Basic Medical Research

Trehalose preincubation increases mesenchymal (CD271$^+$) stem cells post-cryopreservation viability

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ABSTRAK

Latar belakang: *Dimetil sulfoksida (Me_2SO) adalah krioprotektan yang umum digunakan dalam kriopreservasi sel. Me_2SO diketahui menyebabkan perubahan epigenetik yang mempengaruhi perkembangan sel punca dan diferensiasi sel. Oleh karena itu diperlukan upaya untuk mengembangkan tehnik kriopreservasi yang dapat melindungi fungsi sel dan menghindarkan efek samping Me_2SO. Trehalosa diketahui dapat melindungi organisme dalam kondisi ekstrem seperti dehidrasi dan suhu dingin. Penelitian ini bertujuan melihat efek proteksi pre-inkubasi trehalosa dalam prosedur kriopreservasi.*

Metode: *Penelitian ini bersifat ekperimental. Sel punca mesenkim (CD271$^+$) dari biorepositori Universitas YARSI digunakan dalam eksperimen. Pre-inkubasi trehalosa dilakukan selama 1 jam, internalisasi trehalosa kemudian dikonfirmasi dengan pemeriksaan FTIR-ATR. Viabilitas sel dalam kultur dibandingkan antara kelompok yang terdiri dari (1) kriopreservasi tanpa pre-inkubasi trehalosa, (2) kriopreservasi dengan pre-inkubasi trehalosa dan (3) tidak mengalami kriopreservasi (kontrol) setelah 24 jam dibekukan. Absorbansi dari setiap kelompok diperoleh pada panjang gelombang 450 nm. Analisis statistik dilakukan menggunakan* student t test.

Hasil: *Viabilitas sel punca mesenkim (CD271$^+$) pada kelompok yang mendapat pre-inkubasi trehalosa lebih tinggi ($p<0,05$) dari kelompok yang tidak mendapat pre-inkubasi trehalosa. Viabilitas yang lebih baik pada kelompok yang mendapat pre-inkubasi trehalosa dibandingkan kelompok kontrol mengindikasikan adanya proteksi terhadap tripsinisasi. Sel punca mesenkim (CD271$^+$) yang diinkubasi dengan medium yang mengandung 100 mM trehalosa menghasilkan efisiensi internalisasi trehalosa sebanyak 15%.*

Kesimpulan: *Hasil menunjukkan efek proteksi prosedur pre-inkubasi trehalosa dalam kriopreservasi. Penelitian selanjutnya diarahkan untuk menerangkan mekanisme internalisasi trehalosa ke dalam sitoplasma dan mekanisme proteksi yang berperan dalam kriopreservasi.*

ABSTRACT

Background: Dimethyl sulfoxide (Me_2SO) is a common cryoprotective agent widely used in cell preservation system. Me_2SO is currently known to cause epigenetic changes which are critical in stem cells development and cellular differentiation. Therefore, it is imperative to develop cryopreservation techniques that protect cellular functions and avert Me_2SO adverse effect. Trehalose was able to protect organism in extreme condition such as dehydration and cold. This study aimed to verify the protective effect of trehalose preincubation procedure in cryopreservation.

Methods: The study was conducted using experimental design. Thawed mesenchymal (CD271$^+$) stem cells from YARSI biorepository were used for the experiment. Trehalose preincubation was performed for 1 hour, internalized trehalose was confirmed by FTIR-ATR measurement. Three groups consisted of (1) cryopreserved without trehalose preincubation, (2) cryopreserved with trehalose preincubation, and (3) did not undergo cryopreservation were evaluated after 24 hours in LN2 for viability in culture. The absorbance from each group was measured at 450 nm. The analysis performed using paired student t test.

Results: Viability of thawed mesenchymal (CD271$^+$) stem cells that undergo trehalose preincubation prior cryopreservation was significantly higher ($p<0.05$) compared to group without trehalose preincubation. Higher viability observed between group with trehalose preincubation compared with controlled group suggests protection to trypsinization. Mesenchymal (CD271$^+$) stem cells incubated for 1 hour in 100 mM trehalose supplemented medium results in 15% trehalose loading efficiency.

Conclusion: These findings confirm the protective effect of trehalose preincubation in cryopreservation. Future research should be directed to elucidate the trehalose internalization mechanism and eventually the protective mechanism of trehalose in mammalian cell cryopreservation.

Keywords: cryopreservation, mesenchymal (CD271$^+$) stem cells, trehalose preincubation

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Development of regenerative medicine using stem cells as an approach to replace damaged and worn out cells and tissues warrants a storage solution that protects stem cells unique ability to differentiate into many types of cells of the body. Current method of cell cryopreservation incorporated the use of dimethyl sulfoxide (Me_2SO) as an intracellular cryomedium. This practice is currently under criticsm as described by Diaferia et al[1] in their review. Me_2SO is known to cause adverse effects and toxicity to patient, unexpected changes in cell fate, affects epigenetic control by acting on DNA methyltransferases, and loss of pluripotency in human embryonal stem cells.[1] Despite the adverse effect of Me_2SO, it is still commonly used as cell protectant thus we used Me_2SO-based cryopreservation in this study.

Therefore, it is necessary to develop alternative procedure that can be adapted to cryopreservation workflow and avert the potential adverse effect of using Me_2SO as an intracellular cryopreservant. Among other such as sucrose utilized by embryologist for ovum and sperm preservation, trehalose (378.33 g/mol) is a glucose disaccharides that has been recognized to be able to protect cells,[2] organism[3] and stabilize intracellular protein in extreme condition such as dehydration and cold.[4] Cellular toxicity of trehalose was also compared in this study to give a picture of the extent of toxicity posed in trehalose preincubation to mesenchymal stem cells along with other disaccharides such as sucrose and known toxic chemicals such as Me_2SO and hydrogen peroxide which is a potent oxydator and caused damage to organic compound. Preincubation was known to lead to trehalose internalization into the cytoplasm. Several methods of trehalose internalization requires cell poration using adenosine triphosphate (ATP) and the use of liposomes is being investigated.[5–7]

Mesenchymal stem cell is a heterogeneous population recently discovered to contain not only multipotent stem cell but also non-tumorigenic pluripotent stem cell subpopulation known as the multi-lineage differentiating stress enduring (MUSE) cells.[8,9] The ability to preserve functional properties of such unique subpopulation will greatly contribute to the development of regenerative medicine.

Trehalosa as a cryoprotectant has been evaluated in several types of cells such as mesenchymal stem cells-derived from bone marrow, erythrocytes, platelets, and umbilical cord blood stem cells.[10–12] Suggested method of trehalose internalization was by fluid phase endocytosis after 1–24 hours of trehalose pre-treatment via clathrin-mediated endocytosis.[12] However, this was suggested as cell-type specific because the endocytosis mechanism involved.[13] This study aimed to verify the protective effect of trehalose-preincubation in Me_2SO-based cryopreservation of mesenchymal ($CD271^+$) stem cells derived from peripheral blood mononuclear cells which were a potential source of MUSE cells.

METHODS

Research design

The present study conducted using experimental design from December 2014 to March 2016 at YARSI University Cell Culture Facility, Jakarta, Indonesia. Ethical approval received from Komisi Etik Lembaga Penelitian Universitas YARSI (No. 016/KEP-UY/BIA/II/2016).

Cell culture

Mesenchymal stem cells were derived from plastic-adherent mononuclear cells of peripheral blood (PBMC) obtained from Laboratorium Terpadu Universitas YARSI Biorepository. Purified using CD271 magnetic sorting magnetic-activated cell sorting (MACS); Miltenyi, expanded and cultured in Dulbeco's modified eagle's medium (DMEM) low glucose (Gibco) supplemented with 10% fetal bovine serum (FBS) heat inactivated (Gibco). 1% penicillin-streptomycin and fungizone (Gibco) added to prevent bacterial and fungal contamination. Mesenchymal stem cells used for experiments were from passage four to six in exponential phase. All experiment described below were performed in triplicate.

Toxicity of trehalose, sucrose, Me_2SO and hydrogen peroxide were compared using $CD271^+$ mesenchymal stem cells. The study was conducted in a 96 microwell plate with as much as 20,000 viable cell cultured. Incubation of the investigated chemicals at 0 mM, 12.5 mM, 25 mM, 50 mM, and 100 mM of trehalose, 0 mM, 18.75 mM, 37.5 mM, 75 mM, and 150 mM of sucrose, 0%, 0.06%, 0.12%, 0.25%, 0.5%, and 1% of Me_2SO and 0 µM, 31.25 µM, 62.5 µM, 125 µM, 250 µM, 500 µM hydrogen peroxide for one hour and continued

with 30 minutes water-soluble tetrazolium-1 (WST-1) proliferation assay (Roche) incubation to assess cell viability. Measurement conducted using microplate reader at 450 nm. Long-term toxicity was assessed in seven-days-culture, viability was measured at three different time point (day *in vitro*/DIV two, four, and seven) also using WST-1 proliferation assays in 96 microwell plate. Osmolarity measurement of medium supplemented by trehalose and sucrose were conducted at Faculty of Veterinary Medicine, Bogor Agricultural University, Embryology Laboratory.

Trehalose measurement by FTIR-ATR

Mesenchymal ($CD271^+$) stem cells cultured at 80% confluency using tissue flask 25 sqcm were incubated with 100 mM trehalose mixed in culture medium for one hour. Cells were harvested by trypsinization using trypsin / ethylenediaminetetraacetic acid (EDTA) 0.05% for five minutes and washed by centrifugation twice. Resulting pellet were counted using tryphan blue exclusion method and adjusted for 1,000,000 viable cell resuspended in 50 µl phosphate buffer saline (PBS), Gibco for FTIR measurement. Intracellular trehalose detection performed using fourier transform infrared spectroscopy (FTIR) – attenuated total reflectance (ATR).

Cell pellet were dried using deoxyribonucleic acid (DNA) concentrator (MiVac) at 37°C for 45 minutes. Infrared spectra were generated by putting dried cell pellet on top ATR germanium crystal. 32 scan performed by FTIR at 1,100–900 wavenumber/cm, peak at 995–991 wavenumber/cm as reported by Sakurai et al[14] was considered as trehalose. Trehalose concentration was determined using simple Beer's law principle by generating trehalosa standard curve in FTIR-ATR (Nicolet-Bruker). Omnic software was used to measure peak height and area under peak curve to generate a standard curve of known trehalose concentration with a linear fitting.

Cryopreservation

Trehalose-incubated mesenchymal ($CD271^+$) stem cells described previously were cryopreserved using standard slow-freeze technique and cryoprotectant (70% culture medium, 20% Me_2SO, and 10% fetal bovine serum). Three groups were established consist of (1) cryopreserved without trehalose preincubation-slow freeze 721 (SF721) (group 1), (2) cryopreserved with trehalose-preincubation-SF721-Tre (group 2), and (3) did not undergo cryopreservation (control). Cells were stored in -80°C overnight and transferred to liquid nitrogen (LN_2) the next day. After 24 hours in LN_2 cells were thawed and cultured in 96 microwell plate at 10,000 viable cells per well for functional evaluation using WST-1 proliferation assay. Absorbance of attached and viable cell after 60 minutes plating were measured at 450 nm.

Data analysis

Blank absorbances were subtracted from the original data. Analysis was performed using Excell (version 2013; Microsoft). Statistical significance between group 1 and control group and between group 2 and control group were both determined using paired student t test. Phosphate buffer saline spectra were subtracted from all spectra obtained to construct the trehalose standard curve.

RESULTS

Toxicity studies

Toxicity profile of Me_2SO, trehalose, and sucrose hydrogen peroxides were illustrated in Figure 1. These data suggest that 100 mM of trehalose was at comparable toxicity to 1% Me_2SO which was currently accepted as a safe level for cell exposure. A minimum of 150 mM of sucrose was also comparable but the osmolality limited it used. The level of toxicity posed by these dissacharides is comparable with a very low level of hydrogen peroxide concentration.

Further evaluation of the disaccharides were conducted using 50 mM, 25 mM, and 12.5 mM of trehalose and sucrose. Compared to control (0mM) group, within each time points both disaccharides reduced cells viability in a dose-dependent manner. However, observation of each dose group from three different time points during seven days of culture showed that the cells continued to have significant proliferation capacity despite the presence of disaccharides in the medium (Figure 2). Disaccharides toxicity was considered related to cellular hydration status. Figure 3 shows the changes in culture medium osmolality after supplementation with trehalose and sucrose. Increase in medium osmolality caused a relative hyperosmolarity in

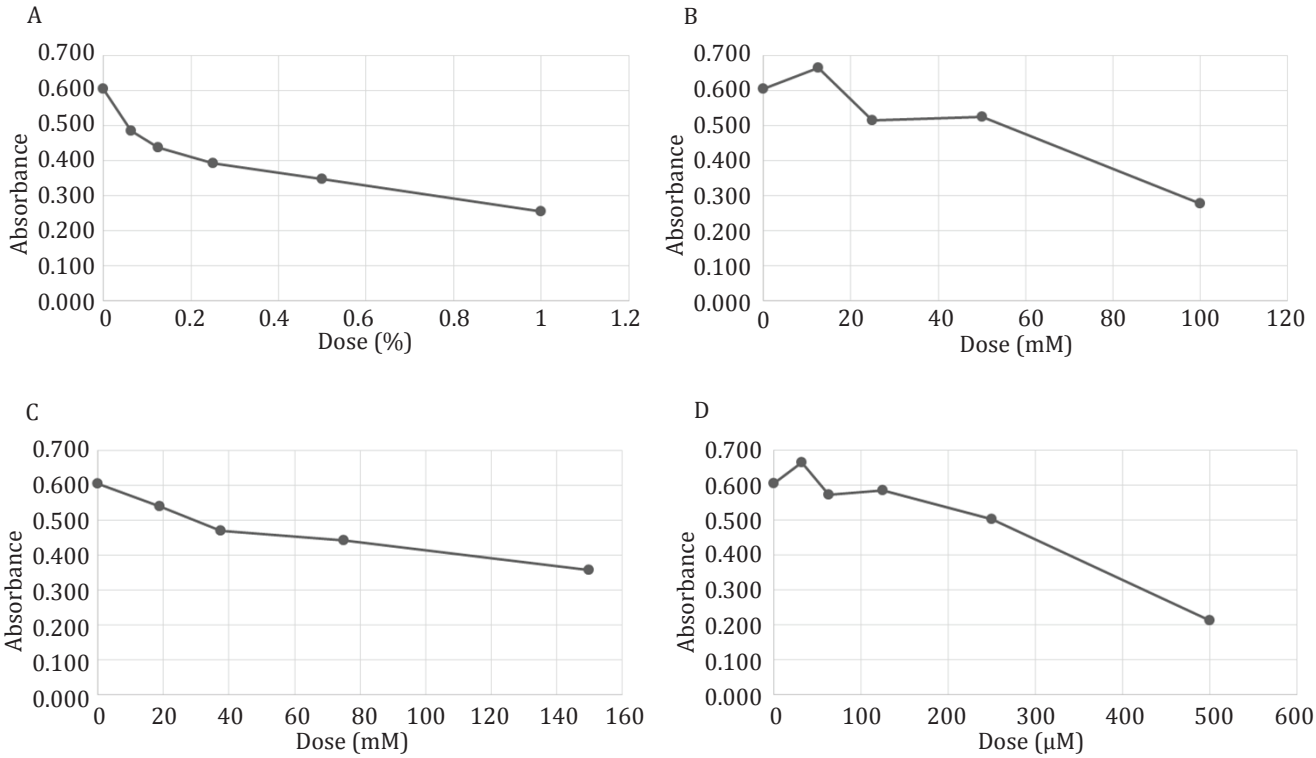

Figure 1. Toxicity profile of Me_2SO (A), trehalose (B), sucrose (C), and hydrogen peroxide (D) to mesenchymal ($CD271^+$) stem cells. Toxicity of 100 mM of trehalose is relative comparable to 1% Me_2SO, 160 mM sucrose and 500 µM of hydrogen peroxide. No significant difference ($p>0.05$) between trehalose (100 mM) and Me_2SO (1%)

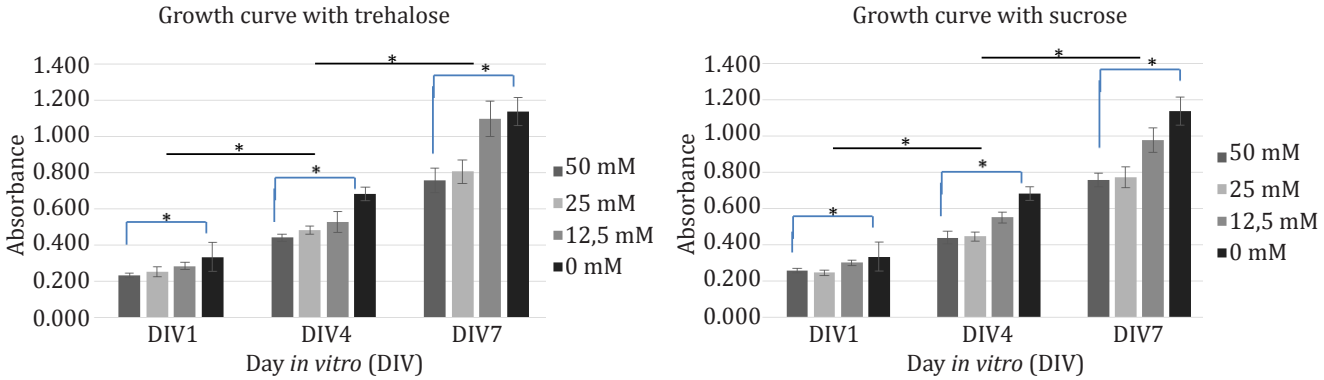

Figure 2. Growth curve of mesenchymal ($CD271^+$) stem cells with trehalose (left) and sucrose (right) as suplementation in culture medium. * $p<0.05$

extracellular environment and triggered water efflux from intracellular compartment that caused dehydration and cell death.

Intracelullar trehalose measurement

Internalization of trehalose into cytoplasm was confirmed by FTIR measurement. Figure 4 shows a spectrum recorded from washed and dried cell pellet with characteristic peak at the fingerprint region (below 1,500/cm) for trehalose at 991–992 /cm. Absorbance level of trehalose derived from infrared spectra were used to construct trehalose standard curve for intracellular trehalose quantitation. (Figure 5) the standard curve allowed quantitation of trehalose within the cytoplasm which gives a resultof 15% loading efficiency. 15.07 mM of trehalose were estimated within the cytoplasm of approximately 1,000,000 viable cells that were measured by FTIR-ATR.

Cryopreservation study

Trehalose-incubated cells showed a greater ($p<0.05$) viability and attachment after 24 hours in liquid nitrogen compared with non-trehalose-incubated cells and control group which was not cryopreserved. These findings confirmed that intracellular trehalose were able to protect mesenchymal ($CD271^+$) stem cells in cryopreservation. Figure 6 shows significant viability retained by trehalose-incubated cells compared to non-cryopreserved cells (control group). These data suggested a certain protection against stress evoked by trypsinization process.

DISCUSSION

Our data showed that extracellular trehalose could cause a toxic effect on cells by inducing osmolality imbalance. Lynch et al[15] reported approximately 60% of cells death recorded after cells loaded with over 200 mM of internal trehalose concentration were resuspended in phosphate buffer saline of physiologically osmolality (300 mOsm). Our toxicity data showed a low dose

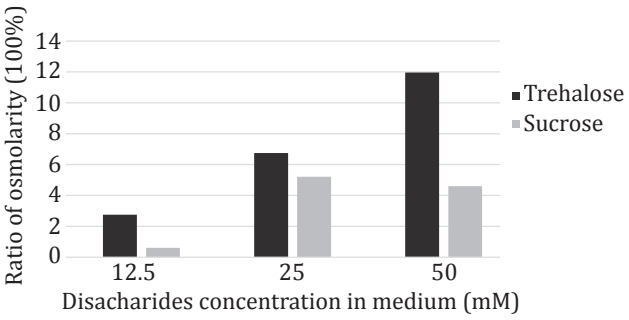

Figure 3. Changes in medium osmolarity after disacharides suplementation

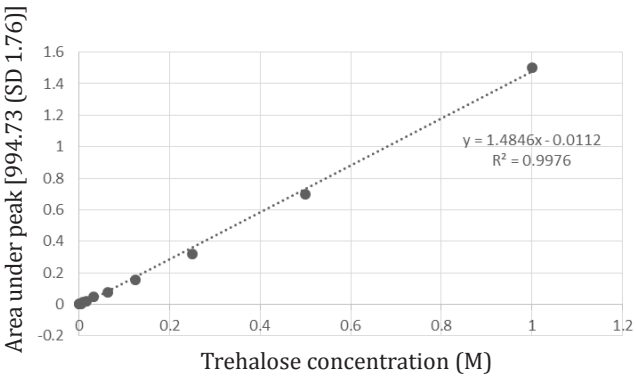

Figure 5. Trehalose standard curve with linear fitting

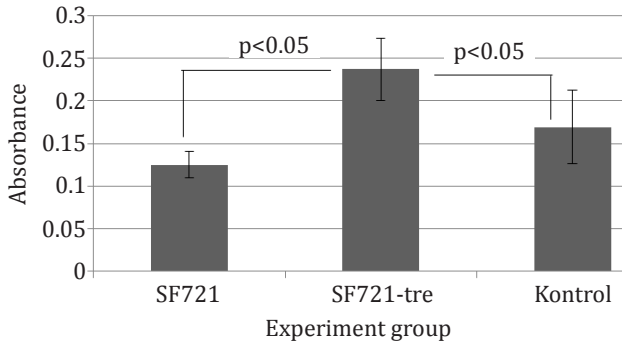

Figure 6. Viability of attached cells in culture after thawing. SF721: Cryopreserved without trehalose preincubation; SF721-Tre: Cryopreserved with trehalose preincubation; SF721: slow-freeze with 70% culture mediun, 20% FBS and 10% Me_2SO

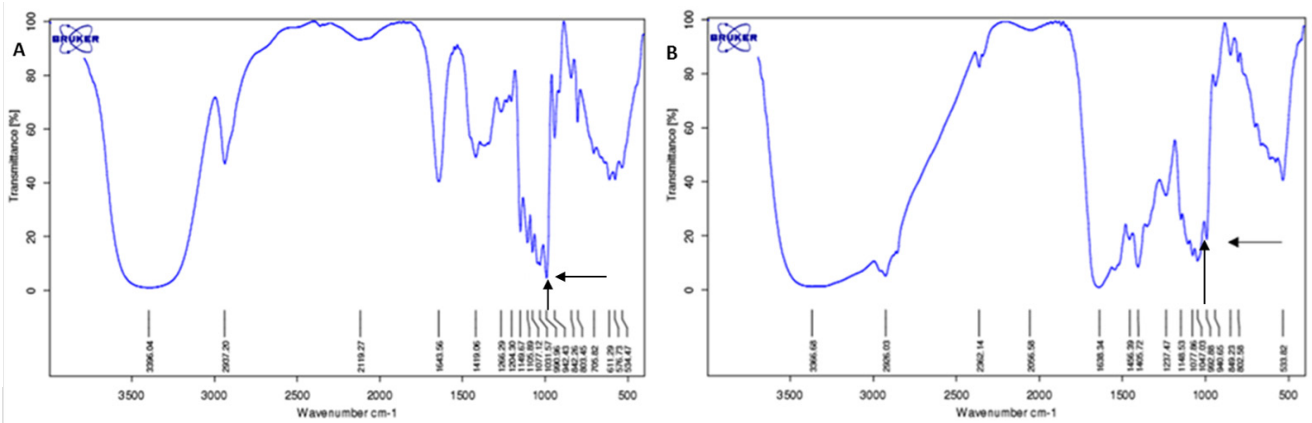

Figure 4. A) Spectrum recorded from 250 mM trehalose solution shows a characterisitic peak of trehalose at 990.96 /cm wavenumber (arrow); B) Spectrum of dried lysate cell derived from 100 mM trehalose preincubated cell culture shows trehalose peak at 992.65 /cm wavenumber (arrow). The difference in wavenumber attributed to water content from the trehalose solution

of extracellular trehalose (<50 mM) caused reduction in viability at day seven of culture (Figure 2). However, an increase in viability was clearly observed compared to previous time point in the same dose group. This suggests that trehalose toxicity did not affect all of the cells population and the mesenchymal stem cells population still retained the proliferative capacity.

Zhou et al[2] used 50 mM trehalose incubated for four hours results in 13 mM intracellular trehalose concentration which quite similar with our data (15.07 mM). Zhang et al[16] also have a similar result of 14.57 mM while Oliver et al[12] reported at 19 mM. The use of ATP as a poration agent greatly increased the loading kinetics such as intracellular concentration of 50 mM reached after 90 minutes incubation.[6] These data were derived from different cell and different method of quantitation such as anthrone reaction and HPLC measurement. As far as we know our study is the first that used FTIR-ATR for intracellular trehalose quantification.

Our study suggests that trehalose-preincubation which results a 15 mM of intracellular trehalose concentration alongside the standard slow-freezing, Me_2SO-based cryomedium were sufficient to protect mesenchymal ($CD271^+$) stem cells from cryoinjury. Although to achieve this we used 100 mM of trehalose with one hour preincubation protocol which caused significant reduction in cell viability. This condition warrants a further investigation in trehalose internalization mechanism. Furthermore, our data suggest that intracellular trehalose was benefit in preventing trypsinization-induced cell injury. Trypsin, although widely used in passaging procedure, is in fact a protease that can cause stress and cell death. Trehalose was known to help retain cellular integrity and prevent protein denaturation.[4] Therefore, intracellular trehalose may prevent cell injury during trypsinization resulting in a higher viability compared to the control group.

Different trehalose loading kinetics reported by comparing different study that has been conducted on platelets, erythrocytes, and stem cells using trehalose incubation method, suggest there is a cell-type specific of internalization method. Although Oliver et al[12] reveals bone-marrow derived mesenchymal stem cells used clathrin-mediated endocytosis for trehalose internalization, this not necessarily the same for other cell type. Elucidating the mechanism of trehalose internalization of different cell type can shed a new understanding of cell membrane regulation.

In conclusion, this study concluded that trehalose-preincubation procedure was able to protect mesenchymal ($CD271^+$) stem cells from cryoinjury results from slow-freeze cryopreservation procedure. Further study should be directed to elucidate factors that regulate trehalose internalization kinetics in different cell types. Research could also be directed to verify trehalose cryoprotection in different type of cryopreservation such as vitrification.

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Conflict of interest
Funding was provided by Lembaga Pengelola Dana Pendidikan (LPDP) scholarship scheme.

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