In this issue, Kusuma et al suggest the potential benefit of trehalose preincubation prior to cryopreservation of CD271+ mesenchymal stem cells (MSCs). Kusuma et al's study showed that preincubation with trehalose increased cell viability compared to control that was not preincubated, and control without cryopreservation medium. However, this study only addressed viability after 24 hours in liquid nitrogen, and did not address other functional variables that play a role in the success of cell therapy, such as differentiation capacity, clonogenic efficiency, immune-modulation properties, and senescence after thawing.

A review on many cryopreservation studies that used various concentrations of dimethylsulfoxide (DMSO) or other cryoprotectants without trehalose preincubation for various periods including 24 hours showed that in most studies, viability and differentiation capacity were not impaired. Most studies assessed viability directly after thawing, while the study of Kusuma et al assessed post thaw viability after one hour in culture. Cells usually undergo apoptosis or necrosis through various pathways after hours to two days after thawing, and reduced viability is usually evident after three days in culture; thus post thaw viability assessment directly or after one hour in culture does not reflect real viable cell number, and if the cells are intended to be used in cell therapy directly after thawing, cell death may occur in the patient, and cell necrosis may cause inflammatory responses.

Moreover, viable after cryopreservation does not necessarily mean functional, as re-culture (passage) after cryopreservation needs more cells to be seeded compared to re-culture of fresh cells, which shows that though viable, most cryopreserved cells have lost their attachment and proliferation capacity. Therefore, measuring clonogenic efficiency is more appropriate to describe cell viability and proliferation capacity, as post thaw clonogenic efficiency is associated with better engraftment and outcome in cell therapy.

In addition, Kusuma et al study measured viability by water-soluble tetrazolium-1 (WST-1) proliferation assay, which might give a higher viability result, as WST-1 measured metabolic activity, and cryopreserved cells were reported to have increased metabolic activity. Moreover, WST-1 proliferation assay in Kusuma et al study showed absorbance that was not interpolated to viable cell numbers, thus could not be compared to other cryopreservation studies that measured viable cell numbers using trypan blue exclusion. Therefore, viability benefit of trehalose preincubation before cryopreservation needs further assurance using trypan blue exclusion method and clonogenic efficiency testing. In terms of differentiation, further studies are needed to show whether trehalose preincubation is superior compared to direct cryopreservation using DMSO containing cryopreservation medium.

Mesenchymal stem cells have immunomodulation properties and are promising to treat graft versus host, or autoimmune diseases. However, several studies showed that cryopreservation caused impairment in post thaw MSC immunomodulation properties due to activation of a heat shock program, but the impairment was reversible after 24 hours of re-culture. Whether impairment of immunomodulation properties may be restored in vivo after transfusion into patients is not known, and needs further studies.

The use of MSC with impaired immunomodulatory function due to cryopreservation is hypothesized
to play a role in the failure of MSC therapy in acute steroid-resistant graft-versus-host disease.\(^6\) The failure was supposed to arise from the fact that the MSCs (Prochymal from Osiris Therapeutics Inc) were derived from a mass production from a volunteer donor, which was expanded to 10,000 doses, while other clinical trials expanded only to five, or maximal to ten doses. Moreover, most cryopreserved MSCs are thawed and transfused into patients within a few hours, without re-culture to reverse cryopreservation deleterious effects.\(^6\) Over expansion may cause cell senescence due to cumulative population doublings that exceed the Hayflick limit.

Moreover, impaired immunomodulatory function might be due to high numbers of pre-freeze and 48-hours post thaw senescent cells.\(^7\) When a high number of post thaw senescent cells are re-cultured, they lose their proliferation capacity and force non-senescent cells to proliferate more to become confluent, thus increases their cumulative population doublings to reach their Hayflick limit, and so non-senescent cells will become senescent, increasing the senescent cell population. A study showed that post thaw clonogenic efficiency of umbilical cord blood hematopoietic progenitor cells after cryopreservation in DMSO containing cryo-medium was 22%,\(^3\) which means that only 22% of post thaw cells can proliferate. Reduced post thaw clonogenic efficiency might play a role in increased proportion of senescent cells, and thus their functional performance. In terms of immunomodulation properties and proportion of senescent cells after thawing, whether trehalosa pre incubation is superior compared to direct cryopreservation using DMSO containing cryopreservation medium is unknown, and therefore, further studies are needed.

In conclusion, pre incubation with thehalosa increased cell viability, but functional variables that play a role in the success of cell therapy, i.e. differentiation capacity, clonogenic efficiency, immunomodulation properties, and senescence after thawing need to be addressed.

**REFERENCES**