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Exploring novel cell cryoprotectants based on neutral amino acids

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ABSTRACT

Cryoprotectants play a key role in cell cryopreservation because they can reduce cryoinjuries to cells associated with ice formation. To meet the clinical requirements of cryopreserved cells, cryoprotectants should be biocompatible, highly efficient and easily removable from cryopreserved cells. However, integration of these properties into one cryoprotectant still remains challenging. Herein, three biocompatible neutral amino acids, including β -alanine, γ -aminobutyric acid and ε -aminocaproic acid, are first reported to have the potential as such ideal cryoprotectants. The results demonstrate that they can inhibit ice formation and reduce osmotic stress to provide extracellular and intracellular protection, thereby ensuring high cryopreservation efficiency for both anuclear and nucleated cells. More importantly, due to the remarkable osmotic regulation ability, the neutral amino acids can be rapidly removed from cryopreserved cells via a one-step method without causing observable damage to cells, superior to the current state-of-the-art cryoprotectants—dimethyl sulfoxide and glycerol. This work provides a new perspective to develop novel cryoprotectants, which may have dramatic impacts on solvent-free cryopreservation technology to support the cell-based applications, such as cell therapy and tissue engineering, etc.

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1. Introduction

Living cells are key elements for modern cell-based medical applications, such as cell therapy, tissue engineering and transfusion medicine, etc. [1–3]. To achieve optimal outcomes, the collected cells should be well preserved to maintain their viability and functionality [4]. Cryopreservation is a commonly used technology for long-term cell storage, because it enables cells to enter a ‘suspended animation’ state at ultra-low temperature [5,6]. Currently, the cryopreservation technology relies on the use of cryoprotectants (CPAs) to protect cells from ice-related mechanical injury and osmotic injury during cryopreservation process (Fig. 1a) [7]. Dimethyl sulfoxide (DMSO) and glycerol are two state-of-the-art CPAs because of their high cryoprotection efficacy [8–10]. However, notable drawbacks significantly restrict their clinical use. For example, their intrinsic toxicity can cause damage to cells as well as clinical safety concerns. Numerous studies have shown that DMSO is correlated to dysfunctionality of enzymes, apoptosis and uncontrolled differentiation of stem cells [11,12]. Meanwhile, it has been reported that DMSO and glycerol can induce life-threatening intravascular hemolysis [11,13]. To address these problems, many approaches have been developed to reduce the toxicity induced by these CPAs. For

instance, some researchers have proposed toxicity cost functions to optimize the CPA addition and removal procedures, which may minimize the toxicity [14,15]. Another drawback is that the poor membrane penetrability of DMSO and glycerol requires stepwise washing procedures to completely remove them from cryopreserved cells. It is time-consuming and may cause significant cell loss (~ 10%) during each washing step, hindering the urgent supply of cells for lifesaving treatments [16,17]. Therefore, developing CPAs that are simultaneously biocompatible, highly efficient and easily removable from cells is of great significance.

During the last few decades, researchers have developed many kinds of CPAs to replace or reduce the content of DMSO or glycerol, including antifreeze (glyco)proteins [AF(G)Ps] (and their mimics) [18,19], sugars (e.g. trehalose, sucrose) [20,21], alcohols (e.g. ethylene glycol, propylene glycol) [22] and small molecule phenolic-glycosides [23,24], etc. Particularly, AF(G)Ps have received significant attentions due to their inhibition effects on ice recrystallization [25,26]. However, AF(G)Ps are cytotoxic and immunogenic as foreign proteins [27]. Meanwhile, it is difficult to obtain AF(G)Ps from natural resources or biosynthesize them, limiting their large-scale use [19]. Recently, some researchers have reported that osmoprotectants (e.g. betaine, proline), which are accumulated in many plants to resist cold stress, can be used as promising CPAs [28,29]. It was because that they could balance the intra- and extracellular osmotic pressure and stabilize the protein functions during freezing [30,31]. Interestingly, both AF(G)Ps and osmoprotectants have

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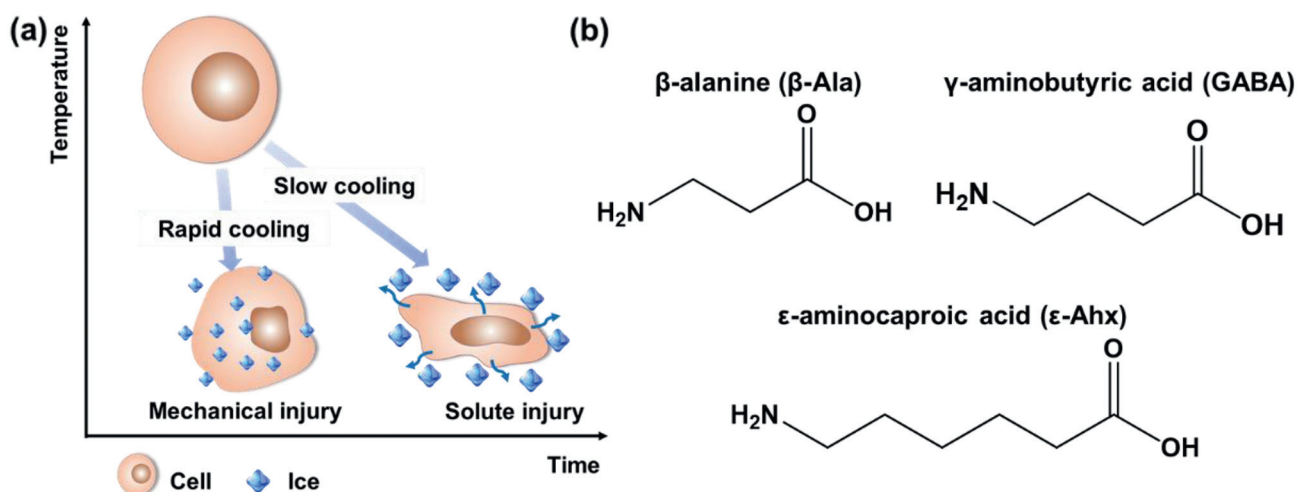


Fig. 1. Mechanisms of cryoinjuries and proposed cell cryoprotectants based on neutral amino acids. (a) Schematic drawing of cryoinjuries (mechanical and osmotic injuries) to cells during cryopreservation process. (b) Molecular structures of three neutral amino acids, including β -alanine (β -Ala), γ -aminobutyric acid (GABA) and ϵ -aminocaproic acid (ϵ -Ahx).

some connections with neutral amino acids (nAAs). The structure analysis shows that AF(G)Ps contain large amounts of neutral amino acid (nAA) residues. For example, AFGPs are highly conserved with a repeated nAA-based tripeptide (alanine–alanine–threonine) linked by a disaccharide [32]. The type I AFPs is reported to consist of ~70% neutral alanine residues [33]. In terms of osmoprotectants, many of them are nAAs or the derivatives of nAAs [31,34].

According to previous studies, there are many nAA transporters (e.g. system A, system L and system N) on cell membrane, endowing them with rapid uptake/release ability to counteract osmotic injury to cells [35–40]. Besides, the nAAs comprise an amino-group and a carboxy group that can form hydrogen bonds with water molecules, thus might disturb the interactions between water molecules and impede ice formation. Based on this, nAAs might have the ability to reduce the ice-related osmotic injury and mechanical injury to cells during the cell cryopreservation process. However, to the best of our knowledge, we have not found any literatures that systematically investigate the role of nAAs in cell cryopreservation.

Therefore, the objective of the present study was to investigate the potential of nAAs as novel CPAs. To this end, β -alanine (β -Ala), γ -aminobutyric acid (GABA) and ϵ -aminocaproic acid (ϵ -Ahx) were selected as three representative nAAs (Fig. 1b). Their capacities of inhibiting ice formation and reducing osmotic stress to cells were tested. Afterward, two types of model cells, including anuclear red blood cells (RBCs) and nucleated cells (Hela cells, GLC-82 cells and smooth muscle cells), were cryopreserved to study the cryoprotection efficacy of nAAs. In addition, a simple and fast one-step method was applied to remove nAAs from cryopreserved cells. Lastly, cytotoxicity tests were conducted to evaluate their biocompatibility.

2. Materials and Methods

2.1. Materials

β -Ala (>99%), GABA (>99%) and ϵ -Ahx (>99%) were all purchased from Sigma-Aldrich. Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute-1640 (RPMI-1640), Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12) and fetal bovine serum (FBS) were all obtained from Gibco. Sucrose (>99%) was purchased from Adamas. Glycerol (>99%) was obtained from Alfa Aesar. Penicillin–streptomycin (P/S), trypsin–EDTA (0.025%–0.01%), DMSO (>99%) and phosphate–buffered saline (PBS) were all purchased

from Beijing Solarbio Science and Technology Co., Ltd. Live/Dead viability kits were obtained from Invitrogen. Milli-Q water ($18.2 \text{ M}\Omega \cdot \text{cm}$) was used in all experiments.

2.2. Differential scanning calorimetry (DSC) assays

Water solutions (5–10 mg) with varied concentrations of β -Ala, GABA or ϵ -Ahx were sealed in aluminum crucibles and transferred to calorimeter sample chambers (TA Q2000). Then, the samples were cooled from 10°C to -40°C at a rate of $-10^\circ\text{C} \cdot \text{min}^{-1}$, held for 5 min, and warmed back to 10°C at a rate of $2^\circ\text{C} \cdot \text{min}^{-1}$. The heat flows were monitored in real time.

The total water content (w_{tc}), freezable water content (w_{f}) and nonfreezable water content (w_{nf}) of each sample were determined by the following equations [41,42].

$$w_{\text{tc}} = m_{\text{w}}/m \quad (1)$$

$$w_{\text{f}} = \Delta H/\Delta H_{\text{w}} \quad (2)$$

$$w_{\text{nf}} = w_{\text{tc}} - w_{\text{f}} \quad (3)$$

where m_{w} and m were the mass of water and whole sample, respectively; ΔH was the melting enthalpy of each sample during warming process, determined by integration of the endothermic peak; ΔH_{w} was the specific heat of water fusion equal to $334 \text{ J} \cdot \text{g}^{-1}$.

2.3. Cell preparation

Sheep RBCs were obtained from Nanjing SenBeiJia Biological Technology Co., Ltd., and stored at 4°C for less than 7 days. Before the experimental use, they were washed by PBS solutions and centrifuged at $2000 \text{ r} \cdot \text{min}^{-1}$ and 4°C for 10 min (Sigma, 3 K15) to remove the dead cells and preservation solutions. The pelleted RBCs were used within 2 h at room temperature.

The culture medium for Hela cells was DMEM medium with 10% FBS and 1% P/S, and that for GLC-82 cells and smooth muscle cells (SMCs) were RPMI 1640 medium and DMEM/F-12, respectively, with 10% FBS and 1% P/S. All cells were cultured at 37°C under an atmosphere of 5% CO_2 . At a ~90% confluence, cells were trypsinized using trypsin–EDTA and washed with PBS solutions for two times. Finally, they were collected by centrifugation ($800 \text{ r} \cdot \text{min}^{-1}$, 4 min) and resuspended in culture medium for further use.

2.4. Osmotic regulation tests

RBCs were exposed to PBS solutions containing 0.2 mol·L⁻¹ sucrose (Suc), 0.3 mol·L⁻¹ Suc, 0.2 mol·L⁻¹ Suc + 0.1 mol·L⁻¹ β-Ala, 0.2 mol·L⁻¹ Suc + 0.1 mol·L⁻¹ GABA and 0.2 mol·L⁻¹ Suc + 0.1 mol·L⁻¹ ε-Ahx, respectively. After exposure at 37 °C for 24 h, the RBC morphology was observed by a microscope (Nikon Eclipse Ti-S) and cell area was calculated by NIS-elements software.

Hela cells were exposed to culture mediums containing 0.2 mol·L⁻¹ sucrose (Suc), 0.3 mol·L⁻¹ Suc, 0.2 mol·L⁻¹ Suc + 0.1 mol·L⁻¹ β-Ala, 0.2 mol·L⁻¹ Suc + 0.1 mol·L⁻¹ GABA and 0.2 mol·L⁻¹ Suc + 0.1 mol·L⁻¹ ε-Ahx, respectively. After exposure at 37 °C for 24 h, Hela cells were stained using a Live/Dead viability kit to determine the cell survival rate. The morphology of survived cells attached onto substrates was observed by the microscope.

2.5. Measurement of cell survival rate

The measurement of RBC survival rate was referred to previous reports [18,28]. An aliquot of 100 μL fresh RBCs added to 1 mL PBS solution was positive control (100% RBC survival), and that added to 1 mL water was negative control (0% RBC survival). After centrifugation (2000 r·min⁻¹, 4 °C, 10 min), the supernatant absorbances of positive control (A₁), negative control (A₀) and detected samples (A) were measured at 450 nm by a microplate reader (Tecan Infinity M200pro). RBC survival rate was calculated according to the following equation.

$$\text{RBC survival rate} = 1 - (A - A_1) / (A_0 - A_1) \times 100\% \quad (4)$$

A live/dead staining assay using the Live/Dead viability kits was utilized to determine the survival rate of nucleated cells (Hela cells, GLC-82 cells and SMCs) [43,44]. Cells were introduced to a 96-well microplate with 100 μL calcein-AM/ethidium homodimer-1 reagent mixture according to the manufacturer's instructions. After incubated away from light at room temperature for 30 min, cells were observed by an inverted fluorescence microscope (Nikon Eclipse Ti-S). The cell survival rate was calculated by counting the numbers of live cells (green) and dead cells (red).

2.6. Preparation of CPA solutions

Different concentrations (wt%) of β-Ala, GABA, ε-Ahx, glycerol or DMSO were respectively dissolved in PBS solutions (for anuclear RBC cryopreservation) or cell culture mediums (for nucleated cells). Then, the solutions were sterilized using a Millex-GP syringe filter unit (0.22 μm, Millipore Millex) for further use.

2.7. Cell cryopreservation tests

For RBC cryopreservation, a 100 μL aliquot of pelleted fresh RBCs was vertically added in cryovials (Corning, 430659). Then, 1 mL of CPA solution was gently added to the cryovials and the final RBC density was 3.8 × 10⁹ mL⁻¹. After a gentle inversion for 5 min at room temperature, the cryovials were directly immersed in a liquid nitrogen tank. For nucleated cell cryopreservation, 1 mL of CPA solution was added in cryovials with a cell density of 1 × 10⁶ mL⁻¹. After a gentle mix for 5 min at room temperature, the cryovials were directly immersed in a liquid nitrogen tank.

After storage in liquid nitrogen tank for 1 day, the samples were rapidly thawed in a 37 °C water bath. Afterward, the measurement of cell survival rate was immediately performed as described above to determine the cryopreservation efficiency of CPAs.

2.8. Washing tests via a one-step method

A one-step washing test was conducted to compare the ability of nAAs, DMSO or glycerol to be removed from cells. An 80 μL aliquot of fresh sheep RBCs was added in 3 mL of PBS solutions containing 2%, 6%, 10% β-Ala, GABA, ε-Ahx, glycerol or DMSO, respectively. After a gentle mix, they were incubated at 37 °C with 5% CO₂. All samples were prepared in duplicate. After an incubation period of 24 h, one duplicate was centrifuged (2000 r·min⁻¹, 4 °C, 10 min) to measure the RBC survival rate. The other was directly added with 3 mL of PBS solutions. Then the mixture was centrifuged (2000 r·min⁻¹, 4 °C, 10 min) to measure the RBC survival after washing.

2.9. CPA removal from cryopreserved cells via a one-step method

RBCs were cryopreserved with β-Ala, GABA, ε-Ahx or glycerol at their optimal concentrations, respectively. All samples were cryopreserved in duplicate. After thawing, one duplicate was centrifuged (2000 r·min⁻¹, 4 °C, 10 min) to measure the post-thaw RBC survival rate. The other was directly added with equal amount of PBS solution and the mixture was centrifuged (2000 r·min⁻¹, 4 °C, 10 min). Then the supernatant absorbance was measured to determine the post-thaw-wash RBC survival rate.

Hela cells were cryopreserved with β-Ala, GABA, ε-Ahx or DMSO at their optimal concentrations in duplicate as RBCs did. After thawing, one duplicate was immediately used to measure the post-thaw cell survival rate. The other was directly added with equal amount of culture medium and the mixture was centrifuged to remove the supernatant. Then, Hela cells were stained by a Live/Dead viability kit to determine the post-thaw-wash cell survival rate.

2.10. Morphology of post-thaw-washed RBCs

After washing, three circular fields were randomly selected to observe the morphology of post-thaw-washed RBCs by an inverted microscope. Post-thaw-washed RBCs that could maintain their biconcave shape as fresh RBCs were defined as normal shaped RBCs.

2.11. Proliferation tests of post-thaw-washed Hela cells

The post-thaw-washed Hela cells were resuspended in culture mediums. Then, they were reseeded onto culture flasks and cultured at 37 °C under an atmosphere of 5% CO₂. After a determined period of culturing, three circular fields were randomly selected to observe cell morphology. Meanwhile, cell numbers were counted daily to determine the proliferation rate.

2.12. Cytotoxicity evaluation tests

RBCs or Hela cells were exposed to PBS solutions or culture mediums containing 2% (w/w) β-Ala, GABA or ε-Ahx, respectively, at 37 °C under an atmosphere of 5% CO₂. After exposure for 24 h, the cell survival rate was measured as indicated above. Meanwhile, the morphology of Hela cells attached onto substrates was also observed.

2.13. Data analysis

The treatments for osmotic regulation tests, cryopreservation tests, CPA removal tests, and cytotoxicity tests were repeated in triplicate. Then, the replicated samples were collected to determine the mean values and errors. A two-tailed homoscedastic Student's t test with a 95% confidence interval was used for statistical analysis, a *p* value less than 0.05 was considered as statistically significant [44,45].

3. Results and Discussion

3.1. Ability to prevent ice formation

Inevitable ice formation and growth during freeze–thaw cycles are original causes of detrimental cryoinjuries to cells. In 1970s, Mazur proposed the two-factor cryoinjuries, as shown in Fig. 1a [7]. When cells are slowly cooled, the extracellular ice formation leads to water efflux, thus increasing the concentration of intracellular solutes to a detrimental level and resulting in cell death (osmotic injury). When cells are rapidly cooled, the intracellular ice formation disrupts delicate subcellular structures, reducing cell survival due to mechanical damage (mechanical injury). Therefore, CPAs that are capable of inhibiting ice formation and growth are essential to reduce cryoinjuries to cells during cryopreservation.

In this work, the influences of nAAs on water crystallization were investigated according to DSC tests. As shown in Fig. 2a, all samples displayed endothermic peaks due to ice melting. Compared to pure water, the nAA solutions showed smaller melting peaks, suggesting the inhibition effects of nAAs on ice formation. Besides, nAAs could depress water freezing points (Fig. 2b) and increase the nonfreezable water contents of solutions (Fig. 2c). As is well known, ice formation is related to the aggregation of water molecules linked by hydrogen-bonded network [46]. It was reported that the amino-groups and carboxy groups of nAAs could bind to water *via* hydrogen bonds. Consequently, nAAs disturbed the hydrogen-bonded network structure of water and impeded the ice formation [47–49]. Therefore, the ice inhibition ability of nAAs showed their potential to reduce ice-related cryoinjuries to cells.

3.2. Ability to reduce osmotic stress

As water freezes, the increased concentrations of intra- and extracellular solutes induce considerable osmotic shock to cells and affect the cell survival [10]. Notably, the nAAs have been reported to balance the intra- and extracellular osmotic pressure and maintain cell volume [40,50–52]. It is attributed to that they can be taken up or released by cells *via* multiple membrane transporters under osmotic stress [35–40]. Herein, the ability of β -Ala, GABA and ϵ -Ahx to protect RBCs and Hela cells from osmotic injury was studied (Fig. 3).

As is well known, sucrose is impenetrable to the cell membrane. Therefore, the sucrose added to isotonic solutions would cause osmotic stress to cells due to the increased extracellular osmotic pressure. As shown in Fig. 3a and c, compared to control group (physiological pressure), RBCs exposed to PBS solutions supplemented with 0.2 or 0.3 mol·L⁻¹ sucrose (65% or 97% higher than physiological pressure) were shrunk with smaller RBC area due to the hypertonic stress.

Interestingly, when RBCs were exposed to 0.2 mol·L⁻¹ sucrose with 0.1 mol·L⁻¹ nAAs, the cell area was significantly increased. It was because that β -Ala, GABA and ϵ -Ahx could be taken up by RBCs under osmotic stress *via* multiple membrane transporters, such as system A, system L and system N, thus balancing extra- and intracellular osmotic pressure and regulating RBC volume [35–40]. Besides, no significant difference in cell area could be observed between β -Ala and GABA, suggesting β -Ala possessed a similar uptake ability with GABA to counteract hypertonic stress to RBCs.

A similar tendency could also be observed for Hela cells. As shown in Fig. 3b, when Hela cells were exposed to culture medium containing 0.2 or 0.3 mol·L⁻¹ sucrose, cell survival rate decreased to 51% or 3% respectively. Meanwhile, compared to cells exposed to culture mediums (control), Hela cells in 0.2 or 0.3 mol·L⁻¹ sucrose groups showed abnormal attachment morphology (Fig. 3d). Interestingly, when cells were exposed to 0.2 mol·L⁻¹ sucrose with 0.1 mol·L⁻¹ nAAs, the cell survival rate was significantly improved, albeit to a different extent (β -Ala > GABA > ϵ -Ahx). Meanwhile, the cells showed similar adherence ability with that of control group (Fig. 3d). It was attributed to the uptake of β -Ala, GABA and ϵ -Ahx by Hela cells *via* membrane transporters to balance extracellular and intracellular osmotic pressure (Fig. 3e) [35–40], which was highly favorable to reduce osmotic injury and provide intra- and extracellular protection to cells during cryopreservation.

3.3. Cell cryopreservation

After investigating the abilities of nAAs to inhibit ice formation and reduce osmotic injury, we next studied their cryoprotection efficacy to anuclear RBCs and nucleated cells (Hela cells, GLC-82 cells and SMCs), which are two types of model cells used in cryopreservation studies (Fig. 4). In this work, the rapid-freezing protocol, which meant directly putting samples in a liquid nitrogen tank, was selected due to its simple and time-saving procedure compared to the slow-freezing method.

For RBC cryopreservation, glycerol is considered as the golden standard CPA in many clinical settings [53,54]. Therefore, it was used as a control CPA to compare with nAAs. As shown in Fig. 4a, the optimal cryopreservation efficiency of β -Ala, GABA and ϵ -Ahx to RBCs was 77%, 73% and 58% respectively, which was significantly higher than that of the blank control group (~5%). Meanwhile, the post-thaw RBC survival rate of β -Ala, GABA was comparable to that of glycerol using the same rapid freezing procedure (~82%).

Because anuclear RBCs have no complex subcellular structures, such as nucleus and other organelles, the cryoinjuries might be less pronounced. Therefore, we next cryopreserved the nucleated cells with nAAs. Meanwhile, DMSO as a state-of-the-art CPA for nucleated cells was selected for comparison. As shown in Fig. 4b, the cryopreservation efficiency to Hela cells of β -Ala, GABA and ϵ -Ahx at optimal

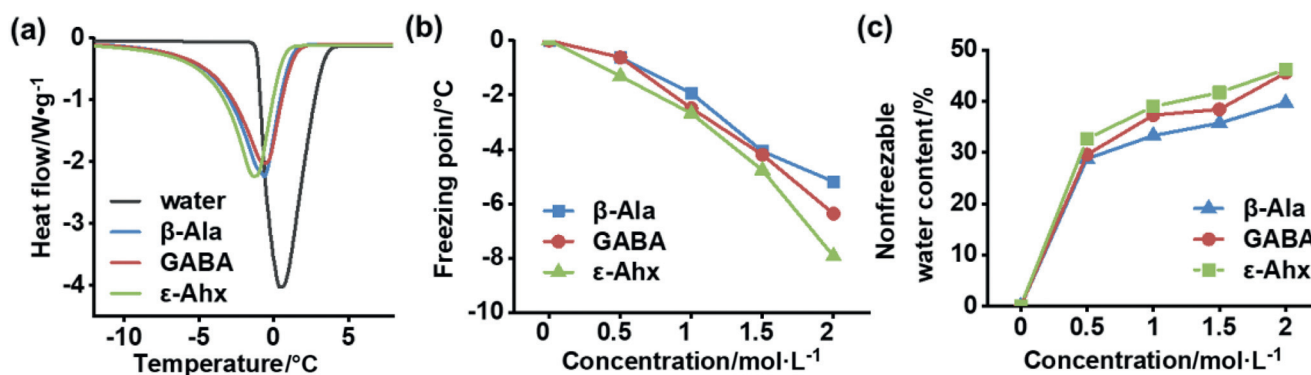


Fig. 2. DSC tests. (a) Heat flow curves of pure water (grey), water solutions with 0.5 mol·L⁻¹ β -Ala (blue), 0.5 mol·L⁻¹ GABA (red) or 0.5 mol·L⁻¹ ϵ -Ahx (green) during melting process. (b) Freezing points and (c) nonfreezable water contents of pure water, water solutions with varied concentrations of β -Ala (blue), GABA (red) and ϵ -Ahx (green). β -Ala denoted β -alanine, GABA denoted γ -aminobutyric acid and ϵ -Ahx denoted ϵ -aminocaproic acid.

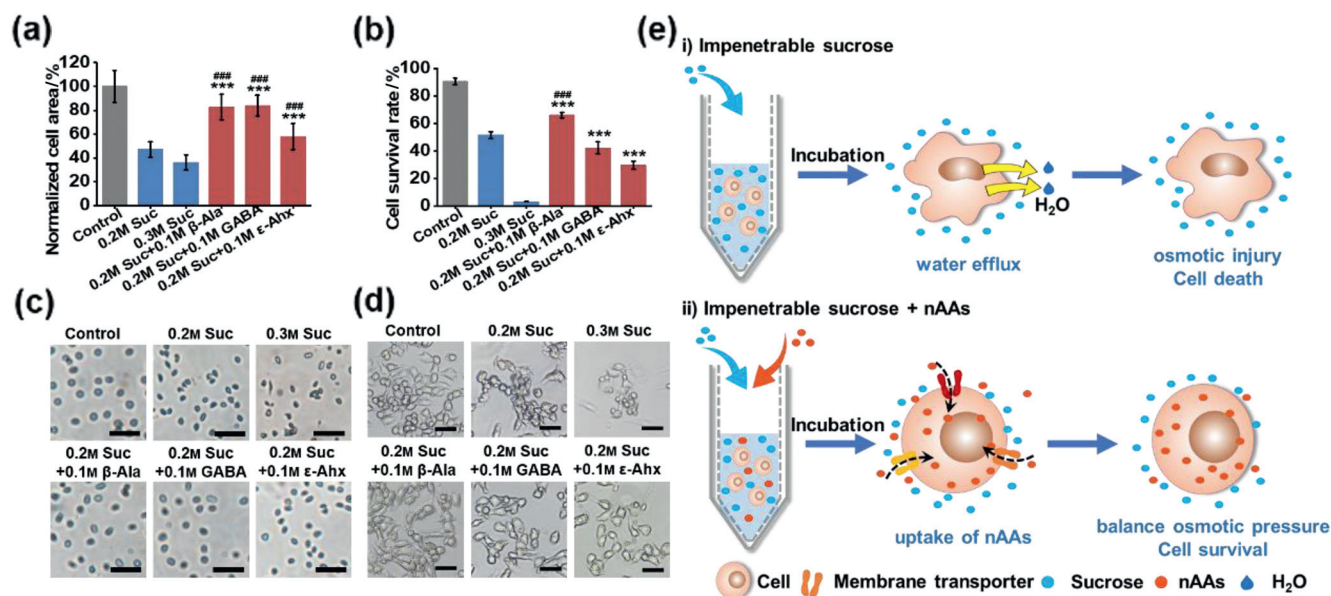


Fig. 3. Osmotic regulation tests. (a) Normalized cell area and (c) morphology of RBCs after exposing to PBS (control), or containing $0.2 \text{ mol} \cdot \text{L}^{-1}$ sucrose (Suc), $0.3 \text{ mol} \cdot \text{L}^{-1}$ Suc, $0.2 \text{ mol} \cdot \text{L}^{-1}$ Suc + $0.1 \text{ mol} \cdot \text{L}^{-1}$ β-Ala, $0.2 \text{ mol} \cdot \text{L}^{-1}$ Suc + $0.1 \text{ mol} \cdot \text{L}^{-1}$ GABA, $0.2 \text{ mol} \cdot \text{L}^{-1}$ Suc + $0.1 \text{ mol} \cdot \text{L}^{-1}$ ε-Ahx, respectively, for 24 h. The RBC area in control groups was normalized as 100%. Scale bar = $20 \mu\text{m}$. (b) The survival rate and (d) morphology of Hela cells attached onto substrates after exposing to culture medium (control), or containing $0.2 \text{ mol} \cdot \text{L}^{-1}$ sucrose (Suc), $0.3 \text{ mol} \cdot \text{L}^{-1}$ Suc, $0.2 \text{ mol} \cdot \text{L}^{-1}$ Suc + $0.1 \text{ mol} \cdot \text{L}^{-1}$ β-Ala, $0.2 \text{ mol} \cdot \text{L}^{-1}$ Suc + $0.1 \text{ mol} \cdot \text{L}^{-1}$ GABA, $0.2 \text{ mol} \cdot \text{L}^{-1}$ Suc + $0.1 \text{ mol} \cdot \text{L}^{-1}$ ε-Ahx, respectively. Scale bar = $50 \mu\text{m}$. (e) Scheme drawing of uptake of sucrose and nAAs by cells. nAAs denoted the neural amino acids, including β-alanine (β-Ala), γ-aminobutyric acid (GABA) and ε-aminocaproic acid (ε-Ahx). Values were means $\pm$ the standard deviation ($n = 3$). Number signs indicated comparisons with $0.2 \text{ mol} \cdot \text{L}^{-1}$ Suc. Asterisks indicated comparisons with $0.3 \text{ mol} \cdot \text{L}^{-1}$ Suc, $***p < 0.001$. $***p < 0.001$ (M: $\text{mol} \cdot \text{L}^{-1}$).

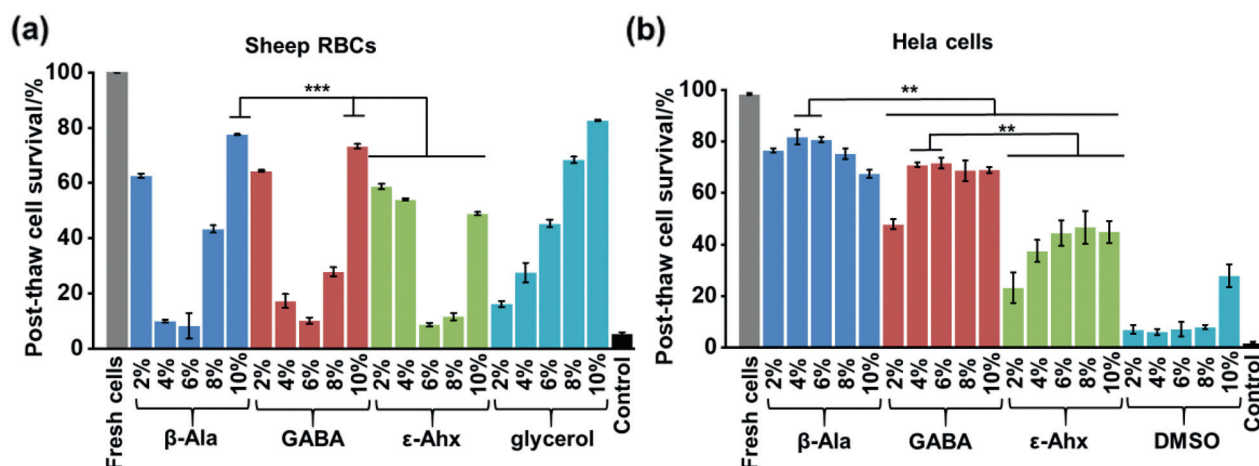


Fig. 4. Cell cryopreservation tests. Post-thaw cell survival of (a) sheep RBCs and (b) Hela cells cryopreserved with β-alanine (β-Ala), γ-aminobutyric acid (GABA), ε-aminocaproic acid (ε-Ahx), glycerol or DMSO, respectively, at varied concentrations. Cells cryopreserved without cryoprotectants were control samples. Values were means $\pm$ the standard deviation ($n = 3$). $***p < 0.001$. $**p < 0.01$.

concentrations could achieve to 81%, 71% and 46%, respectively. Whilst, only 1% and 27% of post-thaw cell survival could be found in control and DMSO groups respectively. Commonly, DMSO requires a stepwise slow-freezing protocol with a freezing rate of $\sim 1 \text{ }^{\circ}\text{C} \cdot \text{min}^{-1}$, so that it can penetrate cell membrane with a sufficient amount during this interval to provide intracellular protection [10]. According to a previous report, under this optimal slow-freezing condition, DMSO could achieve $\sim 92\%$ cryopreservation efficiency to Hela cells [55]. Herein, with the rapid freezing protocol (freezing rate was $\sim 180 \text{ }^{\circ}\text{C} \cdot \text{min}^{-1}$) [28], DMSO could not provide sufficient intracellular protection due to the lack of time to enter cells (few minutes) and therefore showed inferior cryoprotection efficacy. While nAAs (especially β-Ala and GABA) performed well in cryopreservation of Hela cells with a rapid freezing protocol, suggesting they could promptly enter cells to offer intracellular

protection. Besides, nAAs with lower number of methylene groups showed higher cryoprotection efficacy (β-Ala > GABA > ε-Ahx), possibly because of the stronger ability to enter cells for intracellular protection [7,56].

To further validate the versatility of nAA-based CPA solutions, nucleated GLC-82 cells and SMCs were cryopreserved and also exhibited effective cryopreservation performance (Figs. S1 and S2).

3.4. One-step removal of CPAs from cryopreserved cells

Prior to clinical use of cryopreserved cells, a CPA removal procedure is required to avoid any side effects to human body. One-step method, i.e. directly adding isotonic solutions to thawing cell suspensions followed by a centrifugation procedure, is a simple and fast way for

CPA removal [57]. However, this method cannot be used for the removal of two state-of-the-art CPAs—DMSO and glycerol. Because the intracellular DMSO and glycerol diffuse out of cells slowly, the high osmolality changes at the dilution point during the one-step washing procedure induce significant osmotic shock to cells [58,59]. Currently, a multistep method, *i.e.* adding a series of washes with progressively decreasing saline concentrations into thawing cell suspension, is used for DMSO and glycerol removal. This time-consuming and multistep washing procedure has become the bottleneck problem of DMSO and glycerol in clinical cryopreservation technology [16,45,60].

Herein, considering the multiple membrane transporters of nAAs, the intracellular nAAs might have the potential to be rapidly removed from cells to mitigate osmotic changes during the one-step washing process. To verify this, the osmotically sensitive RBCs were exposed to PBS solutions containing 2%–10% β -Ala, GABA and ϵ -Ahx, respectively, for 24 h. Then, RBC survival was measured after removal of nAAs *via* the one-step method. RBCs that were exposed to 2%–10% DMSO or glycerol were used as control groups. As shown in Fig. S3a–d, severe RBC hemolysis occurred during one-step removal of DMSO and glycerol even at a concentration of 2%, consistent with previous reports [41,60,61]. While the same procedure did not cause observable RBC hemolysis during the removal of 2%–10% nAAs. It was because intracellular DMSO or glycerol diffused out of cells slowly, RBCs suffered dramatic osmotic changes, which caused large amount of water influx and excessive cell volume excursions (Fig. S3e) [58,59]. Meanwhile, a greater extent of RBC hemolysis was found in glycerol group than in DMSO group, possibly because of the lower membrane permeability of glycerol [62]. By stark contrast, the nAAs could be rapidly released by cells *via* multiple membrane transporters to counteract osmotic changes, thus avoiding the extreme volume changes and lysis of RBCs (Fig. S3e) [63,64].

With this information to hand, RBCs or Hela cells were cryopreserved using nAAs at the optimal concentration, and their cryopreservation efficiency after the one-step washing procedure was investigated. As shown in Fig. 5a, after thawing, the immediate one-step washing procedure did not cause observable decrement on RBC survival in nAA groups. By stark contrast, despite the high post-thaw cryoprotection efficacy of glycerol, severe hemolysis (more than 24%) occurred after washing, resulting in significantly lower post-thaw-wash RBC survival than that of in β -Ala, GABA groups. A similar tendency could also be observed for Hela cells. As shown in Fig. 5b, no significant decrease of cell

survival after washing could be observed in nAA groups. While a 23% decrease was found in the DMSO group. These results demonstrated that β -Ala, GABA and ϵ -Ahx could be rapidly removed from cryopreserved cells and reduce the osmotic stress to cells during washing process, which was important to provide cells for timely lifesaving treatments.

3.5. Functionalities of post-thaw-washed cells

Normal functionality of cryopreserved cells is a key factor to successful therapeutic outcome for cell-based medical treatments. Therefore, we next investigated the functionality of post-thaw-washed cells.

The anuclear RBCs could not attach onto substrate or proliferate, we therefore observed their morphology for the functionality test. As shown in Fig. 6a and c, the post-thaw-washed RBCs in nAA groups could maintain their biconcave shape as that of fresh RBCs. For nucleated Hela cells, the cryoinjuries caused during cryopreservation process may disrupt cellular enzyme or mitochondrial functions, thus further affecting the adherence and proliferation abilities of cells. As shown in Fig. 6b, compared with fresh cells, the recovered Hela cells could adhere onto substrates with normal morphology. Meanwhile, they also exhibited similar proliferation ability (Fig. 6d). Therefore, β -Ala, GABA and ϵ -Ahx could protect the functionalities of cells during cryopreservation process.

3.6. Cytotoxicity tests

Biocompatibility is one of the criteria of ideal CPAs to ensure clinical therapeutic safety and avoid adverse effects to patients of post-cryopreserved cells [12]. Therefore, cytotoxicity evaluation of novel CPAs is of great significance.

It has been reported that β -Ala can be used as a dietary supplement to enhance the performance of soldiers and athletes [65,66]. GABA is also reported as a food supplement to alleviate anxiety and improve sleep quality [67]. Meanwhile, ϵ -Ahx has been approved by FDA for acute bleeding treatment. To validate their biocompatibility, RBCs or Hela cells were exposed to PBS solutions or cell culture mediums containing 2% β -Ala, GABA or ϵ -Ahx. As shown in Fig. 7a–b, no significant hemolysis of RBCs could be observed in nAA groups. Meanwhile, Hela cells in nAA groups also exhibited similar survival ability as well as

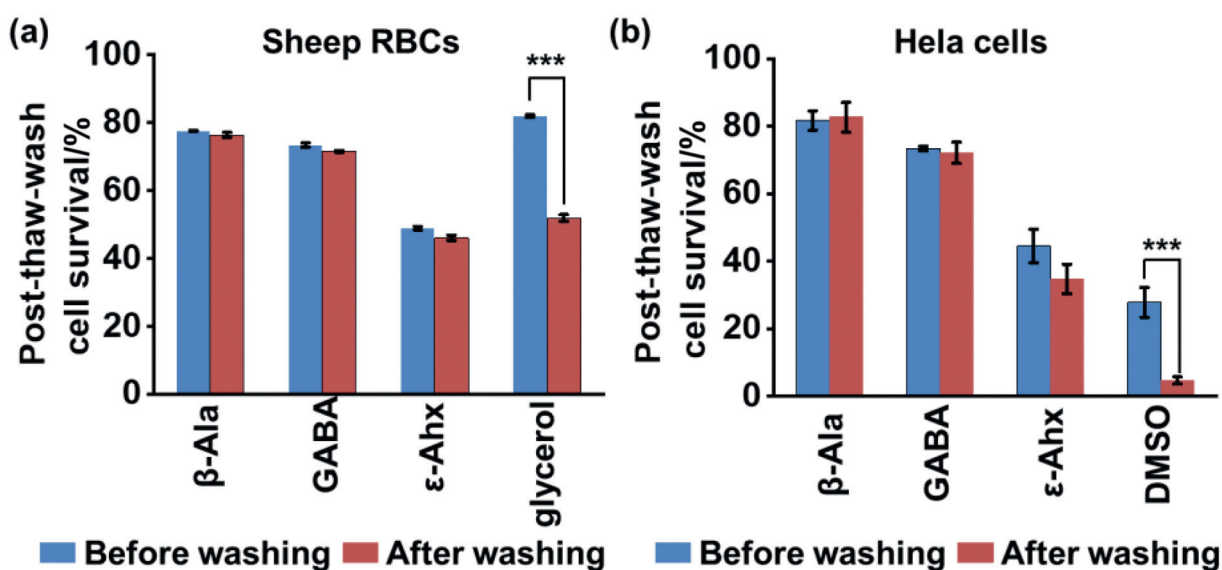


Fig. 5. Removal of cryoprotectants from cryopreserved cells *via* a one-step method. (a) Post-thaw-wash survival of sheep RBCs cryopreserved using β -alanine (β -Ala), γ -aminobutyric acid (GABA), ϵ -aminocaproic acid (ϵ -Ahx) or glycerol at the optimal concentrations. (b) Post-thaw-wash survival of Hela cells cryopreserved using β -alanine (β -Ala), γ -aminobutyric acid (GABA), ϵ -aminocaproic acid (ϵ -Ahx) or DMSO at the optimal concentrations. Values were means $\pm$ the standard deviation ($n = 3$). *** $p < 0.001$.

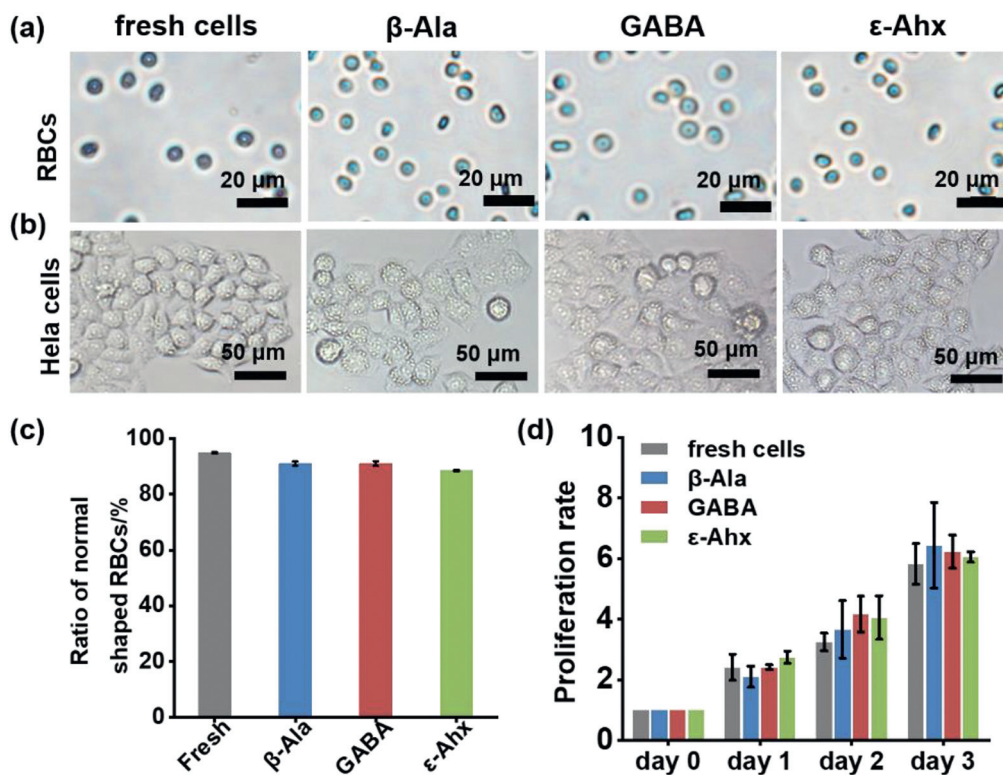


Fig. 6. Functionalities of recovered cells. Morphology of recovered (a) RBCs or (b) Hela cells. (c) Ratio of normal shaped RBCs cryopreserved with β -alanine (β -Ala), γ -aminobutyric acid (GABA) or ϵ -aminocaproic acid (ϵ -Ahx), respectively, at the optimal concentrations. (d) Proliferation rate of recovered Hela cells cryopreserved with β -alanine (β -Ala), γ -aminobutyric acid (GABA) or ϵ -aminocaproic acid (ϵ -Ahx), respectively, at the optimal concentrations.

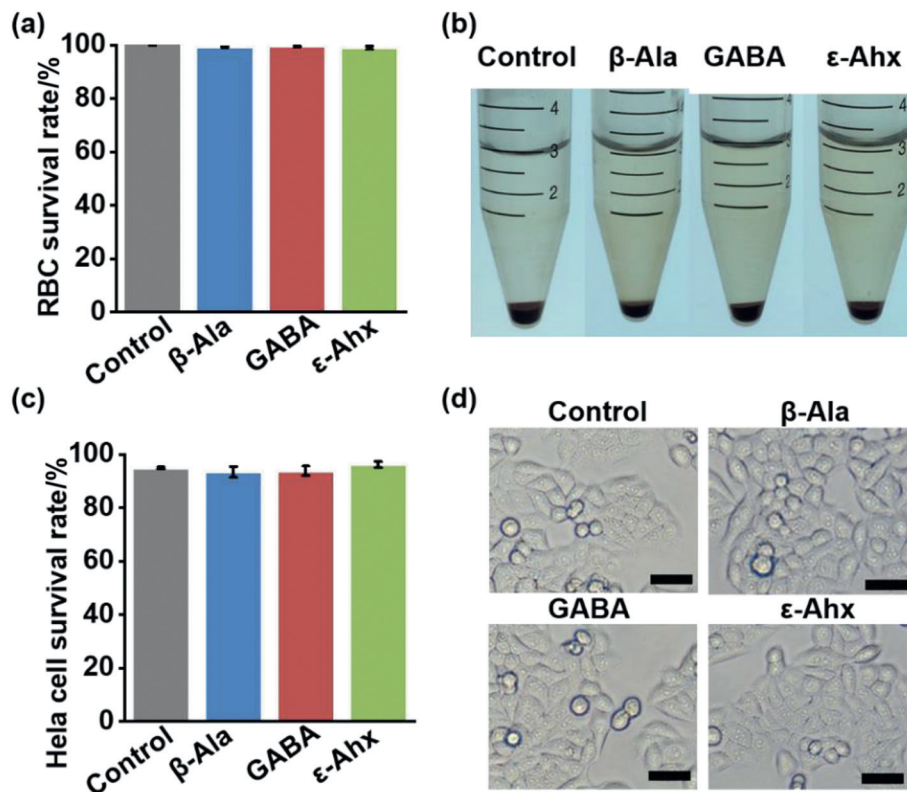


Fig. 7. Cytotoxicity tests. (a) The survival rate and (b) images of hemolysis of RBCs after exposing to PBS solutions with 2% β -alanine (β -Ala), γ -aminobutyric acid (GABA) or ϵ -aminocaproic acid (ϵ -Ahx) for 24 h. PBS solution was used as control. (c) Cell survival rate and (d) attachment morphology of Hela cells after exposing to culture mediums with 2% β -alanine (β -Ala), γ -aminobutyric acid (GABA) or ϵ -aminocaproic acid (ϵ -Ahx) for 24 h. Culture medium was used as control. Scale bar = 100 μ m.

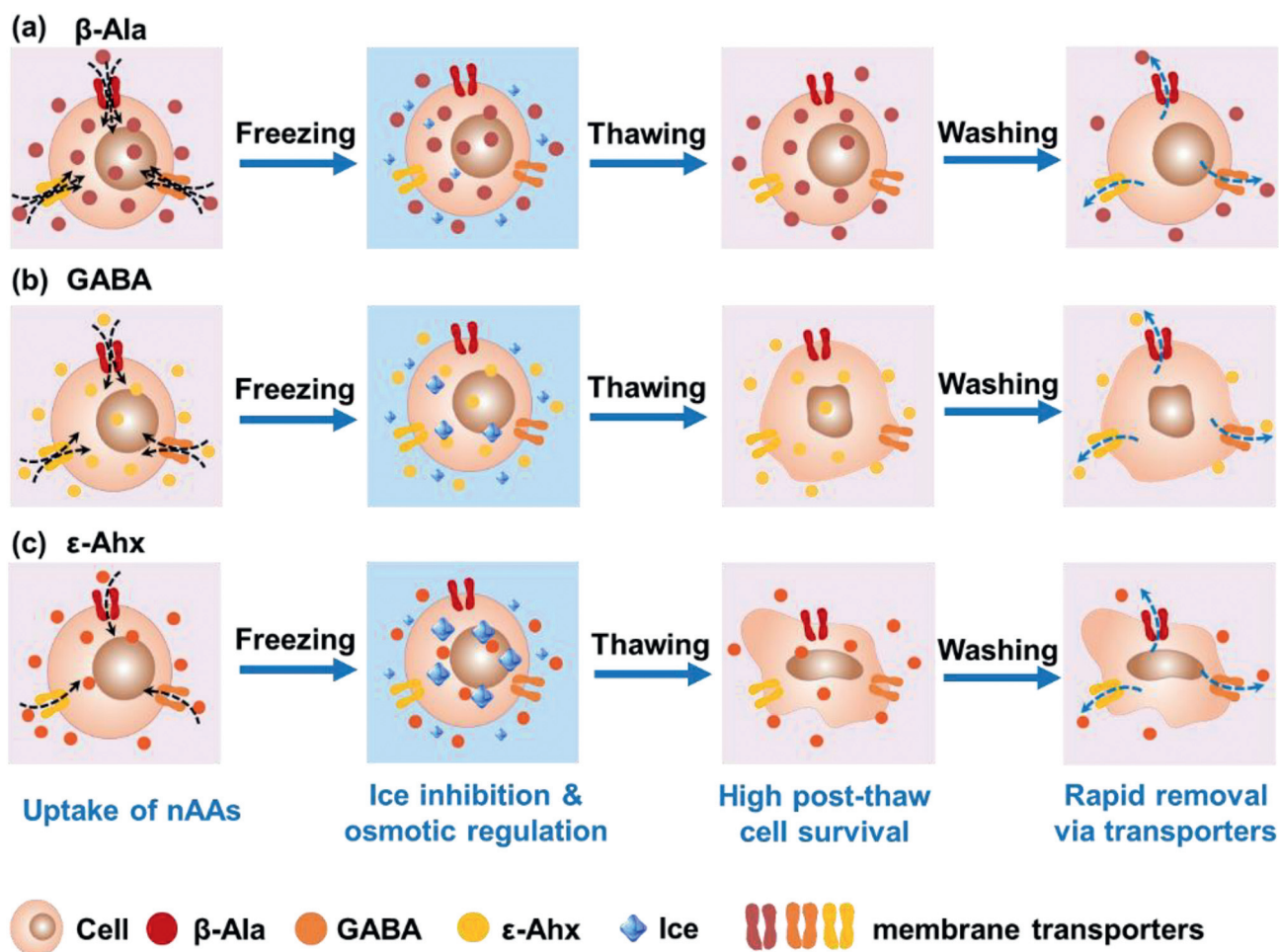


Fig. 8. A proposed mechanism of the roles of (a) β -alanine (β -Ala), (b) γ -aminobutyric acid (GABA) and (c) ϵ -aminocaproic acid (ϵ -Ahx) in cryopreservation.

normal morphology as that of in control groups (Fig. 7c-d). These results indicated the good biocompatibility of β -Ala, GABA and ϵ -Ahx.

3.7. Proposed mechanisms

On the basis of these above results, we proposed a mechanism to illustrate the roles of β -Ala, GABA and ϵ -Ahx in cryopreservation process, as shown in Fig. 8.

Firstly, as demonstrated by the osmotic regulation tests (Fig. 3), when cells were exposed to hypertonic nAA-based CPA solutions, β -Ala, GABA and ϵ -Ahx could be taken up by cells via multiple membrane transporters (e.g. system A, system L and system N) under osmotic stress [38–40]. Meanwhile, β -Ala and GABA showed stronger uptake ability by cells than ϵ -Ahx. Secondly, according to the DSC tests (Fig. 2) and osmotic regulation tests (Fig. 3), the intra- and extracellular nAAs could inhibit ice formation and mitigate osmotic stress, thus reducing the associated cryoinjuries to cells during the rapid freezing procedure. Thirdly, cells could be preserved at the ultralow temperature (-196°C) and recovered at 37°C with high post-thaw survival rate, which were proved by the cryopreservation tests (Fig. 4). Because intracellular ice formation and growth dominated the major cryoinjury with the rapid-freezing protocol [7,56], β -Ala and GABA that possessed higher uptake ability by cells could provide stronger intracellular protection and ensure higher cryopreservation efficiency than ϵ -Ahx. Lastly, from the one-step removal tests (Fig. 5), when post-thaw cells were washed by a simple and fast one-step method, the intracellular nAAs could be rapidly removed via membrane transporters [35–40],

which was important to provide therapeutic cells in urgent cases for lifesaving treatment.

4. Conclusions

In summary, three nAAs (β -Ala, GABA and ϵ -Ahx) were first reported as novel CPAs, which could integrate properties of biocompatibility, high cryoprotection efficacy and rapid removal from cryopreserved cells. The high cryoprotection efficacy of nAAs for both anuclear and nucleated cells was attributed to their abilities to reduce cryoinjuries by inhibiting ice formation and regulating osmotic pressure. Meanwhile, β -Ala, GABA showed higher cryoprotection efficacy than ϵ -Ahx because of their higher uptake ability by cells for stronger intracellular protection. More importantly, the rapid removal of nAAs from cryopreserved cells via a one-step washing method was of great significance to provide therapeutic cells for lifesaving treatments in urgent cases. This work offered a new perspective to develop ideal CPAs, and might have dramatic impacts on solvent-free cryopreservation technology to support cell-based applications, such as cell therapy and tissue engineering, etc.

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Supplementary Material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cjche.2020.07.009>.

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