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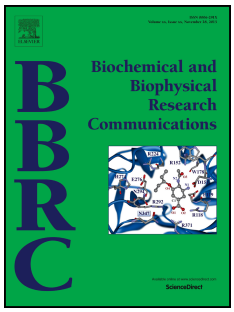

Natural zwitterionic L-Carnitine as efficient cryoprotectant for solvent-free cell cryopreservation

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Natural zwitterionic L-Carnitine as efficient cryoprotectant for solvent-free cell cryopreservation

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Abstract

Organic solvents, such as dimethyl sulfoxide (DMSO) and glycerol, have been commonly used as cryoprotectants (CPAs) in cell cryopreservation. However, their cytotoxicity and need of complex freezing protocols have impeded their applications especially in clinical cell therapy and regenerative medicine. Trehalose has been explored as a natural CPA to cryopreserve cells, but its poor cell permeability frequently results in low cryopreservation efficacy. In this work, we presented that a natural zwitterionic molecule—L-carnitine—could serve as a promising CPA for solvent-free cryopreservation. We demonstrated that L-carnitine possessed strong ability to depress water freezing point, and with ultrarapid freezing protocol, we

studied the post-thaw survival efficiency of four cell lines (GLC-82 cells, MCF-7 cells, NIH-3T3 cells and Sheep Red Blood Cells) using L-carnitine without addition of any organic solvents. At the optimum L-carnitine concentration, all four cell lines could achieve above 80% survival efficiency, compared with the significantly lower efficiency using organic CPAs and trehalose. After cryopreservation, the recovered cell behaviors including cell attachment and proliferation were found to be similar to the normal cells, indicating that the cell functionalities were not affected. Moreover, L-carnitine showed no observable cytotoxicity, which was superior to the organic CPAs. This work offered an attractive alternative to traditional CPAs and held great promise to revolutionize current cryopreservation technologies, to benefit the patients in various cell-based clinical applications.

Keywords: cryopreservation, cryoprotectant, L-carnitine, zwitterionic, ultrarapid freezing

1. Introduction

With the continued development of cell-based applications, there are burgeoning demands of living cells for human healthcare [1-3]. For example, due to their capacity of self-renewal and multilineage differentiation, stem cells have been explored to treat a broad spectrum of human diseases, including cancer and cardiac disease, etc [4]. However, stem cells are susceptible to tumorigenicity or heterogeneity in incubated flasks at physiological conditions, so cell preservation pausing the “life clock” of living cells is critical technique for cell-based applications [5]. For another example, in the U.S. there are 2.2 million units of blood required per year [6], but ~1.7 million units of blood from donors have to be discarded mainly due to short shelf-life (i.e., 42 days), leading to an undesired waste (~382 million USD annually) in clinical therapy

(National Blood Collection and Utilization Survey Report) [2]. Therefore, effective preservation methods for cells are highly desired and hold the potential to improve the outcome of clinical cell-based applications.

Cryopreservation is an essential and effective technology for the long-term storage of biological samples, such as cells, tissues and organs, etc [7, 8]. Biological systems can be cryopreserved, because their metabolism will be halted at the cryogenic temperatures (-80℃or -196℃), and then resumes at the physiological temperature after a proper thawing process. However, during the freezing process, cells could be damaged due to ice formation, which results in two types of cryoinjuries: ice injury and solute injury [9, 10]. To solve this problem, CPAs have been developed to protect cells from cryoinjuries, and now they have become the key to successful cryopreservation. The current state-of-the-art strategies for cryopreservation require the addition of large amounts of organic solvents, such as DMSO and glycerol, to prevent ice formation and maintain cell integrity. However, the organic solvent CPAs have shown several problems. Glycerol is used as a CPA at the earliest time for successful cryopreservation of fowl spermatozoa in the late 1940s by Polge [11], but a major disadvantage of glycerol is its poor penetration ability into most types of cells, leading to the difficulty of intracellular protection. Subsequently, Lovelock and Bishop reported the use of DMSO, which has the advantage of rapid penetration into cell membrane, and currently it has been widely applied due to its high cryoprotective efficacy [12]. However, its intrinsic cytotoxicity remains as the bottleneck of its clinical applications. It has been reported that DMSO could affect metabolism, enzymatic activity and cell cycle [13], which have a direct impact on cellular function and growth. Moreover, in cell-based therapeutic applications, DMSO is believed to be

the main causal agent of various adverse reactions, including allergic/cardiovascular complications, and renal/hepatic dysfunctions, etc [14, 15]. For example, after infusion of DMSO-cryopreserved hematopoietic stem cells, 14 out of 17 patients were found to develop cardiac arrhythmia [16]. Another disadvantage of traditional organic CPAs is their need of complex and time consuming protocol. They require controlled-rate freezing process and washing steps for CPA removal, as well as well-developed equipment. In addition, the requirement of high CPA concentration (commonly 10%-20%) also increases the risk of cell damage during their introduction or removal, because the cells will suffer from osmotic injury. Therefore, an ideal CPA should be effective, while can avoid any detrimental effect.

Many efforts have been made to search ideal CPAs for solvent-free cryopreservation, but no significant progress has been achieved. For example, antifreeze proteins (AFPs) found in some living organisms (polar fish, insects and plants) are linked to freezing avoidance [17], but it suffers from low availability from natural sources, limited post-freezing recovery rate and potential immunogenicity [18-20]. For another example, trehalose, a natural disaccharide of glucose found in organisms [21], has been applied to the bioprotection of various biologicals, such as proteins, vaccines and bacteria. However, trehalose cannot provide sufficient intracellular protection, which results from the impermeability of trehalose into cell membranes. So, it is often necessary to add the supplementary organic CPAs when using trehalose to cryopreserve cells [22, 23].

Inspired by nature, a zwitterionic molecule—L-carnitine—has attracted our attention as a promising CPA (Fig.S1). L-carnitine is a vitamin-like molecule, and is correlated with cold tolerance in many biological systems. For example, after exposed

to a cold shock, the dietary L-carnitine supplementation could significantly improve the survival rate of ornamental cichlid fish [24]. In addition, as an osmoprotectant, L-carnitine can protect cells from osmotic injury, when the cells are exposed to the hyperosmotic environment [25]. More importantly, L-carnitine is natural and non-toxic, which can be found in microorganisms, plants, and most animals, it is an essential metabolite and a 70kg human body can synthesize about 11-34 mg L-carnitine per day. These findings motivate us to explore the potential of L-carnitine as a non-toxic CPA for solvent-free cell cryopreservation[26-28]. In this work, with ultrarapid freezing protocol, we cryopreserved several different cell lines (GLC-82 cells, MCF-7 cells, NIH-3T3 cells and SRBCs) using L-carnitine as the sole CPA and evaluated the functionalities of post-thaw cells, including cell attachment and proliferation.

2. Materials and methods

2.1 Materials

L-carnitine was purchased from Dalian Meilun Biotech Co. Ltd. Trehalose and glycerol were all purchased from Alfa Aesar. DMSO, penicillin-streptomycin (PS), trypsin- ethylenediamin tetraacetic acid (trypsin-EDTA), and phosphate buffered saline (PBS) were all purchased from Beijing Solarbio Science and Technology Co. Ltd. 0.22um filters were purchased from Millipore. Roswell Park Memorial Institute-1640 (RPMI-1640), Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were all obtained from Gibco.

2.2 Cell culture

GLC-82 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, streptomycin (100 UI/mL), and penicillin (100 UI/mL). MCF-7 cells and NIH-3T3 cells were cultured in DMEM medium supplemented with 10% FBS, streptomycin (100 UI/mL), and penicillin (100 UI/mL). All the three types of cells were incubated at 37℃with 5% CO2 (Model HERA cell-BB15; Thermo).

2.3 Cryopreservation solutions

Cryopreservation solutions were prepared as follows: the experiment group was L-carnitine solution of different concentrations (0.5%, 1%, 2%, 4%, 6%, 8%, 10%, 20%, 50%); the control groups were the blank medium (RPMI-1640/DMEM medium containing 10% FBS), DMSO, trehalose, and glycerol respectively. L-carnitine, DMSO, trehalose and glycerol were added to the medium containing 10% FBS and dissolved to obtain the designated cryopreservation solutions. All the cryopreservation solutions were sterilized with 0.22 μm filters.

2.4 Differential scanning calorimetry tests

The influences of L-carnitine, DMSO, trehalose and glycerol on ice formation were studied by Differential scanning calorimetry (DSC) (Mettler-Toledo DSC 1/500). 5~10 mg solution was sealed in an aluminum pan, which was set on the calorimeter sample chamber. The measurements were carried out using a Mettler-Toledo DSC 1/500 with the cooling rate of 10℃/min and the heating rate of 2℃/min. Calorimetric measurements were performed in the range from -50 to 10℃.

2.5 Cytotoxicity assay

NIH-3T3 cells suspended in 0.1 mL DMEM medium at a concentration of 1.0 x 104 cells/mL were seeded in 96-well TCPS plates. After incubating for 24 h at 37°C, 0.1 mL cryopreservation solution of different concentrations was added to each well. Then, the samples were incubated by 72 h. To measure the cell viability, the supernatant was discarded from each of the well, then 10μL 3-(4,5-dimethylthial-2-yl)-2, 5-diphenyltetrazalium bromid (MTT) and 90μL DMEM medium were added to the cultured cells, followed by further incubating for 4 h at 37°C with 5% CO2. After removing the remaining medium, 0.1 mL formazan solution was added to each well to dissolve the precipitation. The resulted color intensity, which was proportional to the number of viable cells, was measured by a microplate reader (Infinite M200 pro, Tecan) at 490 nm.

2.6 Cell cryopreservation with ultrarapid freezing protocol

When the cells grew to 70%-80% confluence, they were digested with trypsin-EDTA and suspended in phosphate buffered saline (PBS). All the cells were counted using a hemocytometer and the cells were diluted to a density of 1.0 x 106 cells/mL. The cryovials containing 1.5 mL of various cryopreservation solutions and 1.0 x 106 cells were directly immersed into liquid nitrogen (LN2).

2.7 Cell recovery

After overnight preservation, samples were taken out from LN2 and immediately placed into a 37℃water bath with gently shaking until completely thawed. Cell suspensions were transferred to sterile centrifuge tubes (15 mL, Corning, USA) and diluted in the RPMI-1640/DMEM medium. After 4 mins of centrifugation (800 RPM), the cells were collected for further analysis.

2.8 Cell viability assay

The viability of post-thaw cells was detected by a Live/Dead Viability kit (Life Technologies). The Live/Dead Viability kit included two kinds of molecular probes, named calcein-AM and ethidium homodimer (EH). The excitation setting for calcein-AM was 488 nm and EH was 543 nm; the emission setups were 500–530 nm and 560 nm, respectively. The cryopreserved cells and dye were added to 96-well TCPS plates (n=3, per sample), incubated 30 mins in the dark, and then observed with an inverted fluorescence microscope (Nikon Eclipse Ti-S). The living cells were stained as green, whereas the dead cells were stained as red. The survival efficiencies were the ratios of living cells numbers over total cell numbers (n=3).

2.9 Post-Thaw hemolysis tests

All SRBC samples were thawed at the 37℃water bath with gently shaking. Cell recoveries (hemolysis) were assessed by a microplate reader at 450 nm. 100% cell recovery (0% hemolysis) was prepared by the addition of PBS to SRBCs suspension and left unfrozen. 0% cell recovery (100% hemolysis) was prepared by osmotic shock through addition of water to SRBCs suspension. Cell recovery was calculated by subtracting the attained hemolysis (%) from 100 (%) giving a figure for cell recovery (%) (n=5).

2.10 Cell attachment and proliferation

After cryopreservation, the samples were thawed at the 37℃water bath with gently shaking, then the cells were transferred to 24-well TCPS plates, and cultured in the incubator. After 12 hours, cell attachment and the morphologies were observed using the inverted microscope. To study cell proliferation properties after cryopreservation,

the NIH-3T3 cells were seeded at the cell number of 1.0 x 104 (the seeded number of GLC-82 cell was 5.0 x 104) in 48-well TCPS plates containing 1 mL medium each well (n = 5). After seeding, the cell number was counted at 24, 48, 72, 96 and 120 h respectively. According to the results, we plotted the growth curve, calculated the doubling time and evaluated the cell proliferation properties.

3 Results and Discussions

3.1 Influences on phase transition

CPA can protect cells from damage caused by ice formation during the freeze-thawing cycle. CPAs, such as DMSO, glycerol and trehalose, are commonly hydrophilic solutes that can strongly interact with water molecules, leading to a significant decrease of freezing point and inhibition of ice formation [29, 30]. Therefore, to study the potential of L-carnitine as a CPA, we tested its influence on the ice formation by DSC (Fig. 1). The aqueous solutions of L-carnitine showed an endothermic peak upon thawing, and the peak value decreased with the increasing solution concentration, similar to DMSO, glycerol, and trehalose. However, L-carnitine samples showed the smallest value of melting peak at the same concentration, indicating that L-carnitine possessed the strongest ability to inhibit ice formation. Moreover, the freezing point of L-carnitine was significantly lower than the other solutions at corresponding concentrations, suggesting the strongest ability of L-carnitine to depress the freezing point (Fig. 1E). In addition, the amount of unfrozen water, which is the key factor to increase cryopreservation efficiency [31, 32], is determined by the integration of the endothermic peak, and as was shown in Fig. 1A–D, the unfrozen water content of L-carnitine solutions was more than the other solutions. In summary, compared with DMSO, glycerol and trehalose, L-carnitine

showed an advantage of the inhibition of ice formation. We supposed the reason was that DMSO, glycerol and trehalose prevented water molecules from attending the ice crystallization through hydrogen bonds [33, 34], whereas zwitterionic L-carnitine could inhibit ice formation by strong electrostatically induced hydration [35, 36].

3.2 Cytotoxicity assessments

Currently, the cytotoxicity of CPAs is still a major obstacle to the widespread application of cryopreservation. The toxicity may cause the alteration of cellular morphology, the failure of the cell attachment, and the change of cell growth rate, or even the cell death [37]. In this work, the cytotoxicity assessments of L-carnitine and DMSO were performed by MTT test [38], and Fig. 2A showed the microscope images of NIH-3T3 cells incubated for 3 days with increasing concentrations (0-3%) of L-carnitine or DMSO. Both the cell number and morphology of L-carnitine group were similar to those of the normal cells, and were superior to the DMSO group. Fig. 2B presented the results of the MTT test, which were in accordance with the microscope images. NIH-3T3 cells exposed to different concentrations of L-carnitine for 3 days showed no remarkable difference from normal cells in cell viability and growth rate. On the contrary, DMSO induced a significantly decreased cell viability even at the low concentration of 0.5%. At the concentration of 3%, the cell viability was down to 40.19%. These results suggested that L-carnitine was highly biocompatible and nontoxic, while DMSO was cytotoxic to affect cell proliferation, at low concentration.

3.3 Post-thaw cell viability

The optimized cryopreservation protocol is highly important for cell recovery viability. Several strategies have been reported: slow-freezing, fast-freezing,

vitrification, and ultrarapid freezing. Ultrarapid freezing means that samples are frozen by direct immersion into liquid nitrogen. It is an effective cryopreservation method due to its high time-efficiency and low costs of equipment and labor [39]. In this work, four types of cells (GLC-82 cells, MCF-7 cells, NIH-3T3 cells and SRBCs) were selected. Results showed that, with the ultrarapid freezing protocol, L-carnitine could achieve high cryopreservation efficiency, which was significantly better than those of traditional CPAs. Fig. 3A showed representative images of the Live/Dead assay of GLC-82 cells, MCF-7 cells and NIH-3T3 cells after thawing and Fig. 3B presented the survival efficiency of the GLC-82 cells at different concentrations of L-carnitine, DMSO, glycerol and trehalose. As was shown in Fig. 3B, the survival efficiency of the optimum was 89.42 % at 6% of L-carnitine, which was much higher than the three other CPAs. At the optimal survival efficiencies of DMSO, glycerol and trehalose were only 56.64%, 4.06% and 68.33%, respectively, and the blank control was only 17.86%. Fig. 3C-D presented the survival efficiencies of MCF-7 cells and NIH-3T3 cells. The optimal cell recovery of MCF-7 cells was 77.91% at 10% of L-carnitine, and that of the NIH-3T3 cells was 77.80% at 8% of L-carnitine. Similarly, the survival efficiencies of the three other CPAs were significantly lower than L-carnitine. Effective preservation of RBCs was important for patients suffering from severe blood loss. Clinical cryopreservation protocols utilized high concentrations of glycerol (up to 40wt%) as the CPA[39]. However, deglycerolization procedures were time-consuming after thawing. In this study, we used natural zwitterionic L-carnitine to replace glycerol as the CPA, and the SRBCs recoveries were shown in Fig. 3E. At 8% of L-carnitine, the SRBCs recovery was up to 83.99%, showing distinct advantage over the other CPAs.

3.4 Evaluation of post-thaw cell behaviors

Besides viability, it is also important that post-thaw cells can well retain their functionalities. During freezing process, cellular membranes and enzyme systems are damaged by the osmotic shock and the ice crystal formation, which directly impacts cell attachment and proliferation abilities [40]. Cell attachment is a fundamental cell function and can also indicate cell damage. To further study the influence of cryopreservation on post-thaw cell, we studied cell attachment. As was shown in Fig. 4A, GLC-82 cells cryopreserved by L-carnitine could attach to the TCPS and present similar morphology to normal cells. In contrast, GLC-82 cells cryopreserved with three other CPAs almost completely lost their attaching ability. As was shown in Fig. S2A, there was also no negative impact on the MCF-7 cells and NIH-3T3 cells cryopreserved by L-carnitine. Among the various functionalities relating to cryopreserved cells, CPA could affect proliferation potential of the post-thaw cells. Cell proliferation ability was evaluated by measuring cell doubling time, which impacted the quantity of nucleolar proteins and protein B23 in human cancer cells. Fig. 4B and Fig. S2B showed the proliferation curves of the GLC-82 cells and NIH-3T3 cells after cryopreservation by L-carnitine with the ultrarapid freezing protocol. Cells cryopreserved by L-carnitine maintained growth ability, without any loss of proliferation potential. Fig.4C and Fig. S2C presented doubling times of GLC-82 cells and NIH-3T3 cells respectively. Doubling times were comparable between unfrozen cells and cryopreserved by L-carnitine cells, indicating that cryopreserved by L-carnitine did not affect the proliferative capacity.

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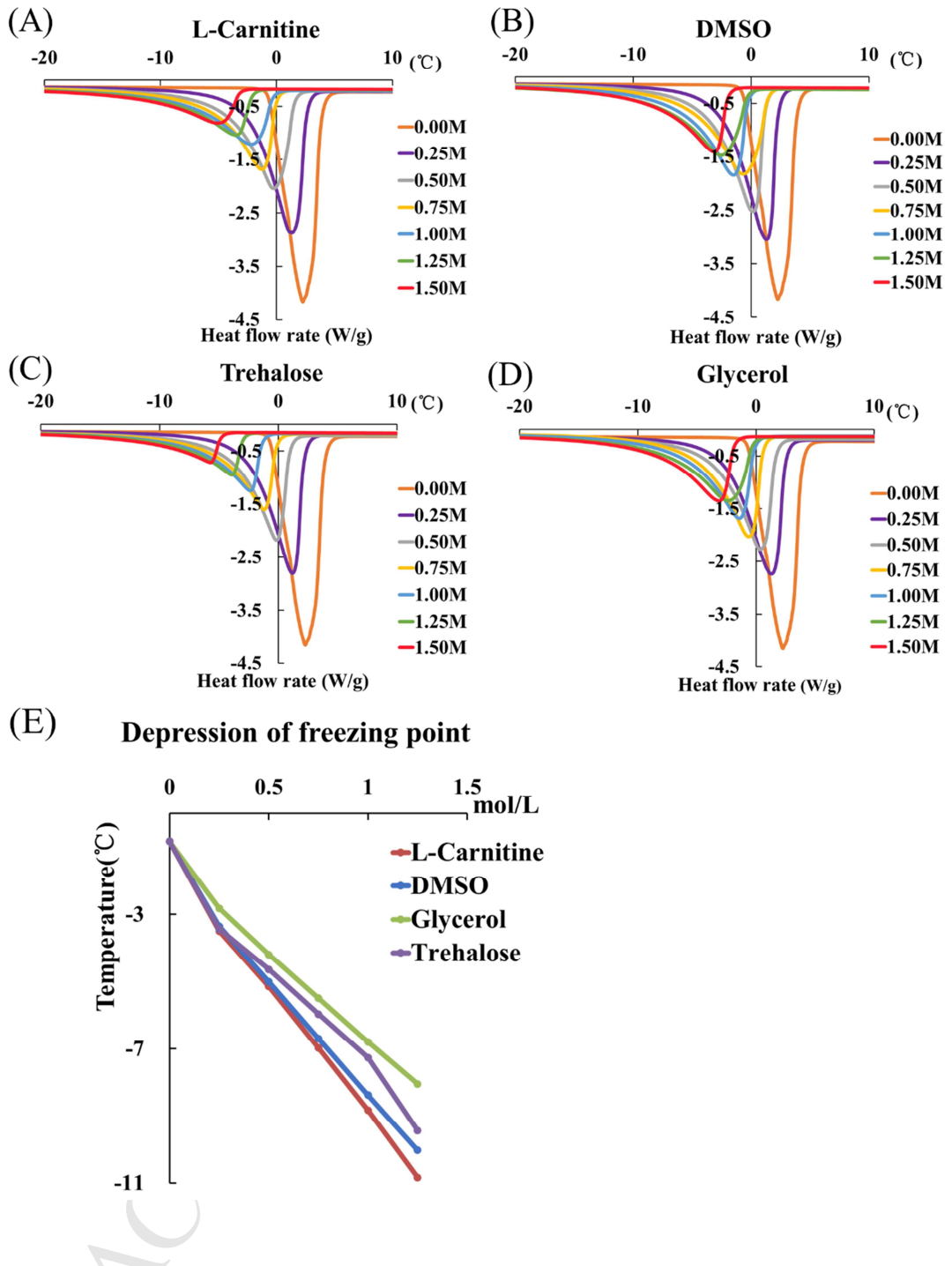

Fig.1. Effect on phase transition. DSC thermograms of different concentrations of (A) L-carnitine; (B) DMSO; (C) Glycerol; (D) Trehalose. (E) Freezing points of different concentrations of L-Carnitine and three other traditional CPAs.

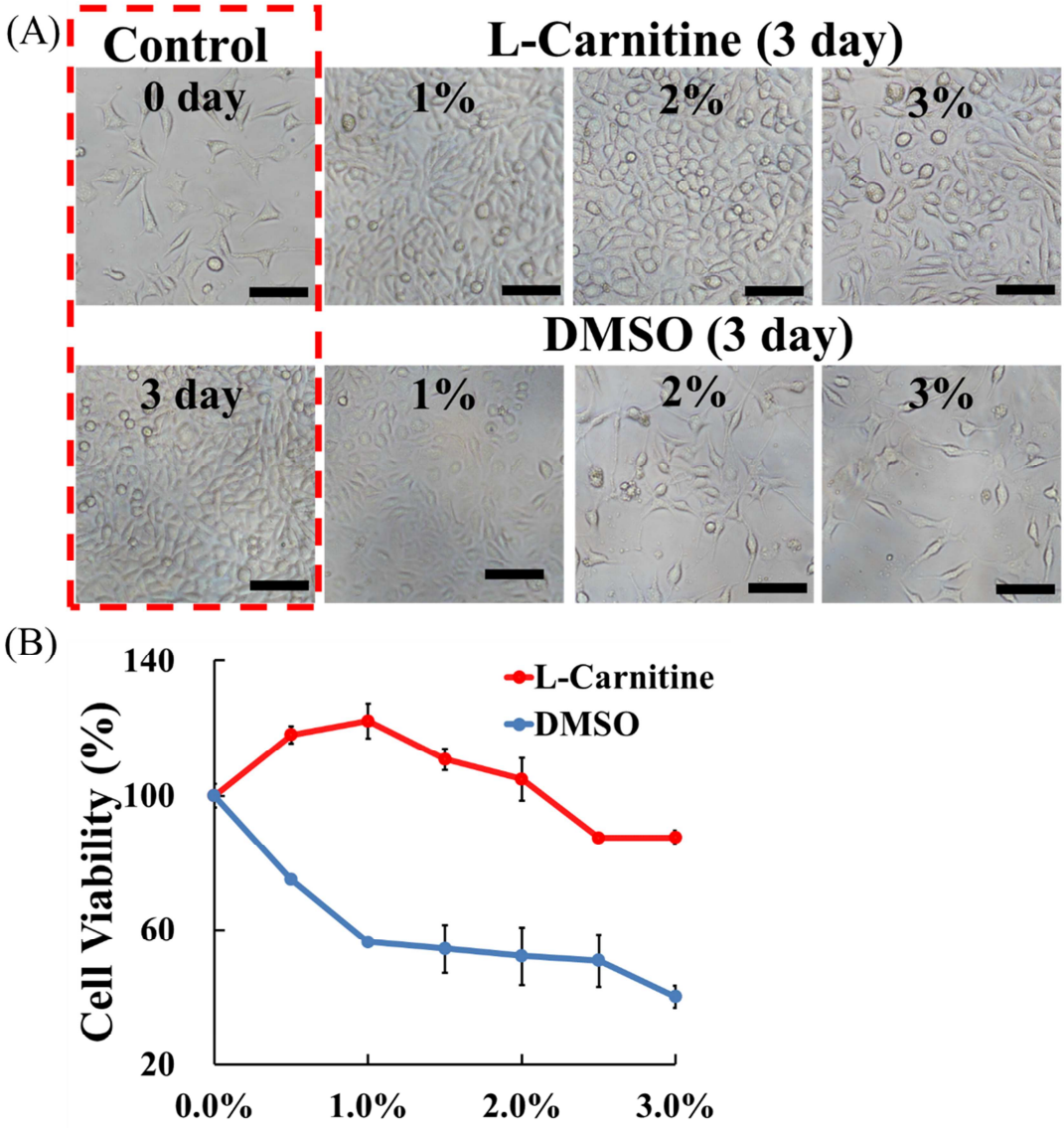

Fig. 2. Cytotoxicity of L-carnitine and DMSO. (A) microscope images of NIH-3T3 cells incubated 3day with the indicated concentration of L-carnitine and DMSO. (B) Cytotoxicity of DMSO and L-carnitine, followed by MTT assay. n=4. Scale bar: 50 μm.

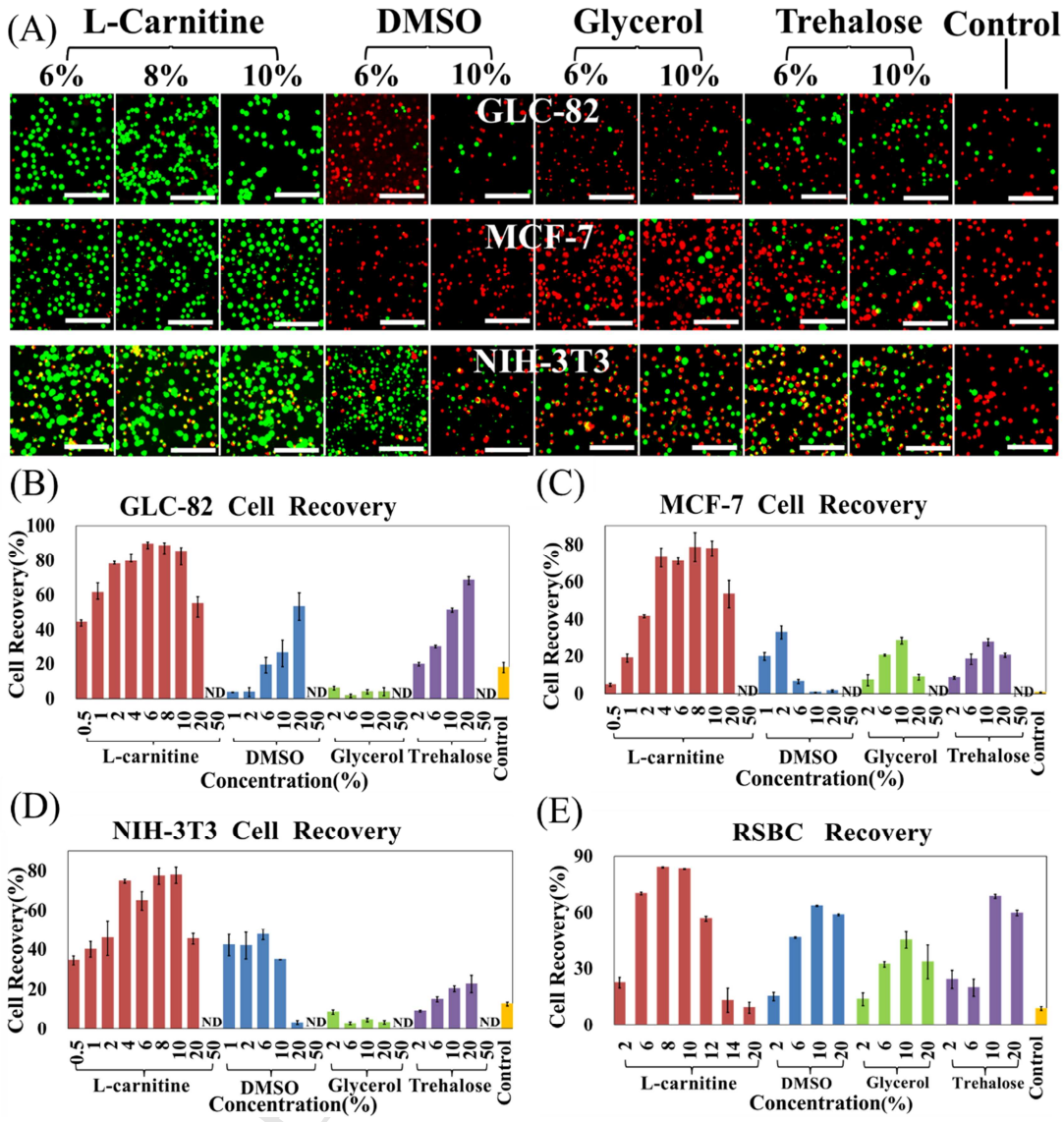

Fig.3. (A) Representative LIVE/DEAD images of GLC-82 cells (the first row), MCF-7 cells (the second row), and NIH-3T3 cells (the third row). Cell viabilities of the cryopreserved groups and the controls were detected after thawing. Green: live cells. Red: dead cells. Post-thaw cell recovery of (B) GLC-82 cells (C) MCF-7 cells (D) NIH-3T3 cells (E) RSBCs. ND: not detected. Scale bar = 50 μm. n≥3.

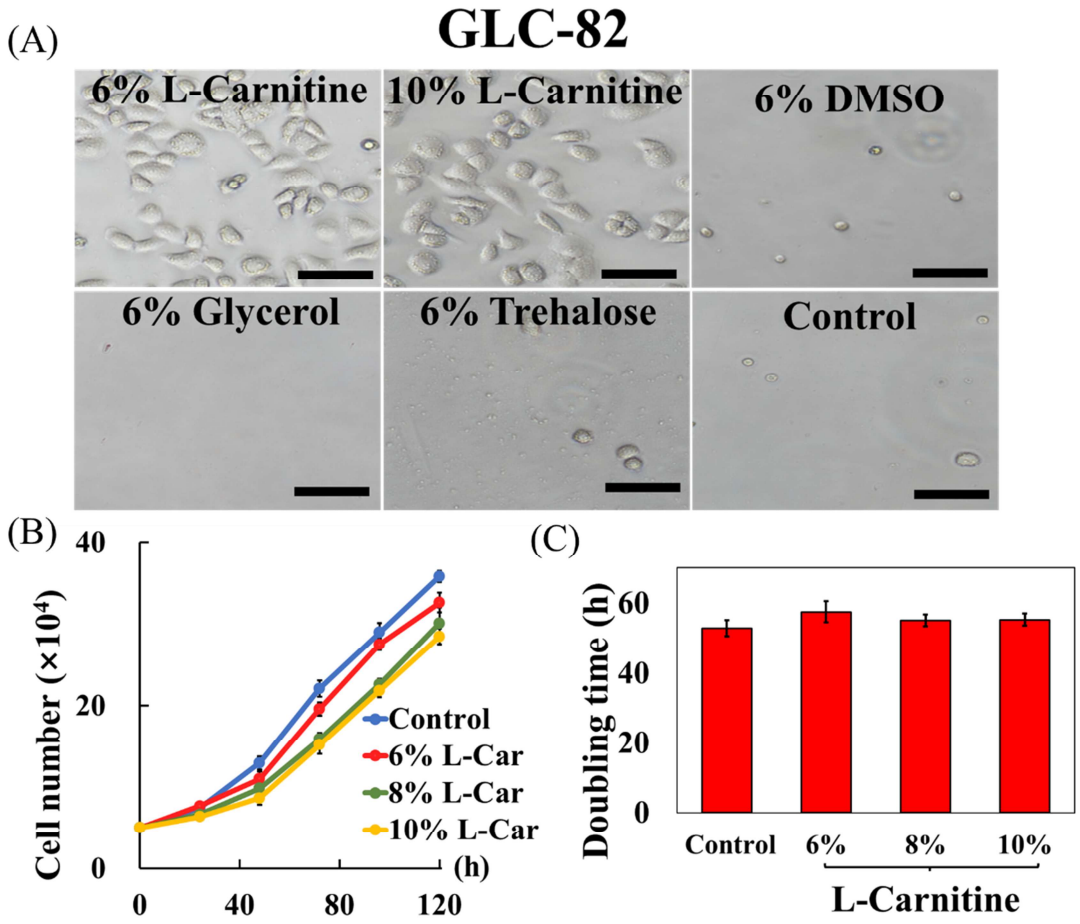

Fig.4. Cell attachment and proliferation properties after cryopreservation. (A) Representative microscopy images of GLC-82 cells cryopreserved with 6% L-carnitine, 10% L-carnitine, 6% DMSO, 6% Glycerol, 6% Trehalose and control and cultured after thawing. (B) Growth curves of GLC-82 cells in different groups. (C) Doubling times of GLC-82 cells in different groups. Scale bar: 50 μm. n=5.

Supplementary Information

Natural zwitterionic L-Carnitine as efficient cryoprotectant for solvent-free cell cryopreservation

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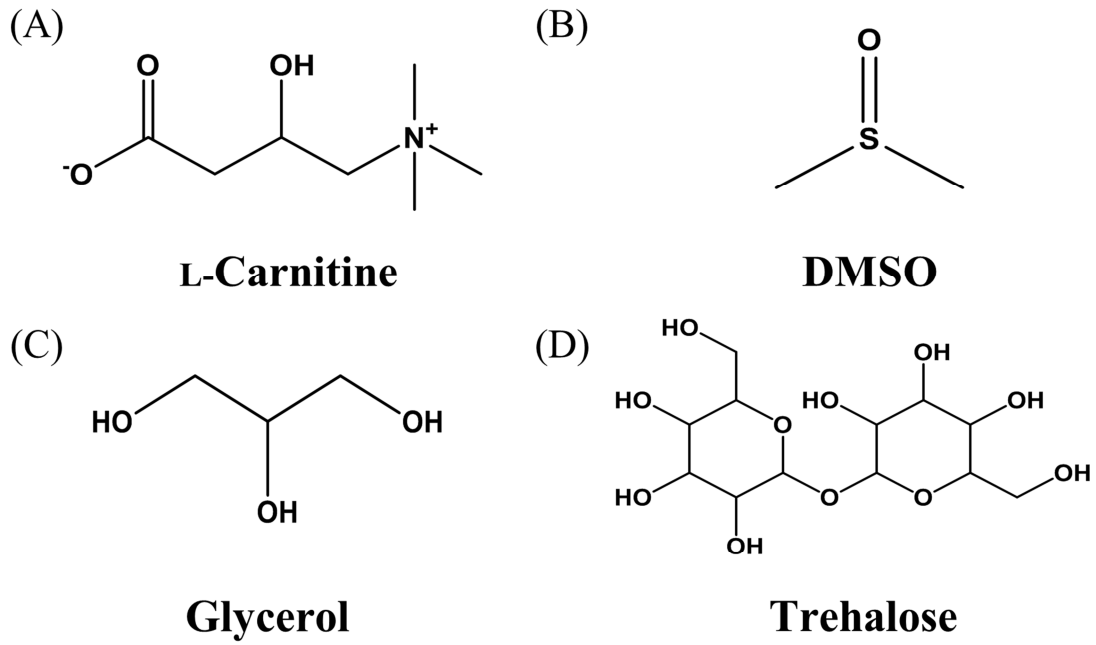

Fig S1. Molecular structures of (A) L-Carnitine, (B) DMSO, (C) Glycerol and (D) Trehalose.

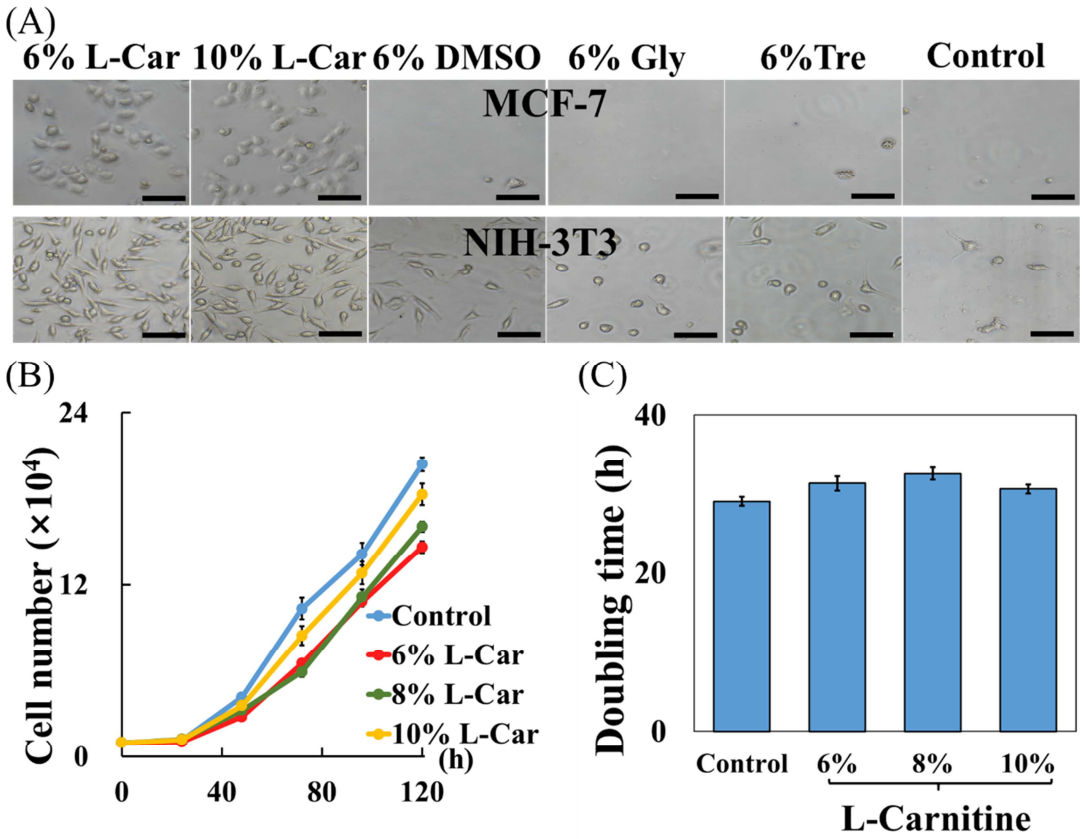

Fig.S2. Cell attachment and proliferation properties after cryopreservation. (A) Representative microscopy images of MCF-7 cells (the first row) and NIH-3T3 cells (the second row), cryopreserved with 6% L-carnitine, 10% L-carnitine, 6% DMSO, 6% Trehalose, 6% Glycerol and control and cultured after thawing. (B) Growth curves of NIH-3T3 cells in different groups. (C) Doubling times of NIH-3T3 cells in different groups. Scale bar: 50 μm. n=5.

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Highlights

Natural zwitterionic L-carnitine can as an efficient cryoprotectant for solvent-free cell cryopreservation is presented.

- For the first time, L-carnitine is proven as an efficient CPA without addition of any organic solvent CPAs.
- L-carnitine shows no observable cytotoxicity.
- L-carnitine enables cell cryopreservation with ultrarapid freezing protocol which is much simpler and more timesaving than traditional stepwise freezing using organic solvent CPAs.