Answers to Reviewers, Editors and Editorial Office Director

RESPONSE TO REVIEWER#1:

Dear Reviewer #1, thank you very much on your comments and recommendation for the acceptance for publication of our manuscript entitled "The effects of simultaneously applied endothelial and osteogenic differentiated adipose-derived stem cells combined with platelet-rich plasma on vascularization and osteogenesis in ectopically implanted bone tissue-engineered construct" in the respectable *World Journal of Stem Cells*. Minor English language polishing has been done throughout the manuscript. Please, find these changes within the revised manuscript marked in font color red.

RESPONSE TO REVIEWER#2:

Dear Reviewer #2, many thanks on your comments related to our manuscript entitled "The effects of simultaneously applied endothelial and osteogenic differentiated adipose-derived stem cells combined with platelet-rich plasma on vascularization and osteogenesis in ectopically implanted bone tissue-engineered construct" submitted to the respectable *World Journal of Stem Cells*. We have carefully read and responded to all your comments and marked all demanded changes in font color red. The changes made by authors as a consequence of the demanded changes are also marked in font color red throughout the manuscript. Please, find our detailed answers point by point listed below.

Reviewer #2, comment #1 In introduction, the authors should cite and discuss the previous studies on BTE constructs containing endothelial cells and osteoblasts, and discuss the novelty of this study.

Response to comment #1 According to your suggestion, we have provided data regarding previous studies on BTE constructs containing endothelial cells and osteoblasts, and discussed the novelty of this study in the Introduction section of our manuscript as following:

Page #5: from line 21 to line 29

Page #6: from line 1 to line 28

Reviewer #2, comment #2 The authors should provide data for expression of endothelial-related genes in ASCs after 12 days of *in vitro* endothelial differentiation induction.

Response to comment #2 Since endothelial-related genes in ASCs after 12 days of *in vitro* endothelial differentiation induction in the presented study showed the same expression patterns as in our previously published article "**Najdanović JG**, Cvetković VJ, Stojanović S, Vukelić-Nikolić MĐ, Stanisavljević MN, Živković JM, Najman SJ. The influence of adipose-derived stem cells induced into endothelial cells on ectopic vasculogenesis and osteogenesis. *Cell Mol Bioeng* 2015; 8(4): 577-590 [DOI 10.1007/s12195-015-0403-x]." it would be the repetition of the same results. Thus, we added citation of this article at relevant place in the text of the manuscript, at page #10, from line 7 to line 14.

Reviewer #2, comment #3 Page 20: "Relative expression levels of endothelial-related genes in implants were generally higher in BPUI compared to BC and BPEO implants. One of the reasons for such expression pattern in BPUI implants was probably the presence of biological factors released upon PRP activation since these factors accelerate and improve ASCs *in vivo* differentiation." Do the authors mean that these endothelial-related genes are highly expressed in ASCs undergoing endothelial differentiation, but slightly expressed in mature endothelial cells and ASCs after 12 days of *in vitro* endothelial differentiation induction?

Response to comment #3 By the sentence quoted above, the authors meant that relative endothelial-related genes expression was generally higher in implants that contained uninduced ASCs (BPUI implants) compared to the implants without ASCs in their composition (BC implants) and implants that contained ASCs *in vitro* induced into ECs and OBs (BPEO implants). Further, the authors have found that such expression pattern is supported with a literature data cited at manuscript page # 21, from line 7 to line 11. Our results are in accordance with these references in a sense that biological factors released from PRP can accelerate and improve differentiation of previously uninduced ASCs during *in vivo* experimental period. In addition, our opinion is that the biological situation in the mentioned constructs, which are composed not only from the ASCs, is more complex and it could not be observed only on the cell component. The authors observed the whole construct composition where the cells are one of the components.

Reviewer #2, comment #4 Page 25: “In spite of all favorable features if BPEO implants, these implants had strong regression of the tissue between BMM granules...” Which figure shows this sign?

Response to comment #4 In order to show tissue regression between BMM granules in group BPEO at 8-week observation point, the authors have added additional image into Figure 3 and marked it as 3G. Strong regression of the tissue between BMM granules in 8-week-old BPEO type of implants is marked in Figure 3G with red ellipse.

Reviewer #2, comment #5 Figure 2a: Why expression level of *Vwf* gene in all the implants was consistently lower than that in the calibrator sample from week 1 to week 8?

Response to comment #5 Besides ECs, *Vwf* gene is also highly expressed in platelets and megakaryocytes, which is supported with reference #56 cited at #22 of our manuscript (lines 13 and 14). In the present research, we have used P03-1d cells combined with PRP as a calibrator sample (page #12, from line 11 to line 13). The concentration of platelets was $1.89 \pm 0.5 \times 10^6$ per microliter of PRP. High concentration of the applied platelets indicates that *Vwf* gene expression was also high in the calibrator sample. In addition to that, we have measured relative gene expression levels. Altogether, those facts explain consistently lower *Vwf* gene expression in all the implants regarded to the calibrator sample.

Reviewer #2, comment #6 Figures 6-8: I don’t find any obvious differences between the groups in terms of immunoexpression levels of VEGFR-2, CD31 and osteocalcin based on the figures given. The authors should also provide the quantitative data and perform statistical analysis.

Response to comment #6 In order to quantify immunoexpression of VEGFR-2, CD31 and osteocalcin in the tissue sections, the authors have performed IHC optical score analysis in ImageJ free software version 1.53 (National Institute of Health, Bethesda, Maryland) (Java 1.8.0_172), by using the open source plugin IHC profiler in ImageJ software. The applied method was decribed in *Materials And Methods* section entitled *Immunohistochemistry* (page #14 from line 15 to line 29 and page #15 from line 1 to line 7). Obtained quantive data were presented in *Results* section entitled *Immunohistochemical analysis* as following:

page #19 from line 16 to line 27

page #20 from line 8 to line 10

page #20 from line 12 to line 25

Obtained results were analyzed throughout *Discussion* section.

Reviewer #2, comment #7 There are a few typo errors. For example, “B implants” (page 21) and “B group” (pages 22 and 24).

Response to comment #7 The authors have corrected these typo errors in the following manner: “B implants” are changed into “BC implants” and “B group” into “BC group”. Also, the authors have changed B into BC throughout the manuscript.

RESPONSES TO SCIENCE EDITOR

Dear Science Editor, thank you very much on your comments and suggestions for our manuscript entitled “The effects of simultaneously applied endothelial and osteogenic differentiated adipose-derived stem cells combined with platelet-rich plasma on vascularization and osteogenesis in ectopically implanted bone tissue-engineered construct” subjected to the respectable World Journal of Stem Cells. We have carefully read and responded to all your comments and marked all demanded changes with font color red. The changes made by authors as a consequence of the demanded changes are also marked with font color red through the manuscript. Please, find our detailed answers point by point listed below.

Science Editor, comment #1 The abbreviation of adipose-derived stem cells should be ASCs.

Response to comment #1 The authors have changed the abbreviation ADSCs to ASCs throughout the manuscript.

Science Editor, comment #2 In Figure 1, the four groups (*Vwf, Egr1, Flt1, Vcam1*) should be distinguished by different colors or patterns.

Response to comment #2 The authors have changed Figure 1 so that four markers (*Vwf, Egr1, Flt1, Vcam1*) are now represented with different colors.

Science Editor, comment #3 In figure 2, the error bar of *Vwf* of BC in 1w, *Flt1* of BPEO in 2w, *Sppl* of BPEO in 2w were very high, which proves that the data needs to be validated.

Response to comment #3 As it was described in the section *Results, Gene expression analysis in implants*, at page #15 from line 23 to line 25, and page #16 from line 1 to line 8, for relative expression levels of all examined endothelial-related genes and one bone-related gene in BC, BPUI and BPEO groups of implants, there were six samples (n = 6) per each group, per experimental period (n(BC) = 6, n(BPUI) = 6, n(BPEO) = 6). In addition, we would like to emphasize that each sample was placed in two wells (duplicate) of the 96-well plate used for Real-time PCR analysis. Therefore, twelve replicas were used for obtaining the mean value for relative expression of each gene per group, per experimental period. First of all, the authors have no cDNA samples left from the experiment for validation by Real-time PCR analysis. Therefore, the authors tried to exclude the highest and the lowest value out of these replicas for statistical analysis of *Vwf* of BC in 1w, *Flt1* of BPEO in 2w, *Spp1* of BPEO in 2w, but this did not lead to significant changes of already calculated standard deviations and statistical significances. Over and above, at this point of time, it would be impossible to set this whole experiment again. Specifically, obtaining of the samples for new validation of relative gene expression analysis encompasses numerous steps described in the *Materials and methods* section of our manuscript, from purchasing the animals, period of adaptation of animals to the new environment, isolation and cultivation of cells, implantation procedure, waiting for experimental period to pass in order to isolate implants. Then, the whole procedure from getting cDNA out of isolated RNA and, finally, performing Real-time PCR analysis. Of course, the time for purchasing all necessary materials for the experiment and uncertainty regarding COVID virus situation should also be kept in mind. Therefore, the authors would like to keep the results shown in Figure 2 as they are shown now.

Science Editor, comment #4 In figure 5, the error bar of vascularization BPUI and BPEO in 8w were very high, which proves that the data needs to be validated.

Response to comment #4 As it was described in the section *Results, Histology and histomorphometry*, at page # 18, from line #23 to line #27, percentage of vascularization in BC, BPUI and BPEO groups of implants was determined in implants extracted two and eight weeks after implantations. Four samples (n = 4) per each group, per experimental period (n(BC) = 4, n(BPUI) = 4, n(BPEO) = 4) were taken for this analysis. For each group, for both experimental periods, results are presented as mean value ± SD. Histomorphometrical parameters were

determined in the NIS-Elements software version 3.2 (Nikon, Tokyo, Japan) on H&E stained tissue sections. We have checked again the obtained results and recalculated significant differences and found no differences in comparison with the results presented in the Figure 5. Also, at this point of time it would be impossible to set this whole experiment again. Specifically, obtaining of the samples for new validation of percentage of vascularization encompasses numerous steps described in the *Materials and methods* section of our manuscript, from purchasing the animals, period of adaptation of animals to the new environment, isolation and cultivation of cells, implantation procedure, waiting for experimental period to pass in order to isolate implants. Then, the whole tissue processing procedure from decalcination of explanted tissue to the application of H&E staining methods and analysis described in section *Materials and methods, Histology and histomorphometry* requires additional time. Of course, the time for purchasing all necessary materials for the experiment and uncertainty regarding COVID virus situation should also be kept in mind. Therefore, the authors would like to keep the results shown in Figure 5 as they are shown now.

Science Editor, comment #5 Sometimes were used “2-week-old”, sometimes were used “2 week-old”, please modified “2 week-old” into “2-week-old”.

Response to comment #5 The authors have modified “2 week-old” into “2-week-old” throughout the manuscript. The authors have also corrected this type of typo errors throught the manuscript.

Science Editor, comment #6 In the figure legends, the author not only marked “objective magnification”, but also marked the “Scale bar”. This is repeated markers for the magnification. The authors should be only described as “Scale bar”.

Response to comment #6 The authors have deleted marker “objective magnification” from figure legends and left only “Scale bar” marker in all figure legends that contained repeated markers.

Science Editor, comment #7 The questions raised by the reviewers should be answered.

Response to comment #7 The authors have answered to all questions raised by the reviewers and marked the changes in the manuscript in red font color.

Science Editor, comment #8 Supplementary comments: This is an invited manuscript. The study was supported by Ministry of Education, Science and Technological Development of the Republic of Serbia. The topic has not previously been published in the WJSC. Issues raised: The authors did not provide the approved grant application form(s). Please upload the approved grant application form(s) or funding agency copy of any approval document(s).

Response to comment #8 Thanks for Your comment. At this moment, we will submit the content of the page from the website of the Ministry of Education, Science and Technological Development of the Republic of Serbia with the name and description of the project III41017, as well as links to those pages.

Science Editor, comment #9 The authors did not provide original pictures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor.

Response to comment #9 The authors have now provided original pictures within PowerPoint document entitled "58180-Figures.ppt".

Science Editor, comment #10 The "Article Highlights" section is missing. Please add the "Article Highlights" section at the end of the main text.

Response to comment #10 The "Article Highlights" section is added at the end of the main text. Please find this section in the main text of our manuscript, pages # 29, #30, and page #31 from line 1 to line 15.

RESPONSES TO EDITORIAL OFFICE DIRECTOR

Dear Editorial office director, thank you very much on your comments and suggestions for our manuscript entitled "The effects of simultaneously applied endothelial and osteogenic differentiated adipose-derived stem cells combined with platelet-rich plasma on vascularization and osteogenesis in ectopically implanted bone tissue-engineered construct" subjected to the respectable World Journal of Stem Cells. We have carefully read your comments and marked all demanded changes by using *Track changes* option. The changes made by authors as a consequence of the demanded changes are also marked by using *Track changes* option through the manuscript.

Editorial office director comment #1 Please don't include any *, #, †, §, ‡, ¥, @….in your manuscript.

Response to comment #1 The authors have deleted * sign from the manuscript.

Editorial office director comment #2 Please use superscript numbers for illustration; and for statistical significance, please use superscript letters. Statistical significance is expressed as $^{a}P < 0.05$, $^{b}P < 0.01$ ($P > 0.05$ usually does not need to be denoted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used, and a third series of P values is expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$

Response to comment #2 The authors have changed numbers in figures and figure legends in the manner that numbers are now written in superscript. We have also changed letters used for statistical significance and used superscript letters in reviewed version of our manuscript.

ADDITIONAL COMMENTS FROM AUTHORS TEAM

In addition to the demanded responses regarding the issues raised by Editorial office director, Science Editor and the reviewers, the authors have also made this changes:

1. Figures are arranged according to the Guidelines_and_Requirements_for_Manuscript_Revision_Basic_Study
2. Figure legends were changed according to the Guidelines_and_Requirements_for_Manuscript_Revision_Basic_Study
3. The sections of the structured abstract and Core Tip was shortned according to the request from Guidelines_and_Requirements_for_Manuscript_Revision_Basic_Study: ".
4. The running title was added at first side of the manuscript, according to the Guidelines_and_Requirements_for_Manuscript_Revision_Basic_Study.
5. Since new parts of the manuscript are added according to comments made by reviewer #2, new references are added. Therefore, the reference list is renumbered.

6. All changes made according to comments made by Editorial office director, Science Editor and reviewers are marked in red. All changes that were made as a consequences of the demanded changes are also marked in red.