Cryobiology 73 (2016) 15–19

Contents lists available at ScienceDirect

Cryobiology

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

The effect of magnetic field during freezing and thawing of rat bone marrow-derived mesenchymal stem cells

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ARTICLE INFO

Article history:
Received 19 February 2016
Received in revised form
16 June 2016
Accepted 21 June 2016
Available online 23 June 2016

Keywords:
MSCs
Cryopreservation
Thawing
Magnetic field
Bone regeneration

ABSTRACT

Previous studies showed that a programmed freezer with magnetic field can maintain a high survival rate of mesenchymal stem cells (MSCs). The purpose of this study was to evaluate the influences of magnetic field during freezing and thawing on the survival of MSCs isolated from rat bone marrow. The cells were frozen by a normal programmed freezer or a programmed freezer with magnetic field (CAS-LAB1) and cryopreserved for 7 days at −150 °C. Then, the cells were thawed in the presence or absence of magnetic field. Immediately after thawing, the number of surviving or viable cells was counted. The cell proliferation was examined after 1-week culture. Cryopreserved MSCs which were frozen by a normal freezer or a CAS freezer were transplanted into bone defects artificially made in calvaria of 4-week-old rats. Non-cryopreserved MSCs were used as a control. The rats were sacrificed at 8, 16, or 24 weeks after transplantation and the bone regeneration area was measured. Proliferation rates of MSCs after 1 week were significantly higher in the CAS-freezing-thawing group than in the CAS-freezing group. The extent of new bone formation in the CAS-freezing-thawing group tended to be larger than in CAS-freezing group 24 weeks after transplantation. These results suggest that a magnetic field enhances cell survival during thawing as well as freezing.

1. Introduction

Regenerative medicine for lost and damaged organs and tissues due to injury or disease has progressed significantly in recent years. Particularly, cell transplantation treatment with mesenchymal stem cells (MSCs) which can be induced to differentiate into multiple cell lineages is recognized for regeneration of various organs and tissues [6]. Our previous study showed that a large amount of new bone formation was observed in MSCs grafting at calvarial defects in rats [12]. Also in a human study, it was revealed that the MSCs with bone substitute can be useful for bone regeneration of alveolar cleft [3]. However, the percentage of MSCs which are isolated from a patient's bone marrow is only about 0.01% of the mononuclear cells [18,23,24,27], and in some cases, several-fold is needed for transplantation, because the size of tissue defect is too large or engraftment of transplanted cells is minimal. Therefore, it is necessary to develop a safe and useful cell cryopreservation technique for regenerative medicine.

Recently, it was reported that a programmed freezer with a magnetic field which is called the Cells Alive System (CAS) was effective for cell cryopreservation because it can reduce intracellular ice formation. This is due to the function of magnetic field vibration which inhibits water molecules from making clusters during the freezing process [10]. Lee et al. reported that whole teeth which were cryopreserved by a CAS freezer can be used for autotransplantation and provide a viable source of human dental pulp stem cells [17]. Lin showed that human embryonic stem cells can maintain their pluripotency capacity and undifferentiated state after cryopreservation by a CAS freezer [19]. It was shown that a CAS freezer is useful for cryopreservation of human induced pluripotent stem cell-derived neural stem/progenitor cells [25]. Our previous study also showed that percent cell viability and proliferation rates of MSCs were significantly higher in the CAS freezer group than in the non-magnetic freezer group. Alizarin positive reaction, a large amount of calcium quantification, and greater

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http://dx.doi.org/10.1016/j.cryobiol.2016.06.006
0011-2240/

alkaline phosphatase activity were shown in the CAS freezer group after osteogenic differentiation. Furthermore, positive reaction of Oil Red O staining and high amounts of PPARγ and FABP4 mRNAs were shown in CAS freezer groups after adipogenic differentiation. These findings suggested that a CAS freezer can maintain high survival and proliferation rates of MSCs and maintain both adipogenic and osteogenic differentiation abilities [13]. However, proliferation rates of MSCs in the CAS group was still significantly less than that of non-cryopreserved MSCs. So, it was necessary to improve the CAS system for MSCs cryopreservation.

On the other hand, previous studies showed that thawing of cryopreserved tissues by electromagnetic heating is also important to prevent ice crystal formation [8,28]. However, there is no available data for the effect of a magnetic field during thawing for MSCs cryopreservation. The purpose of this study was to evaluate percent cell viability and proliferation rates of MSCs which were frozen and thawed with a magnetic field. Furthermore, we examined bone regeneration of rat calvaria defects after transplantation of these cryopreserved MSCs.

2. Material and methods

2.1. Cell culture

Isolation of rat MSCs was performed by dissecting the femora from four 4-week-old Fischer 344 male rats, cutting the ends of the bones and extruding the marrow with 5 ml of Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) using a 24 gauge needle and 1 ml syringe. Bone marrow cells (3×10^5) were seeded in poly-L-lysine treated 10 cm^2 polystyrene culture dishes (CORNING, New York, NY, USA) and maintained in DMEM with 10% fetal bovine serum (FBS: Biological Industries, Kibbutz Beit Haemek, Israel) at 37 °C in a humidified atmosphere consisting of 5% CO_2. After 24 h, nonadherent cells were removed by replacing the medium. The medium was replaced every 2–3 days while the cells were grown to confluency [1]. MSCs were characterized by their adhesiveness and fusiform shape. Adherent cells were removed using 0.1% Trypsin-EDTA (Difco, Detroit, MI, USA) and replated in a new dish for subculture. All cells from four rats were mixed together at passage 3. All experiments were performed in accordance with protocols approved by the Committee of Research Facilities for Laboratory Animal Science at Hiroshima University School of Medicine.

2.2. Cell freezing

Three hundred thousand cells were placed in a tube with 1 ml preservation media containing 10% dimethyl sulfoxide (Me_2SO), 5% albumin, 0.2% D-glucose, 0.6% NaCl, 0.03% glutamine, 0.2% $NaHCO_3$ without any serum (Bambanker, Lymphotec, Tokyo, Japan). In this study, we used a newly-developed programmed freezer (CAS-LAB1, ABI Corporation Ltd., Nagareyama, Japan), which can apply magnetic field as needed during both freezing and thawing. The cells were frozen by CAS-LAB1 with or without (control group) 0.1 mT magnetic field and cooled to −30 °C at −0.5 °C/min according to previous studies [13–15]. Then, the cells were cryopreserved for 7 days in a deep freezer at −150 °C.

2.3. Cell thawing

After 7 days cryopreservation, the cells (CAS-freezing group and CAS-freezing-thawing group) which were frozen with a magnetic field were thawed in the 37 °C compartment of CAS-LAB1 freezer with or without 0.1 mT magnetic field. During thawing with 0.1 mT magnetic field, 75 mA electric field was also induced. The cells which were frozen without a magnetic field were thawed without magnetic field at 37 °C (control group).

2.4. Percent cell viability and proliferation rate

Immediately after thawing, the cells were stained by 0.5% trypan blue and the number of viable cells was counted. Next, the cells were cultured in DMEM containing 10% FBS for 1week. The cells were then treated with 0.1% trypsin-EDTA and the number of viable cells was counted. The proportion of surviving cells to the number present immediately after thawing was calculated.

2.5. Transplantation of the cryopreserved MSCs into rat calvaria

1. Creation of sagittal suture defects in cranial bone and transplantation of MSCs

Sixty 4-week-old Fischer 344 rats were used in this experiment. They were divided into four groups (n = 15 per group): (1) parietal bone defect and non-frozen MSCs transplantation (control group) (2) parietal bone defect and normal programmed-freezing and normal-rewarming MSCs transplantation (non-magnetic group) (3) parietal bone defect and CAS-freezing and normal-thawing MSCs transplantation (CAS-freezing group) (4) parietal bone defect and CAS-freezing and CAS-thawing MSCs transplantation (CAS-freezing-thawing group).

Animals were anesthetized with sodium pentobarbital (50 mg/kg) and local anesthesia of 1% lidocaine at the scalp and pericranium. A midline skin incision was made and the periosteum was cut at the midline. The scalp and pericranium were reflected laterally, and the parietal bone and sagittal suture were exposed. Then, a bone and suture defect with a diameter of 6.0 mm was made using a trephine bur (Implatex Co., Ltd., Tokyo, Japan), according to a previous study [12].

2. Transplantation of MSCs

5×10^4 cells and 2.2×10^4 cells (800 cells/mm^2) from each group were suspended and transplanted onto the upper (9 mm in diameter) and lower (6 mm in diameter) bioresorbable membranes (GC MEMBRANE; GC Corp., Tokyo, Japan), respectively, and cultured for 24 h in DMEM before transplantation into the calvaria defect according to a previous study [12].

2.6. Histological examination

Five rats in each group were sacrificed at 8, 16, or 24 weeks after surgery with an overdose of pentobarbital (120 mg/kg, i.p.). Animals were fixed in 4% paraformaldehyde and rinsed in distilled water. Specimens were then decalcified in 14% ethylenediaminetetraacetic acid (pH 7.4) for 28 days and embedded in paraffin.

The parietal bones, including the sagittal suture, were cut into 7 μm frontal sections. These sections were stained with hematoxylin and eosin (H & E) and observed under a light microscope. Five sections at 35 μm intervals within the most central section of each specimen were subjected to morphometric analysis. Bone structures that existed within the original bone defect with a diameter of 6.0 mm were regarded as new bone formation and were measured on digitized photomicrographs (NIH Image, Bethesda, MD, USA.). The mean and standard deviation of the new bone area were then calculated, and the data were analyzed.