Cryobiology 68 (2014) 139–146

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Boron increases the cell viability of mesenchymal stem cells after long-term cryopreservation ☆

Selami Demirci, Ayşegül Doğan, Burcu Şişli, Fikrettin Sahin *

Department of Genetics and Bioengineering, Faculty of Engineering and Architecture, Yeditepe University, 26 Agustos Campus, Kayisdagi Cad., Kayisdagi, TR-34755 Istanbul, Turkey

ARTICLE INFO

Article history:
Received 19 August 2013
Accepted 15 January 2014
Available online 21 January 2014

Keywords:
Boron
Cryopreservation
Stem cell
Me_2SO
Cryoprotectant
Dental stem cell

ABSTRACT

The field of stem-cell biology has emerged as a key technology for the treatment of various disorders and tissue regeneration applications. However, a major problem remains in clinical practice, which is the question of whether stem cells preserve their self-renewal and differentiation potential in the culture conditions or not. In the current study, effects of boron on the cryopreservation of human tooth germ stem cells (hTGSCs) were evaluated for the first time. The impacts of various boron concentrations (sodium pentaborate pentahydrate (NaB)) were tested on characterized hTGSCs viability for different time intervals (24, 48, and 72 h). 20 μg/ml NaB with lower Me_2SO concentration was found to display positive effects on hTGSCs during repeated freezing and defrosting cycles, and long-term cryopreservation. After thawing, cells were analyzed for their surface antigens and differentiation capacity. hTGSCs were successfully cryopreserved without any change in their mesenchymal stem cell characteristics as they were treated with boron containing freezing medium. In addition, fatty acid composition was examined to demonstrate membrane fatty acid profiles after freeze-thawing. Besides, NaB treatment extended osteogenic and chondrogenic differentiation of hTGSCs remarkably after long-term cryopreservation with respect to control groups. The study clearly suggests that NaB has a protective role on the survival of hTGSCs in short- and long-term cryopreservation. Due to the possible storage of hTGSCs at early ages, development of a functional and reliable cryopreservation media can be designed as a future solution to the dental stem cell banking.

Introduction

Stem cells have been proposed to be promising for future therapies for various diseases where current therapies are unsatisfactory, including genetic disorders, severe injuries, metabolic or neurological disorders. Cell replacement by using stem cells or activating the internal stem cells has been recommended to construct a fully integrated functional tissue. Stem cell source, in this concept, is the main key factor that directly affects the adequacy and functionality of the tissue restoration or improvement as different stem cells possess various characteristics and lineage specific differentiation capacity [20]. Mesenchymal stem cells (MSCs) have attracted remarkable interest in recent years among other stem cell types including embryonic stem cells (ESCs) because of their safety, ability to greatly expand in culture, self-renewal and differentiation capacity [26]. Although bone marrow is the most elucidated and primary MSC cell source, the difficulty of isolation procedure, and contamination risk has created great demand for alternative cell sources. In addition, dental stem cells have been proven to consist more potent neurogenic and endothelial precursor cells, and proliferate faster *in vitro* than bone marrow derived stem cells [19,29].

Human tooth germ stem cells (hTGSCs), derived from the third molar of young adults, start the organogenesis process at the age of six, therefore self-renewal and multipotency of these cells are higher with respect to other MCSs [34]. Thus, exploring the therapeutic approaches to these cells has raised interest in usage of hTGSCs in tissue regeneration studies.

Widespread requirement of MCSs in long-term clinical applications have forced scientists to find practical methods for stem cell banking [32]. Cryopreservation of stem cells is important to provide storage of high cell numbers, ease transport and banking multipotent cells for long periods [16]. Although several methods have been published for isolation, and expansion of stem cells *in vitro*, medical applications are limited due to loss of stem cell properties during storage conditions. It has been claimed that long-term utilization of stem cells in clinical practice is limited

☆ *Statement of funding:* This work was funded by Yeditepe University, Kayisdagi, Istanbul, Turkey.

* Corresponding author. Fax: +90 (216) 578 0829.
E-mail address: fsahin@yeditepe.edu.tr (F. Sahin).

0011-2240/$ - see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cryobiol.2014.01.010

because of reduced multipotency and proliferation capacity after cryopreservation [32]. Hence, improvement of effective cryoprotectants and cryopreservation procedures are highly required to protect cells from freeze–thaw stress.

After the discovery of boron as an essential element for plant, accumulating evidences suggest that boron is also vital micromineral for higher organisms including human and animals [24]. Boron has been associated with the mammalian brain function, immune regulation, bone and hormone metabolism [22]. Although molecular mechanisms of boron effect on mammalian cell are still not completely understood, they have been well documented particularly for the plant biology. In several studies, boron has been claimed to be involved in membrane integrity of plant cells Boron deficiency in plant cells is characterized by disruptions of cell wall and membrane unity [13]. Boron and calcium metabolism in the plant cell membrane is important for cell signaling events [4]. Moreover, boron is able to form diester borate complexes with membrane glycoproteins and glycolipids acting as calcium chelators or redox regulators which is important for membrane structure and function [15,31]. However, the exact role and effect of boron on cell viability and membrane integrity during cryopreservation have not been studied for any cell type. In the current study, sodium pentaborate pentahydrate (NaB) as a boron source was used to overcome freeze–thaw stress during hTGSC cryopreservation for the first time. Due to the possible storage of hTGSCs at early ages, development of a functional and reliable cryopreservation medium can be proposed as future solution to the dental stem cell banking. The fundamental objective of our study is to develop improved storage conditions providing prolonged functional efficacy to the cells.

Materials and methods

Isolation and characterization of hTGSCs

hTGSCs were isolated from the tooth germ of a fifteen years old patient and characterized according to the protocol described previously by our group [10,35]. Briefly, cells were cultured in Dulbecco's modified essential medium (DMEM) (Invitrogen, Gibco, UK) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Gibco, UK) and 1% PSA (Penicillin/Streptomycin/Ampicillin) (Invitrogen, Gibco, UK) in an incubator (Thermo, US) at 37 °C with 5% CO2 and 95% humidity. Primary antibodies against) CD34 (SC-51540), CD45 (SC-70686), CD90 (SC-53456), CD105 (SC-71043), CD 14 (SC-7328), CD166 (SC-53551) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), CD29, (BD556049) and CD73 (BD550256) (Zymed, San Francisco, CA) were used in the study for characterization of mesenchymal stem cells. The flow cytometry analyses of the cells were completed using Becton Dickinson FACS Calibur (Becton Dickinson, San Jose, CA, USA, model No. 342975) flow cytometry system.

Cytotoxicity assay

NaB was kindly provided by National Boron Research Institute-BOREN (Ankara, Turkey), and it was dissolved in the culture medium at a 0.1 g/ml stock concentration. The stock solution was filtered through a 0.2-μm filter (Sartorius AG, Göttingen, Germany) and diluted to lower concentrations in Dulbecco's modified Eagle's medium. 13 separate NaB concentrations were prepared in cell culture medium. hTGSCs at passage number 2 were seeded onto 96-well plates (Corning Glasswork, Corning, NY) at a concentration of 5000 cells/well followed by the addition of NaB. Cell viability was measured by the 3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS)-assay (CellTiter96 Aqueous One Solution; Promega, Southampton, UK) according to the manufacturer's instructions as described previously [30].

Cryopreservation of hTGSCs in the presence of NaB

20 μg/ml NaB, determined according to the toxicity analysis, was used for further experiments. hTGSCs were cryopreserved in freezing medium containing 20 μg/ml NaB, 20% FBS and 1% of PSA, and five different dimethyl sulfoxide (Me_2SO) (Sigma, USA) concentrations (10%, 7%, 5%, 3%, 0%). Standard freezing medium (DMEM with 20% FBS, 10% Me_2SO and 1% of PSA) was used as negative control. During cryopreservation studies, 1×10^6 cells were suspended in 1.0 ml freezing medium with or without NaB, and put into 2 ml cryo-vials. The cryo-vials were kept at −80 °C in Nalgene cryo 1 °C freezing containers (Thermo Fisher, Waltham, MA, USA) to provide slow freezing. The vials were transferred to the −196 °C liquid nitrogen tank 1 day after freezing. Cells were thawed in a 37 °C water bath immediately and washed with growth medium to remove harmful effect of Me_2SO. Cell viability was analyzed using trypan blue (T-8154, Sigma–Aldrich) and hemocytometer. Repeated freeze–thaw cycles and long-term cryopreservation were applied to the cells in order to test the effects of cryopreservation mediums on cell viability. Subsequent freeze–thaw cycles are required to mimic routine cell culture applications, and long-term cryopreservation is required to simulate cell banking for long periods of time. Cryopreserved cells were thawed 1 day or 6 months after each cryopreservation cycle for short- and long-term cryopreservations, respectively. 1×10^6 cells were cryopreserved for each freeze–thaw cycle when the cells reached 80% confluence after thawing.

Differentiation studies

Osteogenic, chondrogenic, and adipogenic differentiation of hTGSCs were induced to confirm MCS characteristics of long-term cryopreserved cells. After thawing, long-term frozen control and NaB treated groups of hTGSCs were seeded in 12 well plates (BIOFIL, TCP, Switzerland) at a concentration of 15×10^3 cells/well followed by addition of pre-made differentiation media. The differentiation media were replaced every other day, and the cells were incubated in a humidified (95%) incubator at 37 °C with 5% CO2 for 7–10 days [10].

von Kossa staining

von Kossa staining was performed to show calcium deposition as a marker of osteogenic differentiation. Shortly, the cells were fixed with 2% (w/v) paraformaldehyde (Sigma, US) at 4 °C for 30 min. Cells were rinsed with distilled water and stained with von Kossa kit (Bio optica, Italy) according to manufacturer's instructions.

Alcian blue staining

Alcian blue staining was conducted to visualize chondrogenic differentiation. After fixation with 2% (w/v) paraformaldehyde, cells were stained with Alcian blue staining solution, prepared in 3% (v/v) acetic acid, by incubating for 30 min. Staining solution was removed and the cells were washed with PBS for three times followed by observation under light microscope [38].

Oil red staining

Oil red staining was performed to show lipid droplets visible as a parameter of adipogenic differentiation After fixation with 2% (w/v) paraformaldehyde for 30 min cells were rinsed with PBS