Editors' Choice

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Mesenchymal stem/stromal cells generated from induced pluripotent stem cells are highly resistant to senescence

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ABSTRACT

The use of mesenchymal stem/stromal cells (MSCs) has attracted attention in the field of regenerative medicine based on their anti-inflammatory and tissue repair-promoting effects. Bone marrow is widely used as a source of MSCs; however, the performance of bone marrow (BM)-MSCs deteriorates as the cells age along with cell passaging. Recently, it has been reported that MSCs can be generated from induced pluripotent stem cells (iPSCs), which is expected to represent a new source of MSCs. However, few studies have investigated aging in iPSC-derived MSCs (iMSCs) and their functions. In this study, we investigated whether iMSCs overcome cellular senescence compared to that in BM-MSCs. Cellular senescence was quantitatively evaluated by staining iMSCs and BM-MSCs with fluorescein di-β-D-galactopyranoside (FDG) and following flow cytometer analysis. The hepatocyte growth factor (HGF) concentration in the culture supernatant was also measured as a factor in the therapeutic efficacy of nephritis. The iMSCs did not reach their proliferation limit and their morphology did not change even after 10 passages. The FDG positivity of BM-MSCs increased with passaging, whereas that in iMSCs did not increase. The HGF concentration increased with passaging in iMSCs. In conclusion, our results suggest that iMSCs may be less susceptible to senescence than BM-MSCs and may be used in clinical applications.

Keywords: induced pluripotent stem cell-derived mesenchymal stem/stromal cells, mesenchymal stem/stromal cells, induced pluripotent stem cells, cellular senescence, fluorescein di-β-D-galactopyranoside

Abbreviations:
MSC: mesenchymal stem/stromal cell
iPSC: induced pluripotent stem cell
iMSC: induced pluripotent stem cell-derived mesenchymal stem/stromal cell
BM-MSC: bone marrow-derived mesenchymal stem/stromal cell
FDG: fluorescein di-β-D-galactopyranoside

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HGF: human hepatocyte growth factor

INTRODUCTION

Mesenchymal stem/stromal cells (MSCs) are stem cells that exhibit self-renewal and differentiation potential, defined by their osteogenic, chondrogenic, and adipogenic properties and surface markers.[1] MSCs exist in vivo in the bone marrow, adipose tissue, and umbilical cord. The use of MSCs has attracted attention in the field of regenerative medicine owing to their anti-inflammatory effects and tissue repair-enhancing effects via proliferation of progenitor cells, angiogenesis, and anti-apoptotic effects. We have also demonstrated the therapeutic effects of MSCs in nephritis and acute kidney injury.[2-4]

As advances in clinical applications of MSCs have progressed, several critical issues have emerged. First, cellular senescence is a concern in the clinical application of MSCs, since it has been reported in vitro that MSCs have a decreased proliferation rate[5] and a decreased differentiation potential[6] with further cell passaging. Second, cellular heterogeneity is a potential bottleneck in ensuring sufficient cell numbers for therapy. MSCs exhibit different proliferative and differentiation capacities depending on their source.[7-9] As a possible solution to these problems, MSCs can be generated from induced pluripotent stem cells (iPSCs).[10-13] iPSC-derived MSCs (iMSCs) are expected to represent a new source of MSCs based on the possibility of obtaining a large amount of cells showing relatively high uniformity and low invasiveness in cell harvesting. Therefore, we investigated the characteristics of iMSCs. We determined whether cellular senescence observed in normal MSCs is overcome in iMSCs since there are few studies that evaluate cellular senescence. In the present study, we successfully demonstrated that iMSCs are more resistant to cellular senescence than bone marrow-derived MSCs (BM-MSCs) by staining cells with fluorescein di-β-D-galactopyranoside (FDG), which can quantitatively evaluate the cellular senescence,[14] after continuous passaging and analyzing cell proliferation potential and cell morphology. We also evaluated human hepatocyte growth factor (HGF), which is thought to be involved in the therapeutic efficacy of MSCs.[2]

MATERIALS AND METHODS

Preparation of MSCs

iMSCs were derived from the 1231A3 strain of iPSCs (reprogrammed using episomal vectors, kindly provided from Yamanaka laboratory). The iMSCs were induced using a previously described method.[10] Briefly, iPSCs were cultured on an iMatrix-511 (Nippi, Tokyo, Japan)-coated cell culture dish containing StemFit AK03N (Ajinomoto, Tokyo, Japan) for 4 d and then cultured in StemFit Basic03 (equivalent to AK03N without basic fibroblast growth factor, Ajinomoto) containing 10 μM SB431542 (Fujifilm Wako, Osaka, Japan) and 1 μM CHIR99021 (Axon Medchem, Reston, VA, USA) for 10 d to induce neural crest cell (NCC) differentiation. NCCs were selectively harvested using a cell sorter and CD271 as a marker, and the sorted cells were seeded into human fibronectin (Thermo Fisher Scientific, Waltham, MA, USA)-coated dish containing Basic03 supplemented with 10 μM SB431542, 20 ng/mL epidermal growth factor (Fujifilm Wako), and fibroblast growth factor 2 (Fujifilm Wako) and expanded. Subsequently, differentiation into iMSCs was induced using PRIME-XV MSC Expansion XSFM medium (Fujifilm Wako). BM-MSCs were purchased from Lonza (Lot: 19TL168853, 19TL191055; Basel, Switzerland).

MSC culture

The same culture medium was used for both iMSCs and BM-MSCs. The maintenance medium was PRIME-XV MSC Expansion XSFM (Fujifilm Wako) containing penicillin/streptomycin. Plates were coated with human fibronectin (Thermo Fisher Scientific). Cells were passaged at intervals of 3–7 d. The number of cells seeded during passages was 5×10^5 to 1×10^6 cells/10 cm dish. Cells were cryopreserved in STEM-CELLBANKER GMP grade (Zenogen Pharma, Koriyama, Japan).

Flow cytometry

FACS Canto II (BD Biosciences, Franklin Lakes, NJ, USA) was used to confirm the surface markers of iMSCs. Antibodies against CD73-fluorescein isothiocyanate (FITC) (5 μL/1 × 10^6 cells; BioLegend, San Diego, CA, USA), CD105-PE (5 μL/1 × 10^6 cells; BD Biosciences), human leukocyte antigen DR isotype (HLA-DR)-APC (5 μL/1 × 10^6 cells; BioLegend), CD44-PE (1.25 μL/1 × 10^6 cells; BioLegend), and CD45-FITC (20 μL/1 × 10^6 cells; BD Biosciences) were used to confirm that the cell surface markers met the MSC criteria. For FDG analysis, fluorescence intensity was evaluated using FACS Canto II (BD Biosciences). FDG (Thermo Fisher Scientific) was used as the reagent.

FDG analysis

Cryopreserved cells were thawed in a water bath at 37 °C and collected in a 15 mL tube containing phosphate-buffered saline (PBS). After centrifugation at 430 × g for 10 min, the supernatant was removed and cells were resuspended in PBS. Subsequently, 100 μL of the cell suspension was aliquoted into a flow cytometer tube. The cell suspension was preheated in a 37 °C water bath for 10 min and mixed with 100 μL of 2 mM FDG, which was preheated at 37 °C. After heat treatment in a 37 °C water bath for 1 min, 1.8 mL of 4 °C PBS was added to stop the reaction. Zombie NIR (BioLegend) was used to stain dead cells. Cell suspensions were kept on ice until flow cytometry analysis. The flow cytometer was set up according to standard procedures. Unstained iMSCs and BM-MSCs were used to correct for background autofluorescence, respectively.

HGF level measurement

Cells were seeded at 1.0×10^6 cells/well in 2 mL/well of Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum in 6-well plates coated with human fibronectin. The supernatant was collected at 24 h after cell seeding. The collected medium was centrifuged and the supernatant was harvested as the sample. HGF level was measured by SRL, Inc (Tokyo, Japan). HGF level was measured using an enzyme-linked immunosorbent assay and the detection limit was 0.3 ng/mL. Sample concentrations below the detection limit were considered 0 ng/mL.

Data analysis

Numerical data were analyzed using the Kruskal-Wallis test. P value < 0.05 was considered significant. If the Kruskal-Wallis test indicated a significant difference, the two groups were compared by Mann-Whitney U test and adjusted by Bonferroni. Significant differences between groups are indicated by an asterisk (*). Results obtained using flow cytometry were analyzed using FlowJo ver. 10.6.1 (BD Biosciences).

RESULTS

Generation of MSCs from iPSCs

The generated iMSCs had a fine spindle shape and adhered to plastic dishes, and they were morphologically similar to previously reported MSCs (Fig. 1A).[15] The cell surface markers were consistent with those reported on MSCs (CD73+, CD44+, CD105+, HLA-DR-, and CD45-) (Fig. 1B).

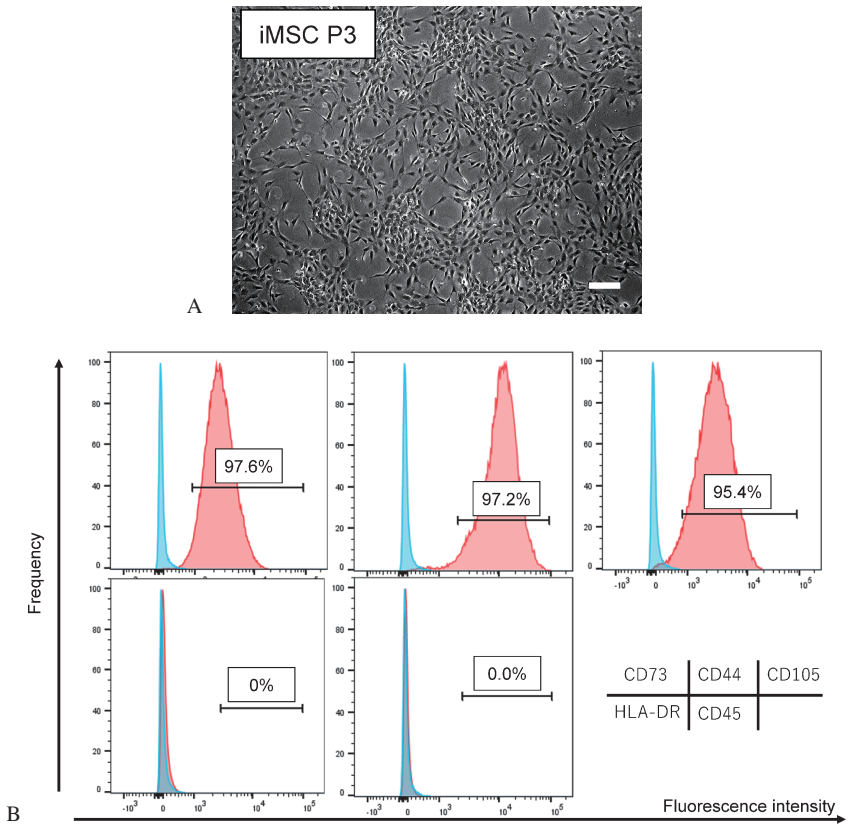

Fig. 1 iMSC from iPSCs

Fig. 1A: Light microscopic images of iMSCs generated from iPSC strain 1231A3. Scale bar = 100 μm.

Fig. 1B: Expression of surface markers on iMSCs. Flow cytometry analysis of each surface marker on the iMSCs.

iMSC: induced mesenchymal stem cell

PSC: pluripotent stem cell

Morphological changes in iMSCs and BM-MSCs after passaging

BM-MSCs showed a reduced proliferative potential and their size increased in diameter as passaging progressed (Fig. 2A, B). Although Fig. 2A was not able to show significant differences because the number of experiments was small (n = 2), cell proliferation of BM-MSCs tended to be slower and it was more difficult to obtain sufficient cell numbers for passage. However, iMSCs continued to proliferate with each passage, and their cell morphology did not change significantly until at least passage 13 (P13) (Fig. 2C).

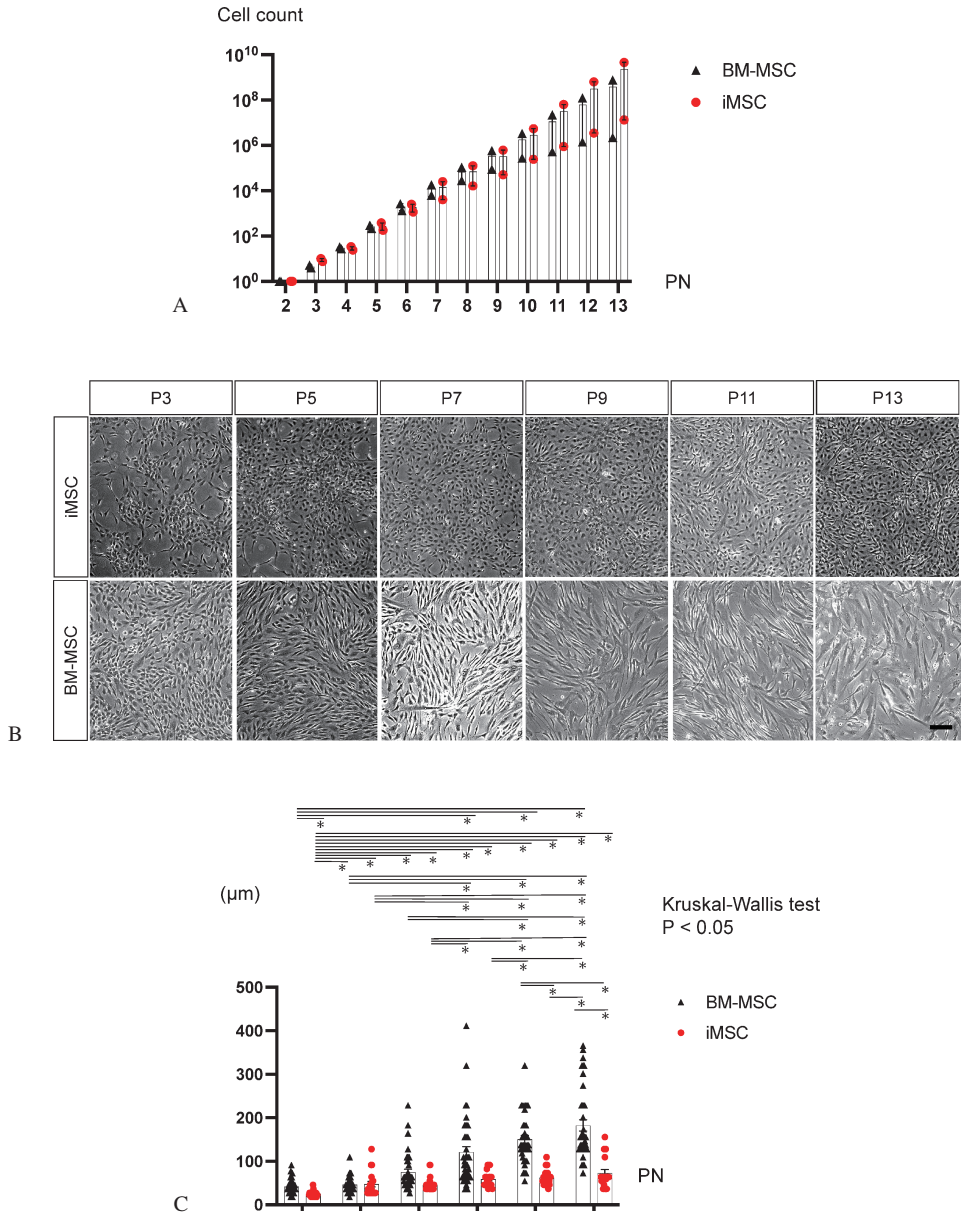

Fig. 2 Cell proliferation of MSCs along with passaging

Fig. 2A: Mean and standard error of the numbers of cells of iMSCs (n = 2) and BM-MSCs (n = 2) after passaging are shown.

Fig. 2B: Morphology of iMSCs after passaging. Changes in iMSC and BM-MSC morphology with passaging from P3 to P13. The upper lane shows iMSCs and the lower lane shows BM-MSCs. Scale bar = 100 μm.

Fig. 2C: The largest diameter of the cells. The mean and standard error of the largest diameter of 20 cells at each passaging number is shown.

PN: passage number

iMSCs: induced mesenchymal stem cells

BM-MSCs: bone marrow-derived MSCs

Changes in FDG positivity of iMSCs and BM-MSCs along with passages

The FDG positivity of iMSCs and MB-MSCs was examined via flow cytometry as passaging progressed (Fig. 3A). iMSCs did not show an increasing trend in FDG signal as passaging progressed, while BM-MSCs showed an increasing trend in FDG signal as passaging progressed. A change in FDG signal from the positivity rate from P3 was observed (Fig. 3B), and the signal tended to increase with passaging in BM-MSCs. However, the analysis of P13 BM-MSCs was considered less accurate because the proliferation of BM-MSCs was slow and a large number of P13 cells could not be obtained.

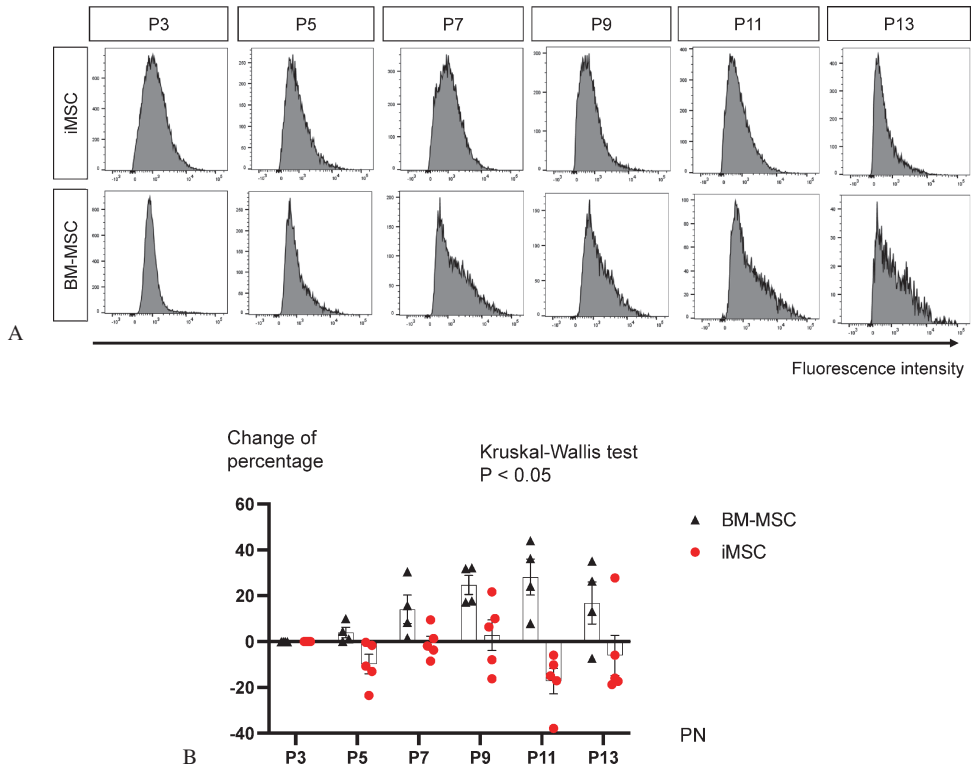

Fig. 3 Senescence of MSCs along with passaging

Fig. 3A: Change in population of FDG-positive cells along with passaging. Flow cytometry results are shown for FDG from P3 to P13 of iMSCs and BM-MSCs.

Fig. 3B: Rate of transition of FDG-positive cells along with passaging. Mean and standard error of the FDG positivity of iMSCs (n = 5) and BM-MSCs (n = 4), using P3 as a reference, and how it changed as passaging progressed from P3.

PN: passage number
iMSCs: induced mesenchymal stem cells
BM-MSCs: bone marrow-derived MSCs
FDG: fluorescein di-β-D-galactopyranoside

HGF levels in culture supernatants of iMSCs and BM-MSCs

HGF levels increased in the culture supernatant of iMSCs with passaging (Fig. 4) and reached a plateau at P9. In contrast, many BM-MSCs had undetectable values.

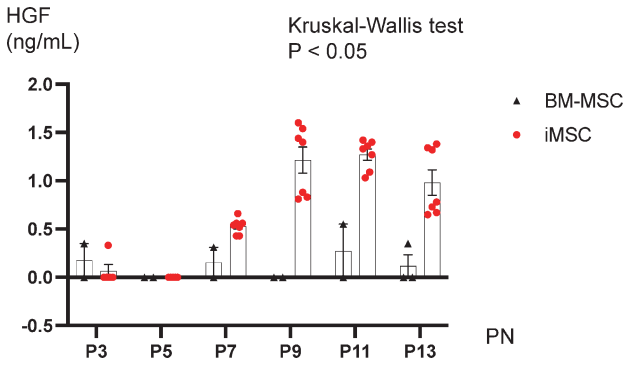

Fig. 4 Change in concentration of HGF with passaging
Mean and standard error of HGF concentrations in culture supernatants of iMSCs (n = 7) and BM-MSCs (n = 2–3).
PN: passage number
iMSCs: induced mesenchymal stem cells
BM-MSCs: bone marrow-derived MSCs
HGF: hepatocyte growth factor

DISCUSSION

In this study, we investigated the morphological changes in iMSCs after passaging and the change in proportion of FDG-positive cells with passaging, which were compared with changes in BM-MSCs. iMSCs showed a minor morphological change with passaging. A minor change in proportion of FDG-positive cells was observed. Therefore, iMSCs were considered to show a minor progression in aging even after cell passaging. In contrast, the concentration of HGF, which is thought to be involved in the therapeutic effect in nephritis, increased over time, suggesting that the therapeutic effect per cell may increase as passaging proceeds.

MSCs are present in various tissues in vivo, including bone marrow, adipose tissue, and umbilical cord. The applications of BM-MSCs are affected by difficulties in maintaining the necessary cell count and the invasiveness of the procedure, which requires general anesthesia for BM-MSC collection. The use of iPSCs as a cell source may solve these problems. iPSCs are capable of unlimited proliferation, and a large number of cells can be obtained at the time of iPSC production. In addition, the same batch of cells can be obtained stably and in large quantities at both the induced NCC and iMSC stages. This eliminates the need for repeated invasive procedures as is the case with BM-MSCs. In the case of BM-MSCs, it is necessary to check the donor or the obtained cells for infection or contamination each time the cells are harvested. It is also necessary to check the quality of the obtained cells. These efforts and costs are extremely large when considering clinical applications, as they must be performed using cells of a high quality. In contrast, if a large number of iMSCs can be obtained from the same batch, then these are a homogeneous group of cells, and the quality and infection checks can be performed only once. This reduction in labor and cost represents a remarkable advantage for clinical applications. Furthermore, the cells are a homogeneous group without any variations in quality; thus therapeutic efficacy is unaffected when used for treatment, which is also important in terms of quality control.

One of the advantages of using iMSCs is that a large population can be cultured and the same donor-derived iMSCs can be continuously used for treating the same patient. iMSCs have low immunogenicity and are not easily eliminated in xenografts or xenotransplants. However,

while such findings have been observed, the production of antibodies against MSC HLA has also been reported.[16] The continued random use of MSCs from various donor origins for treating chronically progressive renal disease may result in the development of donor-specific antibodies at the time of renal transplantation when the patient eventually reaches end-stage kidney failure, thus limiting the options for kidney transplantation. iMSCs also have the advantage of allowing the use of MSCs from the same donor until HLA antibodies are produced.

MSCs have different proliferative, growth factor secretory, and differentiation capacities depending on the organ from which they are harvested and the culture environment.[7-9] In our previous studies, we focused on HGF as a factor related to the therapeutic efficacy of MSCs. We found that MSCs exert high ischemia-related tissue repair and immunomodulatory effects,[3] and we also examined the conditions under which HGF levels increase.[17] In our iMSCs, HGF secretion did not decrease after passaging, but rather increased. Therefore, it is possible that HGF secretion does not decrease or even increase with passaging in the iMSCs. Therefore, the therapeutic effect of HGF on nephritis is also expected to be retained with passaging.

Limitations

This study had a few limitations. This study conducted an in vitro experiment demonstrating that iMSCs may be less susceptible to aging; however, the actual therapeutic effect needs to be tested in vivo in the future. Additionally, experiments were performed using iMSCs generated via a specific induction method. Results may differ for MSCs induced using other methods.

CONCLUSION

MSCs derived from iPSCs were considered to be less susceptible to aging than BM-MSCs. Furthermore, it was suggested that the therapeutic effect may not decrease or even increase with passaging to a certain extent (to P13).

AUTHOR CONTRIBUTION

TA and AT contributed equally as first authors to this work.

DISCLOSURE STATEMENT

There are no conflicts of interest to declare.

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