Cryobiology 76 (2017) 140–145

Contents lists available at ScienceDirect

Cryobiology

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

Cryopreservation of bovine somatic cells using antifreeze polyamino-acid (carboxylated poly-L-lysine)

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ARTICLE INFO

Article history:
Received 30 November 2016
Received in revised form
30 January 2017
Accepted 31 January 2017
Available online 1 February 2017

Keywords:
Cryopreservation
Carboxylated poly-L-lysine
Dimethyl sulfoxide
Bovine
Somatic cells

ABSTRACT

Carboxylated poly-L-lysine (CPLL) is an ampholytic polymer compound, obtained by converting 65 mol% of amino groups to carboxyl groups after synthesizing ε-poly-L-lysine aqueous solution and succinic anhydride. CPLL has cryoprotective properties similar to those of anti-freeze protein. The addition of CPLL to freezing medium has been reported to improve the post-thawing survival rate of murine cells, human induced pluripotent stem (iPS) cells, embryonic stem (ES) cells and embryos. In this study, investigating CPLL for its effectiveness as a new cryoprotective material is aimed. In experiments with bovine somatic cells, CPLL was suggested to have an equal or superior cryoprotective effect to dimethyl sulfoxide (DMSO), the conventional material for cellular frozen storage, based on the results for post-thawing cell survival and proliferation rates. CPLL was demonstrated to have another advantage; thawed cells can be cultured without removing the cryopreservation medium when CPLL is used, but not when DMSO is used. These results suggest that CPLL could be used as cryoprotective material for bovine cells. It is also expected that CPLL can be applied to embryo and oocytes storage for cattle, and similar functions for cells and embryos of other animal species.

1. Introduction

Cryopreservation is used for long-term preservation of biological materials containing cell and tissue. Stem cells such as ES cells and iPS cells are important to development for regenerative medicine and clarification of the disease mechanism [26,29], somatic cells are important to commercial application in gene bank [2,4]. Therefore, to cryopreserve them effectively is beneficial for various studies. Although many chemical substances such as DMSO, glycerol, ethylene glycol, propylene glycol were used as cryoprotective materials for cells and tissues [5,8,9,14,20,22], these materials permeate into the cytoplasm, protecting cell due to suppress ice crystallization. Although DMSO is widely used for various type of cells, it is reported that it has a cytotoxic effect caused by high osmotic pressure, it found to be one of the factor affecting the differentiation of cells [13,31]. Furthermore, DMSO was found to alter the epigenetic DNA methylation profile of mouse embryoid bodies, and to be a strong inducer of DNA hydroxymethylation in pre-osteoblastic MC3T3-E1 cells [1,7,11,27]. Therefore, it is necessary to remove quickly cryopreservation medium after thawing. To reduce this failure, methods for mixing multiple cryoprotective materials, reducing cryopreservation medium concentrations adding high molecular compound or saccharides have been developed. Adding ficoll [10] or trehalose [6,15] with cryopreservation medium is more effective for cryopreservation. However, these substances do not permeate into cytoplasm, therefore, low freezing capacity, not enough to be used alone. Although many research already have reported that cryopreservation of cells with DMSO, glycerol, ethylene glycol, propylene glycol, it is necessary to develop new cryoprotective materials with less toxicity and better cryopreservation efficiency than currently available cryoprotective materials.

Carboxylated poly-L-lysine (CPLL) is the ampholytic polymer compound, obtained by converting 65 mol% of amino groups to carboxyl groups after synthesizing ε-poly-L-lysine aqueous solution and succinic anhydride. CPLL is reported to have high cryoprotective properties as other cryoprotective materials [16–18,25], it

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http://dx.doi.org/10.1016/j.cryobiol.2017.01.010
0011-2240/

shows higher cryopreservation efficiency and lower cytotoxicity than DMSO. The aim of this study is to investigate the efficacy of CPLL as new cryoprotective material for somatic cells such as bovine fibroblast cell and bovine cumulus cell in comparison with DMSO.

2. Material and methods

2.1. Carboxylated ε-poly-L-lysine (CPLL)

Previously, it was reported the synthesis of the polymeric cryoprotectant CPLL (0.65) [17]. To synthesize CPLL (0.65), 25% (w/w) ε-poly-L-lysine aqueous solution (JCN Corp., Tokyo, Japan) and succinic anhydride (198-04355, Wako Pure Chem. Ind. Ltd., Osaka, Japan) were mixed and reacted at 50 °C for 1 h to convert 65% amino groups to carboxyl groups.

2.2. Cell preparation and culture

Bovine fibroblast cells and cumulus cells were prepared by modifying the previously described reports [3,12]. Briefly, primary culture of skin fibroblast cells was established from ear tissues of Japanese Black cattle. The ear tissue pieces were washed with Phosphate buffered saline (PBS) (05913, Dulbecco's PBS (-) "Nissui", Nissui pharmaceutical Co., Ltd, Tokyo, Japan) and onto plated 60 mm plastic culture dishes (353002, Tissue Culture Dish, Corning International K.K., NY, USA). Fibroblast cells were cultured in Dulbecco's modified Eagle's medium (05915, Dulbecco's Modified EAGLE MEDIUM "Nissui", Nissui pharmaceutical Co., Ltd, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (26140-079, Fetal Bovine Serum, qualified, US origin, Thermo Fisher Scientific K.K., Kanagawa, Japan) at 38.5 °C under 5% CO2 in a humidified atmosphere. When the attached fibroblast cells reached 80% confluence, cells were dissociated with 0.1% (w/v) of trypsin (27250-018, Trypsin (1:250), Thermo Fisher Scientific K.K., Kanagawa, Japan) containing 0.02% (w/v) of EDTA (345-01865, Ethylenediamine-N,N,N',N'-tetraacetic Acid Disodium Salt Dihydrate (2NA), Wako Pure Chemical Industries, Ltd., Osaka, Japan) in PBS and seeded onto new plastic culture dishes.

Primary culture of cumulus cells was collected from ovary of Japanese Black cattle by aspirating cumulus oocyte complexes. Cumulus cells were dissociated from cumulus oocyte complexes by 1% hyaluronidase (H3506, Hyaluronidase from bovine testes, Sigma-Aldrich Co. LLC, St. Louis, USA) and plated onto 60 mm plastic culture dishes. Cell culture of cumulus cells was also carried out with the previously mentioned methods for fibroblast cells. In this study, fibroblast cells and cumulus cells were used until five passages.

2.3. Cryopreservation protocol and evaluation of survival

In bovine fibroblast cells, the cryopreservation media were prepared as follows: no cryoprotectant, 10% (v/v) DMSO, 5% (v/v) DMSO + 5% (w/v) CPLL, 5% (w/v) CPLL, 7.5% (w/v) CPLL and 10% (w/v) CPLL were dissolved in DMEM with 75% FBS and pH was adjusted to 7.4 using HCl or NaOH. Fibroblast cells was counted and

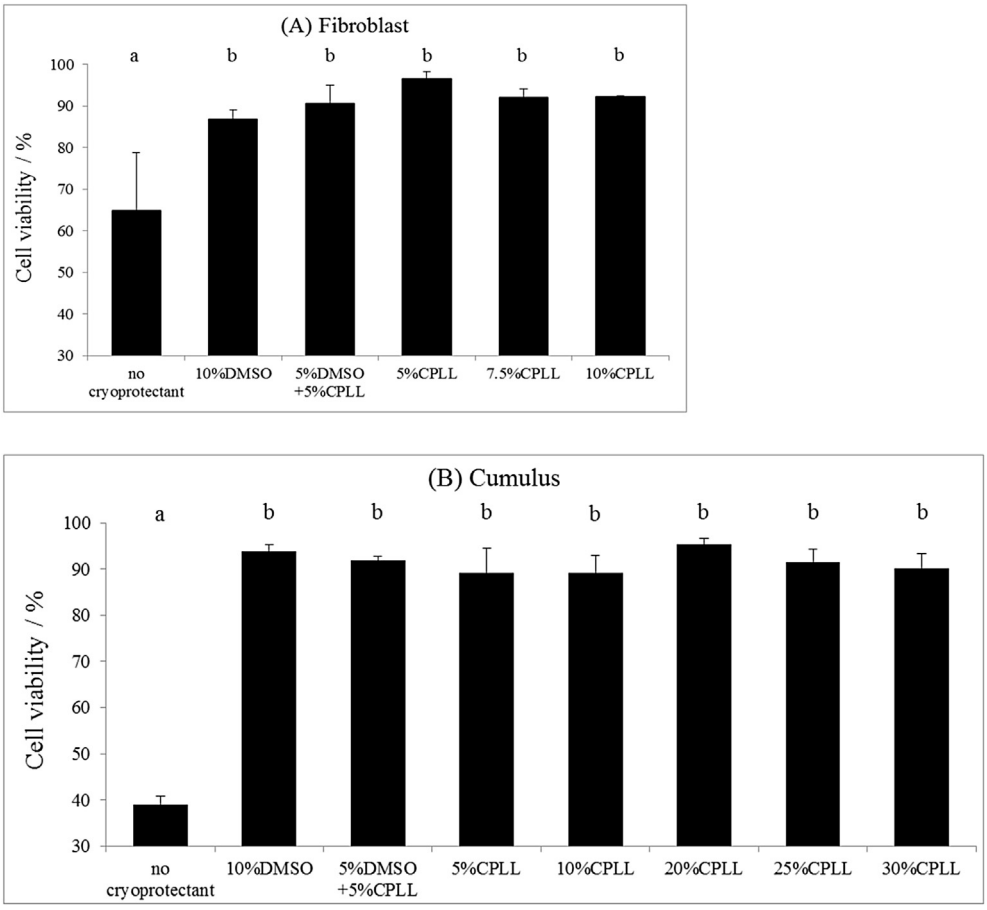

Fig. 1. Post-thaw viability of bovine fibroblast cells (A) and bovine cumulus cells (B). Results are mean ± SD. The error-bars indicate SD. Different lowercase letters represent statistically significant differences (a,b: $P < 0.01$). (replication = 3; (A) n = 18, (B) n = 24).