferase into oxidized glutathione, the amount of glutathione transformed being directly proportional to the amount of glutathione S-transferase enzyme that transforms glutathione linked with a luciferin precursor into oxidized glutathione linked to luciferin which emits light. The more intense the light, the more glutathione was transformed, so more glutathione was synthesized, so the cell was more stressed. Oxidative stress was assessed using the Glutathione Fluorescent Detection Kit (Invitrogen, ThermoFischer Scientific, USA).

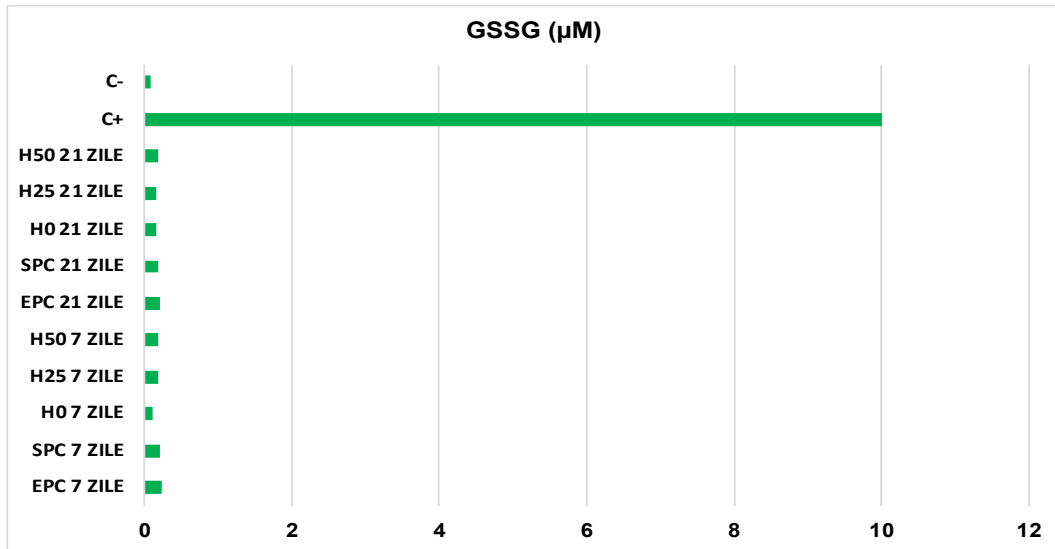


Fig. 4 Assessment of the oxidative stress of EPC and SPC in H0, H25 and H50 hydrogels, at 7 days and 21 days using GSH assay

The results showed that EPC and SPC grown in vascular constructions for 7 days and 21 days are not subjected to major oxidative stress. The highest concentration of oxidized glutathione (GSSG) was in cells grown in 2D system ($0.22 \mu\text{M}$ for EPC and $0.21 \mu\text{M}$ for SPC). For the H0 hydrogel the GSSG values were $0.11 \mu\text{M}$ and $0.16 \mu\text{M}$ at 7 days and 21 days respectively. The H25 hydrogel recorded GSSG values of $0.18 \mu\text{M}$ and $0.16 \mu\text{M}$, and in the H50 hydrogel the GSSG level was $0.18 \mu\text{M}$ at 7 days and $0.17 \mu\text{M}$ at 21 days from cultivation (Fig. 4).

I.3 Analysis of the secretory profile of EPC and SPC incorporated in the hydrogel

In order to demonstrate the ability of EPC and SPC to participate in vascularization, the production of VEGF and nitric oxide in the hydrogels was determined. The production of VEGF was quantified by the ELISA technique and that of nitric oxide using the Griess test.

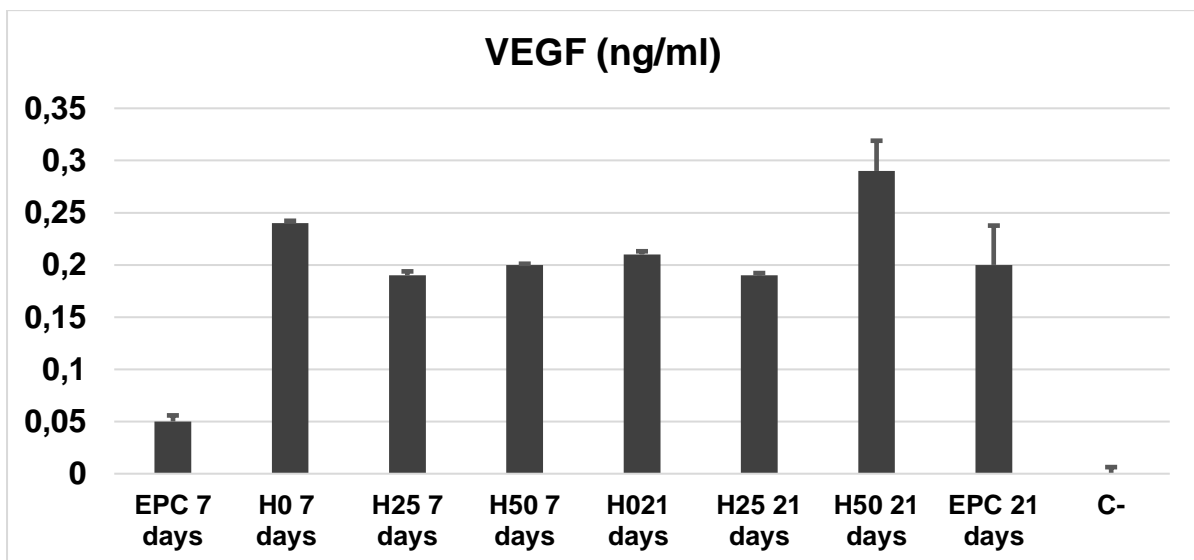


Fig. 5 VEGF concentration (ng/ml) in vascular constructions H0, H25 and H50, at 7 days and 21 days using ELISA assay

The results showed that in vascular constructions the level of VEGF is higher than in 2D system at 7 days. After 21 days of cultivation, the concentration of VEGF in vascular constructs H50 was 0.29 ng / ml, in H25 0.19 ng / ml and in H0 0.21 ng / ml comparable to EPCs raised in 2D system (0.2 ng / ml). Also, the detection of VEGF in the EPC cultures obtained from AFSC shows that the differentiation towards the endothelial line occurred (Fig. 5).

Nitric oxide (NO) is a molecular mediator of many physiological processes, including vasodilation, inflammation, thrombosis, immunity and neurotransmission. There are a number of methods for measuring NO in biological systems. One of these methods involves the use of the Griess diazotization reaction to detect spectrophotometrically the nitrite formed by the spontaneous oxidation of NO under physiological conditions. Detection and quantification of nitric oxide was performed with the Griess Reagent kit (Molecular Probe, ThermoFischer Scientific, USA). The sulphanilic acid is converted into a diazonium salt by reaction with nitrite in acid solution. The diazonium salt is then coupled to N-(1-naphthyl)-ethylenediamine, forming an azo dye that can be spectrophotometrically quantified based on its absorption at 548 nm.

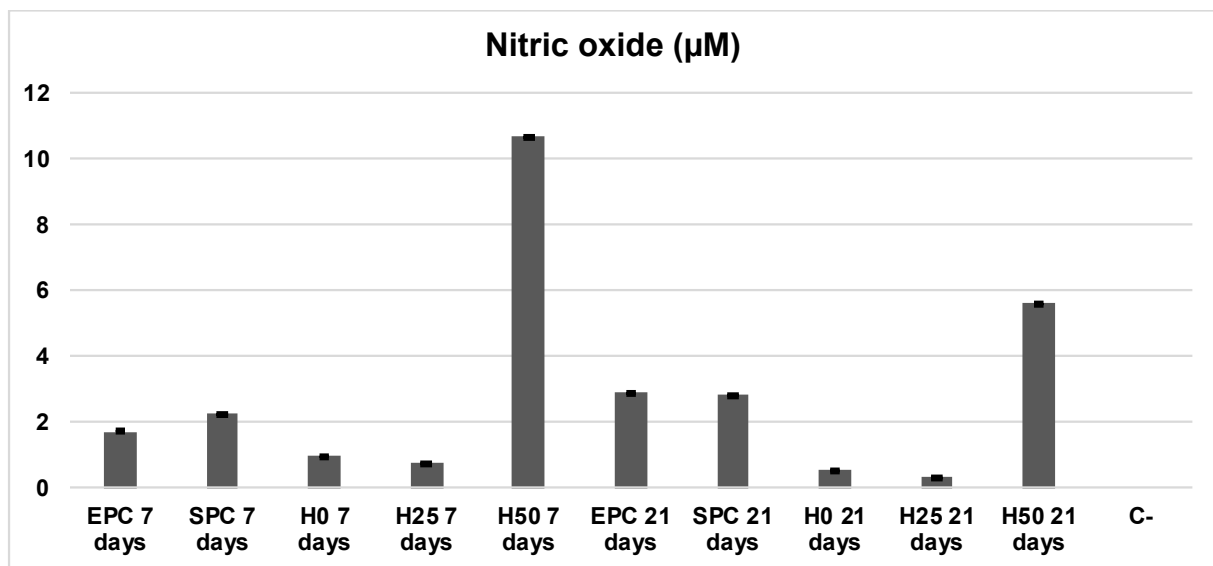


Fig. 6 Assessment of nitric oxide level in H0, H25 and H50 hydrogels, at 7 days and 21 days using the Griess test

Nitric oxide is synthesized by endothelial cells and plays a key role in maintaining vasorelaxation by exerting effects on smooth vascular muscle cells, whose contractile state defines vascular tone. NO produced in the cytosol of endothelial cells diffuses rapidly into adjacent smooth muscle cells, exerting paracrine effects by activating soluble guanylyl-cyclase to increase the synthesis of cyclic 3,5-guanosine monophosphate (cGMP) [9].

NO also decreases cytosolic Ca^{2+} concentration by inhibiting voltage-activated Ca^{2+} channels and activates protein kinases that phosphorylate proteins in the sarcoplasmic reticulum as well as Ca^{2+} -dependent potassium channels. Reduction of cytosolic Ca^{2+} concentration leads to inhibition of the calcium-calmodulin- light chains myosin-kinase complex in smooth muscle vascular cells, favouring vasorelaxation [10].

The obtained results showed that the differentiated cells from AFSC are endothelial progenitor cells because they express VEGF and nitric oxide. The highest amount of NO was identified in the H50 hydrogel reaching values of 10.7 μM and 5.65 μM at 7 days and 21 days respectively. (Fig. 6). EPCs grown in 2D system showed a NO level of 1.74 μM and 2.93 μM at 7 days and 21 days of cultivation (Fig. 6).

II.1 Analysis of the rearrangements of actin filaments and microtubules by immunofluorescence

To create a vascular structure, communication between different cell types is required. These interactions are accomplished through cell junctions, the extracellular matrix and the cytoskeleton. The adhesion of cells occurs through the interactions between the adhesion molecules such as CAMs ("cell adhesion molecules": integrins, selectins, cadherins), the transmembrane proteins located on the surface of the cell and the actin filaments, intermediate filaments and microtubules. Through these cell adhesion processes, cell migration and tissue formation are controlled [11,12]. Integrins are transmembrane receptors that facilitate communication between cells and the extracellular matrix (ECM). When ligand binds to integrins activate signal transmission pathways that mediate a number of cellular processes, such as cell cycle regulation, cytoskeleton organization, and the movement of new receptors to the cell membrane. Integrin ligands include fibronectin, vitronectin, collagen and laminin. Together with the signals from the receptors for soluble growth factors, such as VEGF, EGF, integrins contribute to cellular decisions on biological actions, such as adhesion, movement, death or differentiation. Cell attachment occurs through the formation of cell adhesion complexes, which consist of integrins and many cytoplasmic proteins, such as talin, vinculin, paxillin and act-actinin [13]. Cadherins are Ca^{2+} -dependent homophilic glycoproteins. Classical cadherins (endothelial, neural, and platelet) are concentrated at the level of cellular junctions, and they bind to the actin filament network through specific binding proteins called catenins. Selectins are a family of heterophilic proteins that are dependent on fucosylated carbohydrates for binding.

The three members of the family are E-selectin (endothelial), L-selectin (leukocyte) and P-selectin (thrombocyte). Selectins have many roles, but particularly important are those in the immune system, helping in the trafficking and adhesion of leukocytes [14].

Flow cytometry using fluorescent labelled specific antibodies (Gallios, Beckman-Coulter) was used to measure adhesion molecules and cellular interactions. Cells (1×10^5 cells / marker) were labelled with fluorochrome-conjugated primary antibodies (FITC - Fluorescein-isothiocyanate and PE - Phycoerythrin) for CD11b (α_M integrin), CD29 (β_1 integrin), CD31 (PECAM-1), CD44 (HCAM), CD49e (α_5 integrin), CD54 (ICAM-1), CD56 (NCAM), CD73, CD90 (Thy-1), CD105 (endoglin), CD106 (VCAM-1), CD117 (kit-c), CD133 (prominin), CD146 (MCAM), VEGFR1, FOS ALK, and HLA-ABC (Beckman-Coulter). AFSC, EPC and SPC were detached using acutase and washed in phosphate saline (PBS). The cells were then incubated with primary antibodies at room temperature and in the dark for 30 minutes. Further, the cells were washed and centrifuged at 400g, 10 min, in PBS with 1% BSA. For negative controls, cells were stained with isotype IgG antibodies (Beckman-Coulter). Flow cytometry data were analysed using Gallios software (Beckman-Coulter).

Table 1. Results obtained from AFSC and progenitor cells immunophenotyping by flow cytometry

No	MARKER	AFSC	EPC	SPC
1.	CD11b PE (integrin α_M)	4.2%	1.3%	0.08%
2.	CD29 PE (integrin β_1)	28%	4%	0.2%
3.	CD31 FITC (PECAM-1)	6.5%	1.3%	0.45%
4.	CD44 FITC (HCAM)	100%	100%	100%
5.	CD49e PE (integrin α_5)	100%	99%	94%
6.	CD54 FITC (ICAM-1)	12.5%	25%	5.7%
7.	CD56 PE (NCAM)	13%	38%	1.8%
8.	CD73 FITC	99%	90%	98.5%
9.	CD90 FITC (Thy-1)	97%	95%	100%
10.	CD105 FITC (endoglin)	95.5%	2.5%	1.7%
11.	CD106 PE (VCAM-1)	0%	1.5%	0.14%
12.	CD117 PE (c-kit)	5%	1.7%	0%
13.	CD133 APC (prominin)	5%	1%	0.29%
14.	CD146 PE (MCAM)	98%	70%	2.2%
15.	VEGFR1 PE	7%	3.5%	1.6%
16.	FOS ALK PE	7.5%	3%	3.5%
17.	HLA-ABC PE	100%	1.3%	100%

The results showed that EPC and SPC express CD44 (HCAM) at 100% and CD49e ($\alpha 5$ integrin) at 99% for EPC and 94% for SPC. EPC shows increased expression for the CD54 (25%), CD56 (38%), CD105 (95.5%) markers. Also, markers CD73, CD90, and CD146 are expressed by both EPC and SPC in percentages between 70-100% (Table 1).

II.2 Evaluation of cell morphology by scanning / transmission electron microscopy

Scanning electron microscopy (SEM) was used to observe the behaviour of EPC and SPC in collagen and hyaluronic acid hydrogels. EPC and SPC were homogenized in H0, H25 and H50 hydrogels and bioprinted according to the blood vessel model created in BioCAD. At 7 days and 21 days, constructions were analysed by SEM. They were washed with PBS and fixed in 2.5% glutaraldehyde for 1 hour at room temperature. Then glutaraldehyde was removed and successive dehydration with ethanol (40%, 60%, 70%, 80%, 90% and 100%) was performed for 10 minutes each. The SEM analysis was performed with a HITACHI S2600N electron microscope, at 25 keV, on samples coated with a thin layer of silver.

The results showed that the cells adhere and distribute evenly in the collagen and hyaluronic acid hydrogels, which demonstrates the biocompatibility of these hydrogels.

The H0 hydrogel has a porous structure, forms large pores with a diameter between 80-150 μ M, H25 has smaller pores, between 40-120 μ M and H50 between 80-150 μ M. In the presence of the H0 hydrogel, the cells have a fusiform morphology and emit filopods that allow the cells to interact with each other and with the collagen fibres. The H25 and H50 hydrogels allow a good growth of cells, forming a rich extracellular matrix which allows a good adhesion and the possibility of organizing in a tissue (Fig. 7).

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