Reviewer Comments

Reviewer ID: 00906911

Reviewer's Comments

Generally, the composition of this paper appears to be sound. But it would be needed to present the analysis comparing the results of this study between those using common protocols related to the topic of this study. The supplementation might improve the originality and value of this article.

Dear Reviewer,

We have performed the manuscript revision according to your comments. Below you can find our responses towards to your comments

Reviewer's Comments
But it would be needed to present the analysis comparing the results of this study between those using common protocols related to the topic of this study.

Author's Response	
According to reviewer's comment, we have added to the discussion section the comparison between our study and other common studies.	
Discussion (page 24)	This low expression of ACTA2 in WJ-MSCs has been reported previously by others [35].
Page 25	It has been shown in literature, that contractile VSMCs genes increased their expression under stress-strain conditions, contributing to the adaption of the contractile phenotype [23]. In our study, no stress-strain conditions were applied, and this might explain the *SOX9* expression by the differentiated VSMCs.
Page 25	Our results seemed to be comparable with other previously published studies [37 -43], where early, intermediate and late VSMCs markers such as ACTA2 and MYH11, have been expressed. However, most of these studies are using more complicated and sophisticated approaches including the use of iPSCs technology, or the prolonged exogenous supplementation of growth factors [24-27]. Moreover the use of iPSCs technology in humans is under strictly control. Modern research is focused on the production of safe viral free iPSCs clones, in order to be used in clinical trials. On the other hand, vascular tissue engineering demands enormous number of cells, which are very difficult to be obtained from patients. Most times, vessel tissue biopsy is needed for the isolation of VSMCs. However, patient's condition and age are important factors that may hamper the VSMCs isolation. In our study, it was proposed the production of VSMCs from MSCs. MSCs can be efficiently isolated from several human tissues, including bone marrow and adipose tissue. Additionally, MSCs are pluripotent stem cells that can be expanded in wide numbers and then can be differentiated to the desired cell population, such as

	VSMCs. Taken together all this data, it is proposed an alternative way for obtaining functional VSMCs, even from seriously diseased patients, that could be used in vascular tissue engineering.
Page 26	DNA content of decellularized vascular grafts was significantly reduced and was below than 50 ng/mg of dry tissue as has been proposed by Crapo *et al* [44], confirming further the successful decellularization of the hUAs.
Page 26	These results were in accordance with previous published studies in vessels or other tissues, and the reduction of the above macromolecules were mostly attributed by the SDS reagent of the decellularization protocol [3,4,17,45].
Page 27	It is known, that SOX9 in combination with RUNX2 and MSX2 could contribute to the synthetic conversion of VSMCs, resulting in collagen and sGAG synthesis. Although, in our study where only static seeding conditions were applied, the proper maturation of VSMCs and the adaption of contractile phenotype onto the decellularized vessels may require other approaches such as the use of dynamic seeding conditions. Indeed, a pulsatile vessel bioreactor could mimic the blood flow of human body with specific stress-strain conditions and contribute to the adaption of contractile phenotype by the VSMCs.
Conclusion (page 28)	Until now, several complicated and expensive approaches are used for the production of vascular populations and small diameter vessels conduits [26, 27, 28, 47] . Unlike to these approaches, our proposal relied on the use the hUC and its derivatives as an alternative approach for blood vessel engineering.

Reviewer's Comments
The supplementation might improve the originality and value of this article.
Author's Response
Supplementary data of the manuscript has been uploadaded.

We think that we have performed the revisions according to your comments. If anything else is needed by our side, please notify us. Also, we have checked and corrected the whole manuscript for any grammar or syntax errors.

Thank you for your time spending.

Yours sincerely,

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Reviewer Comments

Reviewer ID: 00031349

This study is novel and important.

The following addition experiments might be helpful to further support the conclusion.

Minor comments: Figure 1. Checked senescent cells in Passage 4. Please determine the percentage of tri-lineage cell types. Figure 2. Determine VSMC marker protein levels using immunoblotting and analyze the capacity of VSMC proliferation, migration or invasion. Figure 3. locate the SMC markers such as alpha-smooth muscle action and calponin etc. Figure 3. Determine Cell cycling related protein of PCNA and Ki67 in VSMCs using dual labeling.

This study is novel and important. The following addition experiments might be helpful to further support the conclusion. Minor comments:

Reviewer's Comments
Figure 1. Checked senescent cells in Passage 4.

Author's Response	
In this study, the MSCs characteristics regarding the total cell number in each passage, CDT, PD and % viability was performed. The total number of MSCs were appeared to be increased over passage 4 as it was indicated by the data of Figure 1(A-E). No morphological differences or apoptotic cells were appeared during all passages. Furthermore, the determination of cell viability was performed with the use of Trypan blue. Trypan blue is the reference method for cell viability estimation. In addition, we performed crystal violet assay in MSCs from passage 1 to 4 according to manufacturer's instructions. To quantify and crosscheck the trypan blue stain results. The experimental procedure has been added to the main manuscript.	
Materials and Methods – Growth kinetics and Cell Viability of WJ-MSCs (pages 10-11)	Additionally, the determination of cell viability was performed with Crystal Violet assay (ab232855, Abcam, Cambridge, United Kingdom) according to manufacturer's instructions. Briefly, 2 x 105 MSCs from passages 1 to 4 were added to each well of 96 well plate (Costar, Corning Life, Canton, MA, USA). DMSO vehicle was used as background control and doxorubicin was added in a well contained MSCs as prolifereation inhibitor. All MSC samples were cultured for 72 hours at 5% CO2 in a humidified atmosphere. Then, the culture medium was removed, the cultures were washed and 50 µl of Crystal Violet was added to each well for 20 min at room temperature. Then, washing step was performed for four times, the 96 well plate air dried, followed by addition of 100 µl Solubilization solution to each well. Finally, absorbance at 595 nm was measured and the % cytotoxicity was calculated based on the determination of the optical density (OD) using the following equation:

	% Cytotoxicity =(OD (DMSO)-OD(Sample))/(OD (DMSO)) x 100% Where, OD (DMSO) was the DMSO control after background correction and OD (Sample) was the OD of the sample after background correction.
Results – Characteristics of isolated WJ-MSCs (pages 19-20)	Cell viability of WJ-MSCs, determined either with Trypan blue or Crystal Violet, in passages 1 to 4 was above 90% (Figure 1E and S1).
Crystal violet assay can determine effectively, the cell viability and senescence, according to the publication of Sliwka *et al.* The Comparison of MTT and CVS Assays for the Assessment of Anticancer Agent Interactions. PLoS One. 2016 May 19;11(5):e0155772. For this reason, we preferred to use Crystal Violet assay.	

Reviewer's Comment

Author's Response	
Please determine the percentage of tri-lineage cell types.	
In general the percentage of tri-lineage differentiation of MSCs is not determined accurately. There some assays that could help for the determination of tri-lineage differentiation. For this purpose, quantification methods based on stain intensity were applied. Osteocyte differentiation and Ca^{2+} were estimated with Alizarin Red S quantification method according to manufacturer's instructions. This assay, was performed in MSCs from passages 1 to 4.	
Materials and Methods - WJ-MSCs trilineage differentiation assay (pages 11 – 12)	In addition, Alizarin Red S quantification assay (ECM815, Millipore) was used for determination of Ca^{2+} deposits, according to manufacturer's instructions. Furthermore, the chondrogenic differentiation was further assessed with the Bern Score. Specifically, three dependent observers evaluated the chondrogenic differentiation based to an already published protocol [31].
Results - Characteristics of isolated WJ-MSCs (pages 19 – 20).	Moreover, "osteocytes" were produced Ca2+ deposits more than 0.9 mM (Figure S2). In regards to the "chondrogenic" differentiation, 3D cultures of WJ-MSCs were characterized by production of proteoglycan aggregations, as it was indicated by Alcian blue and Bern Score (Figure 1F and Table S1).
The above data clearly suggested that a high percentage of MSCs were successfully differentiated either to "osteocytes" or to "chondrocytes". In addition, Oil Red O staining, revealed the successful differentiation of MSCs to "adipocytes"	
Unfortunately, the estimation of "adipogenic" differentiation has not be performed. Currently, all methods used for lipid droplets quantification lead to contradictory	

results. For this purpose we did not perform any quantification assay regarding the estimation of "adipogenic" differentiation.

Reviewer's Comments
Determine VSMC marker protein levels using immunoblotting and analyze the capacity of VSMC proliferation, migration or invasion.

Author's Response	
Reviewer suggested the performance of immunoblotting to quantify better the successful VSMCs differentiation. Unfortunately, in our laboratory we cannot perform this experimental procedure. We think that immunofluorescence using smooth muscle α-actin (ACTA2) and myosin heavy chain (MYH11) in order to determine the differentiation of MSCs into VSMCs is accurate as experiment. The results, clearly showed the elevated expression of the above proteins in VSMCs compare to MSCs Furthermore, quantification based on mean fluorescence intensity (MFI) was also performed using ImageJ.	
Materials and Methods – Immunofluorescence of VSMCs (page 14)	Furthermore, mean fluorescence intensity (MFI) of WJ-MSCs and VSMCs was determined using ImageJ 1.46r (NIH, USA).
Results – Evaluation of VSMCs differentiation (page 21)	In addition, MFI of ACTA2 and MYH11 was determined in both WJ-MSCs and VSMCs (Figure S4). Statistically significant differences were observed in the ACTA2 ($p<0.01$) and MYH11 ($p<0.01$) expression between WJ-MSCs and VSMCs (Figure S4), indicated in this way the successful differentiation of VSMCs.
Supplementary Data	Figure S4. Determination of ACTA2 and MYH11 MFI of WJ-MSCS and VSMCs. A) MFI of ACTA2 in WJ-MSCs and VSMCs. Statistically significant difference was observed in ACTA2 expression between WJ-MSCs and VSMCs ($p<0.01$). B) MFI of MYH11 of WJ-MSCs and VSMCs. Statistically significant differences were observed in MYH11 between WJ-MSCs and VSMCs ($p<0.01$).
The estimation of VSMC proliferation was performed by using ATP assay (MAK190, Sigma-Aldrich). We preferred to use ATP assay instead of ATP-ADP ratio for the determination of cell proliferation. The ATP-ADP ration, is mostly used for the determination for the cell viability and that was the actual reason why we used in repopulated hUAs. On the other hand, as the reviewer suggested and in order to better determine the VSMC proliferation, we performed the ATP assay.	
Materials and Methods – Estimation of cell proliferation using ATP assay (page 14)	Cell proliferation was determined with ATP assay (MAK190, Sigma-Aldrich, Darmstadt, Germany) according to manufacturer's instructions. Briefly, 100.000 WJ-MSCs of VSMCs were plated in 24-well plate (Costar). The next day, the cells were lysed with the use of 100 μl ATP Assay buffer. Then, 20 μl of cell lysates were transferred to 96-well plates, followed by the addition of reaction buffer. All samples were incubated for 30 min at room temperature. Finally, the absorbance were measured to photometer at 570 nm.

	Determination of ATP concentration was achieved by interpolation to a standard curve. The standard curve was consisted of 0 (blank), 5, 10, 20, 50, 100, 150, 200 nM standards.
Results - Evaluation of VSMCs differentiation (page 20)	The WJ-MSCs and VSMCs proliferation was performed using the ATP assay. Both cellular populations were characterized by equal amount (17 ± 3 nM and 18 ± 3 nM) of ATP (Figure 2C).
	Figure 2. Differentiation of WJ-MSCs towards VSMCs. Morphological features of untreated WJ-MSCs and differentiated to VSMCs (A1, A2). PCR results regarding the expression of VSMCs specific genes, such as ACTA2, MYOCD, MYH11 and TGLN, and pluripotency related genes, including NANOG and OCT4 in untreated WJ-MSCs and differentiated VSMCs. GAPDH was the desired house-keeping gene for current analysis (B). Determination of WJ-MSCs and VSMCs proliferation by performing the ATP assay. Indirect immunofluorescence against the early VSMC marker ACTA2 and late VSMC marker MYH11 in untreated WJ-MSCs (D1, D5, D9 and D2, D6, D10) and differentiated VSMCs (D3, D7, D11 and D4, D8, D12) in combination with DAPI, respectively. Images A1-2 were presented with 10x original magnification and scale bar 100 μm. Images D1-12 were presented with 20x original magnification and scale bar 50 μm.
Discussion (page 25)	Both WJ-MSCs and VSMCs were characterized by equal production of ATP, suggesting in this way the retention of VSMCs' proliferation properties.

Reviewer's Comment
Figure 3. locate the SMC markers such as alpha-smooth muscle action and calponin etc.

Author's Response	
As the reviewer suggested we performed immunofluorescence for ACTA2 and MYH11 in combination with DAPI both in non decellularized and decellularized vascular grafts. Also immunofluorescence against MYH11 in combination with DAPI was initially available in Figure 4, which was served as negative control. No signal from MYH11 or DAPI was evident in decellularized hUAs.	
Materials and Methods – Histological Analysis of hUAs (pages 15 -16)	In addition, indirect immunofluorescence against ACTA2 and MYH11 in combination with DAPI staining was applied. Non decellularized and decellularized hUAs were fixed with 10% v/v formalin buffer (Sigma-Aldrich, Darmstadt, Germany), dehydrated, blocked and sectioned at 5 μm. Then, the slides were deparaffinized, rehydrated, blocked followed by the addition of monoclonal antibody ACTA2 (1:500, Catalog MA1-744, ThermoFischer Scientific, Massachusetts, USA) or MYH11 (1:1000, Catalog MA5-11971,

	ThermoFischer Scientific, Massachusetts, USA). Secondary FITC conjugated antibody (1:100, Sigma-Aldrich, Darmstadt, Germany) was added. Finally, DAPI (Sigma-Aldrich, Darmstadt, Germany) stain was added, the slides were glycerol mounted and proceed for examination under fluorescent microscope (LEICA SP5 II fluorescent microscope, Leica, Microsystems, Wetzlar, Germany).
Results – Decellularization of hUAs (page 21)	In addition, TB stain was appeared to be less dense in decellularized hUAs compared to non decellularized vessels. Signal detection of ACTA2, MYH11 and DAPI was evident only in non decellularized hUAs, confirming in this way the successful decellularization procedure (Figure 3A).
	Figure 3. Histological and biochemical analysis of decellularized hUAs. Histological analysis involved the H&E (A1,2), SR (A3,4) and TB (A5,6) in non decellularized and decellularized hUAs. Indirect immunofluorescence against ACTA2 (A7,8) and MYH11 (A9,10) in combination with DAPI was performed in non decellularized and decellularized hUAs. Biochemical analysis involved the determination of total hydroxyproline (B), sGAG (C) and DNA content (D) in non decellularized and decellularized hUAs. Statistically significant differences were observed in total hydroxyproline ($p<0.05$), sGAG ($p< 0.001$) and DNA ($p< 0.001$) content between non decellularized and decellularized hUAs, Images A1-6 were presented with original magnification 10x and scale bars 100 μm. Images A7-10 were presented with original magnification 20x and scale bars 50 μm. Non decel hUA: Non decellularized human umbilical artery, decell hUA: decellularized human umbilical artery.
Discussion (page 26)	Indirect immunofluorescence results indicated no presence of ACTA2 or MYH11 in decellularized hUAs, confirming further the cell elimination.

Reviewer's Comments
Figure 3. Determine Cell cycling related protein of PCNA and Ki67 in VSMCs using dual labeling.

Author's Response
In the beginning, I think that the reviewer might wanted to perform the detection og PCNA and Ki67 in VSMCs in reopoulated hUAs, which are demonstrated in figure 4 and not in figure 3. The detection of PCNA and Ki67 is very important in order to determine the proliferation of VSMCs in repopulated hUAs. Following the reviewer's comment, we have performed immunohistochemistry against Ki67 and PCNA. In addition, the proliferation of VSMCs was further

<table>
<tr><td colspan="2">confirmed with DNA quantification, which was also illustrated in Figure 4. The corrections regarding the above comment are the following</td></tr>
<tr><td>Materials and Methods - Repopulation of hUAs with VSMCs (pages 17 – 18)</td><td>In addition, immunohistochemistry against Ki67 and proliferating cell nuclear antigen (PCNA) was performed in repopulated hUAs. Briefly, the slides were deparafinized, rehydrated and the whole procedure was performed using the Envision Flex Mini Kit, high pH (Cat # K802421-2J, Agilent Technologies, California, USA) according to manufacturer's instructions. Ki67 (1:50, Cat # 305504, Biolegend, San Diego, USA) and PCNA (1:100, ab 18197, Abcam, Cambridge, United Kingdom) were used for the detection of cell proliferation in repopulated hUAs. Decellularuzed hUAs were served as negative control both in indirect immunofluorescence and immunohistochemistry assays.</td></tr>
<tr><td>Results – Repopulation of hUAs with VSMCs (page 22)</td><td>. In addition, immunohistochemistry results indicated the expression of key proliferation markers such as Ki67 and PCNA in VSMCs of repopulated hUAs (Figure 4A).</td></tr>
<tr><td></td><td>Figure 4. Repopulation of decellularized hUAs with VSMCs. Histological analysis with H&E of decellularized hUAs (A1, A5), repopulated hUAs after 1st week (A2, A6), 2nd week (A3, A7) and 3rd week (A4, A8). Indirect immunofluorescence against MYH11 in combination with DAPI of decellularized hUAs (A9), repopulated hUAs after 1st week (A10), 2nd week (A11) and 3rd week (A12). Immunohistochemistry against Ki67 and PCNA of decellularized hUAs (A13, A17), and repopulated hUAs after 1st week (A14, A18), 2nd week (A15, A19) and 3rd week (A16, A20). Images A1-4 were presented with original magnification 10x, scale bars 100 μm. Images A5-12 were presented with original magnification 20x and scale bars 50 μm. Images A13-20 were presented with original magnification 40x and scale bars 20 μm. Total hydroxyproline (B) and sGAG (C) quantification of hUAs before and after repopulation with VSMCs. Evaluation of DNA content (D) and ADP/ATP ratio (E). Statistically significant differences in total hydroxyproline, sGAG, DNA content and ADP/ATP ratio were observed between the study groups ($p<0.05$). Decel hUA: decellularized human umbilical artery, repop hUA: repopulated human umbilical artery.</td></tr>
<tr><td>Discussion (page 27)</td><td>Repopulated hUAs were positive for Ki67 and PCNA, as it was indicated by the immunohistochemistry, confirming the successful proliferation of VSMCs.</td></tr>
<tr><td colspan="2">Our initial thought was to perform immunofluorescence against both Ki67 and PCNA, but unfortunately this would take much time, and may overpass the time limit for the revisions given from the journal (9 days). For this purpose we performed immunohistochemistry assay. We think that the results of this assay hopefully satisfies the reviewer.</td></tr>
</table>

We think that we have performed the revisions according to your comments. If anything else is needed by our side, please notify us. Also, we have checked again and correct the whole manuscript for any grammar or syntax errors.

This is a very important work for us and our willing is to have positive outcome. For this purpose, we are willing to perform any additional changes if they are required, in order the manuscript to get published.

Yours sincerely,

Mallis Panagiotis, MSc, PhD

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