Answering Reviewers

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Column: Basic Study

Title: Influence of donor age on differentiation and division capacity of human adipose-derived stem cells

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Reviewer #1:
Scientific Quality: Grade D (Fair)
Language Quality: Grade B (Minor language polishing)
Conclusion: Major revision
Specific Comments to Authors: Authors compared the differentiation and division capacity of ADSCs derived some young and old donors. Although the topic is interesting, major revisions requested:

1. Authors compared the differentiation and division capacity of 2 kinds of ADSCs (y and o ADSC) at passage 4 and passage 7. However, I think that the difference will be displayed in the further passages, at 10th or 15th.

Our experiments were conducted before passage 8, as standard established in our laboratory, since cells do not enter in the senescence process before that. So, we can guarantee the use of cells in good quality and full performance. We believe at 10^{th} or 15^{th} passages ADSC may be already showing senescence signals. Senescence is well known as an influencer factor of cell activity (division and differentiation capacity) as reviewed by Turinetto, 2016.

We could have evaluated the senescence process of our cells. But, in the present study, our intention was not to evaluate the senescence process after isolation, which would be related to the process and protocols to keep, expand, freeze, and maintain the cells. Here, we would like to know if the age of the donor would influence the cells in their full performance. That is why we conducted the experiments before the cells started to show senescence signals, reducing the variables which could be influencing cell division and

differentiation. Thus, we were able to observe a cleaner response by observing the influence of donor age.

2. To compare the division capacity, authors should compare the Population Doubling Level (PDL), not PDT.

Thank you for your concerns. In fact, we agree that PDL is a more reliable parameter than PDT to state cellular age considering their proliferative capacity under cultivation. However, PDT is also a very common measurement applied to evaluate growth kinetics in general papers (Alt, 2012; Choudhery, 2014; Ye, 2016).

Despite that, we did the comparison of PDL between yASCs and oASCs. We used hASCs from four young and four old donors and maintained it in culture during 4 passages. Each cell culture was realized in triplicate and the number of cells were counted using Trypan Blue stain and a hemocytometer (Neubauer chamber). The PDL was calculated using the following formula: PDL = 3.32 (log N - log N_0) + PDL_0, as previously described by Hayflick L. (1973). The results were plotted in the figure attached to this document (figure 1) and showed that we could not find differences in PDL between yASCs and oASCs. We also included this result as Figure S2 of the manuscript.

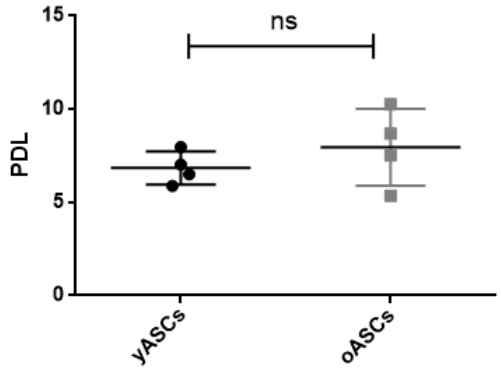

Figure 1 Population doubling level (PDL) of hASCs from different age groups.

Determination of PDL of hASCs from both age groups over four passages in cell culture. The results are expressed as the mean ± SD. Data were compared using a t test, ns = nonsignificant differences.

3. Please compare the differentiation as well as division capacity of ADSCs at the same PDL.

Thank you for your suggestion and despite we understand the reviewer's point of view, we have not evaluated the PDL of the cells when the differentiation experiments were runned. But, as demonstrated in the graphic above (figure 1), no differences were found in the PDL of cells isolated from young and old donors (same cells used in the differentiation experiments). So, the experiments of differentiation and division capacity were conducted in cells with similar PDL values.

Reviewer #2:
Scientific Quality: Grade C (Good)
Language Quality: Grade A (Priority publishing)
Conclusion: Major revision
Specific Comments to Authors: This manuscript describes the influence of donor age on phenotype, proliferation and differentiation potential of human adipose-derived mesenchymal stem cells (hASCs) isolated from adipose tissues by lipoaspiration. The authors found that there were no significant difference between young hASCs (yASCs) and old hASCs (oASCs) in terms of surface marker expression, proliferation, adipogenic and osteogenic potential. These results demonstrated that donor age does not affect phenotype, proliferation and differentiation potential of hASCs isolated from lipoaspirated tissues. Overall, the manuscript is well-written. However, there are several comments that the author needs to address.

1. There are many studies reporting the influence of donor age on proliferation and differentiation potential of hASCs. What was the new approach that the authors take to perform this study?

The first question we asked ourselves was: does the age of hASC donors interfere with their performance, since there are changes in the microenvironment of an aging organism? But, and what about the intrinsic performance of these cells after isolation and when maintained in the same conditions (without environmental alterations)?

Then, looking in literature, we observed many contradictory evidences about how the donor age influences the hASCs performance. So, this was the stimulus to perform our study. We observed that while some studies indicated that old MSC had lower proliferation rates (Alt et al, 2012), others showed no difference (Schipper et al, 2008). Regarding differentiation it is the same: adipogenesis, for instance, was shown to improve (Marwdziac et al, 2016), decrease (Alt et al, 2012) or stayed in the same levels (Choudhery et al, 2014) when compared among hASC from different donors. The differences could be related to many factors such as: method for MSC isolation, body area of harvesting, type of origin tissue, methodology of assays (e.g. proliferation or differentiation quantification), differentiation induction media, age range, number of cell donors, and many others. Here we focus on hASC derived from lipoaspirates, using 9 donors for each age range (young x old) with no differences in IMC. Also, to avoid differences in induction medium we choose to use commercial ones. In addition, the quantification of adipogenesis or osteogenesis was performed with two methodologies, providing greater reliability to the data.

We also have observed the age increase in lipoaspirate donors along the time. It might be related to the social condition of the donors, among other factors, which could represent a worldwide tendency causing the discharge of those samples, without any firm conclusion from the literature data related to poor quality and/or greater variability of those samples. Additionally, most of the countries, including Brazil have been facing an increase in life expectancy (Camargos et al 2019, Roh et al. 2019), and the properties of samples from elderly donors should be addressed in more detail. The huge variety of results from different studies is probably due to different studies designs, body area of harvesting, or type of origin fat tissue. No study in the literature was able to consistently state that cells from young or old donors have dubious quality to be applied to research or clinical trials.

This work will contribute to the understanding of the influence of age on the performance of hASC when grown in vitro, under specific and controled conditions.

2. One of the minimal criteria of defining human mesenchymal stem cells (hMSCs) is hMSCs are capable of undergo adipogenic, osteogenic and chondrogenic differentiation. Therefore, the authors should also provide data for chondrogenic induction of hASCs used in this study.

We appreciate your concerns. Indeed, based on ISCT, MSC were characterized based on its fibroblast-like morphology, the expression of a set of surface markers and the trilineage capacity (adipogenesis, osteogenesis and chondrogenesis) (Dominici M, et al 2006). Our group has been working with MSC isolation for a more than a decade and we follow the same protocol as first stablished (Rebelatto et al 2008), whereas all the ISCT have been addressed. The aim of the current work was, after selecting the cells that had the immunophenotypic profile described for MSC (avoiding differences in this regard), to evaluate differences specifically in the adipogenesis and osteogenesis potential of these cells. Some studies indicated that adipogenesis and osteogenesis are opposite processes and that, if one happens, the other is inhibited (James, 2013). Besides that, looking at the literature many of the studies evaluating the differences between young and old donors of hASCs compare cell functionality choosing one or two differentiation protocols as parameters (Schipper, 2008; de Girolamo, 2009; Khan, 2009; Zhu, 2009). Chondrogenic differentiation is less addressed in these types of comparative studies. Maybe, it could be explained by the fact that chondrogenic properties of hASCs seem to be less robust when compared to bone marrow MSCs (Bourin, 2013). Regardless of these reasons, for the present study we decided to follow the trend of this type of articles, focusing on adipogenesis and osteogenesis potential of these cells.

3. I would suggest the authors to assess gene expression of osteogenic markers in yASCs and oASCs upon osteogenic induction.

As suggested by the reviewer, the expression of the osteogenic marker Runx2 was evaluated among yASCs and oASCs submitted to osteogenic differentiation. The results were included in figure 4 of the manuscript.

4. It would be great if the authors can explain why they selected RNA polymerase II as a housekeeping gene in this study.

The RNA polymerase II (RNAPII) gene has already been described as an efficient housekeeping gene, with low transcription variation among different tissues and experimental conditions (Radonić et al, 2004). It was also demonstrated that RNAPII represents a stable reference gene under different culture conditions of placenta-derived mesenchymal stem cells, and was previously used by our group for normalization of gene expression of adipose-derived mesenchymal stem cells (Minervini et al, 2009; Shigunov et al, 2012). Several studies have been published comparing the stability of reference genes of mesenchymal stem cells, but to the best of our knowledgement, no concerns about the use of RNAPII as reference gene were described (Fink, et al, 2008; Li, et al, 2015; Dessels, et al, 2019). Considering the reasons stated here, we consider that RNAPII is an appropriate housekeeping gene to be used in our experiments.

5. Is there any study reporting the influence of donor age on proliferation and differentiation potential of hASCs isolated from adipose tissues by lipectomy?

Yes, there are some studies evaluating hASCs from subcutaneous adipose tissue by lipectomy. However, sometimes, the isolation process or the region of the body from where the fat was collected is not clearly described. In general, the results are also varied. As examples, we could cite the study from Alt and colleagues (2012) where they analysed hASCs from abdominal adipose tissue specimens and found differences in proliferation and differentiation potential between different aged groups. Kawagishi-Hotta and colleagues (2017) analysed cells isolated from subcutaneous adipose tissue from various body parts (abdomen, groin, lower limb, back and buttock) and although they did find that adipogenic potential declined with age, no alterations were find in the proliferation and osteogenic profile of hASCs from different aged groups. Schipper and colleagues (2008) also evaluated hASCs isolated from an heterogenous source of fat from body contouring procedures (mainly from upper arm and medial thigh). They verified differences in adipogenic potential of cells from mid-aged donors (40-45 yrs), but not between the youngest (25-30 yrs) and the oldest (55-60 yrs) groups. They also did not see differences in the proliferation profile between the groups.

References:

Choudhery MS, Badowski M, Muise A, Pierce J, Harris DT. Donor age negatively impacts adipose tissue-derived mesenchymal stem cell expansion and differentiation. *J Transl Med* 2014; **12:** 8 [PMID: 3895760 DOI: 10.1186/1479-5876-12-8]

Ye X, Liao C, Liu G, Xu Y, Tan J, Song Z. Age-related changes in the regenerative potential of adipose-derived stem cells isolated from the prominent fat pads in human lower eyelids. *PLoS One* 2016; **11:** e0166590 [PMID: 5113966 DOI: 10.1371/journal.pone.0166590]

Turinetto V, Vitale E, Giachino C (2016) Senescence in human mesenchymal stem cells: functional changes and implications in stem cell based therapy. Int J Mol Sci 17:1164 [PMID: 27447618; DOI: 10.3390/ijms17071164]

Alt EU, Senst C, Murthy SN, Slakey DP, Dupin CL, Chaffin AE, Kadowitz PJ, Izadpanah R. Aging alters tissue resident mesenchymal stem cell properties. *Stem Cell Res* 2012; **8:** 215-225 [PMID: 22265741 DOI: 10.1016/j.scr.2011.11.002]
Kawagishi-Hotta M, Hasegawa S, Igarashi T, Yamada T, Takahashi M, Numata S, Kobayashi T, Iwata Y, Arima M, Yamamoto N, Yagami A, Nakata S, Uzawa T, Matsunaga K, Sugiura K, Akamatsu H. Enhancement of individual differences in proliferation and differentiation potentials of aged human adipose-derived stem cells. *Regen Ther* 2017; **6:** 29-40 [PMID: 6134902 DOI: 10.1016/j.reth.2016.12.004]
Schipper BM, Marra KG, Zhang W, Donnenberg AD, Rubin JP. Regional anatomic and age effects on cell function of human adipose-derived stem cells. *Ann Plast Surg* 2008; **60:** 538-544 [PMID: 4160894 DOI: 10.1097/SAP.0b013e3181723bbe]
Bourin P, Bunnell BA, Casteilla L, Dominici M, Katz AJ, March KL, Redl H, Rubin JP, Yoshimura K, Gimble JM. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). *Cytotherapy* 2013; 15: 641-648 [PMID: 23570660; DOI: 10.1016/j.jcyt.2013.02.006]
de Girolamo L, Lopa S, Arrigoni E, Sartori MF, Baruffaldi Preis FW, Brini AT. Human adipose-derived stem cells isolated from young and elderly women: Their differentiation potential and scaffold interaction during in vitro osteoblastic differentiation. *Cytotherapy* 2009; 11: 793-803 [PMID: 19878065 DOI: 10.3109/14653240903079393]
Khan WS, Adesida AB, Tew SR, Andrew JG, Hardingham TE. The epitope characterisation and the osteogenic differentiation potential of human fat pad-derived stem cells is maintained with ageing in later life. *Injury* 2009 Feb;40(2):150-7. doi: 10.1016/j.injury.2008.05.029.
Zhu M, Kohan E, Bradley J, Hedrick M, Benhaim P, Zuk P. The effect of age on osteogenic, adipogenic and proliferative potential of female adipose-derived stem cells. J Tissue Eng Regen Med 2009; 3: 290-301 [PMID: 19309766 DOI: 10.1002/term.165]
Radonić, A.; Thulke, S.; Mackay, I.M.; Landt, O.; Siegert, W.; Nitsche, A. Guideline to reference gene selection for quantitative real-time PCR. *Biochem. Biophys. Res. Commun*. 2004, 313, 856–862, [DOI:10.1016/j.bbrc.2003.11.177].
Minervini, C.F.; Izumi, M.; Miki, T. Effect of culture conditions on reference genes expression in placenta-derived stem cells. *Int. J. Stem Cells* 2009, 2, 69–75, [DOI:10.15283/ijsc.2009.2.1.69].
Shigunov, P.; Sotelo-Silveira, J.; Kuligovski, C.; De Aguiar, A.M.; Rebelatto, C.K.; Moutinho, J.A.; Brofman, P.S.; Krieger, M.A.; Goldenberg, S.; Munroe, D.; et al. PUMILIO-2 is involved in the positive regulation of cellular proliferation in human adipose-derived stem cells. *Stem Cells Dev.* 2012, 21, 217–227, [DOI:10.1089/scd.2011.0143].
Fink, T.; Lund, P.; Pilgaard, L.; Rasmussen, J.G.; Duroux, M.; Zachar, V. Instability of standard PCR reference genes in adipose-derived stem cells

during propagation, differentiation and hypoxic exposure. *BMC Mol. Biol*. 2008, 9, 98, [DOI:10.1186/1471-2199-9-98].
Li, X.; Yang, Q.; Bai, J.; Xuan, Y.; Wang, Y. Identification of appropriate reference genes for human mesenchymal stem cell analysis by quantitative real-time PCR. *Biotechnol. Lett*. 2015, 37, 67–73, [DOI:10.1007/s10529-014-1652-9]
Dessels, C.; Pepper, M.S. Reference Gene Expression in Adipose-Derived Stromal Cells Undergoing Adipogenic Differentiation. *Tissue Eng. - Part C Methods 2019*, 25, 353–366, [DOI:10.1089/ten.tec.2019.0076]
Hayflick L. (1973) Tissue Culture Methods and Applications, P.F. Kruse, Jr. and M.K. Patterson, Jr. eds., p. 220 (Academic Press, New York)
Marędziak M, Marycz K, Tomaszewski KA, Kornicka K, Henry BM. The Influence of Aging on the Regenerative Potential of Human Adipose Derived Mesenchymal Stem Cells. *Stem Cells Int*. 2016; 2016:2152435. [DOI: 10.1155/2016/2152435]
Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy* 2006; **8:** 315-317 [PMID: 16923606 DOI: 10.1080/14653240600855905]
James, A. W. Review of Signaling Pathways Governing MSC Osteogenic and Adipogenic Differentiation. *Scientifica* (Cairo), 2013, 684736. [DOI:10.1155/2013/684736]
Roh, D. S., Panayi, A. C., Bhasin, S., Orgill, D. P., & Sinha, I. Implications of Aging in Plastic Surgery. *Plast Reconstr Surg Glob Open*, 2019, 7(1), e2085. [DOI:10.1097/GOX.0000000000002085]
Rebelatto, C. K., Aguiar, A. M., Moretao, M. P., Senegaglia, A. C., Hansen, P., Barchiki, F., Correa, A. Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. *Exp Biol Med (Maywood*), 2008, 233(7), 901-913. [DOI:10.3181/0712-RM-356]

Point-by-point authors changes

1- All figures were updated.
2- Section D was added at figure 4.
3- References and in text citation were reviewed.
4- Article Highlights were added.

Minor changes:

Page 3, line 19: space removed after "≥"

Page 3, line 25: "PPARγ2 and CEBPA" replaced by "PPARγ2, CEBPA and Runx2 "

Page 4, line 8: “PPARγ2 and CEBPA” replaced by “PPARγ2, CEBPA and Runx2“

Page 5, line 29: “et al.” replaced by “et al”

Page 6, line 11: space removed after “≥”

Page 6, line 12: space removed after “≤”

Page 8, line 16: Added: “and 14 days of osteogenesis”

Page 8, line 17: “an” replaced by “a”

Page 8, line 20: Added: “and osteogenesis”

Page 8, line 26: Added: “Runx2 (runt-related transcription factor 2, forward primer 5’- ACTGGCGCTGCAACAAGAC -3’ and reverse primer 5’- CCCGCCATGACAGTAACCA -3’) was used to asses osteogenic differentiation.”

Page 9, line 10: “p” replaced by “P”

Page 9, line 21: “table” replaced by “Table”

Page 10, line 6: Added “in a comparable range of population double level (PDL) (Figure S2)” after “cells”

Page 10, line 30: “p” replaced by “P”

Page 10, line 30: “p” replaced by “P”

Page 11, line 15: Added “to” after “able”

Page 11, line 15: Added “than” after “better”

Page 11, line 16: Excluded “.” after “cells”

Page 11, line 18: “p” replaced by “P”

Page 11, line 18: “p” replaced by “P”

Page 11, line 19: Added: As an osteogenic marker, we used Runx2. This gene presents higher expression levels at the 14th day of osteogenesis[16]. For this reason, that differentiation time was chosen for Runx2 expression quantification. We verified that osteogenesis-induced cells presented higher marker expression when compared to noninduced cells. Nonetheless, no differences were found between induced yASCs and oASCs (Figure 4D).

Page 13, line 19: “space” removed after “>”

Page 22, line 11: “$^{a}P < 0.05$” replaced by “$^{a}P<0.05$”

Page 26, line 1: Added: “D: Expression levels of Runx2 mRNA after osteogenic induction of yASCs and oASCs. Data are represented as the mean ± SD and are compared using a *t* test. ns = nonsignificant differences.”