Peer Review File

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Reviewer A: This study investigates the use of CD34+ bone marrow stem cells injected into diabetic retinas to assess their revascularization potential. The study is well designed and with a clear scientific rationale. Images are high quality and the authors' attention to quality control of retinal images is exemplary. They conclude that human CD34+ cells have a protective effect on the three retinal capillary plexus in a mouse model of diabetic retinopathy.
Response: Thank you for your positive review of our manuscript.

Here some comments the authors need to address:

Comment 1: The quantitative analysis is unclear in relation to the experimental unit. In methods, authors described that 9 diabetic retinas were used, but the figures 4 and 5 show more than 30 samples. It seems images have been used as the experimental unit for quantitative analysis, which may inflate the p value. Authors should consider the use of SuperPlots (PMID: 32346721) and use the single eye as independent experimental unit.
Response 1: Thank you for this excellent suggestion. We have re-analysed the study data using SuperPlots and the single eye as independent experimental unit. In addition, we found an error in the sample size for the PBS group (n=12, not 13) which was corrected in the updated manuscript. After making these changes, the study conclusions remain unchanged for the superficial plexus layer but no significant difference was noted for the intermediate and deep plexus layers. The manuscript and figures have been updated to reflect the revised analysis. The abstract and discussion have been edited to reflect the new study findings.

Comment 2: In methods, an EGFP labelling procedure is described for CD34+ cells before delivery. This will enable cell tracking and a key question is what happens with the CD34+ cells. Do they remain in the vitreous? Do they cross the inner limiting membrane? Can they reach the deep retinal capillary plexus? Do they differentiate into endothelial cells or microglia? Assessing the EGFP expression in retina will

provide important insights to answer these questions.

Response 2: In our previous publication, we tracked the EGFP labeled CD34+ cells in the diabetic eyes following intravitreal injection using in vivo retinal imaging and immunohistochemistry (Yazdanyar et al. Exp Eye Res, 2020). We demonstrated rapid retinal homing and integration of these cells into the inner layers of the retina. Some of these cells integrated into the retinal vascular wall while others were identified in adjacent avascular retina.

Comment 3: The decrease in vascular density at early stage diabetic retinopathy in the STZ model (5 months) is debatable. This was used as the major readout and therefore, authors should discuss the mentioned benefit for the increased vascular density in the retina when a decrease is not evident in non-diabetic mice.

Response 3: Although the intravitreal injection was performed at 5 to 5.5 months after induction of diabetes, the eyes analyzed for vascular density were harvested 4 weeks after intravitreal injection, i.e. 6 to 6.5 months after induction of diabetes. At this time point, retinal vascular changes consistent with diabetic retinopathy have been observed by histology (Feit-Letchman, R.A., Kinouchi, R., Takeda, M., Fan, Z., Mohr, S., Kern, T.S., et al., 2005. Vascular damage in a mouse model of diabetic retinopathy: relation to neuron and glial changes. Invest. Ophthalmol. Vis. Sci. 46, 4281–4287). We also confirmed the development of areas of capillary nonperfusion using in vivo retinal imaging at this time point (i.e. OCT angiography and Fluorescein angiography; Yazdanyar et al. Exp Eye Res, 2020). The updated manuscript includes the fluorescein angiogram findings at these time points.

Reviewer B: The authors have examined the protective effects of intravitreal injection of human CD34+ bone marrow stem cells on all the retinal capillary layers in a streptozotocin-induced mouse model of diabetic retinopathy. Using flat-mount retinal immunohistochemistry and confocal microscopy suing ImageJ software, retinal vascular density and vascular length density were measured in all three layers of retinal capillaries. Both these parameters were found to be significantly increased in the treated eyes compared to PBS-treated or untreated eyes. Thus, the authors conclude that these stem cells have a protective effect in all there retinal capillary plexuses. As capillary nonperfusion is a hallmark of diabetic retinopathy and several

studies using OCTA have shown that the macular capillary density is impaired in DR patients, the current study has enormous clinical implications. A clinical trial by the group has already established the safety and feasibility of intravitreal injection of autologous CD34+ BMSC in eyes with retinal ischemia and retinal degeneration. The present study further establishes the efficacy of the technique in restoring the integrity of the all the three retinal capillary plexuses in a murine model of diabetic retinopathy. The methods were all well designed, results properly analyzed and conclusions were well drawn.

Comment 1: The authors have used the intravitreal injection of BMSC in mice with diabetes for five months. As they have labeled it as the murine model of diabetic retinopathy, it would be important for the readers to know if there were any signs of DR in this model like acellular capillaries or increased permeability or any other DR lesion. This model has been well established in the literature for studying DR, and any further study in another model of DR is not really needed.
Response 1: Thank you for pointing this out. Our prior publication (Yazdanyar et al., Exp Eye Res, 2020) reported that we observed retinal vascular leakage on late phase fluorescein angiography and patches of capillary dropout (i.e. retinal ischemia) on early phase fluorescein angiography and OCT angiography. These are all signs of retinal vascular damage from diabetic retinopathy observed in mice and human eyes. The information has been added to Methods and Discussion.

Comment 2: The authors have labeled the BMSC with GFP. What percentage of BMSC were labeled with GFP after transduction?
Response 2: Although a majority of the CD34+ cells were labeled with EGFP, we did not perform flow cytometry to quantitate the exact percentage. In our prior publication (Yazdanyar et al. Exp Eye Res, 2020), we identified some human cells in the retina that were not labeled with EGFP. Thus, the transfection was not 100%. For the current study, we concentrated on the effect of CD34+ cells on retinal vascular density and length density. We did not try to localize the CD34+ cells using EGFP.

Comment 3: If the CD34+ cells home into the avascular retina in addition to the retinal vessels, what are the consequences of such integration? Formation of retinal

vessels in the avascular retina? Such integration may have the immense potential in many retinal vascular diseases.

Response 3: Since we found CD34+ cells in avascular retina in our previous publication, we anticipate these cells are having paracrine trophic effects on the damaged retinal vasculature. Whether these cells are actively forming new vessels is unknown at the present time.