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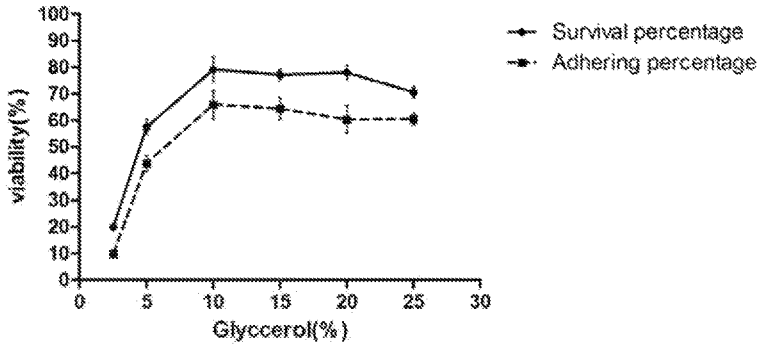

FIG. 2

(57) **Abstract:** Cell preservation and preparative medium and methods for using the medium are provided. The medium, as well as the methods, enables flexible (i.e., rapid or slow) freezing and thawing of cells while maintaining high survival rate, protecting cell viability and multipotent properties. The medium is effective in protect and ameliorate against freezing and thawing injury, in the absence of common ingredients of DMSO, proteins such as albumin, and serum.

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CELL PRESERVATION AND PREPARATIVE MEDIUM AND METHOD FOR USING THE SAME

TECHNICAL FIELD

This invention relates to the field of cell preservation medium and cell preservation method. In particular, the invention relates a cell preservation and preparative medium comprising poloxamerthat is highly protective and may be free of protein/serum and DMSO, and methods for efficiently protecting cell survival through freezing and thawing using the same medium.

BACKGROUND

The rapid progress and wide application of cell therapy have put focus on the need for steady supply of cells, in particular, the need to cultivate and supply constant qualities of cells. Given the limited cell division capacity, propensity for mutation, and finicky reequipment for cell culture, cell freezing is simply an indispensable aspect in securing constant supply of cells. Humaninduced pluripotent stem cells/embryonic stem cells (iPS/ES cells) are low in survival rate after cryopreservation, which poses a problem. Cell therapy, gene therapy, and tissue regeneration all requires technologies of cryopreservation of cells, especiallyiPS/ES cells.

Therapeutic cells, such as mesenchymal stem cell (MSC), induced pluripotent stem cells (iPS), hemoiestatic stem cell (HSC), lymphocytes, natural killer cell (NK), dendrite cell (DC), neural stem cell (NSC), and Retina precursor (RP) cells, have great potentials for clinical application, in which HSC and MSC and lymphocytes have already been widely used. MSC are a group of cells with immunomodulatory, multipotent and fast proliferative ability and widely used for immune-modulatory therapy, bone and cartilage regeneration, myocardium regeneration. The NSC are potentially used for treating a number of neurological disorder such as Parkinson's disease and Huntington's disease and anti-aging therapy. Since HSC possess the ability to self-renew and differentiate into all types of blood cells, human bone marrow enriching HSCs have been used to treat blood and immune disorders many decades ago, and transplantation of HSC becomes a routine therapeutic method of modern medicine. Therapeutic lymphocytes such as lymphokine-activated killer (LAK), chimeric antigen receptor T lymphocyte (CAR-T) and tumor infiltrating lymphocytes (TIL) have been used for tumor therapy. Human embryonic stem cells are potentially used for diabetes, Parkinson's diseases and many other diseases. RP cells have been used for treatment of human retina diseases.

Because therapeutic cells are living material, its clinical application face several critical difficulties including effective cryopreservation, convenient delivery and safety. For an effective cryopreservation, a proper cryopreservation protectants (CPA) is required.

Conventional cryopreservation protectants include DMSO, serum, glycol and its derivatives, polysacchrides, and polymers.

Among these cryopreservation protectants, DMSO is widely used for the preservation of therapeutic cells as a standard ingredient. However, several lines of evidence suggest that DMSO is toxic to cells and may induce cell differentiation. Reducing or replacing DMSO would have been beneficial.

Polymers are used for entrapping the protectants within a capsule during cell resuspension so as to decrease the size of formed ice crystals and avoid the diffusion limitation. Such polymers include synthetic nonpenetrating polymers and vinyl-derived polymers. Protein and serum are also widely used as cryoprotectants. For example, Sericin, a water-soluble sticky protein isolated from silkworm cocoon, may replace fetal bovine serumor DMSO in culturing human adipose tissue-derived stem cells or hepatocytes. Due to possible contamination from animal serum or protein and batch to batch variations of serum/protein products, replacing serum and protein would be desired for clinic application for therapeutic cells.

The presence of DMSO, serum and proteins as cryoprotectants also interferes with the next step of using the cells. Current method for delivering therapeutic cells is thawing the frozen cells followed by wash out the cryopreservation solution prior to intravenous administration. This creates additional step(s) that reduces cell vitality, promotes cell aggregation, and introduces contamination. Although direct injection of the thawed cell mixture containing DMSO and human AB serum or human albuminhas beendone, the safety of this procedure has been questioned. It would have been desirable to eliminate the step of wash out the cryopreservation solution.

Cells are damaged during freezing and thawing by the presence of water crystalline, thus methods for preventing or ameliorating the damages have been directedtowards reducing or minimizingcrystallization. Different approaches have been used. For example, one method for cryopreservation of human iPS/ES cells is to use a cryofreezing solution with high centration solute (2 M of DMSO, 1 M of acetamide and 3M of propylene glycol) and rapid freezing (10 second, in liquid nitrogen), both factors contributing to the vitrification of water. Vitrification is the transformation of a substance into a glass, that is to say a non-crystalline amorphous solid. Thawing has to be performed quickly by using warm media to wash out the cryofreezing solution, since the high centration solute are toxic to cells.

Paradoxically, some methods attempt to reduce crystalline by slow-freezing. For example, Ha SY and others reported a slow-freezing method for human iPS/ES cells in which human ES cells (SNUhES-3 strains) were subjected to cell dissociation by using a 0.25% (w/v) trypsin, 0.53 mM EDTA solution and, thereafter a cryopreservation (5% DMSO, 50% FBS (fetal bovine serum), 10% EG, and 5% DMEM/F12) was slow-frozen and rapidly thawed. None of these methods are high survival rate (<50%). The requirement for rapid or slow freezing/thawing creating extra burden in the application of

cells. The presence of the solute such as DMSO also prohibits direct injection of cell mixture.

Therefore, there remains a need for a cell preservation and preparative medium, and a method for using the cell preservation and preparative medium, to achieve high cell survival rate, flexible freezing and thawing tempo, and even direct injection of the thawed cell mixture into a subject.

SUMMARY

Problems to be Solved by the Invention

As described so far, the present cell preservation and preparative media, especially those used for stem cells, have low survival rate, troublesome and exacting operating procedures, and burdensome follow-up steps for cleanup.

In view of the above-described situation, an object of the present invention is to provide a preservation medium of cells, especially stem cells, and a preservation method therefor, which can be used as a flexibly-timed method, high in cell survival rate, simple in operation and fully compatible with direct intravenous injection.

Means to Solve the Objects

A protein and DMSO-free medium (PDF medium) is disclosed that comprises poloxamer. Unexpectedly, inventors found and optimized apoloxamer-contain solution that is able to achieve excellent protections for cells during freezing and thawing cycle or during routine cell culture and preparation, especially for various stem cells, even in the absence of DMSO and protein/serum. The discovery is significant not only because the protective effects, as measured by cell survival rate and adhesion rate, are high compared to existing methods, but also because DMSO and protein/serum may induce stem cells differentiation and interfere with cell functions. In addition, cells processed with the protein and DMSO-free medium (PDF medium) can be injected directly into a subject, obviating the step of separating DMSO and protein within traditional media.

In at least one embodiment, a cell preservation and preparative medium for that has protective effect for the cells is provided. The medium comprisesa poloxamer and a simple polyol,the poloxamer is present in a concentration range of 0.5% to 10% (v/v); and the simple polyol is present in a concentration range of 5% to 25% (v/v).Theprotective effect includes a high survival rate (>50%, >60%, or >70%) for cells undergo freezing and thawing cycle.

In a preferred embodiment, the poloxamer concentration may be 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%or 15% (v/v). In a preferred embodiment, the simple polyol concentration may be in a range of 5-10%, 10-15%, 15-20%, and 20-25% (v/v).

In at least one embodiment, the poloxamer is F68/poloxamer 188.

In at least one embodiment, the simple polyol is selected from the group of glycerol and propylene glycol.

In at least one embodiment, the cell preservation and preparative mediumfurther comprises a buffer.

In at least one embodiment, the buffer is selected from PBS, HEPES, Tris.HCl, Multiple Electrolytes Injection (MEI), and normal solution (0.9% NaCl).

In at least one embodiment, the cell preservation and preparative medium described above is free of dimethyl sulfoxide (DMSO), serum, a substitute component of serum, a protein component, and an animal-derived component. DMSO is toxic to cells and can act to induce cell differentiation. Animal-derived components introduces additional risk and variations when delivered into a subject. By eliminating DMSO, serum, substitute component of serum, protein component, and animal-derived component, this cell preservation and preparative medium are made from components wildly and safely used clinically for injection into animals and humans, thus the cells/medium mixture can be injected into animals/humans directly, obviating the need to separating the medium from the cells prior to injecting the cells.

In at least one embodiment, additional additives can be included in the cell preservation and preparative medium. These additional additivesmay include glucose, sucrose, trehalose, heparin, and ATP rare minerals, growth factors, peptides, and other components that promotes cell viability, stability, functionality, and consistency. Other types of additives are possible.

In at least one embodiment,a cell preservation and preparative medium is disclosed, wherein the cell preservation and preparative medium is used for storing or preparing embryonic stem cells, hematopoietic stem cells, tissue stem cells, induced pluripotent stem cells, mesenchymal stem cells, retina precursor cells, cancer cells, primary cell lines, immortalized cell lines, sperms, eggs, pancreatic islets, and tissues.

In at least one embodiment,a cell preservation and preparative medium is disclosedthat can be used for storing or preparing cells in gene therapy, cell therapy, drug preparation of therapeutic cells, cell cryopreservation, and tissue cryopreservation.

In at least one embodiment,the cell preservation and preparative medium is effective in preventing cell injury during freezing and thawing, wherein a cell survival rate is at 50% higher, irrespective of the speed of freezing and thawing.

In at least one embodiment,acell preservation and preparative method for freezing and rapidly thawing cells is disclosed, including: a suspension step for suspending cells in a cell preservation and preparative medium, wherein the medium comprises a poloxamer at a concentration of 0.5% to 15% (v/v) and a simple polyol at a concentration of 5% to 25% (v/v);a freezing step for freezing the resuspended cells; anda thawing step to thaw the frozen cells after the freezing step without significant loss of cell number.

In at least one embodiment, thedisclosedcell preservation and preparative method further includes that, wherein the freezing step can be performed either rapidly or slowly to allow a flexible timeframe without significant reduction of cell survival rate; wherein the thawing step can be performed slowly or rapidly by adding a warm culture medium, by incubating in a water bath, or by coming into contact with a warmer object.

In at least one embodiment, the poloxamerused in the method is F68/poloxamer 188.

In at least one embodiment, the simple polyol is selected from the group of glycerol and propylene glycol.

In at least one embodiment, the cell preservation and preparative medium comprises a buffer.

In at least one embodiment, the buffer used in thecell preservation and preparative methodis selected from PBS, HEPES, Tris.HCl, Multiple Electrolytes Injection (MEI), and normal solution (0.9% NaCl).

In at least one embodiment,the cell preservation and preparative medium used in the method is free of any one of: dimethyl sulfoxide (DMSO), serum, a substitute component of serum, a protein component, and an animal-derived component.

In at least one embodiment, the cell preservation and preparative method disclosed may be applied to freeze cells in a range of 1to 1×10^7 per mL of the preservation and preparative medium.

In at least one embodiment, when using the preservation and preparative method, the cell preservation and preparative medium further comprises at least one of glucose, sucrose, trehalose, heparin, and ATP.

In at least one embodiment, a cell preservation and preparative method is disclosed that is effective in preventing freezing and thawing injury to cells and eliminates the need for washing cells. The method comprises a suspension step for suspending cells in a cell preservation and preparative medium, wherein the stem cell preservation medium comprises a poloxamer, a simple polyol, and a buffer; and injection step to inject the cells into a subject without separating the cell preservation and preparative medium from the cells.

In at least one embodiment,thecell preservation and preparative method disclosed may have the freezing step performed either rapidly or slowly, allowing a flexible timeframe without significant reduction of cell survival rate. By slowly, the thawing time may be 30 seconds, 1 minutes, 10 minutes, 30 minutes, 1 hours, 2 hours, 4 hours, 8 hours, 24 hours, 48 hours, and any time in between. The slow tempo may be achieved through an equipment or device.

In at least one embodiment,the cell preservation and preparative method disclosed above may have apoloxamer 188 concentrationin the range of 0.5% to 10% (v/v). In a preferred embodiment, the poloxamer 188 concentration may be 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%,10% or 15%.

In at least one embodiment, the cell preservation and preparative method disclosed above contains glycerol or propylene glycol in a concentration range 5-25% (v/v). In a preferred embodiment, the glycerol or propylene glycol concentration may be in a range of 5-10%, 10-15%, 15-20%, and 20-25% (v/v).

Still other embodiments of the present invention will become readily apparent to those skilled in the art from the following detailed description, wherein is described embodiments of the invention by way of illustrating the best mode contemplated for carrying out the invention. As will be realized, the invention is capable of other and different embodiments,

and its several details are capable of modifications in various obvious respects, all without departing from the spirit and the scope of the present invention. Accordingly, the drawings and detailed description are to be regarded as illustrative in nature and not as restrictive. It is to be noted that various changes and modifications practiced or adopted by those skilled in the art without creative work are to be understood as being included within the scope of the present invention as defined by the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig.1 is a graph showing the protective effect and the optimization of F68 on cryopreservation of MSC. The MSC cells were mixed with PDF basal medium containing different concentration of F68 and then subjected to cryopreservation and evaluation of viability of the thawed cells. MSCs were cryopreserved for one week at liquid nitrogen. Survived cells were determined by trypan blue, and the percentage of adhering cells were calculated 24 hours after initial expansion in culture media.

Fig.2 is graph showing the protective effect and the optimization of glycerol on cryopreservation of MSC withinthe PDF medium. MSC were cryopreserved in PDF basal medium containing 5% F68, PBS and different concentrations of glycerol at liquid nitrogen for one week. Viabilities were measured by trypan blue, and the percentage of adhering cells were calculated within 24 hours following initial expansion in culture media.

Fig.3 is graph showing the protective effect and the optimization of propylene glycol on cryopreservation of MSC within the PDF medium. MSC were cryopreserved in PDF basal medium containing 5% F68, PBS, and one of: 1) glycerol, 2) propylene glycol, and 3) DMSO at liquid nitrogen for one week. Viabilities were measured by trypan blue, and the percentage of adhering cells were calculated within 24 hours following initial expansion in culture media.

Fig.4 is a graph showing the protective effect and the optimization of buffers on cryopreservation of MSC within the PDF medium. MSCs were cryopreserved with 10% glycerol/5% F68 mixture with different buffers including PBS (pH7.0-7.4), HEPES (pH7.0-7.4), MEI,Tris.HCl (pH7.0-7.4) and normal solution (0.9% NaCl). MSCs were cryopreserved for one week at liquid nitrogen. Survival was measured by trypan blue, and the percentage of adhering cells were calculated following initial expansion in culture media.

Fig.5 shows the uninterrupted differentiation of PDF-cryopreserved MSCs into osteocytes, adipocytes and chondrocytes. The undifferentiated cells (low panels) and differentiated cells were respectively stained with Alizarin Red S (a), Oil Red O(b) and Alcian Blue (c).

Fig.6 is a graph showing unaltered expansion of PDF-cryopreserved lymphocytes after short-term cryopreservation. The fresh lymphocytes and PDF-preserved lymphocytes were cultured at X-VIVO15 containing 5% FCS, 2000iu/ml INF-γ and 2000iu/ml IL 2. The cells were counted every other day, and fresh medium was added to adjust the cell density to $\sim 1\times10^{6}$/mL.

Fig.7 shows normal clone growth of mouse ES cells after cryopreservation. The cloning formation efficiency of ES cells were statistically equal between cells frozen with PDF media(a) and frozen in the presence of DMSO

Fig.8 shows normal electrocardiogram and pulse rate in mice after injection with reasonable amount of PDF-MSCs, in comparison with PBS.

Fig.9 shows comparisons of electrocardiograms of dogs after and before injection of PDF-MSC, revealing no significant change of wave patterns as well as pulse rate. To assess the toxicity of the MSCs prepared by PDF medium, adult beagles were treated for four times at roughly two-week intervals with PDF-MSC injections.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Embodiments of the present invention will be described in detail below with reference to the drawings. The present invention is not limited to the following embodiment and examples of shown in the figure, and the present invention can be variously changed in design.

Poloxamers ($HO{\cdot}(C_2H_4O)m{\cdot}(C_3H_6O)n{\cdot}H$) are nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)). Poloxamers are also known by the trade names Synperonics, Pluronics, and Kolliphor.

Because the lengths of the polymer blocks can be customized, many different poloxamers exist that have slightly different properties. For the generic term "poloxamer", these copolymers are commonly named with the letter "P" (for poloxamer) followed by three digits: the first two digits multiplied by 100 give the approximate molecular mass of the polyoxypropylene core, and the last digit multiplied by 10 gives the percentage polyoxyethylene content (e.g. P188 = poloxamer with a polyoxypropylene molecular mass of 1,800 g/mol and an80% polyoxyethylene content). For the Pluronic and Synperonictradenames, coding of these copolymers starts with a letter to define its physical form at room temperature (L = liquid, P = paste, F = flake (solid)) followed by two or three digits, The first digit (two digits in a three-digit number) in the numerical designation, multiplied by 300, indicates the approximate molecular weight of the hydrophobe; and the last digit x 10 gives the percentage polyoxyethylene content (e.g., F68 indicates a polyoxypropylene molecular mass of 1,800 g/mol and an80% polyoxyethylene content). In the example given, poloxamer 188 (P188) are equivalent toPluronicF68. Pluronic F68 is abbreviated to F68.

The term “polyol” refers to a compound comprising two or more hydroxyl groups, and by convention, polyols do not refer to compounds that contain other functional groups. Simple polyol refers to a polyol with less than seven carbons.

Protective effect for a cell preservation and preparative medium refers to, after undergoing one free-thaw cycle, 1) the cell survival rate is above 30%, or preferably above 40%, or more preferably above 50%, or even more preferably above 60%, or even more preferably above 80%; or 2) the cell adhesion rate is above 30%, or preferably above 40%,

or more preferably above 50%, or even more preferably above 60%, or even more preferably above 80%; or 3) both.

Embodiment 1

Design and screening of a ready-to-use protein and DMSO-free medium.

A protein/DMSO-free medium (PDFmedium) offers many advantages, and was contemplated, designed, and screened. A large array of medicinally or medically tested chemicals/reagents that can be administrated by injection were screened for their cryopreservation activity. A group of chemicals/reagents were found to demonstrate a detectable level of protective effect on cryopreservation. These chemicals/reagents include F68 and its derivatives, PVP, Dextran, Glycerol, PEG, propylene glycol, and carbohydrates, among others.

After undergoing screenings on a substantial combination of these chemicals/reagents, glycerol, F68 and carbohydrates were shown to possess the greatest protective effect for cell cryopreservation. Glycerol and F68 were selected for further testing. Glycerol, F68, and PBS buffer were used to form a basal medium (PDF-basal). PDF basal medium is prepared by 0.5-10% F68 synthetic materials and 2.5- 25% glycerol and PBS buffer. A broad PDF-basal medium is one that uses derivatives/substitutes of the Glycerol, F68, and PBS buffer. Table 1 describes the components of PDF basal medium. The complete PDF is designed by adding nutrient additives into the PDF basal medium.

Table 1. Components of PDF basal medium.

Components	Derivatives	Concentrations
F68	Poloxamers with different molecular weights	0.5-10%
Glycerol	PEG, propylene glycol	2.5-25%
PBS buffer	HEPES, Tris.HCl, Normal solution, Multiple Electrolytes Injection	0.1-200mM

Embodiment 2

PDF Basel Medium protects mesenchymal cells during freezing and thawing.

For clinical application, MSCs are usually expanded in a large scale and then cryopreserved under -80°C or liquid nitrogen. To obtain enough fresh mesenchymal stem cells, we collected umbilical cord from hospital with a permission. The umbilical cords were monitored without infectious pathogens and subjected to consequent experiments within 2 hours. The blood vessels and miscellulous tissue were removed carefully and then cut the Wharton's jelly into small cubes (2x2x2 mm^3) followed by washing with PBS twice. The resultant tissue cubes were cultured in complete culture medium containing 15% FBS, 80% DMEM/F12 at 37°C. 14 days after inoculation, the mesenchymal cells were spread out in a typical shuttle shape. Subculture for these cells were conducted when the cell confluency reached up to 80%. Based on our previous observations, these MSC cells within 6-7 passages have potent ability to differentiate into adipocytes, Chondrocytes and

osteoblasts, and the cell marker is always CD90, CD105, and CD73 positive, CD34, CD45, CD11b, CD19, and HLA-DR negative, but the cell shape and fate changed gradually afterward. Thus, to optimize the composition of PDF medium, we used human mesenchymal stem cells within P3-P5 passages as target cells.

Fig.1 is a graph showing the protective effect and the optimization of F68 on cryopreservation of MSC. To optimize the best concentration of F68 in PDF medium, we added different amount of F68 to 10% glycerol/PBS buffer, and then mixed with $2x10^6$MSC cells. The mixtures were then cooled down from 4°C (1 hour) to -80°C (2 hours) and to liquid nitrogen (one week). At the 8th day after frozen, the cell mixtures were thawed rapidly in 37°C water incubation followed by brief centrifugation at 1000 rpm x 5 minutes. The cell pellets were re-suspended with PBS and the survival cells were counted with a Trypan blue exclusion method. To test if the cells are capable of adhering growth, we seeded the total cells into culture dishes containing 10% FBS DMEM/F2 medium. 24 hours after culture, both the dead cells and adhered cells were harvested and counted under a microscope. The percentage of adhering cells over the total cells were calculated. When F68 concentration less than 0.5%, less than 50% cells survived and about 30% cells adhered to the culture dish. As the F68 concentration increased, the ratio of survived and adhered cells increased. When F68 concentration increased up to 5%, about 80% cells survived and 70% cells could adhere. However, when the F68 concentration increased to 10%, the adhered cell percentage reduced significantly to 40-45%. These observations showed that effective protection (>60% survival)can be achieved at the concentration range from 0.5 % to 10% for F68, and the highest protection were observed at theconcentration of 5% for F68.

Fig.2 is graph showing the protective effect and the optimization of glycerol on cryopreservation of MSC within the PDF medium. To optimize the best concentration of glycerol in PDF medium, we added different amount of glycerol to 5% F68 in PBS. The protective effect of the media on the MSC cells were assessed with the same method as described in Fig.1. When the glycerol concentration less than 2.5%, only ~20% thawed cells survived and ~10% cell could adhere to the culture dish. As the concentration increased up to 10%, about ~81% cells survived and ~65% with ability to adhere. At the concentration of 15%, the medium showed a similar efficacy to 10% (Fig. 2). This result demonstrated that 5% to 25% of glycerol in PDF medium are protective, and 10% glycerol gave the best protective effect.

Fig.3 is graph showing the protective effect and the optimization of propylene glycol on cryopreservation of MSC within the PDF medium. To assess the protective effect of propylene glycol (PREG), a derivative of glycerol, we prepared PDF medium with 10% propylene glycol (PREG)or 10% glycerol (GLY) or DMSO. The protective effect of these three media on the MSC cells were assessed with the same method as described in FIG.1. The resultant PDF medium showed a comparable efficacy for cell survival percentage, but the ability to adherewas decreased for 10% propylene glycol. The medium with 10% propylene glycol can still be used for non-adhering cells.

Fig.4 is a graph showing the protective effect and the optimization of buffers on cryopreservation of MSC within the PDF medium. To select a proper buffer system for PDF medium, we used 10% glycerol/5% F68 mixture as a basal medium and added different buffers such as PBS (pH7.0-7.4), HEPES (pH7.0-7.4), Tris.HCl (pH7.0-7.4), Multiple Electrolytes Injection (MEI) and normal solution (0.9% NaCl). The basal medium containing normal solution showed a relative poor protective effect on the MSC cryopreservation, while all the PBS, HEPES and Tris.HCl buffer showed good efficacy (>60% survival). Thus, the protective medium disclosed in the invention has a wide tolerance for commonly used buffers in cell preparation. As PBS is a cheaper and widely used buffer in clinical application, PBS buffer is a preferred option for preparation of PDF basal medium.

Embodiment 3

PDF basal medium cryopreserved embryonic stem cells, MSC, lymphocytes, RP, CHO and tumor cell lines with high viability.

To be consistent with clinical application of therapeutic cells, $5x10^6$ cells were suspended with 1 ml of PDF mediums (PDF-cells) and then cooled at 4°C for 60 minutes, -20°C for 30 minutes and -80°C for 4 hours and finally to liquid nitrogen. A week after freezing, the PDF-cells were thawed by 37°C water incubation with soft shaking. The viability of the resultant cells was measured with a trypan blue excursion method and adhering growth as described above. The viability was evaluated by the survival percentage and adhering percentage.

The results of this experiment were shown in Table 2. The results demonstrated that PDF basal medium can be used for cryopreservation of different cells, and survival percentages ranged from 70-90% while the adhering percentages ranged from 55-85%.

Table 2. Viability of the cells cryopreserved by PDF basal medium.

Cell	Survival percentage	Adhering percentage
Embryonic Stem Cell	75-80	n/a
MSC	70-80	55-65
RP	80-84	70-75
CHO	75-85	80-85
Lymphocytes	85-90	n/a
Hela S3	85-90	80-85
HepG2	80-90	75-83

Embodiment 4

To further improve the effect of PDF basal medium, we designed protective additives. After a substantial screening, we selected five ingredients used as additives. These are glucose, sucrose, trehalose, heparin, and ATP. In this design, 1-50mM glucose was used for energy supply; 1-50mM sucrose and trehalose; 0.1-100ug/ml heparinsulphate was used

for recovery of cell growth; 0.1-100mM ATP was used for down regulation of cell metabolism. The complete PDF medium is prepared by mixing PDF basal medium with the additives. The protective effect was assessed in the same as in Fig.1.

The results of this experiment were shown in Table 3. The results demonstrated the additives were able to enhance the cryopreservation efficiency of PDF basal medium appreciably.

Table 3. Viability of the cells cryopreserved by complete PDF medium

Cell	Survival percentage	Adhering percentage
Embryonic Stem Cell	90-95	
MSC	85-95	78-90
RP	84-93	80-90
CHO	88-90	80-85
Lymphocytes	86-90	Not tested
Hela S3	85-93	85-95
HepG2	85-92	80-87

Embodiment 5

PDF medium did not affect the function of the cryopreserved MSC.

Fig.5 shows the uninterrupted differentiation of PDF-cryopreserved MSCs into osteocytes, adipocytes and chondrocytes. The undifferentiated cells (low panels) and differentiated cells were respectively stained with Alizarin Red S (a), Oil Red O(b) and Alcian Blue (c).

The impact of cryopreservation on the phenotype of MSC were studied. We examined the differentiation ability of the Cryo-MSC in vitro. 30 days after freezing, the MSC were thawed at 37°C and cultured in 8% FBS (Gibco) DF-12 medium for 24 hours prior to differentiation assay (CorinaVater, Philip Kasten et al, Acta Biomaterialia,2011; FatemehHendijani, HojjatSadeghi-Aliabadi et al, Cell Tissue Bank,2014). The methods we used may be described as: 1. Cells were harvested and reseeded in 6-well plates at a density of 5,000 cell/cm2 in DF-12 culture medium supplemented with 8 % FBS in a humidified atmosphere with 5 % CO_2 at 37°C. For Chondrogenic differentiation, micromass cultures were generated by seeding 20 uL droplets of the cell suspension ($5x10^6$ cells/ml) in the tube. 2. After 3 hours, the medium was replaced with induction medium in the differentiation experiment wells. 3. Differentiation medium was changed with fresh medium every 3 days. Exposure to adipogenic differentiation medium resulted in formation of lipid droplets in MSCs that stained with Oil Red. Exposure to osteogenic differentiation conditions resulted in calcium deposits formed within MSCs which stained with Alizarin Red S. Cartilage- like tissue formation was observed in clusters of cells after exposure to differentiation medium as indicated by glycosaminoglycan staining by Safranin O for chondrogenic cells.

Embodiment 6

PDF medium did not affect the expansion of cryopreserved IL-2-activated lymphocytes.

Fig.6 is a graph showing unaltered expansion of PDF-cryopreserved lymphocytes after short-term cryopreservation. Lymphocytes are a group of important therapeutic cells for tumor therapy and immunotherapy. We isolated human periphery lymphocytes from healthy volunteers with a Ficoll isolation solution. The PBMC were harvested by centrifugation and then adjust cell concentration to 10^7/ml followed by mixing with equal volume of 2xPDF complete medium. The mixture was then frozen gradually to liquid nitrogen for 2 weeks. To assess the protection effect of the PDF complete medium, we thawed the cells and subjected to expansion experiment. The thawed cells were cultured in T75 flask coated with anti-CD3/anti-CD28 antibody. X-VIVO15 was used as basal medium,supplemented with 5% FCS, 2000iu/ml INF-γand 2000iu/ml IL2. During the culture period, the cells were counted every other day and until the 14th day after culture. The result showed that PDF medium-preserved lymphocytes have amplified about 38 folds with 14 days, which was comparable to fresh lymphocytes.

Embodiment 7

PDF medium did not affect the clone growth of mouse ES cells.

Fig.7 shows normal clone growth of mouse ES cells after cryopreservation, specifically, the cloning formation efficiency of ES cells were statistically equal between cells frozen with PDF media(a) and cells frozen in the presence of DMSO (b).

Clone growth ability is an important parameter reflecting the stemness of mouse ES cells. To assess the possible functional affection of ES cells after application of PDF medium, we harvested mouse ES cells from the culture. The cells were digested with trypsin and harvested by brief centrifugation. $2x10^6$ cells were mixed with 1.5 ml complete PDF medium and cooled from 4°C, -20°C, -80°C to liquid nitrogen. 1 week after freezing, the cells were thawed by 37°C water bath. The recovered cells were seeded on culture dish plating with MEF feeder cells and cultured at 37°C and 5% CO_2. 2 weeks after seeding and refreshing culture medium, the ES clones were counted and photographed. The cloning formation efficiency of PDF-cryopreserved ES ranged from 40-50%, which was comparable and statistically equal to DMSO-containing cryopreservation solution (10% FBS, 10% DMSO in DMEM)(38-48%).

Embodiment 8

Toxicity assay for PDF medium in animals.

Table 4. Toxicity effect of PDF complete medium in mice (C57/BL6). We intravenously injected 200 to 400 ul of complete medium to mice and assessed its toxicity. Upon injection with 200ul PDF medium, the mice (8 weeks old) showed normal appearance, normal physiological activity and behaviors during all the period after injection. Upon once injection with 400ul of PDF medium, the mice also showed normal appearance, normal physiological activities and behaviors. We then injected 400ul of PDF medium to the mice

(n=4) every other day for 2 weeks. The mice showed normal appearance, normal physiological activities and behaviors. The body weights of mice received PDF injection were 23+/-1g which was also comparable to PBS control (22.8+/-1.2g) ($p>0.05$). Macrophenotypic examination for the lung, heart, liver, gut, kidney, spleen and brain showed no apparent alteration in morphology. This result suggested that PDF medium has no apparent toxicity in mice, at least has no acute toxicity.

Table 4. Toxicity effect of PDF complete medium in mice (C57/BL6)

Injections	PBS control	PDF
200ul once (n=4)	Normal appearance Normal physiological activity	Normal appearance Normal physiological activity
400ul once (n=4)	Normal appearance Normal physiological activity	Normal appearance Normal physiological activity
400ul every other day within 2 weeks (n=4)	Normal appearance Normal physiological activity Body weights: 22.8±1.2g	Normal appearance Normal physiological activity Body weights: 23±1g

Embodiment 9

The toxicity assessment of the MSC cells prepared with PDF medium (PDF-MSCs).

Fig.8 shows normal electrocardiogram and pulse rate in mice after injection with reasonable amount of PDF-MSCs, in comparison with PBS. The mice received PDF-MSCs injection showed similar electrocardiogram (B,C,D,E) and heart rate (G)with control group(PBS), except the 400 uL group. On average, mice have around 58.5 ml of blood per kg of bodyweight. A mouse weighing 25 g would therefore have a total blood volume (TBV) of approximately 58.5 ml/kg x 0.025 kg = 1.46 ml. Therefore, 400 uL represent an extreme overload of the circulatory system and likely caused death by ways other than PDF-MSCs.

As shown in Fig.8, since fresh MSC being isolated by trypsin digestion from monolayer culture and cryopreserved MSC by standard DMSO solution may form cell aggregation which primarily contributes to the toxicity of MSC cells, we injected PDF medium-prepared MSCs at 30-40 minutes after thawing in order to assess the aggregation-prevention effect of our PDF solution. For DMSO control group, $2x10^6$ MSCs in 400 ul solution were injected intravenously at 30 minutes after thawing, and 5/6 mice died immediately with repeated twitch, a typical appearance of pulmonary occlusion. However, upon intravenous injection with $2x10^6$ MSCs in 400 ul PDF medium, 4/6 mice appear normal except a slight decrease in pulse per minute after injection, and 2/6 died possibly due to the over dose of cells. The surviving mice receiving the PDF-MSC injection showed normal electrocardiogram and pulse rate (Fig. 8). Injection of less than 300ul MSC suspension showed no toxicity in terms of pulse rate and electrocardiogram (Fig.8). This result indicates attenuated toxicity for PDF medium-prepared MSC cells in mice.

Rabbit has unique responses for nervous, circulation and urinary systems, which may be good for evaluate the safety of PDF-prepared MSC cells. We injected $1x10^7$ cells in 1000 ulPDF-MSCs in ear side veins 40 minutes after thawing. In control group (PBS solution), all

4 rabbits displayed normal behaviors. The urine of these rabbits showed normal yellow, and no protein was detected in the collected urine. In PDF-MSC group, 4 out of 4 rabbits survived after injection without any abnormal appearance and physiological behaviors (Table 5). 1 rabbit showed white trail in urine area, but no protein was detected. This phenomenon is likely caused by a stress impulse on the rabbit and belongs to normal reaction, and not caused by potential toxicity of injection.

Table 5. Observation of clinical indexes in rabbit after MSC injection

Indexes	Normal range	Control group (n=4)	PDF group (n=4)
Abnormal behaviors	Non	Normal	Normal
Feeding	50grams/kg/day	Normal	Normal
Urine color	yellow	Normal	white trail in urine area (1/4) (Normal)
Urine protein	negative	Negative	Negative
Hematuria	negative	Negative	Negative
Urinary volume	10-35ml/kg/day	Normal	Normal
Temperature	38.5-39.5 ℃	Normal	Normal

Fig.9showscomparisons of electrocardiograms of dogs after and before injection of PDF-MSCs, revealing no significant change of wave patterns as well as pulse rate. To assess the toxicity of the MSCs prepared by PDF medium, adult beagles were treated for four times at roughly two-week intervals with PDF-MSC injections.

In terms of body size and functional similarity of many physiological systems including cardiovascular system, respiration system and urine systems, dog is much close to human compared to mice and rabbit model systems. In order to assess the toxicity of the MSC prepared by PDF medium in this animal, we injected $2x10^7$ cells in 2 ml PDF medium intravenously and measured the parameters of peripheral blood and urine, breathe rate, heart beating rate and electrocardiograms etc. All 3 dogs received PDF medium prepared-MSC treatmentsurvived without apparent abnormality of appearance, walking gate, and other physiological behaviors. Comparison of electrocardiograms of dogs after injection for a half hour with that before injection showed no change of wave patterns as well as pulse rate, as shown in Fig.9.

We also measured biochemical parameters in peripheral blood before and after injection with PDF-MSC cell preparations. Table 6 represents the results from the three dogs. The result showed that almost all parameters were in a normal range. Interestingly, after injection with PDF MSC cells, all three dogs showed apparent improvement for liver function as evidenced by reduced GPT and ALP activity.

Table 6. Blood biochemical level for Beagles before or after injection with PDF cell preparations

Indexes	Unit	Reference	D1	D15	D29	D43	D57	D85

GPT-PS	U/L	17-78	32.33±8.39	27.00±5.29	27±6.00	26.50±3.54	25.33±4.04	28.67±6.03
ALP-PS	U/L	13-83	42.67±8.14	31.33±5.13	28.67±6.43	27.50±7.78	20.33±5.03	21.00±3.46
BUN-PS	mg/dl	9.2-29.2	14.50±8.94	16.23±6.50	12.13±4.54	13.50±4.97	13.50±4.97	11.90±1.84
Ca-PS	mg/dl	9.3-12.1	11.57±0.84	11.77±0.47	11.57±0.38	11.25±0.35	10.57±0.32	10.97±0.06
CRE-PS	mg/dl	0.4-1.4	0.50±0.10	0.57±0.06	0.60±0.10	0.60±0.00	0.57±0.15	0.57±0.06
GGT-PS@	U/L	5.0-14.0	4.67±1.15	4.67±1.15	4.67±1.15	5.00±1.41	3.33±1.53	3.00±1.00
GLU-PS	mg/dl	75-128	112.67±5.86	109±5.29	104.00±4.58	103.67±6.81	107.3±36.66	104.67±11.24
TP-PS	g/dl	5.0-7.2	6.97±0.76	4.77±3.87	6.47±0.31	6.73±0.35	6.50±0.20	6.53±0.25
TBIL-PS	mg/dl	0.1-0.5	0.20±0.00	0.17±0.12	0.20±0.00	0.30±0.35	0.20±0.00	0.10±0.00
ALB-PS	g/dl	2.6-4.0	2.90±0.20	4.33±2.14	3.10±0.10	3.47±0.21	3.13±0.12	3,20±0.10
TCHO-PS	mg/dl	111-312	202.67±60.28	196.67±15.18	184.67±25.38	192.67±50.14	191.00±2.67	189.33±62.52
IP-PS	mg/dl	1.9-5.0	2.10±0.60	3.97±0.75	3.27±0.49	3.10±0.46	2.67±0.21	2.90±0.17
GLOB	g/dl		4.07±0.57	3.87±0.12	3.37±0.21	3.27±0.23	3.37±0.12	3.33±0.31
ALB/GLOB			0.73±0.06	0.80±0.00	0.90±0.00	1.07±0.06	0.93±0.06	0.97±0.12
BUN/CRE			31.60±25.25	28.07±9.19	19.77±4.20	28.5±0.99	23.30±3.47	21.07±2.97

While the invention has been particularly shown and described as referenced to the embodiments thereof, those skilled in the art will understand that the foregoing and other changes in form and detail may be made therein without departing from the spirit and scope of the invention.

Claims

1. A cell preservation and preparative medium for preserving cells or preparing cells that has protective effect for the cells, comprising a poloxamer and a simple polyol, wherein,

thepoloxamer is present in a concentration range of 0.5% to 10% (v/v); and the simple polyol is present in a concentration range of 5% to 25% (v/v).

2. A cell preservation and preparative medium for preserving cells or preparing cells according to claim 1, wherein the poloxamer is F68/poloxamer 188,

3. A cell preservation and preparative medium according to claim 1, wherein the simple polyol is selected from the group of glycerol and propylene glycol.

4. A cell preservation and preparative medium according to claim 1, further comprising a buffer.

5. A cell preservation and preparative medium according to claim 4, wherein the buffer is selected from PBS, HEPES, Tris.HCl, Multiple Electrolytes Injection (MEI), and normal solution (0.9% NaCl).

6. A cell preservation and preparative medium according to claim 1, wherein the medium is free of one or more of: dimethyl sulfoxide (DMSO), serum, a substitute component of serum, a protein component, and an animal-derived component.

7. A cell preservation and preparative medium according to claim 1 further comprising any one of the following additives: glucose, sucrose, trehalose, heparin, and ATP.

8. A cell preservation and preparative medium according to claim 1, wherein the cell preservation and preparative medium is used for storing or preparing embryonic stem cells, hematopoietic stem cells, tissue stem cells, induced pluripotent stem cells, mesenchymal stem cells, retina precursor cells cancer cells, primary cell lines, and immortalized cell lines, sperms, eggs, pancreatic islets, and tissues.

9. A cell preservation and preparative medium according to claim 1, wherein the cell preservation and preparative medium is used for storing or preparing cells in gene therapy, cell therapy, drug preparation of therapeutic cells, cell cryopreservation, and tissue cryopreservation.

10. A cell preservation and preparative medium according claim 1, effective in preventing cell injury during freezing and thawing, wherein a cell survival rate is at 60% or higher, irrespective of the speed of freezing and thawing.

11. A cell preservation and preparative method for freezing and rapidly thawing cells, comprising:

a suspension step for suspending cells in a cell preservation and preparative medium; wherein the medium comprises a poloxamer at a concentration of 0.5% to 10% (v/v) and a simple polyol at a concentration of 5% to 25% (v/v);

a freezing step for freezing the resuspended cells; and

a thawing step to thaw the frozen cells after the freezing step without significant loss of cell number.

12. A cell preservation and preparative method according to claim 11, wherein the freezing step can be performed either rapidly or slowly to allow a flexible timeframe without significant reduction of cell survival rate; wherein the thawing step can be performed slowly or rapidly by adding a warm culture medium, by incubating in a water bath, or by coming into contact with a warmer object.

13. A cell preservation and preparative method according to claim 11, wherein the poloxamer is F68/poloxamer 188.

14. A cell preservation and preparative method according to claim 11, wherein the simple polyol is selected from the group of glycerol and propylene glycol.

15. A cell preservation and preparative method according to claim 11, wherein the cell preservation and preparative medium further comprises a buffer.

16. A cell preservation and preparative method according to claim 15, wherein the buffer is selected from PBS, HEPES, Tris.HCl, Multiple Electrolytes Injection (MEI), and normal solution (0.9% NaCl).

17. A cell preservation and preparative method according to claim 11, wherein the cell preservation and preparative medium is free of any one of: dimethyl sulfoxide (DMSO), serum, a substitute component of serum, a protein component, and an animal-derived component.

18. A cell preservation and preparative method according to claim 11, wherein the cells suspended in the cell preservation and preparative medium are present in a range of 1 to 1×10^7 per mLof the medium.

19. A cell preservation and preparative medium according to claim 11, further comprising adding into the medium any one of the following additives: glucose, sucrose, trehalose, heparin, and ATP.

20. A cell preservation and preparative method, effective in preventing freezing and thawing injury to cells and eliminating the need for washing cells, comprising:

a suspension step for suspending cells in a cell preservation and preparative medium; wherein the stem cell preservation medium comprises a poloxamer, a simple polyol, and a buffer; and

injecting the cells into a subject without separating the cell preservation and preparative medium from the cells.

FIG. 1

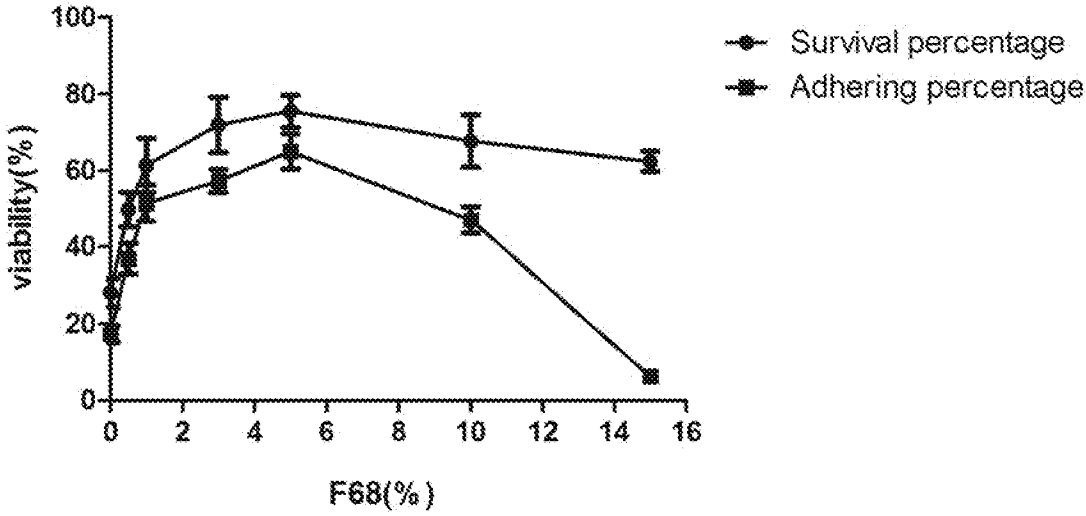

FIG. 1

FIG. 2

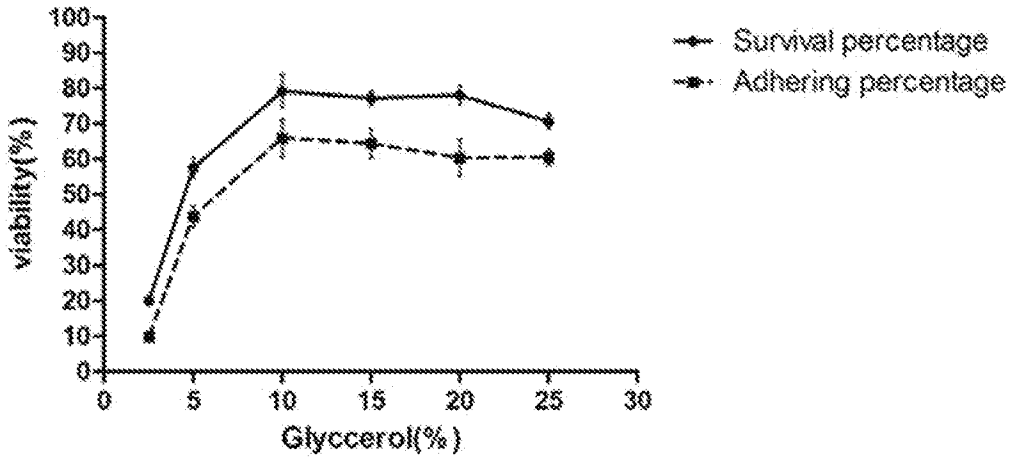

FIG. 2

FIG. 3

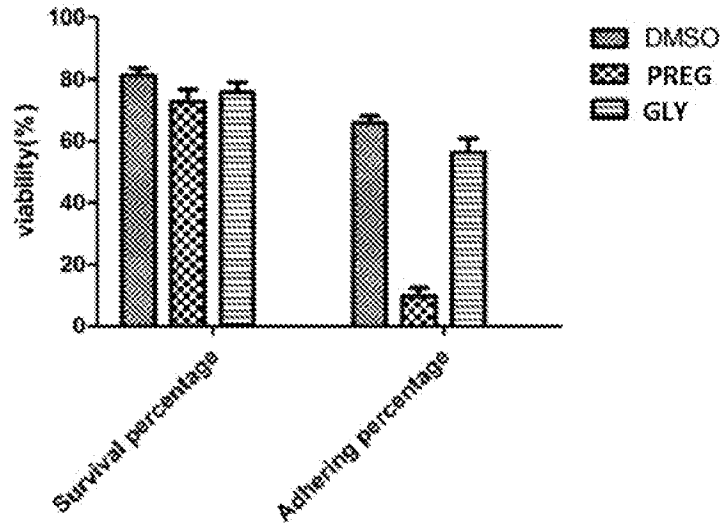

FIG. 3

FIG. 4

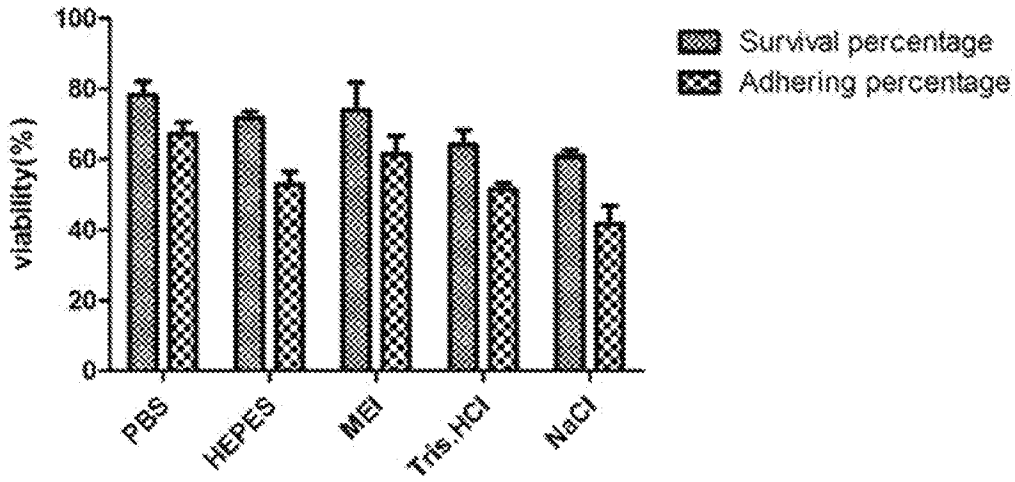

FIG. 4

FIG. 5

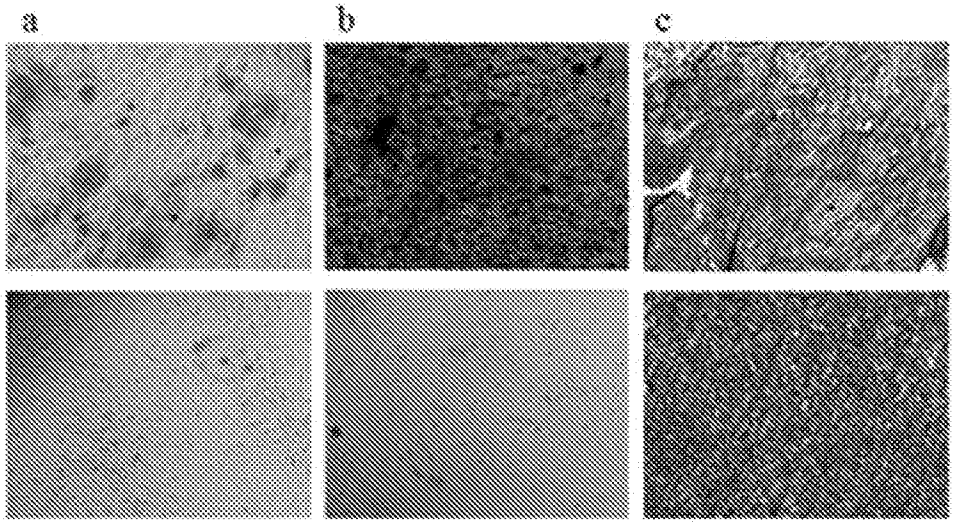

FIG. 5

FIG. 6

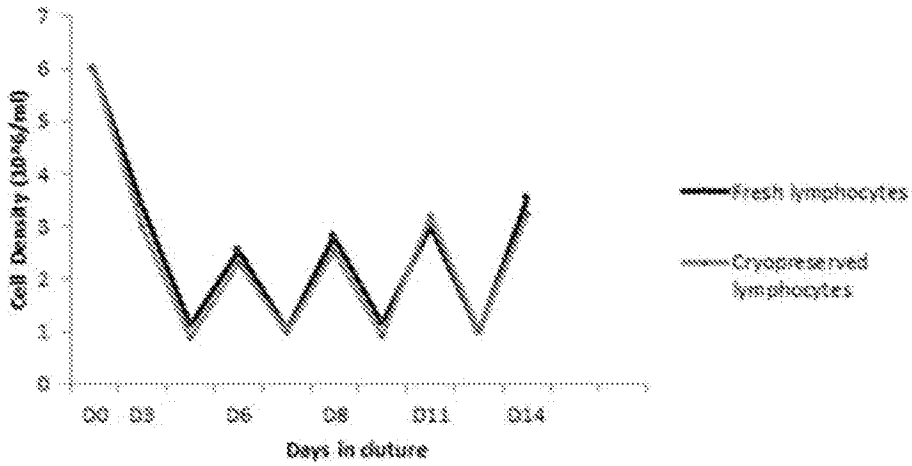

FIG. 6

FIG. 7

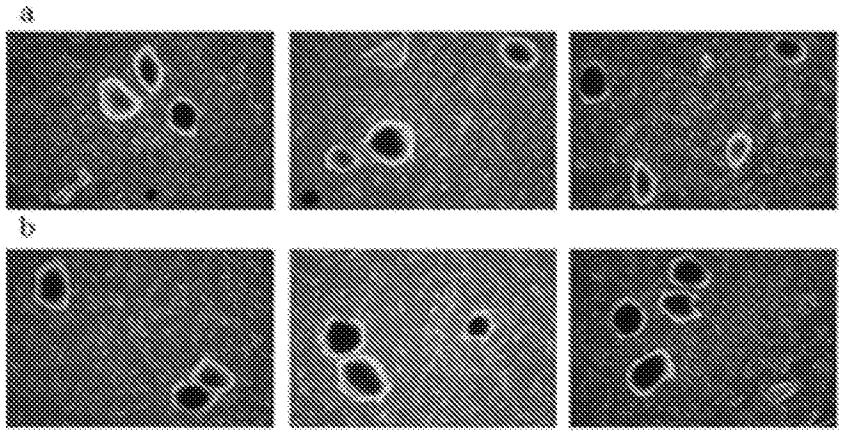

FIG. 7

FIG. 8

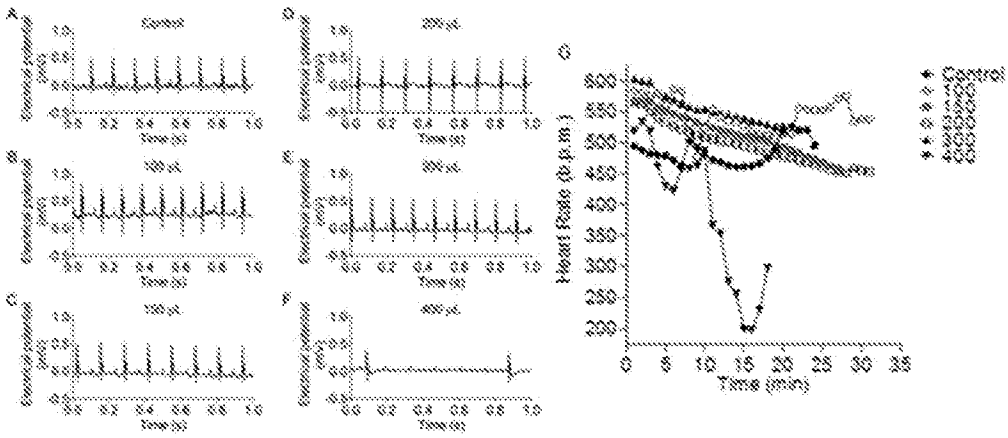

FIG. 8

FIG. 9

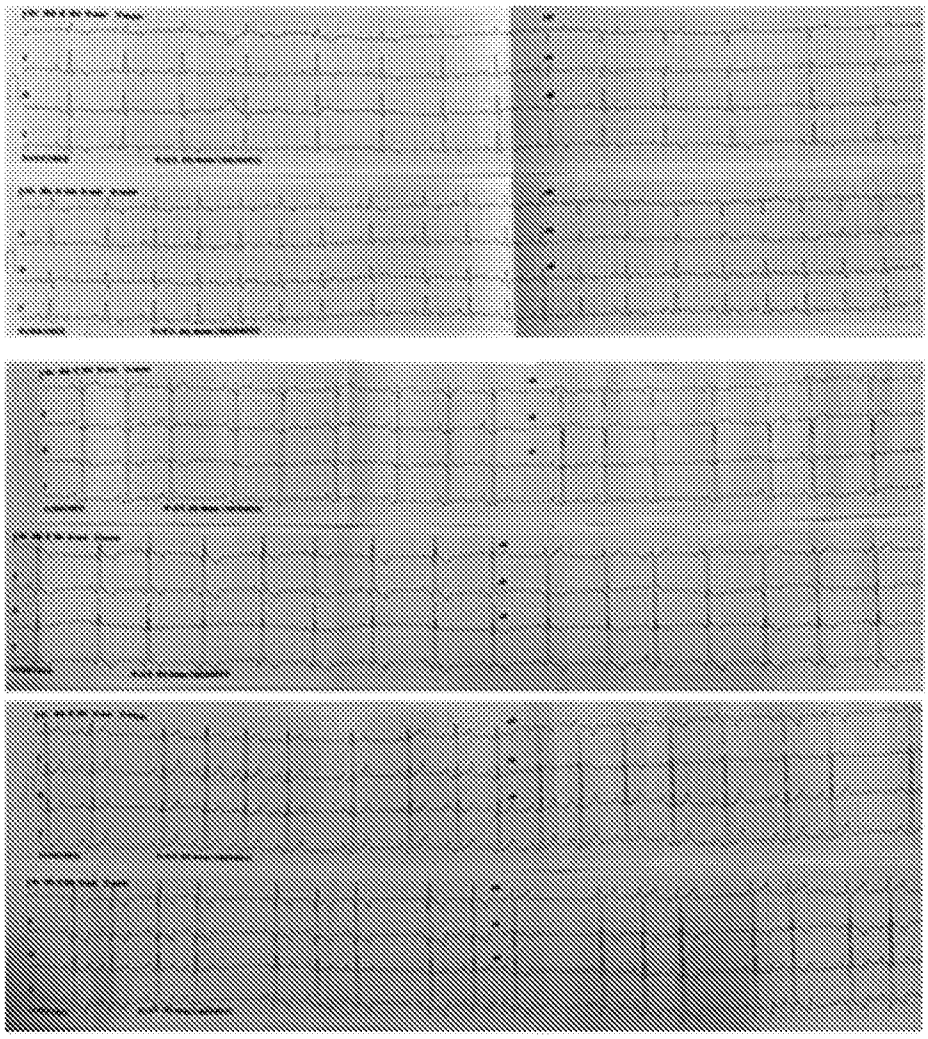

FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2019/093265

A. CLASSIFICATION OF SUBJECT MATTER

A01N 1/02(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CNABS, DWPI, SIPOABS, Wangfang, CNKI, PubMed, ISI web of knowledge:poloxamer, F68, polyol, glycerol, propylene glycol, cell preservation, storage, conservation, cryopreservation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 105961374 A (SHENZHEN HORNETCORN BIO-TECHNOLOGY CO.) 28 September 2016 (2016-09-28) abstract, claims 1-4, paragraphs [0007] and [0020], examples 4and12, figure 2	1-20
X	CN 108902128 A (WENZHOU MEDICAL UNIVERSITY) 30 November 2018 (2018-11-30) claims 1-10, table 1.	1-20
X	CN 106821938 A (HUANG Bing) 13 June 2017 (2017-06-13) claims 1-6	1-20
X	CN 108753683 A (WENZHOU MEDICAL UNIVERSITY) 06 November 2018 (2018-11-06) claims 1-10, table 1	1-20
A	CN 108669070 A (WENZHOU MEDICAL UNIVERSITY) 19 October 2018 (2018-10-19) the whole document	1-20
A	US 2001055809 A1 (HARPAL S.M.) 27 December 2001 (2001-12-27) the whole document	1-20

☐ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 March 2020	**26 March 2020**
Name and mailing address of the ISA/CN **National Intellectual Property Administration, PRC** **6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088** **China** Facsimile No. **(86-10)62019451**	Authorized officer **CAO,Kou** Telephone No. **86-(10)-53962094**

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2019/093265

Patent document cited in search report	Publication date (day/month/year)	Patent family member(s)	Publication date (day/month/year)
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