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Effect of hydroxyapatite nanoparticles on osmotic responses of pig iliac endothelial cells ☆

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ABSTRACT

In order to fully explore the potential applications of nanoparticles in biopreservation, it is necessary to study the effect of nanoparticles on cell membrane permeabilities. The aim of this study is therefore to comparatively evaluate the osmotic responses of pig iliac endothelial cells in the absence and presence of commercially available hydroxyapatite nanoparticles. The results indicate that, after the introduction of 0.01 wt% hydroxyapatite nanoparticles, the dependence of cell membrane hydraulic conductivity (L_p) on temperature still obeys the Arrhenius relationship, while the reference value of the hydraulic conductivity of the cell membrane at 273.15 K (L_{pg}) and the activation energy for water transport across cell membrane (E_{Lp}) change from 0.77×10^{-14} m/Pa/s and 15.65 kJ/mol to 0.65×10^{-14} m/Pa/s and 26.14 kJ/mol. That is to say, the reference value of the hydraulic conductivity of the cell membrane has been slightly decreased while the activation energy for water transport across cell membrane has been greatly enhanced, and thus it implies that the hydraulic conductivity of cell membrane are more sensitive to temperature in the presence of nanoparticles. These findings are of potential significance to the optimization of nanoparticles-aided cryopreservation.

Introduction

Nanoparticles (NPs) are defined as the microscopic particles with at least one dimension less than 100 nm [1]. In recent years, applications of NPs in biomedicine have become more and more popular due to their extraordinary mechanical and electrical properties, and the high specific surface areas. Furthermore, more and more cryobiologists have been attracted to the fields of nanoparticles-aided cryopreservation and cryosurgery [20,36]. Han et al. [12] found that NPs can prominently accelerate ice growth and lower the devitrification temperature of cryoprotectant solutions. Guha et al. [11] showed the complex effect of nanogold particles on the homogeneous nucleation temperature and phase change temperature. Thirumala et al. [32] evaluated the freezing responses of cells in the presence of NPs and found that the NPs increased the measured post-freeze apoptotic response. Lv et al. [22] studied the effects of hydroxyapatite nanoparticles (HA-NPs) on devitrification and recrystallization of two important cryoprotective agent (CPA) solutions. They observed the complex interactions between different solutions, and the sizes and contents of the NPs, and all these factors were found to have significant influences on ice crystal growth and recrystallization. It can be seen that the effects of NPs on thermodynamic and heat transfer behaviors of CPA solutions have been systematically studied, including ice nucleation, devitrification and recrystallization, thermal properties, *etc.*, while the effects of NPs on cell osmotic responses is yet to be explored.

In order to optimize the cryopreservation of cells in cryoprotective agent solution containing NPs, knowing the effects of NPs on cell osmotic responses is the necessary prerequisite. Hydroxyapatite [$Ca_{10}(OH)_2(PO_4)_6$, HA] is the major mineral constituent of vertebrate bones and teeth [26]. Due to its excellent biological compatibility and lower toxicity to cells [30], it has been widely used in biomedical research [43,44]. In this study, the pig iliac endothelial cells (PIECs) are used as model cells, and we shall investigate the effect of HA-NPs on the water transport properties of the cell membrane, due to the following facts: (i) endothelial cells are widely used for the basic research of biology and pharmacology

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[3,25] and cryobiology [4,17,37], (ii) their cryobiological parameters have not yet been fully reported, and (iii) the excellent biological compatibility and lower toxicity of HA-NPs to cells.

Conventionally, there are a number of methods that could be used to measure the transport properties of cell membrane, including the microdiffusion chamber technique [23], the micropipette perfusion [10], the microfluidic perfusion system [5], and the perfusion microscope system [31,38]. These techniques all have their own advantages and drawbacks. We have developed a microperfusion chamber system for measurement of cell membrane permeability [34], and in this study, the system is used with slight modifications. Compared with existing microperfusion chambers, several improvements are successfully introduced: (i) the thermal couple is directly embedded into the microchannel during manufacturing, so as to ensure that the temperature of the extracellular solution could be precisely monitored and acquired; (ii) the time for cell attachment was optimized so that the cells adhered but did not spread out and maintained their original spherical shape; (iii) a slow-mixing technique is used to switch the extracellular solutions while keeping the cells *in situ*, and the corresponding extracellular concentration profile proposed by us [41] in previous studies is used.

In this study, we investigate the effects of HA-NPs on the osmotic properties of PIEC membrane using the improved microperfusion system, including the cell membrane hydraulic conductivity (L_p) at −2, 5, 14 and 23 °C, and its activation energy. These findings are of potential significance to the optimization of nanoparticle-aided cryopreservation.

Materials and methods

Theory for water transport across cell membrane during osmotic shift

Two of the most widely used transport models for the description of water and CPA transport across cell membrane are the K–K model [16,28] and the two-parameter (2-*p*) model [15]. While, it was recently reported that the reflection coefficient introduced by the K–K model actually could be unnecessary or even leads to errors in some aspects [15]. Thus, we prefer to use the 2-*p* model instead.

The 2-*p* model is the most popular one for investigation of cell osmotic responses, and it has been further improved for much wider applications in recent years [8,9,14,40]. In this study, only water transport across cell membrane is focused on, and the 2-*p* model could be simplified into a one-parameter equation in the absence of CPA transport [41,42], shown as follows:

$$\frac{dV_c}{dt} = -L_p ART(C_s^e - C_s^i) \tag{1}$$

where V_c is the cell volume, A is the surface area of the cell, R is the universal gas constant (R = 8.314 J/mol/K), T is temperature. C_s^e and C_s^i are the extracellular and intracellular osmolarity of salt, respectively.

The extracellular osmolarity of salt, C_s^e is commonly assumed to be a step function by most of the existing studies, while Takamatsu et al. [31] experimentally determined the time dependent function for it, and latterly Zhao [41] developed a universal self-adaptive time-varying function according to mathematical approximation. Zhao's method was followed and adopted in this study.

The intracellular osmolarity of salt, C_s^i, could be calculated based on the conservation of the intracellular salt [31]. According to the Boyle van't Hoff relationship, the conservation equation is given by:

$$C_s^i(V_c - V_b) = C_{s,0}^i(V_{c,0} - V_b) \tag{2}$$

where the subscript 0 refers to the values in the isotonic state.

Then, L_p and V_b could be determined by fitting Eq. (1) to the experimentally measured transient cell volumes, as has been described by Zhao [41].

Arrhenius relationship could be used to describe the temperature dependence of L_p [13,18,19]:

$$L_p(T) = L_{pg} \cdot \exp\left[\frac{E_{Lp}}{R}\left(\frac{1}{T_0} - \frac{1}{T}\right)\right] \tag{3}$$

where E_{Lp} is the activation energy for water transport across cell membrane, L_{pg} is the reference hydraulic conductivity at the reference temperature T_0 (=273.15 K), and T is temperature.

As can be seen, there is a linear relationship between the natural logarithm of L_p and the reciprocal of the temperature [34]. In this study, the value of E_{Lp} and L_{pg} were obtained by a linear fitting of the experimental data to the relationship between $\ln[L_p(T)]$ and $1/T$.

Microperfusion system

Fig. 1(a) shows the setup of the microperfusion chamber system. It mainly consists of two syringe pumps (Legato 110P, KD Scientific Inc., USA), a temperature monitor (HP 34970A with 34901A module, Agilent, USA), the self-made microperfusion chamber, the multifunctional type circulation constant temperature water bath (DC-2006, Ningbo scientz biotechnology Polytron Technologies Inc., China), and a beaker used for collection of waste solutions. There is a "T" connector just at the upstream of the inlet for the exchange of the solutions, and in order to effectively decrease the diffusion of the solutions at the interface, the "T" shaped three-way plastic tube with a much thinner inner diameter (0.4 mm) is used.

Fig. 1(b) shows the microperfusion chamber, where a 100 μm thick silicon rubber sheet with a cutout 55 mm long and 2 mm wide is caught in two slide glasses. The upper glass has two holes for the inlet and outlet corresponding to the two ends of the microchannel. Fig. 1(c) shows the circulating coolant cooling unit; it is made of a 10 mm thick plexiglass, with a 9 mm-in-depth flow channel milled for the coolant. The cooling unit is further covered and sealed on the upper slide glass of the microperfusion chamber to control its temperature.

During cell perfusion, as shown in Fig. 1(a), two syringe pumps are used to deliver the isotonic and the hypertonic solutions preloaded in the syringes separately. The osmotic shift is obtained by programmed switching the syringe pump from the isotonic solution to the hypertonic solution. The syringe and the chamber are connected by capillary tubes (0.5 mm and 3 mm in inner and outer diameters, made of Teflon), respectively.

The microperfusion chamber as a whole is then mounted onto the inverted microscope (Ti-FL, Nikon, Japan), and a CCD camera (DS-Ril, Nikon, Japan) with a DS-U3 DS camera control unit (Nikon, Japan) are used to accurately record the cell volume responses during the osmotic shifts.

Cell preparation

The PIECs were cultured with modified RPMI-1640 (Hyclone, USA) at 37 °C in a humidified incubator (Sanyo Electric Co., Ltd, Japan) with 5% CO_2. The cells were harvested once the confluence reached 80–90%: they were firstly washed once with PBS (290 mOsm), and then digested in 0.25 wt% trypsin (HyClone, USA) for 3 min, and the digesting was stopped once the cells were detached. After that the cell suspension was centrifuged at 100*g* for 5 min, then the supernatant was removed, and finally the cells were washed for 3 times using PBS before the cell suspension was ready for the experiments.

Preparation of the suspensions of HA-NPs

The HA-NPs used in this study (Nanjing Emperor Nano Material Co., Ltd, China) had a non-spherical shape with a declared 20 nm