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Short communication

Effect of Rho-associated kinase (ROCK) inhibitor Y-27632 on the post-thaw viability of cryopreserved human bone marrow-derived mesenchymal stem cells

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ABSTRACT

Human bone marrow-derived mesenchymal stem cells (MSC) have previously been reported to be susceptible to cryopreservation-induced apoptosis. A significant fraction of MSC lose their viability during freeze-thawing, which represent a major technical barrier in attaining adequate viable cell numbers for optimal efficacy in transplantation therapy. Recently, it was reported that a Rho-associated kinase (ROCK) inhibitor Y-27632 could enhance the post-thaw viability and physiological function of cryopreserved human embryonic stem cells (hESC). Hence, this study attempted to investigate whether Y-27632 can exert a similar beneficial effect on the post-thaw viability of cryopreserved MSC. A concentration range of 1–100 μM Y-27632 was supplemented in both the cryopreservation medium (10% (v/v) dimethyl sulfoxide), as well as the post-thaw culture medium. The supplementation of Y-27632 had no significant effect on the immediate post-thaw viability, as assessed by trypan blue exclusion. However, 24 h after the frozen-thawed cell suspensions were re-plated on new cell culture dishes (with varying concentrations of Y-27632 within the post-thaw culture media); the MTT assay subsequently showed significant differences in the proportion of adherent viable cells over the concentration range of Y-27632 examined, with a peak at between 5 and 10 μM. At zero concentration of Y-27632, the proportion of viable adherent cells was 39.8 ± 0.9%; and this value peaked at 48.5 ± 1.7% with 5 μM Y-27632 and 48.4 ± 1.8% with 10 μM Y-27632, prior to decreasing to 36.0 ± 0.6% with 100 μM Y-27632. Additionally, it was observed that Y-27632 induced morphological changes in the frozen-thawed MSC. With increasing Y-27632 concentration, the cells displayed more extensive branching of cytoplasmic extensions that gave a 'web-like' appearance. This is consistent with previous reports of Y-27632 stimulating neuronal differentiation of MSC.

1. Introduction

Bone marrow-derived mesenchymal stem cells (MSC) have demonstrated much promise in the emerging field of regenerative medicine (Schäfer and Northoff, 2008; Siddappa et al., 2007; Dezawa, 2008; Urbán et al., 2008). Nevertheless, a major challenge is to obtain sufficient cell numbers for optimal efficacy in transplantation therapy. In this respect, a significant technical barrier is the relatively large fraction of MSC that are lost as a result of cryopreservation-induced apoptosis (Schmidt-Mende et al., 2000) during freeze-thawing. Schmidt-Mende et al. (2000) showed that this is due to artificial cleavage of apoptosis-related proteins within human bone marrow cells during the freeze–thaw process. Subsequently, the same study also demonstrated that cryopreservation-induced apoptosis could be mitigated by the supplementation of broad-spectrum protease inhibitors within the freezing medium and having a much slower thawing rate on ice (Schmidt-Mende et al., 2000).

More recently, a number of studies (Martin-Ibañez et al., 2008; Li et al., 2008a,b) reported the novel use of a Rho-associated kinase (ROCK) inhibitor Y-27632 in enhancing the post-thaw viability and physiological function of cryopreserved human embryonic stem cells (hESC). In addition to significantly increased survival rate of frozen-thawed hESC, there was also observed to be higher efficiency of colony formation and faster recovery of proliferative activity (Martin-Ibañez et al., 2008; Li et al., 2008a,b). Besides hESC, the anti-apoptotic effects of Y-27632 have also been reported on other cell types (Shi and Wei, 2007; Watanabe et al., 2007; Koyanagi et al., 2008; Lingor et al., 2008). Hence, this study attempted to investigate whether Y-27632 can exert a beneficial effect on the post-thaw viability of cryopreserved MSC.

2. Materials and methods

2.1. Cells, culture media, cryopreservation solution and reagents

Bone marrow-derived human mesenchymal stem cells (Cat No. PT-2501, Batch No. 6F4382, cryopreserved at the 2nd passage) were purchased from Lonza Inc. (Walkersville, MD, USA). The cells

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were thawed and cultured up to a further three passages (P5), prior to being utilized for this study. Unless otherwise stated, all reagents and chemicals were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA), while all labware consumables were purchased from Becton-Dickinson Inc. (Franklin Lakes, NJ, USA). The culture media was prepared from the MSCGM® bullet kit (Cat No. PT-3001) purchased from Lonza Inc. (Walkersville, MD, USA), while the cryopreservation solution was composed of culture medium supplemented with 10% (v/v) dimethyl sulfoxide (DMSO). In this study, the culture media and cryopreservation solution would be supplemented with varying concentrations of the ROCK inhibitor Y-27632 (Sigma–Aldrich Inc., St. Louis, MO, USA).

2.2. Cryopreservation and thawing of human mesenchymal stem cells

MSC were seeded in 12-well cell culture dishes with 5.0×10^4 cells per well (≈4.8 cm^2). After 5–6 days of culture, confluent MSC monolayers were attained (1.17×10^5 cells per well), and these were then dissociated with 0.05% (w/v) Trypsin–EDTA. Some wells containing intact confluent MSC monolayers were retained as the control, and were not trypsinized. Subsequently, the dissociated MSC (from each well) were centrifuged and resuspended in 1 ml of cryopreservation solution (supplemented with either 0, 10 or 100 μM Y-27632) within cryovials, and were subjected to slow cooling within a −80 °C refrigerator, through the use of isopropanol freezing containers (Nalgene Inc., Rochester, NY, USA). After 2 h, the frozen cell suspension within cryovials were immersed and stored in the vapor phase of liquid nitrogen for 1 h, prior to thawing within a water bath at 37 °C. The rationale for storing the cryopreserved MSC in liquid nitrogen for just 1 h, instead of 24 h or several days as reported in previous studies, is because the MTT assay utilized control wells seeded at the same cell density as reference in calculating the post-thaw survivability of MSC. Hence, if the cryopreserved MSC were kept in liquid nitrogen for 24 h or longer, the MSC within the reference control wells would undergo proliferation, whereas the cryopreserved MSC cannot proliferate. This in turn will skew the experimental results.

2.3. Characterizing the effects of Y-27632 on immediate post-thaw viability of cryopreserved MSC with the trypan blue exclusion assay

The newly thawed cell suspension from each cryovial were centrifuged and reconstituted in 0.5 ml PBS, placed within accessory sample vials (Cat No. 383721, Beckman-Coulter Inc., Fullerton, CA, USA) and analyzed for cell viability with the trypan blue exclusion assay, by utilizing an automated cell counter (Vi-Cell® XR analyzer, Cat No. 383556; Beckman Coulter Inc., Fullerton, CA, USA) and Vi-CELL® XR Quad Pak Reagent Kit (Cat No. 383198; Beckman-Coulter Inc., Fullerton, CA, USA). The automated cell counter mixes the cell suspension with an equal volume of 0.4% (w/v) trypan blue solution (600 μl), prior to drawing in 50 μl × 20 μl aliquots of the mixture into its counting chamber. Each reading by the automated cell counter is therefore obtained by averaging the results from 50 separate images. For each experimental group, there were four replicate readings. As a control, the initial viability of MSC immediately after trypsinization was also measured, before cell cryopreservation was carried out.

2.4. MTT assay to characterize effects of Y-27632 on the proportion of viable adherent cells upon re-plating of frozen-thawed MSC

The newly thawed cell suspensions from each cryovial were centrifuged and resuspended in culture media supplemented with varying concentrations of Y-27632 (0, 1, 5, 10, 25, 50 and 100 μM), prior to being re-plated on new 12-well culture dishes. For each experimental group, the same concentration of Y-27632 was maintained in both the cryopreservation and post-thaw culture media. After 24 h of culture, the unattached cells were washed off with PBS, and the adherent cells were imaged under phase-contrast microscopy, prior to being subjected to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983). Briefly, this involved placing 1.0 ml of 1 mg/ml MTT constituted in culture media within each well, following by incubation for 3 h at 37 °C in the dark. After incubation, the MTT solution was removed and the stained cells were washed two times in PBS followed by air-drying. The MTT-formazan products were extracted in the dark at room temperature with 0.25 ml of DMSO in each well. 100 μl aliquots of the supernatant in each well were then transferred into a 96-well flat-bottomed cell culture plates, and the absorbance was measured spectrophotometrically at 570 nm using a SpectraMax M5 modular microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). From the absorbance values, the percentage of re-attached viable cells (after dissociation with trypsin and cell-free dissociation buffer) can then be computed by dividing the MTT absorbance values obtained after dissociation with the absorbance reading for the non-dissociated control (after correction for 100 μl DMSO blanks).

2.5. Statistical analysis of data

There were four replicates for each experimental group, and the results from each data set were expressed as mean ± standard derivations. Differences between data sets were assessed by the

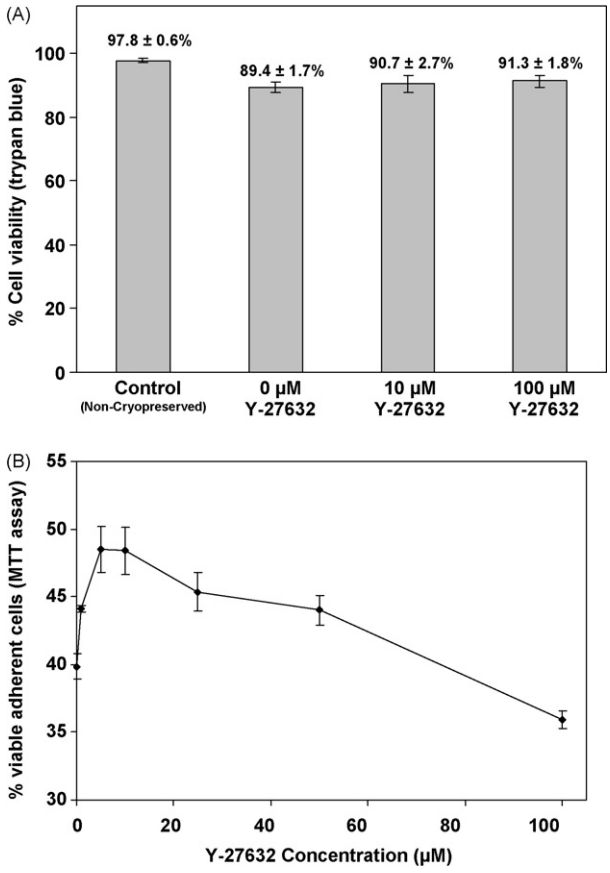

Fig. 1. (A) Immediate post-thaw viability assessed by trypan blue. (B) Proportion of viable adherent cells 24 h after re-plating frozen-thawed MSC on new culture dishes, as assessed by the MTT assay.