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Re-Endothelialized Small Diameter Vascular Grafts Derived from Human Umbilical Arteries as Coronary Artery Substitutes

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Abstract

Background: Primary therapeutic strategy in Cardiovascular Disease (CVD) is the coronary artery bypass. Damaged coronary vessels need to be replaced with small diameter vascular grafts. Tissue engineering has focused on the production of vascular grafts, although major adverse reactions such as graft calcification, low patency rate and lumen occlusion still exist. This study aimed to the development of re-endothelialized small diameter vascular grafts in order to avoid the aforementioned adverse reactions.

Methods: Human umbilical arteries were isolated and decellularized. The evaluation of the decellularization process was assessed by histological analysis. In addition, differentiated Endothelial Cells (ECs) derived from Wharton's Jelly Mesenchymal Stromal Cells (WJ-MSCs) were used for the re-endothelialization process.

Results: Our results indicated the successful decellularization of the vessels. Specifically, human Umbilical Arteries (hUAs) were characterized by absence of cellular and nuclear materials, while their extracellular matrix retained intact. Differentiated ECs successfully expressed *CD31, VEGF-A, VEGF-R, vWF, FLK-1* and were able to form capillary like structures when cultured in Matrigel. Finally, the re-endothelialization of the decellularized hUAs with the differentiated ECs was successful based on the results of indirect immunofluorescence.

Conclusion: In conclusion, the hUAs is a very promising source for the production of small diameter vascular grafts that could be used as coronary artery substitutes.

Keywords: Human umbilical arteries; Decellularization; Vascular graft; Cardiovascular disease

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Introduction

The use of small diameter vascular grafts (<6 mm) is considered the primary therapeutic strategy for CVD. It is estimated that over of 15 million of patients are suffering from CVD, thus demanding surgical operation [1,2]. Until now, several vessels have been used as substitutes of damaged coronary arteries. Among them autologous vessels such as saphenous vein and fabricated synthetic vascular grafts made of Dacron and expanded polytetrafluoroethylene (ePTFE) have been applied in cardiovascular surgeries [2,3]. However, these vascular grafts are accompanied by major adverse reactions, such as thrombus formation, graft calcification and occlusion. In this way, new surgical operation is needed for their replacement. Additionally, only 20% of patients who are suffering from CVD have suitable autologous vessels that can be used in coronary artery bypass [4].

This widely demand of suitable vascular grafts could be overpassed by the use of tissue engineered small diameter vascular grafts [5]. Since the first established report in 1954, regarding the development of functional vessels, major achievements have been performed towards this direction [6]. A great number of studies have focused in successful production of small diameter

vascular grafts by lowering risk factors such as degeneration, infection and immunogenicity [7]. On the other hand, thrombogenicity of decellularized and fabricated vascular grafts is of major importance and still demands considerable attention.

Decellularization approach aims to eliminate the vascular populations such as ECs and vascular smooth muscle cells (VSMCs), without affecting the vessel's ultrastructure. Due to ECs loss, the decellularized vessels are characterized by exposed collagen in their vascular wall, thus increasing the thrombogenicity of the graft. Several groups have tried to modify the exposed vascular wall with the use of chemical anti-thrombogenic factors such as heparin sulfate in order to avoid the platelet aggregation and thrombus formation [8,9]. On the other hand, chemically anti-thrombogenic methods reduce significantly the *in vivo* remodeling properties of the vessels in a similar way as the glutaraldehyde fixed vascular grafts.

Under this scope, we proposed the re-endothelialization of the decellularized vascular grafts, thus avoiding platelet aggregation and thrombus formation. ECs can be isolated from various sources such as peripheral or cord blood and arterial biopsy. However, successful re-endothelialization demands approximately 5×10^5 cells/cm^2, indicating that these numbers are quite difficult to be achieved with the previously mentioned approaches [10]. Most times, insufficient numbers of ECs are obtained from patients with CVD, making their expansion even more challenging.

For this purpose, differentiation of MSCs into ECs could be a solution to address this issue. In this study, decellularization of hUAs was performed according to previous described protocols [4,5], resulting to the production of acellular vessels. Then, re-endothelialization was performed by using differentiated ECs derived from WJ-MSCs in order to produce non thrombogenic small diameter vascular grafts.

Materials and Methods

Isolation of hUAs

Human Umbilical Cords (hUCs), used for the isolation of hUAs, were derived from end term normal and caesarian gestations (gestational ages 38-40 weeks). The isolation of the hUCs was performed by experienced midwives and each specimen was accompanied by signed informed consent from the mothers. The informed consent was in accordance to declaration of Helsinki and the ethical standards of Greek National Ethical Committee. Delivery of the hUCs was performed within 24 hr after the gestation followed by immediate isolation of the hUAs. Briefly, the hUCs were rinsed in phosphate buffer saline 1x (PBS 1x, Sigma-Aldrich, Darmstadt, Germany) for removal of excess blood and blood clots. Then, isolation of hUAs was performed with the use of sterile instruments, while the human umbilical vein and Wharton's Jelly tissue were discarded. Finally, the hUAs were placed in PBS 1x and kept at 4°C until further use.

Decellularization of hUAs

HUAs (n=10) were decellularized according to a previous described protocol with some modifications [4,5]. The decellularization protocol involved the incubation of hUAs in CHAPS buffer (8 mM CHAPS, 1 M NaCl and 25 mM EDTA in PBS 1x, Sigma-Aldrich, Darmstadt, Germany) for 22 hr (h) accompanied by rotational agitation. Then, hUAs were briefly rinsed in PBS 1x for 5 min. The hUAs were further incubated in SDS buffer (1.8 mM SDS, 1 M NaCl and 25 mM EDTA in PBS 1x, Sigma-Aldrich, Darmstadt, Germany) for another 22 hr with rotational agitation, followed by briefly rinses in PBS 1x. Finally, hUAs were placed in a-Minimum Essentials Medium (a-MEM, Sigma-Aldrich, Darmstadt, Germany) supplemented with 40% v/v of Fetal Bovine Serum (FBS, Sigma-Aldrich, Darmstadt, Germany) and incubated at 37°C for 48 hr.

Histological analysis of hUAs

Evaluation of the decellularized hUAs was performed by histological analysis. Non-decellularized (n=10) and decellularized hUAs (n=10) were fixed in 10% v/v formalin (Sigma-Aldrich, Darmstadt, Germany), paraffin embedded and sectioned at 5 μm. Histological analysis involved the use of hematoxylin and eosin (H & E, Sigma-Aldrich, Darmstadt, Germany), for determination of cell nuclei and Extracellular Matrix (ECM) components. Images were acquired in DM L2 light microscope (Leica Microsystems Wetzlar Germany). Indirect immunofluorescence against fibronectin was performed in non-decellularized (n=10) and decellularized hUAs (n=10). Briefly, the slides were deparaffinized, rehydrated and blocked. Then, monoclonal antibody against human fibronectin (1:2000, Sigma-Aldrich, Darmstadt, Germany) was added, followed by secondary FITC-conjugated mouse IgG antibody (1:100, Sigma-Aldrich, Darmstadt, Germany). Finally, the slides were mounted with glycerol (Sigma-Aldrich, Darmstadt, Germany) and images were acquired with LEICA SP5 II microscope equipped with LAS Suite v2 software (Leica, Microsystems, Wetzlar, Germany).

Biochemical analysis of the hUAs

Non-decellularized (n=10) and decellularized hUAs (n=10) were evaluated for their collagen, sulphated Glycosaminoglycans (sGAGs) and DNA content. Total collagen amount was measured based on the quantification of the hydroxyproline content. For this purpose, the Hydroxyproline Assay kit (MAK008, Sigma-Aldrich, Darmstadt, Germany) was used according to manufacturer's instructions. Further biochemical analysis involved the quantification of sGAG content. Non-decellularized (n=10) and decellularized (n=10) hUAs were digested in 125 μg/ml papain buffer (Sigma-Aldrich) at 60°C for 12 hrs, followed by addition of dimethylene blue (Sigma-Aldrich). The sGAG content was quantified photometrically at 525 nm. Final concentration of sGAG content was obtained through interpolation to standard curve. Chondroitin sulfate standards of 12 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml and 150 μg/ml were used for the development of the standard curve.

The amount of DNA was measured in non-decellularized (n=10) and decellularized (n=10) hUAs. All samples were digested in a lysis buffer (0.1 M Tris pH 8, 0.2 M NaCl, 5 mM EDTA in PBS 1x, Sigma-Aldrich, Darmstadt, Germany) supplemented with 30 mg/ml Proteinase K (Sigma-Aldrich, Darmstadt, Germany). The digestion was performed at 56°C for 12 hrs and then inactivation of the enzyme was followed at 90°C for 5 min. Total DNA content was isolated, eluted in 100 μl of DNAse free water (Sigma-Aldrich, Darmstadt, Germany) and quantified photometrically at 260 nm to 280 nm.

ECs differentiation protocol

WJ-MSCs were submitted to differentiation into ECs with the use of VEGF-A. Briefly, WJ-MSCs in a number of 2×10^5 were seeded in 6-well plates (Sigma-Aldrich, Darmstadt, Germany). The following day, EC differentiation medium consisted of a-MEM with 5% v/v FBS and 100 ng/μl of Vascular Endothelial Growth Factor-A (VEGF-A, Sigma-Aldrich, Darmstadt, Germany) was added in the culture. The

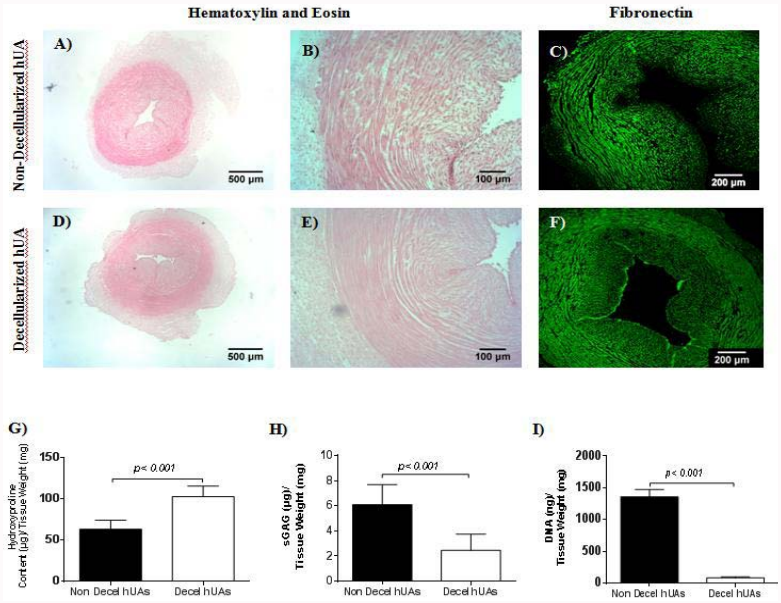

Figure 1: Evaluation of ECM composition in decellularized hUAs. Non-decellularized hUA with H&E (A,B) and indirect immunofluorescence against fibronectin (C). Decellularized hUA with H&E and indirect immunofluorescence against fibronectin (F). Images A and D, original magnification 2.5x, scale bars 500 μm. Images B and E, original magnification 10x, scale bars 100 μm. Images E and F, original magnification 5x, scale bars 200μm. Comparison in hydroxyproline (G), sGAG (H) and DNA (I) content between non-decellularized and decellularized hUAs. Statistical significant differences between non-decellularized and decellularized hUAs observed in all quantifications (*p<0.001*).

EC differentiation medium was changed bi-weekly for a time period of 30 days.

Characterization of differentiated ECs

Gene expression of ECs differentiated from WJ-MSCs was performed by Reverse Transcription (RT)-PCR, PCR and gel electrophoresis. Briefly, total mRNA was extracted using the TRI-reagent (Sigma-Aldrich, Darmstadt, Germany) according to manufacturer's instructions. The quantity and quality of mRNA was analyzed photometrically. Then, 800 ng of RNA was transcribed into cDNA with the use of Omniscript RT kit (Qiagen, Hilden, Germany). Then PCR was performed with Omniscript PCR kit (Qiagen, Hilden, Germany), using specific primers in order to evaluate the gene expression of differentiated ECs (Table 1). All PCR products were analyzed by electrophoresis on 1% w/v agarose gel (Sigma-Aldrich, Darmstadt, Germany). The gene expression of differentiated ECs was compared with undifferentiated WJ-MSCs (negative control group).

Flow cytometric analysis of ECs

Differentiated ECs were analyzed for cell surface antigens by flow cytometry. The ECs samples (n=5) were tested for the expression of CD31 and CD29. More specifically, the cells were labeled with phycoerythrin conjugated anti-CD31 (Beckman Coulter, Marseille, France) and CD29 (Beckman Coulter, Marseille, France). The phenotypes were analyzed in Cytomics FC 500 (Beckman Coulter, Marseille, France) flow cytometer accompanied with CXP Analysis software (Beckman Coulter, Marseille, France). CD marker expression was compared with undifferentiated WJ-MSCs.

Capillary tube formation assay

The capillary tube formation assay was performed in order to evaluate the ability of ECs to form vascular networks. Specifically, Matrigel© (Becton Dickinson, Heidelberg, Germany) was initially thawed on ice overnight according to manufacturer's instructions. Then, 30 μl of the Matrigel© was spread over each well of 24-well plate, followed by incubation for 30 min at 37°C. Differentiated ECs in a number of 3×10^4 cells were seeded in triplicate into each well and 500 μl of culture medium was added in each sample. The culture medium for this assay consisted of α-MEM supplemented with Endothelial Growth Medium-2 (EGM-2, Sigma-Aldrich). Finally, incubation of the samples was performed at 37°C, 5% CO_2. The formation of vascular networks by differentiated ECs was evaluated after 6 hr of the initial culture. As negative control group, non differentiated WJ-MSCs were used. Images of the vascular networks were acquired with inverted DM L2 light microscope (Leica, Microsystems, Wetzlar Germany). Total vascular network length was measured in 5 random areas in each sample using the Image Pro Plus v6 (Media Cybernetics, Washington, USA).

Re-endothelialization of the decellularized hUAs

Segments (n=5) of decellularized hUAs in length of 1 cm were re-endothelialized under static seeding conditions. For the re-endothelialization experiments, initially the decellularized hUAs were placed in 6-well plates. Differentiated ECs at a density of 3×10^5 cells/cm^2 were seeded into the vessel's lumen. Finally, 1 ml of culture medium consisted of a-MEM supplemented with EGM-2, 1% v/v Penicillin-Streptomycin and 1% v/v L-glutamine (Sigma-Aldrich, Darmstadt, Germany) was added in each well and the cultures were incubated at 37°C and 5% CO_2 for a time period of 4 weeks.

Evaluation of the re-endothelialization assay was performed by histological analysis. The segments of hUAs were fixed in 10% v/v formalin buffer (Sigma-Aldrich, Darmstadt, Germany), paraffin embedded and sectioned at 5 μm. Determination, of ECs in vessel's lumen was performed with H & E stain. In addition, indirect immunofluorescence against fibronectin in combination with DAPI (Sigma-Aldrich, Darmstadt, Germany) staining was applied. The slides were deparaffinized, rehydrated and incubated with monoclonal antibody against human fibronectin (1:2000, Sigma-Aldrich, Darmstadt, Germany) followed by secondary FITC-

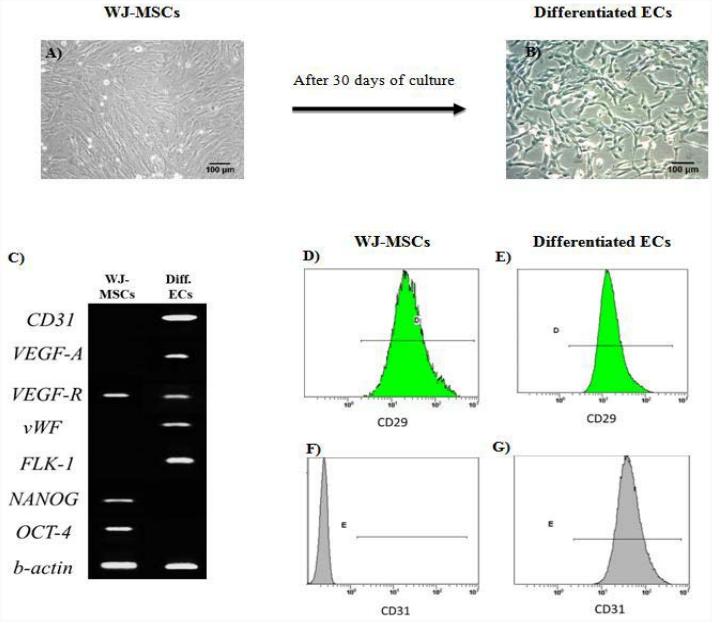

Figure 2: Evaluation of ECs differentiation protocol. Morphology of WJ-MSCs (A) and differentiated ECs (B) after 30 days of culture. Gene expression profile in WJ-MSCs and differentiated ECs (C). Immunophenotypic characterization of WJ-MSCs and differentiated ECs for CD29 (D, E) and CD31 (F,G), respectively.

Table 1: Sequences of primers used in PCR.

Gene	Accession	Forward Sequence	Reverse Sequence	Size
CD31	NM_000442.4	GTCCCCTAAGAATTGCTGCC	TAAAAACAGCTGTGCTGGGG	115
VWF	NM_000552	CCGATGCAGCCTTTTCGGA	TCCCCAAGATACACGGAGAGG	171
VEGFR	NM_001159920	TTTGCCTGAAATGGTGAGTAAGG	TGGTTTGCTTGAGCTGTGTTC	117
VEGFA)	NM_001171627	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA	75
FLK-1	NM_002253	GGCCCAATAATCAGAGTGGCA	CCAGTGTCATTTCCGATCACTTT	109
BETA ACTIN	NM_001017992	GTCTGCCTTGGTAGTGGATAATG	TCGAGGACGCCCTATCATGG	103
OCT-4	NM_001159542	GTGTTCAGCCAAAAGACCATCT	GGCCTGCATGAGGGTTTCT	156
NANOG	NM_024865	TTTGTGGGCCTGAAGAAAACT	AGGGCTGTCCTGAATAAGCAG	116

conjugated mouse IgG antibody (1:100, Sigma-Aldrich, Darmstadt, Germany). Finally, the DAPI stain was applied in order to detect the cell nuclei. Images were acquired with LEICA SP5 II microscope equipped with LAS Suite v2 software (Leica, Microsystems, Wetzlar, Germany).

Scanning electron microscopy

Decellularized and re-endothelialized hUAs (n=5, l=5 mm) were processed for scanning electron microscopy (SEM). Specifically, the samples were fixed with 1% v/v glutaraldehyde solution (Sigma-Aldrich, Darmstadt, Germany) for 30 min, followed by briefly rinses in distilled water. The samples were dehydrated by 10 min submission in 70% v/v, 80% v/v, 95% v/v aqueous ethanol, absolute ethanol and finally placed in hexamethyldisilazane solution (Sigma-Aldrich, Darmstadt, Germany) for 10 min. The samples were air dried and sputter-coated with gold (Cressington Sputter, Coater 108 auto, Watford, United Kingdom) and examined with Phillips XL-30 scanning electron microscope (Phillips, FEI, Hillsboro, OR, USA).

Statistical analysis

GraphPad Prism v 6.01 (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis. Mann Whitney test was applied in order to compare the DNA, collagen and sGAG content between non-decellularized and decellularized samples. Statistical significant difference was considered when p-value was less than 0.05. Indicated values are mean ± standard deviation.

Results

Histological analysis of decellularized hUAs

HUAs were successfully decellularized with the current decellularization protocol. Specifically, decellularized hUAs retained their initial ultrastructure, while no cellular or nuclei materials were evident, as shown by H & E staining (Figure 1). Indirect immunofluorescence results indicated the preservation of fibronectin in decellularized hUAs, while no damage in the vascular wall was observed (Figure 1).

Biochemical analysis

Hydroxyproline, sGAG and DNA quantifications were performed in order to evaluate thoroughly the impact of decellularization process in the vessel's ECM. Non-decellularized hUAs characterized by 63 ± 10 μg hydroxyproline/mg of tissue weight, while decellularized hUAs characterized by 102 ± 11 hydroxyproline/mg of tissue weight. Furthermore, sGAG content of non-decellularized and decellularized hUAs was 6 ± 2 and 2 ± 1 μg sGAG/mg of tissue weight, respectively (Figure 1). DNA amount in non-decellularized hUAs was 1341 ± 102 ng DNA/mg tissue weight, whereas in decellularized hUAs was

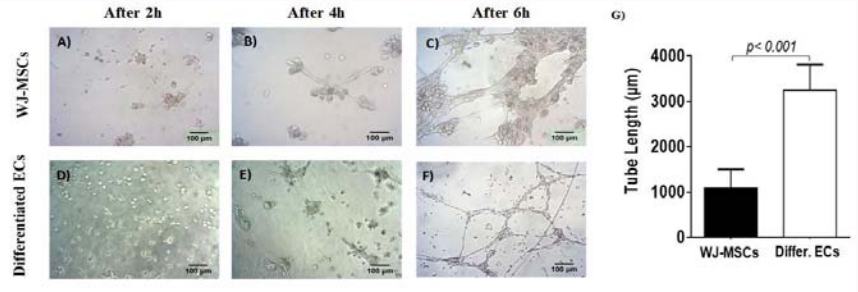

Figure 3: Evaluation of capillary tube formation by differentiated ECs. *In vitro* capillary tube formation by WJ-MSCs and differentiated ECs after 2h (A,D), 4h (B,E) and 6h (C,F), respectively. Original magnification 10x, scale bars 100 μm. G) Tube length measurement in WJ-MSCs and differentiated ECs. Statistical significant difference was observed in tube length between WJ-MSCs and differentiated ECs (*p<0.001*).

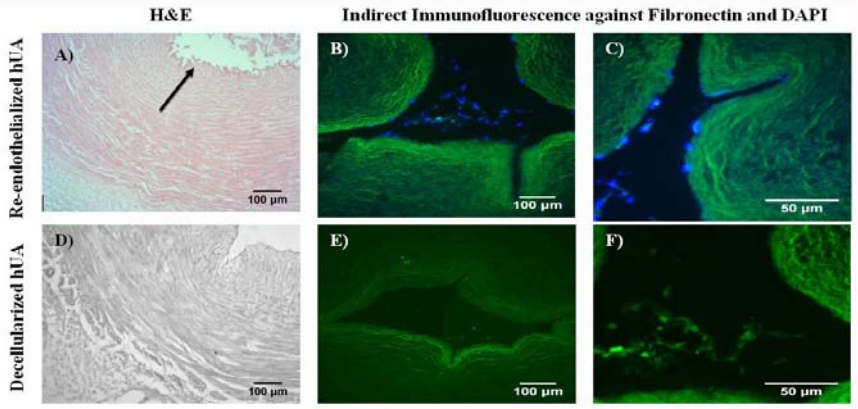

Figure 4: Histological analysis of re-endothelialized hUAs. Re-endothelialized hUA with H&E (A) and indirect immunofluorescence against fibronectin in combination with DAPI stain (B,C). Black arrow in image A indicated the presence of ECs in the vessel's lumen. Decellularized hUA (negative control group) with H&E (D) and indirect immunofluorescence against fibronectin in combination with DAPI stain (E, F). Images A,B,D and E, original magnification 10x, scale bars 100 μm. Images C and F, original magnification 40x, scale bars 50 μm.

82 ± 14 ng DNA/mg tissue weight (Figure 1). Statistical significant difference between non-decellularized and decellularized vessels was observed in all biochemical quantifications (*p<0.001*), used in this study.

Characterization of differentiated ECs

Differentiated ECs were characterized by different shape geometry when compared to WJ-MSCs. Specifically; ECs were smaller in size and were characterized by cobblestone shape, whereas WJ-MSCs had fibroblastic like morphology (Figure 2). After 30 days of differentiation, ECs successfully expressed *CD31, VEGF-A, VEGF-R, vWF, FLK-1*, while totally lost the expression of *OCT4 and NANOG*. On the other hand, WJ-MSCs were able to express only *VEGF-R*, and the key pluripotency genes *OCT4* and *NANOG* (Figure 2). Furthermore, based on the flow cytometric analysis, unlike to WJ-MSCs, differentiated ECs expressed CD31. Specifically, the expression of CD29 for WJ-MSCs and differentiated ECs was 96 ± 4% and 95 ± 3%, respectively. In addition, the expression of CD31 in differentiated ECs was 94 ± 5%, while WJ-MSCs didn't express this protein marker (Figure 2).

Evaluation of capillary tube formation

Differentiated ECs showed a substantial formation of capillary tube structures after 6 hr of cultivation on Matrigel©. WJ-MSCs were able to form a kind of vessel structure but the majority of them failed to produce capillary tubes. On the other hand, differentiated ECs started to form the first capillary tubes after 4 hr of culture in the semisolid medium (Figure 3). After 6 hr, the tube length of the capillaries was greater in differentiated ECs when compared to WJ-MSCs (Figure 3).

Evaluation of re-endothelialized hUAs

Histological analysis showed the successful re-endothelialization of decellularized hUAs. Uniform distribution of ECs differentiated from WJ-MSCs was observed in lumen of hUAs, whereas decellularized hUAs were characterized by totally absence of cellular and nuclear materials. Moreover, ECs were found to be in close proximity with the fibronectin of the vascular wall as shown by indirect immunofluorescence results.

Moreover, the presence of ECs was further confirmed by SEM images. The ECs were attached successfully to the vessel's lumen (Figure 5). On the other hand, only the ultrastructure of the vessel was evident in decellularized hUAs, without the presence of any cellular populations (Figure 5).

Discussion

Development of small diameter vascular grafts (<6 mm) utilizing the tissue engineering approaches including tissue decellularization and vessel fabrication, is a rapidly evolving field. These grafts can be used as substitutes in order to replace damaged vessels such the coronary artery [11]. However, the majority of these substitutes lack of a well-organized endothelium. The use of ECs derived from a vascular biopsy, is a challenging process. Endothelium formation requires a great number of cells that can be efficiently expanded and seeded *in vitro* onto vascular scaffolds. Major achievements have been

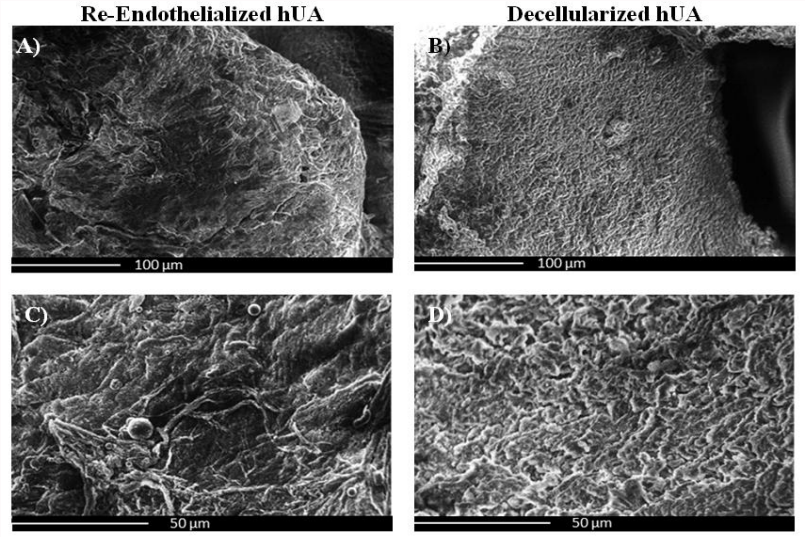

Figure 5: SEM analysis of the re-endothelialized hUAs. Re-endothelialized hUAs, where the seeded cells can be observed clearly (A,C). Decellularized hUAs, where only the vessel's ECM is visible (B, D). No evidence of cellular populations was observed in decellularized hUAs. Images A and B, original magnification 1000x, scale bars 100μm. Images C and D, original magnification 2000x, scale bars 50 μm.

performed towards this direction, involving the isolation of ECs from various sources such as peripheral or cord blood and human umbilical vein [12,13]. However, many studies have controversial results, while the proper seeding of ECs is still a demanding process [12,13].

This aim of this study was to establish and produce re-endothelialized small diameter vascular grafts derived from human umbilical arteries. For this purpose, hUAs were initially decellularized with protocols that utilizes CHAPS and SDS reagents. Histological analysis with H & E, showed the successful decellularization of the hUAs, where no cellular or nuclear materials were evident, while at the same time the arterial ultrastructure was preserved. In addition, these results were further confirmed by DNA quantification, where decellularized hUAs were characterized by a low DNA amount.

Moreover, fibronectin, an abundant structural protein of the vascular wall, was retained in decellularized hUAs as indicated by the indirect immunofluorescence results. Similar observations regarding the preservation of structural proteins in decellularized matrices have been also reported by other groups [4,5,14].

Further evaluation of the decellularization process, involved the biochemical quantification of collagen and sGAGs, which are key elements of the vascular wall. In our study, the hydroxyproline concentration which corresponds approximately to collagen content of the hUAs, was increased in decellularized samples. This elevation in collagen content might be attributed by the loss of cell mass after the decellularization process, leaving only the tissue scaffold. On the other hand, sGAG content was decreased after the decellularization process. It is known, that SDS reagent is an anionic detergent which distracts the non-covalent interactions in proteins, causing in this way the denaturation of proteoglycans and sGAGs [14,15].

Next step of this study was the establishment of the EC differentiation process. WJ-MSCs were submitted to differentiation into ECs by using the *VEGF-A* as a key protein inducer. Differentiated ECs were characterized by elevated expression of *CD31, VEGF-A, VEGFR, FLK-1* and *vWF* while didn't express *OCT4* and *NANOG*. Furthermore, differentiated ECs were characterized by increased levels of CD31 and CD29 markers as indicated by flow cytometric analysis and were capable to produce tube like structure that was stable for at least 6 hours. The ability of MSCs to differentiate into ECs is very promising. Taking into consideration, that patients with CVD don't have suitable vessels for ECs isolation, the differentiation of MSCs could be a solution. Ideally, MSCs can be isolated from bone marrow or adipose tissue of those patients, and then submitted into EC differentiation in order to be used in the re-endothelialization of the vascular grafts.

Once we have established the decellularization protocol in hUAs and the proper differentiation of WJ-MSCs into ECs, then we proceeded in the re-endothelialization of the vessels. Differentiated ECs were capable to be attached in the decellularized vascular wall as shown by the histological results. Indirect immunofluorescence confirmed the presence of ECs into the vessel's lumen, and further showed their connection with the fibronectin of decellularized hUAs. Fibronectin, along with collagens, is a key structural element of the vascular wall, which provides attachment sites for the ECs through its interaction with α5β1 and α4β1 [16]. In addition, it has been reported a possible synergism between integrin interaction, fibronectin and growth factors receptors. ECs are attached in the CS5 domain of fibronectin through collaboration of α5β1 and α4β1, which results to activation of focal adhesion kinase and extracellular regulated kinase (ErK) [16,17]. Prolonged activation of ErK leads to EC proliferation and migration, thus contributing in the endothelium formation [16]. Furthermore, the association between *VEGF-R* and fibronectin interaction, contributes even more in promoting biological responses by ECs, such as vessel homeostasis.

Tissue engineered vascular grafts must be characterized by a proper endothelium, avoiding neointima formation and graft occlusion. Until now, the re-endothelialization process of vessels needs considerable attention. A great number of research groups have focused on improving the re-endothelialization process by treating the vascular grafts with chemical compounds [7-9,18]. Polyelectrolyte multilayer development and immobilization of heparin sulfates in decellularized vessels have been proposed by modifying the amino acids of the vascular wall [8,9]. However, such modifications have negative impact in the vessel functionality. In this way, preventing

the capability of decellularized vessel to be remodeled *in vivo* reduces significantly its function, making it susceptible for calcification development, reduction in patency rate and graft rejection. Especially, when these vascular grafts are intended to be used in pediatric patients, the biological properties of the vessel must not be hampered.

Unlike, to these approaches, in our study was shown that differentiated ECs could successfully be seeded onto decellularized hUAs without treating them with any chemical compounds.

Conclusion

In conclusion, our study focused on the development of a small diameter vascular graft derived from hUAs and its proper endothelialization. The decellularized hUAs could be used as an alternative source for the development of small diameter vascular grafts that could be used as coronary artery substitutes. In addition, these vessels could be used as a model for better understanding the molecular mechanisms of EC differentiation, proliferation and migration in the vascular wall in order to take full advantage of its clinical potential. Future research is needed for the development of biocompatible tissue substitutes derived from decellularized tissues that can potentially be used in clinical and translational medicine.

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