Dear Dr. Fang-Fang Ji,

Thank you very much for your e-mail. We have revised the manuscript no.46612 entitled " Cell source as a major challenge in tissue engineering: aging as a limiting factor " based on the respected reviewers' comments.

The comments of the reviewers have been implemented accordingly and all the corrections and modifications are done using track changes in the revised manuscript. Moreover, the manuscript has been edited and checked by Research Consultation Center (RCC), and the certificate for grade A language is provided.

Comments by the reviewers and answers (A) are as follows:

Responses to the Reviewer #02931898 Comments:

Comment 1:

I think that this paper is too long, particularly since it is centered on the limiting effect of age. The three first chapters should be reduced. This part has been written more like a book (chapter 1 is even entitled "preface"!). The reader expects a more direct introduction.

A:

The "Preface" is changed to "Introduction"

The introduction is rewritten and shortened.

Part 2 is shortened.

Part 3 is shortened.

Comment 2:

I'm surprised by the paragraph entitled "genetically-modified stem cells". This chapter should re-written by including the CRISPR-Cas9 technology, which is major a major breakthrough in the field. Particularly since the recent controversial application to human in vitro fecundation (see Nature, December 6th, 2018, volume 564).

A:

The following sentences are added to part 3.4, page 10 with their respective references:

"The promise of using Clustered regularly interspaced short palindromic repeats (CRISPR) technology brings about new hope as a tool for gene editing of stem cells [37, 38]. Brunger et al., used CRISPR for targeted deletion of the interleukin 1 receptor 1 gene in murine iPSCs to make custom-made inflammation resistant cartilage cells [39]. Genome editing by CRISPR has also been used to correct Duchenne muscular dystrophy patients derived iPS cells successfully to differentiate muscle cells that express functional protein [40]. Moreover, Genetic modification of patient-derived iPSCs using CRISPR and other genetic engineering tools has been used for hemoglobinopathies such as b-thalassemia and sickle-cell anemia [41-44]."

Comment 3:

Furthermore, the ethical point of view should be underlined.

A:

The following sentences are added to part 3.4, page 11 with their respective references:

"Ethical issues of gene editing of stem cells should not be overlooked. Matters like safety and efficacy of gene editing, including off-target mutations, also the concerns regarding human enhancement and eugenics must be closely regulated. All in all, it is a necessity to set boundaries for techniques that have dire consequences [37, 54]."

Comment 4:

In the second sentence of the chapter "embryonic stem cells", the authors say "ESCs are formed from 4 days blastocysts up to the ninth week", which I don't understand. Right after the blastula stage, at the gastrula stage (therefore long before the ninth week) it's impossible to get ESCs!

A:

The following sentence:

"ESCs are pluripotent stem cells obtained from the embryo. These cells form from inner cell mass of blastocysts in 4-5 days' post-fertilization up until the ninth week of gestation."

is changed to:

"ESCs are pluripotent stem cells isolated from inner cell mass of blastocysts up until day 5.5 post-fertilization, right before the stage in which the embryo is ready for gastrulation."

Respective references are added.

Responses to the Reviewer #02398061 Comments:

Comment 1:

It is advised to having at least the first part and the abstract reviewed by a native English-speaking person. Again, both the abstract and the first part are a bit "bumpy." Throughout the manuscript, there are many mistakes, some odd sentences, including choice of words and syntax.

A:

The abstract is rewritten.

The first sections (Introduction and abstract) is rewritten.

The manuscript is checked by a certified English language editor. The following are added to acknowledgments at the request of Research Consultation Center (RCC):

“The authors wish to thank Mr. H. Argasi at the Research Consultation Center (RCC) of Shiraz University of Medical Sciences for his invaluable assistance in editing this manuscript. “

Comment 2:

It is not clear why the discussion of iPSC is done twice in sections IV and 3. It should be in section 3 alone.

A:

Section IV is merged with the section 3.2 and the sentences integrity is rechecked.

Comment 3:

The literature selection could be improved in sections 1-3. Some of the citations are outdated and should be replaced with more recent publications (e.g., macular degeneration and Parkinson’s disease, current standing of ASCs, latest updates on clinical successes, e.g., on gene-engineering SCs, such as sickle cell anemia, …). Altogether, while the principles have been discussed, the text is lacking examples and literature on the most updated standings of SC use both in basic and preclinical research and in clinical application. A much more thorough screening of the updated literature is necessary.

A:

Parts regarding the current use of genetically modified stem cells is added to the manuscript with their respective references. The parts are as follows:

“The promise of using Clustered regularly interspaced short palindromic repeats

(CRISPR) technology brings about new hope as a tool for gene editing of stem cells [37, 38]. Brunger et al., used CRISPR for targeted deletion of the interleukin 1 receptor 1 gene in murine iPSCs to make custom-made inflammation resistant cartilage cells [39].

Genome editing by CRISPR has also been used to correct Duchenne muscular dystrophy patients derived iPS cells successfully to differentiate muscle cells that express functional protein [40]. Moreover, Genetic modification of patient-derived iPSCs using CRISPR and other genetic engineering tools has been used for hemoglobinopathies such as b-thalassemia and sickle-cell anemia [41-44]."

Above and beyond, as our goal was to address the aging and its mechanisms in the stem cell sources, providing a few examples would be satisfactory, regardless of the references' approach toward the cell and molecular biology of stem cells or their clinical use.

Comment 4:

There are no citations in section 4. (Cellular aging is a limiting factor).

A:

Proper references were added (references #55 and #56).

Comment 5:

There should be a better distinction between the different usages of cells for therapeutic application. For example, transplanting cells is fundamentally different than transplanting organs. This also includes the usage of stem cell sources, either as a direct source for transplantation, or as source to produce organs. This should be made clearer in the text.

A:

The following part (in the beginning of section 2) is rewritten in order to clarify the points mentioned by the reviewer:

"While cell transplantation, organ transplantation, and tissue engineering are fundamentally different, there are essentially three varieties of sources: autologous,

allogeneic, and xenogeneic cells, each of which can be subdivided into several types of stem cells including adult and embryonic stem cells."

Comment 6:

Sections 4.1. - 4.6. should include more discussion on pluripotent stem cells. there is a larger literature on "aging" of these cells, both during propagation and differentiation, which has not been touched on.

A:

The following sentences are added to the manuscript, with the citation of their respective references:

At the end of the paragraph 1 of section 4.1:

"This age-associated skewed differentiation is not completely understood for cells like ESCs and iPSCs. For instance, Xie et al. (2011), showed that H9 ESCs have an increased tendency for ectodermal lineages; however, this might have had to do with the culture media composition. However, they observed no difference in teratoma formation between old and young ESCs [87]. iPSCs were found to have a different story; while iPS cell lines have a slightly skewed differentiation capacity, it has no correlation with the cell source they are originated from [88, 89]."

At the middle of the first paragraph of section 4.4:

In fact, iPSCs are reprogramed with regard to the age-, tissue- and senescence-associated, but keep some donor-specific DNA methylation patterns [129].

At the end of the third paragraph of section 4.6:

"Xie et al. (2011), found that the most prominent changes that occur in long-term passaged ESCs have to do with the mitochondria; older passages of H9 and PKU1 hESC

(human ESC) lines have elevated mitochondrial mass, ROS level, and mitochondrial membrane potential [87]. On the other hand, aged iPSCs develop defects in their nuclear envelop [159], which might be the cause of interference in Sirt and NF-kB nuclear transportation and downstream signaling in these cells [160, 161].”

Comment 7:

The title is a bit misleading. I would remove “challenge” and find a better way to reflect the broader nature of the article.

A:

The title of the manuscript is changed from:

“Cell source as a major challenge in tissue engineering: aging as a limiting factor”

to:

“Aging: A Cell Source Limiting Factor in Tissue Engineering”

Responses to the Reviewer #02524648 Comments:

Comment 1:

The English language should be thoroughly and extensively edited for the text to be easily read and understood.

A:

The manuscript is checked by a certified English language editor. The following are added to acknowledgments at the request of Research Consultation Center (RCC):

“The authors wish to thank Mr. H. Argasi at the Research Consultation Center (RCC) of Shiraz University of Medical Sciences for his invaluable assistance in editing this manuscript. “

Comment 2:

Surprisingly, despite the very extensive bibliography, there is a general lack of references through many of the sections in the Ms; this is especially apparent in the first 3 sections. On the other hand, there are other parts (e.g. 4.1) where many references are indicated. In addition, many of the references presented have to do with very specific comments in the text, rather than with the general information the section deals with. Therefore, the reader is left with the feeling that the sentences are not well supported by bibiographical evidence.

A:

The first 3 sections, which are now shorter than before, deal with many general concepts. On the other hand, in the section 4 of the manuscript we discussed many specific phenomena in detail; for this purpose, we extensively dug into many literatures for the fourth section, while sufficed with the current number of references for the general concepts in the first 3 sections.

Comment 3:

Overall, a lot of information is indicated throughout the Ms, but a large part of this information is only briefly mentioned and not substantiated nor the topic adequately introduced or presented in sufficient depth (e.g. sirtuins). On the other hand, the second half of the Ms is full of specific, individual examples (e.g. a lot of information is given on specific properties of MSC and HSC in particular study cases) and the references for these examples.

A:

- The following sentences are added to the manuscript at the beginning of the second paragraph of section 4.6; the appropriate references are cited:

"Sirtuins, (SIRT1–SIRT7) a conserved family of NAD^+-dependent deacetylases, of which SIRT1 is the best known, appears to increase mitochondrial turnover by activation of mitophagy. Activation of sirtuins can considerably extend the replicative capacity of human bone marrow stem cells [151], also human fibroblasts [11, 152]."

- Mentioning specific types of stem cells, e.g. MSCs and HSCs, in the aging part is for the sole purpose of clarification and making it easier for the readers to understand and remember each of the aging mechanisms.

Comment 4:

There is a lot of repetition all throughout the various sections, indicative of an excessive partitioning of the text into small sections that should perhaps have been grouped together, e.g. iPSC appear in two different sections, autophagy is discussed and the information repeated in several parts of the Ms, mitochondria, autophagy, "rejuvenation" avenues should have been brought together or, at least, the information should have been distributed without repetitions, etc. (e.g. the information in section 4.6 is clearly duplicated in section 4.7)

A:

- First iPSC part is merged with the second iPSC part and integrity of this part is rechecked.

- Due to the fact that all the mentioned mechanisms for aging are extremely interlinked, it is inevitable that, for instance, the autophagy is mentioned in the mitochondria part, and vice versa. Moreover, we intended to have each subsection written in a way to be comprehensive without the need to read other parts.

Comment 5:

In section 4.6 (last paragraph) the authors seem to identify the inhibition of tumor growth and lymph metastasis with increased cell viability and there also seems to be some crossing between cell survival and the survival of individuals.

A:

The last paragraph of section 4.6 is changed in a way to clarify the ambiguity of our message; we intended to provide examples on the weak points of the ROS-theory and encourage further research on the reexamination of the basis for this theory. The rewritten part is as follows:

"Above all, some studies contradict ROS as a contributing factor in aging. At the cellular level, Zhu et al. (2014), showed that there is "no evident dose-response effect between cellular ROS level and its cytotoxicity." For instance, they showed that while all three of the piperlongumin (PL), beta-phenylethyl isothiocyanate (PEITC), and Lactic acid (LA) increased ROS in the cultured cells, only PL and PEITC, two ROS-based chemotherapeutic agents, killed the cells and LA "spared them." Additionally, although chemical depletion of glutathione increased ROS much higher than PL and PEITC, it did not affect the cell growth in cultured samples [162]. Gal et al. (2015), showed that administration of the antioxidant N-acetyl cysteine to mouse model of melanoma not only did not increase the survival of the mice but also increased the severity of their tumors by increasing metastasis [163]. Biesalski et al. (2010), meta-analytically reevaluated clinical effectiveness of antioxidants on mortality and health. They showed that micronutrients, including those with anti-oxidant activity, are only effective in those with the deficiencies or the risk of deficiencies, but not effective in individuals with the micronutrients above the minimum required level [164]. All in all, these counterexamples provide suffucuent evidence to raise a reasonable doubt toward ROS-based therapeutics."

Comment 6:

When reading the manuscript, there is perhaps the feeling that genetic modification of stem cells or the use of stem cells/iPSC is considered as a viable avenue towards tissue engineering and transplantation, as well as the idea, especially after reading the conclusion, that aging can in fact be reverted and employed to build transplantable tissues, when this is not really the case and there are instead important hurdles to be overcome.

A:

The conclusion is rewritten to avoid the ambiguity mentioned by the reviewer:

“The way toward the production of tissue engineered products has still serious hurdles to overcome: the choice of cell source, proper biomaterial selection, maintaining blood supply by designing suitable scaffolds and three-dimensional tissue architectures. Combined efforts towards prevailing over these major obstacles are needed to pave the way for achieving tissue engineered products at commercial scales.

In regards to the choice of cell source, aging is a limiting factor. Aging, as inevitable as it seems, is proven to be conquerable. In different cell types the problem of aging is preventable and, to some extents, reversible. As aging is a very complex and dynamic phenomenon, it would be better to approach it from a systems biology point of view to reach the best results; perhaps we need to target multiple pathways to touch down the maximum efficacy. Regardless of the application of the stem cells, i.e., tissue engineering and cell therapy, either way, we have to overcome aging, both in the original cell source and in the *in vitro* proliferation.”

- Also, we now explicitly mentioned the major challenges of the tissue engineering in the abstract:

“Tissue engineering is yet to reach its ideal goal, i.e., creating profitable off-the-shelf tissues and organs; designing scaffolds and three-dimensional tissue architectures that can maintain the blood supply, proper biomaterial selection, and identifying the most efficient cell source for use in cell therapy and tissue engineering are still major challenges.”

- The following sentences are added to the introduction for disambiguation of the role of aging as a challenge in tissue engineering:

“Furthermore, while major challenges of tissue engineering must be addressed at first, aging, as a cell source limiting factor, should not be overlooked.”

“Anatomical and functional complexities of biological systems challenge the artificial construction of viable human tissues and organs. Proper three-dimensional tissue architecture to maintain blood supply is a key constraint on the size of the *in vitro* fabricated tissues [1]. In addition, biomaterial selection and strategies to design tissue scaffolds are vital for regulating cell signaling pathways, which provides appropriate cell-cell interactions such as growth factor delivery, essential for cell differentiation.”

“The fundamentals of this interdisciplinary field not only involves identifying biomaterials and designing scaffolds for *in vivo* cell expansion, but also requires addressing the reliable cell sources.”

Comment 7:

There is not a consensus in the way the subheadings are indicated (e.g. under section 2, the authors use I., II., III…., under section 3, a., b., …, under section 4, 4.1, 4.2, …

A:

The mentioned point is implemented by changing all of the headings and subheadings to a unified format.

Comment 8:

These points need to be re-organised differently to avoid unnecessary duplications of the same pieces of information.

A:

This point is addressed in comment 4 of the same reviewer.

Comment 9:

The general ideas must be clearly supported by a sufficient number of references, while a good number of the specific examples that are discussed in detail should be instead briefly mentioned so that the main messages of the Ms are not lost;

A:

This point is addressed in comment 2 and 3 of the same reviewer.

Comment 10:

The problems that are currently faced by tissue engineering and transplantation, although indeed brought into the Ms, must be more clearly defined, so that there is no subtle message to the reader that the way towards tissue engineering has already been paved by the advent of genetic modifications (e.g. telomerase manipulation), the use of embryonic stem cell / iPSC, and the reversal of aging as a process that does not raise any serious concerns. I would recommend extensive re-writing of the Ms following these guidelines.

A:

This point is addressed in the comment 6 of the same reviewer.

Responses to the Reviewer #03372822 Comments:

Comment 1:

The authors address very relevant issues in their manuscript. However, the overall manuscript is organized as a list of topics with no real link between them. There are even 2 "iPSC" sections that could be blended in a unique section.

A:

The “Introduction”, part 2 and part 3 were rewritten in increase the comprehensiveness and coherence of the manuscript.

First iPSC part is merged with the second iPSC part and integrity of this part was rechecked.

Comment 2:

Hematopoietic stem cells and Mesenchymal stem cells are probably the cells used "routinely" nowadays which present the safest use. These cells are merely described in the section presenting aging-related issues.

A:

Mentioning the HSCs and MSCs was for the sole purpose of citing some examples. We deliberately emphasized on the aging mechanisms to fill this gap in cell sources of tissue engineering.

Comment 3:

"The conclusion is largely biased towards aging and do not summarize the full content of the manuscript."

A:

The conclusion is rewritten:

"The way toward the production of tissue engineered products has still serious hurdles to overcome: the choice of cell source, proper biomaterial selection, maintaining blood supply by designing suitable scaffolds and three-dimensional tissue architectures. Combined efforts towards prevailing over these major obstacles are needed to pave the way for achieving tissue engineered products at commercial scales.

In regards to the choice of cell source, aging is a limiting factor. Aging, as inevitable as it seems, is proven to be conquerable. In different cell types the problem of aging is preventable and, to some extents, reversible. As aging is a very complex and dynamic

phenomenon, it would be better to approach it from a systems biology point of view to reach the best results; perhaps we need to target multiple pathways to touch down the maximum efficacy. Regardless of the application of the stem cells, i.e., tissue engineering and cell therapy, either way, we have to overcome aging, both in the original cell source and in the *in vitro* proliferation."

Comment 4:

"a few typos were detected in the text, but activated English WORD corrector should easily pick them up"

A:

The manuscript is checked by a certified English language editor.

Comment 5:

"4th line of the first iPSCs section, the authors indicate "These factors have been shown to contribute to maintenance of pluripotency in adult cells and are sufficient to generate ...". In this sentence, "adult stem cells" should be substituted by "EMBRYONIC stem cells"."

A:

The comment is implemented and first iPSC section is merged with the second one.

Dear Dr. Ya-Juan Ma,

Thank you very much for your e-mail. We have revised the manuscript no.46612 entitled " Aging: A Cell Source Limiting Factor in Tissue Engineering " based on the respected reviewers' comments.

The comments of the reviewers have been implemented accordingly, and all the corrections and modifications are done using track changes in the revised manuscript.

Comments by the reviewers and answers (A) are as follows:

Responses to Reviewer #02931898 Comments:

Comment 1:

Indeed, I reviewed this paper, In the revised version the authors answered correctly to my remarks.

A:

We appreciate the reviewer's positive assessment of our revised manuscript.

Responses to Reviewer #02398061 Comments:

Comment 1:

One major issue is the reference list that needs to be carefully checked again. Many references don't match the text, in particular starting at page 15 in section miRNAs

A:

All of the references are checked. Appropriate changes are made in the references and the reference list is updated.

Comment 2:

There are also some statements and sentences that are not entirely correct. For example, Page 11, last sentence on iPSC bias: "iPSCs were found to have a different story; while iPS cell lines have a slightly skewed differentiation capacity, it has no correlation with the cell source they are originated from [88, 89]." The statement is incorrect. There is also a literature claiming that the

differentiation capacity of iPSC is biased to their originated cell source, probably an effect of retained epigenetic profiles.

A:

The following sentence:

"iPSCs were found to have a different story; while iPS cell lines have a slightly skewed differentiation capacity, it has no correlation with the cell source they are originated from [88, 89]."

is changed to:

"iPSCs were found to have a different story; while some studies have claimed that iPSCs have skewed differentiation capacity, probably because of their retained epigenetic memory of their original cell lines [89], other studies have reported that iPSCs' differentiation capacity has no correlation with the cell source they are originated from [24,88]."

Respective references are added.

Comment 3:

Page 17, the new text on ROS." For instance, they showed that while all three of the piperlongumin (PL), beta-phenylethyl isothiocyanate (PEITC), and Lactic acid (LA) increased ROS in the cultured cells, only PL and PEITC, two ROS-based chemotherapeutic agents, killed the cells and LA "spared them." Additionally, although chemical depletion of glutathione increased ROS much higher than PL and PEITC, it did not affect the cell growth in cultured samples [162]." - These statements refer to cancer cells and it is unclear if similar mechanisms also occur in stem cells.

A:

The following sentences are added:

“However, these results were achieved in cancer cells, and it is unclear if similar mechanisms also happen in stem cells.”

Comment 4:

Page 15: "… a somatic cell can either be fused with the cytoplasm of an enucleated oocyte…" - this is incorrect, the nucleus of a somatic cell is fused.

A:

The following sentence:

“a somatic cell can either be fused with the cytoplasm of an enucleated oocyte”

is changed to:

“the nucleus of a somatic cell can either be fused with the cytoplasm of an enucleated oocyte …”

Comment 5:

The manuscript has been improved taken into account the comments of the reviewers. It now reads a lot better and provides a comprehensive overview on cell sources for tissue engineering and replacement therapy, aging as a factor in stem cell senescence and functional decline, and some discussion on "geroprotection".

A:

We appreciate the reviewer’s comments that improved our manuscript to a great extent. Also, we appreciate the reviewer’s positive assessment of our revised manuscript.

Responses to Reviewer #03372822 Comments:

Comment 1:

In my opinion the authors have addressed the concerns raised by the reviewers and substantially improved the manuscript.

A:

We appreciate the reviewer's positive assessment of our revised manuscript.

Comment 2:

Some minor English mistakes persist. Overall, the manuscript can be accepted for publication.

A:

The manuscript is checked by a certified English language editor. Additionally, the manuscript is checked by Grammarly to find and correct English mistakes.

Comment 3:

Some minor inconsistencies (such as usage of both denomination s iPSC and iPS cells) persist.

A:

All "iPS cells" denominations are changed to "iPSCs".