

METHOD FOR RECOVERING LIVING CELLS FROM CRYOPRESERVED
CELLS AND SYSTEM FOR THE SAME

Technical Field

5 [0001]

The present invention relates to a method for highly efficiently recovering living cells from cryopreserved cells by thawing, and also to a system for the method.

10

Background Art

[0002]

Efforts have been made in recent years toward the transplantation of various kinds of cells for the healing of damaged tissues or the like. For example, attempts are being made to heal cardiac muscle tissues damaged by ischemic heart disease, such as angina pectoris and cardiac infarction, by means of transplantation with fetal cardiac muscle cells, skeletal muscle blast cells, embryonic stem (ES) cells, and so on.

20

[0003]

Such attempts have led to the development of cell structure formed by the use of scaffold and the development of sheet-like cell culture product in the

form of sheet composed of cells (see Patent Documents 1 to 3, for example).

[0004]

The sheet-like cell culture product is expected to find use in the form of cultured epidermal sheet for damaged or scalded skin, in the form of corneal epithelium sheet for damaged cornea, and in the form of oral mucous membrane sheet for endoscopic resection of esophageal carcinoma.

10 [0005]

Along with the recently established healing method which is based on the regenerative medicine, there are increasing instances for healing in recent years in which artificial tissue or sheet-like cell culture product in the form of three-dimensional structure is made from cryopreserved autologous cells, or in which autologous cells are recovered by thawing cryopreserved cells at the time of direct cell transplantation.

[0006]

20 Cells can be preserved semipermanently by freezing in liquid nitrogen or the like. However, cells are subject to damage due to latent heat that occurs at the time of freezing and damage due to ice crystals which appear in the cells. Thus, it is impossible to entirely

recover living cells from the cryopreserved cells. It is
common practice to secure as many cells as necessary by
culturing and proliferating cells obtained after the
thawing of cryopreserved cells. Since the long term
5 culturing is time-consuming, there has arisen a need for
recovering as many living cells as possible without
laborious steps. Common way to cope with this situation
is by reducing physical damage to cells and increasing
the number of living cells, which will be achieved by
10 improving the methods for freezing and thawing.

Prior Art Document

Patent Documents

[0007]

15 Patent Document 1: JP-T-2007-528755

Patent Document 2: Japanese Patent Laid-open No.
2010-81829

Patent Document 3: Japanese Patent Laid-open No.
2010-226991

20

Summary of Invention

Technical Problems

[0008]

The sheet-like cell culture product should

preferably be prepared from autologous cells from the standpoint of limited rejection. The preparation of sheet-like cell culture product from autologous cells requires time for cell proliferation and differentiation, which limits the rate of preparation. In order to overcome this drawback, there has been proposed a method for preparing the sheet-like cell culture product. The method consists of seeding the cells at a specific density that permits the sheet-like cell culture product to be formed without substantial cell proliferation (see Patent Document 2, for example). Owing to the method, it has become possible to prepare the sheet-like cell culture product, which has a higher physical strength than before, in a shorter time than before.

15 [0009]

The method for preparing the sheet-like cell culture product needs more cells than the conventional one. This makes it necessary to proliferate and culture beforehand the cells collected from the recipient and preserve the proliferated cells cryogenically for use. In the case where the sheet-like cell culture product is prepared by the method, however, the method does not give rise to any satisfactory sheet-like cell culture product, simply resulting in cell proliferation or cell

differentiation, unless cells in a sufficient amount are seeded. The result is that the sheet-like cell culture product is not ready for transplantation if sufficient cells are not secured for the sheet-like cell culture product to be prepared immediately before transplantation. For this reason, it is common practice to recover as many living cells as possible by attempting to minimize physical and chemical damages to cells at the time of freezing and/or thawing cryopreserved cells.

10 [0010]

Thus, it is an object of the present invention to provide a method for recovering living cells highly efficiently from cryopreserved cells and also provide a system for the method. This object is achieved by reducing more than before physical and chemical damages to cryopreserved cells at the time of thawing for the recovery of living cells.

Technical Solution

20 [0011]

The freezing of cells is usually accomplished with the addition of a cryoprotectant so that cells are saved from physical damage due to ice crystals. A common example of the cryoprotectant is dimethylsulfoxide (DMSO).

DMSO is known to exhibit cytotoxicity at approximately 37°C which means that the cytotoxicity appears at the time of thawing the cryopreserved cells. Usually, attempts are made to reduce cytotoxicity as much as possible by quick dilution, for example.

[0012]

In the course of study on the method for efficient recovery of living cells from cryopreserved cells, the present inventors found that the quicker the dilution of cell suspension after thawing for reduction of DMSO's cytotoxicity, the less the number of living cells recovered. This phenomenon is contrary to what has been observed before. As the result of their continued study on this phenomenon, the present inventors further found that the quick dilution greatly damages cells due to rapid change in osmotic pressure and that such damages to cells can be reduced by suppressing the load of osmotic pressure at the time of dilution, which leads to an increased survival ratio of cells. The result of these investigations led to the present invention.

[0013]

The present invention covers the following.

[1] A method for recovering living cells from cryopreserved cells, including: thawing cryopreserved

cells; and diluting the thawed cell suspension with a diluent, wherein the dilution is performed in such a way that the maximum load of osmotic pressure at the time of dilution is equal to or lower than 250 mOsm/second.

5 [2] The method as defined in Paragraph [1] above, wherein the diluent is added in such a way that the maximum load of osmotic pressure at the time of dilution is equal to or lower than 50 mOsm/second.

[3] The method as defined in Paragraphs [1] or [2] above,
10 wherein the diluent is added in such a way that the maximum load of osmotic pressure at the time of dilution ranges from 40 mOsm/second to 50 mOsm/second.

[4] The method as defined in Paragraphs [1] to [3] above, wherein the cells are skeletal muscle blast cells.

15 [5] The method as defined in Paragraphs [1] to [4] above, wherein the diluent contains the rinsing liquid resulting from rinsing of a container for cryopreservation from which the thawed cell suspension has been transferred to another container.

20 [6] A system for thawing cryopreserved cells, including:
(i) an actuating unit configured to inject a diluent; and
(ii) an arithmetic control unit configured to determine the rate at which the actuating unit injects the diluent.

[7] The system as defined in Paragraph [6] above, further

including (iii) a measuring unit configured to measure osmotic pressure of liquid.

[8] A kit including: cells; and a culture substrate used for a method of producing a sheet-like cell culture product without necessity for proliferation culture, the method including a step of seeding at a density suitable to form the sheet-like cell culture product without substantial cell proliferation, wherein cells are those which have been recovered by any one method defined in Paragraphs [1] to [5] above.

Advantageous Effects

[0014]

The present invention is intended to reduce the number of cells dying from an abrupt change in osmotic pressure which has been neglected before, thereby increasing the number of recoverable living cells, by means of controlling the load of osmotic pressure. According to the present invention, it is possible to recover cells which retain high viability even after freezing and thawing. Therefore, the present invention permits to secure sufficient living cells without requiring the step of proliferation culture after thawing.

Modes for Carrying Out the Invention

[0015]

The present invention relates to a method for highly efficiently recovering living cells from
5 cryopreserved cells and also to a system for the method.

The present invention will be described below in more detail with reference to the preferred embodiments thereof.

(1) Method for recovering living cells

10 One aspect of the present invention relates to a method for recovering living cells from cryopreserved cells, which includes steps of thawing cryopreserved cells and diluting with a diluent the suspension of thawed cells. This method is characterized in that the
15 dilution is performed in such a way that the change in osmotic pressure due to dilution is kept sufficiently small.

[0016]

The term "cryopreserved cells" used in the present
20 invention usually means cryopreserved cells themselves. However, it occasionally means a unit of cryopreserved cells. In this case, the term "cryopreserved unit" means a group of cells which are cryopreserved together in a tube, for example. Thus, the "cryopreserved cells" give

upon thawing "a suspension of cells" which had been frozen.

[0017]

The term "load of osmotic pressure at the time of
5 dilution" as used in the present invention means the rate
of change per unit time in the osmotic pressure which
varies with addition of the diluent. The load of osmotic
pressure varies depending on such factors as the rate of
addition of the diluent (or the amount of diluent added
10 per unit time) and the difference in osmotic pressure
between the diluent and the liquid to be diluted (such as
the suspension of thawed cells). The term "maximum load
of osmotic pressure" as used in the present invention
means the maximum value of the load of osmotic pressure
15 which varies as the diluent is added from the start of
dilution to the completion of dilution.

[0018]

The method according to the present invention is
characterized in that the cell suspension obtained by
20 thawing the cryopreserved cells is diluted in such a way
that the change in osmotic pressure is kept sufficiently
small during dilution. The recovery of living cells from
cryopreserved cells by thawing is usually accomplished by
adding a culture medium or the like to the suspension of

thawed cells for dilution in order to lessen the influence of cytotoxic components existing in the suspension of thawed cells. The present inventors found that the dilution at an excessively high rate in the step
5 of dilution causes a rapid change in the osmotic pressure of the suspension, with such a change damaging cells, thereby decreasing the survival ratio of living cells.
[0019]

The dilution with a minimum change in osmotic
10 pressure will reduce damages to cells, thereby increasing the yield of recovery of living cells. The method of reducing the change in osmotic pressure may be achieved by slowing the addition of the diluent or using a diluent which only slightly varies in osmotic pressure from the
15 cell suspension. These techniques are known to persons skilled in the art. What is preferable is to avoid using any diluent with cytotoxicity, thereby excluding any damages other than those caused by the load of osmotic pressure.

20 [0020]

The diluent to be used in the present invention is not specifically restricted so long as it does not cause physical and chemical damages to cells. Examples of the diluent unrestrictedly include liquid culture medium,

such as Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution, buffer solution, such as phosphate buffered saline (PBS), isotonic solution, such as physiological saline, and distilled water. They may
5 optionally be incorporated with additional ingredients such as albumin.

In this specification, the unit "Osm" is used as the unit for osmotic pressure. "1 Osm" denotes an osmotic pressure equivalent to the osmotic pressure possessed by
10 an ideal solution of 1 mol/L. The load of osmotic pressure at the time of dilution is expressed in terms of the rate of change in osmotic pressure per second at room temperature (unit: Osm/second). However, any other arbitrary unit may be selected so long as it can express
15 the magnitude of the change in osmotic pressure. Such units may include the speed of addition of the diluent and the speed of increase in volume or weight.

[0021]

The load of osmotic pressure may be obtained by
20 measuring in real time the change in osmotic pressure or by calculating an average of change per unit time between two measurements made at two different points of time. In the case where a diluent is added at a constant rate, the maximum load of osmotic pressure during dilution may be

obtained from the difference between two osmotic pressures measured at the start of addition and at a point of elapsed time (say, one second in the above-mentioned example of the present invention) after the start of addition. This is based on an assumption that the load of osmotic pressure reaches the maximum value when the addition of diluent starts and then gradually decreases according as the addition of diluent increases.

[0022]

10 The method employed in the embodiment will be described step by step in the following.

<Thawing of cryopreserved cells>

 The step of thawing cryopreserved cells may be accomplished by under any conditions known in the technical field concerned. Usually, thawing should be performed by instantaneous warming with the help of a water bath or the like kept at approximately 37°C. This is because slow thawing tends to cause physical damages to cells due to ice crystals. It is also possible to use any other method than above which is employed in the technical field concerned in order to improve the recovery of living cells.

[0023]

 The method according to the present invention can

be applied to any kind of cells known in the technical field concerned, which are capable of cryopreservation. Preferable examples of such cells include somatic stem cells, such as embryonic stem cells, nerve stem cells, hematopoietic stem cells, and mesenchymal stem cells, and blast cells, such as fibroblast cells, skeletal muscle blast cells, and osteoblast cells, which are used for regenerative medicine. More desirable among them are somatic stem cells and blast cells which can be sampled and proliferated as autologous cells. Skeletal muscle blast cells are most desirable from the standpoint of availability and handleability. It is desirable to use cells in the logarithmic growth phase from the standpoint of high recovery ratio of living cells.

[0024]

The amount of cryopreserved cells may vary according to the capacity of the container used for cryopreservation. Each container should be generally given a suspension of cells for cryopreservation, which has a cell density adjusted to approximately 1×10^5 to 5×10^7 cells/ml. This value may be used as the parameter for the calculation of living cells to be recovered from the unfrozen cell suspension, in view of the fact that all the cryopreserved cells should ideally be recovered

in the form of living cells.

[0025]

The cryopreserving liquid is not specifically restricted so long as it is selected from those used for
5 cryopreservation of cells in the technical field concerned which are sold from a lot of companies. Any ordinary cell culture medium may also be used, which is incorporated with a cryoprotectant, such as dimethylsulfoxide (DMSO) and glycerol, in an amount of
10 approximately 1% to 20%, preferably approximately 5% to 10%. The culture medium may also be replaced by 100% serum.

The containers for cryopreserved cells may be selected from those which are commonly used in the
15 technical field concerned. Their examples include commercial cryovials, ampoules, and cryopreserving bags.

[0026]

<Dilution of cell suspension>

The cell suspension resulting from thawing is
20 likely to contain a cytotoxic component (such as DMSO), as mentioned above. The effect of cytotoxicity can be alleviated by dilution. According to the method of the present invention, this dilution is performed in such a way as to slow the change in osmotic pressure, or to

minimize the load of osmotic pressure, so that the thawed cells survive at a high ratio.

The term "sufficiently slow change in osmotic pressure" as used in the method of the present invention varies in its threshold value depending on the kind of cells and the condition and temperature of thawing. The maximum load of osmotic pressure should be equal to or lower than approximately 250 mOsm/second, preferably equal to or lower than approximately 220 mOsm/second, more preferably equal to or lower than approximately 100 mOsm/second, and most desirably equal to or lower than approximately 50 mOsm/second, in an exemplary case where the cryopreserving liquid is an ordinary one (such as DMEM medium containing approximately 10% DMSO) and the diluent is an ordinary one (such as commercial DMEM medium).

[0027]

The dilution should preferably be performed as rapidly as possible, because excessively slow dilution causes damage to cells due to the cryotoxic component in the cell suspension which invokes other factors than the load of osmotic pressure. The method according to the present invention does not need a specific lower limit for the maximum load of osmotic pressure. However, it

should preferably be equal to or higher than approximately 2 mOsm/second, more preferably equal to or higher than approximately 20 mOsm/second, and most desirably equal to or higher than approximately 40 mOsm/second, in the case where the method employs an ordinary cryopreserving liquid, such as DMEM medium containing approximately 10% DMSO, and an ordinary diluent, such as commercial DMEM medium.

[0028]

10 According to one preferred embodiment of the present invention, the maximum load of osmotic pressure should be 2 mOsm/second to 250 mOsm/second, preferably 2 mOsm/second to 220 mOsm/second, more preferably 20 mOsm/second to 100 mOsm/second, and most desirably 40 mOsm/second to 50 mOsm/second, in view of the amount of recoverable living cells.

[0029]

20 The dilution step may be carried out in such a way that the suspension of thawed cells is held in the container for cryopreservation or transferred to the other container. In the latter case, the container for cryopreservation to which the cell suspension has been transferred should be rinsed with a diluent and the resulting rinsing liquid should be added to the cell

suspension. This procedure is necessary to raise the survival ratio of thawed cells. Such addition of the rinsing liquid is equivalent to the dilution specified in the present invention. Thus, according to one embodiment
5 of the present invention, the diluent includes the rinsing liquid resulting from rinsing the cryopreserving container from which the cell suspension has been transferred to the other container.

[0030]

10 As mentioned above, the method according to the present invention has the maximum load of osmotic pressure affected most easily by the diluent to be added first to the suspension of thawed cells. Therefore, in one embodiment of the present invention, the maximum load
15 of osmotic pressure is defined as that which is observed during the period in which the suspension is diluted thrice. In another embodiment of present invention, the maximum load of osmotic pressure is defined as that which is observed during the period in which the suspension is
20 diluted twice.

[0031]

According to one preferred embodiment of the present invention, the dilution should be performed in such a way that the maximum load of osmotic pressure is

maintained in response to the osmotic pressure measured.

The measurement of osmotic pressure may be performed continuously at all times or intermittently with certain intervals, say, one second, 10 seconds, 30 seconds, or

5 one minute. Any method known in the technical field concerned may be used for the measurement of osmotic pressure. Measurement with an osmometer is one example of such methods.

[0032]

10 (2) System for thawing cryopreserved cells

One aspect of the present invention relates to a system to thaw cryopreserved cells and recover living cells highly efficiently. This system includes (i) an actuating unit for injection of diluent and (ii) an

15 arithmetic control unit to determine and control the rate at which the actuating unit injects the diluent.

[0033]

The actuating unit for injection of the diluent may have any shape so long as it is capable of injection. A
20 desirable shape is one which permits the diluent to drip down. The actuating unit should be capable of adjusting the rate at which the diluent is added according to a signal from the arithmetic control unit.

The arithmetic control unit is intended to

determine and control the rate at which the diluent is injected from the actuating unit. The injection rate may be established according to the previously entered value or calculated from the instantaneous osmotic pressure of the cell suspension. In the latter case, but it is not limited by the following, the initial amount of cell suspension and the osmotic pressure are entered and the existing osmotic pressure and addition rate are determined from the amount of diluent added, osmotic pressure, temperature, and so on. Alternatively, it is also possible to previously program the change in the rate of addition of the diluent.

[0034]

In the case where the rate of addition of the diluent is calculated from the instantaneous osmotic pressure of the cell suspension, the system according to the present invention may further include a measuring unit to measure the osmotic pressure of the liquid. In this case, the arithmetic control unit will be able to determine the rate of addition according to the information entered from the measuring unit.

The system according to the present invention is intended to thaw cryopreserved cells and recover living cells from them. It may include any arbitrary unit to

thaw cryopreserved cells in addition to the foregoing
actuating unit, arithmetic control unit, and measuring
unit. Such additional units would be apparent to those
skilled in the art because the equipment to thaw cryo
5 preserved cells and recover living cells from them are
known in the technical field concerned. Examples of such
additional units unrestrictedly include a thermostat unit
which keeps the system at a constant temperature during
thawing cryopreserved cells, a pipetting unit which
10 transfers the cell suspension from the cryopreserving
container, a spinning unit which centrifuges the cell
suspension, and a counter unit which counts the number of
living cells.

[0035]

15 (3) Method for producing a sheet-like cell culture
product and a kit designed therefor

As mentioned above, those cells which have been
recovered by the recovery method according to the present
invention are adequate for use particularly in the case
20 where they are used as such without any proliferation
step. Thus, one aspect of the present invention relates
to a method for producing a sheet-like cell culture
product from cells recovered by the recovery method of
the present invention.

[0036]

The term "sheet-like cell culture product" as used in the present invention denotes a product composed of cells joined together in sheet form. The cells
5 constituting this product may be joined together directly or indirectly with one another, with cellular elements such as adhesive molecules placed between them (in the former case) and/or an intermediate substance interposed between them (in the latter case). The intermediate
10 substance is not specifically restricted so long as it is capable of physically (or mechanically) joining cells together. It includes, for example, extracellular matrices. The intermediate substance should preferably be one which is derived from cells, particularly one which
15 is derived from cells constituting the cell culture product. The cells should be joined together at least physically (or mechanically); they may also be joined together functionally, such as chemically and electrically. The sheet-like cell culture product may be
20 of monolayer structure or multilayer (laminated) structure (with two, three, four, five, or six layers).

[0037]

The sheet-like cell culture product should preferably be one which is free of scaffold (support).

The scaffold (which is known in the technical field concerned) is used to keep the sheet-like cell culture product physically integral in such a way that cells are allowed to adhere to the surface or inside thereof. A
5 known example of the scaffold is a film of polyvinylidene difluoride (PVDF). The sheet-like cell culture product according to the present invention may be one which remains physically integral without such scaffold. Also, the sheet-like cell culture product should preferably be
10 one which is composed merely of a substance derived from cells constituting the cell culture product and free from other substances.

[0038]

The producing method according to the present
15 invention includes a step of thawing cells which have been cryopreserved by the recovery method according to the present invention, and recovering thawed cells, and a step of seeding thus recovered cells, thereby forming the sheet-like cell culture product.

20 The producing method according to the present invention may further includes a step of cleaning cells after the step of thawing and recovering cryopreserved cells and before the step of forming the sheet-like cell culture product. The washing of cells may be performed in

any known way. Typically, with no restrictions, it is performed by suspending cells in a fluid, centrifuging the suspension, discarding the supernatant, and recovering precipitated cells. The solution for suspension is a culture solution (or culture medium) or physiological buffer solution (e.g., PBS and Hank's balanced salt solution (HBSS)), which may or may not contain a cleaning liquid, such as serum or serum component (e.g., serum albumin). The step of cleaning cells may be performed by repeating suspension, centrifuging, and recovery once or several times (two, three, four, five times, etc.). According to one embodiment of the present invention, the step of cleaning cells is performed immediately after the step of thawing frozen cells.

[0039]

The producing method according to the present invention includes the step of forming the sheet-like cell culture product, which may be performed by any known technique. Some examples of such techniques are described in Patent Documents 1 to 3. The step of forming the sheet-like cell culture product may be carried on a culture substrate. The step of forming the sheet-like cell culture product may include additional steps of

seeding cells on the culture substrate and making the seeded cells into a sheet. In its one embodiment, the producing method according to the present invention does not include the step of proliferating cells between the
5 step of thawing and recovering cells and the step of forming the sheet-like cell culture product.

[0040]

The culture substrate is not specifically restricted so long as it permits cells to form thereon
10 the cell culture product. It includes, for example, containers of various materials and a solid or semisolid surface in the container. The container is not specifically restricted so long as it is made of a structure and a material impervious to such liquid as
15 culture solution. Examples of such a material include polyethylene, polypropylene, Teflon (registered trademark), polyethylene terephthalate, polymethyl methacrylate, nylon-6,6, polyvinyl alcohol, cellulose, silicone, poly-styrene, glass, polyacrylamide,
20 polydimethyl acrylamide, and metal (such as iron, stainless steel, aluminum, copper, and brass). The container should preferably be one which has at least one flat surface. Examples of such a container, which are not specifically restricted, include cell culture dishes and

cell culture bottles. The container may have a solid or semisolid surface therein. Examples of the solid surface include various kinds of plates and containers mentioned above. Examples of the semisolid surface include soft
5 polymer matrices made of gel. The culture substrate may be prepared from the above-mentioned materials or acquired from commercial sources. Preferable culture substrate is not specifically restricted so long as it has an adhesive surface that permits the sheet-like cell
10 culture product to be formed thereon. Typical examples include: a substrate with a hydrophilic surface, such as polystyrene with corona discharge treatment; a substrate which is surface-coated with a hydrophilic compound, such as collagen gel and hydrophilic polymer; a substrate
15 which is surface-coated with an extracellular matrix, such as collagen, fibronectin, laminin, vitronectin, proteoglycan, and glycosaminoglycan, or a cell adhesion factor, such as cadherin family, selectin family, and integrin family. Such substrates are commercially
20 available (e.g., Corning^(R) TC-Treated Culture Dish from Corning).

[0041]

The substrate of the culture substrate may be one which has its surface coated with a various kind of

material which imparts desired properties and characteristics. Known examples of the coating material include polymer, serum, growth factor, and steroid. They may be properly selected by those skilled in the art according to the desired properties and characteristics for the culture substrate. For example, if the substrate surface is required to change in hydrophilicity or hydrophobicity depending on temperature, the coating material should preferably be selected from the following: (meth)acrylamide compounds and N-alkyl-substituted (meth)-acrylamide derivatives (such as N-ethylacrylamide, N-n-propylacrylamide, N-n-propylmethacrylamide, N-isopropylacrylamide, N-isopropylmethacrylamide, N-cyclopropylacrylamide, N-cyclopropylmethacrylamide, N-ethoxyethylacrylamide, N-ethoxyethylmethacrylamide, N-tetrahydro-furfuryl acrylamide, and N-tetrahydro-furfuryl methacrylamide); N,N-dialkyl-substituted (meth)acrylamide derivatives (such as N,N-dimethyl(meth)acrylamide, N,N-ethylmethylacrylamide and N,N-diethylacrylamide); (meth)acrylamide derivatives having cyclic groups (such as 1-(1-oxo-2-propenyl)-pyrrolidine, 1-(1-oxo-2-propenyl)-piperidine, 4-(1-oxo-2-propenyl)-morpholine, 1-(1-oxo-2-methyl-2-propenyl)-pyrrolidine, 1-(1-oxo-2-methyl-2-propenyl)-

piperidine, and 4-(1-oxo-2-methyl-2-propenyl)-morpholine); and temperature-responsive material composed of vinyl ether derivatives (such as homopolymer or copolymer of methyl vinyl ether). There are commercial products of culture dishes coated with a temperature-responsive material (such as UpCell^(R) from CellSeed Inc.). They may be used for the producing method according to the present invention.

[0042]

10 The culture substrate may be coated with serum so that it helps form a sheet-like cell culture product which is densely composed of cells. The term "serum coating" means that the culture substrate is in such a state that its surface bears serum components. This state is not restricted; it may be realized by treating with serum the culture substrate. The treatment with serum may be accomplished by keeping the culture substrate in contact with serum, followed by optional incubation for a prescribed period of time. The serum for coating may be that of the same or different species derived from the seeded cells. The former is preferable. The most desirable serum is autologous serum obtained from individuals derived from seeded cells.

[0043]

The seeding of cells onto the culture substrate may be accomplished by means of any known technique under any conditions. One way of seeding of cells onto the culture substrate is by injection of a cell suspension in which
5 cells are suspending in a culture solution into the culture substrate (or culture container). Injection of cell suspension may be performed by using a dropper or pipette, which is suitable for injection of cell suspension.

10 [0044]

According to a preferred embodiment of the present invention, seeding should be performed with an adequate cell density in such a way that seeded cells form a sheet-like cell culture product without substantial
15 proliferation. The term "adequate cell density in such a way that seeded cells form a sheet-like cell culture product without substantial proliferation" means that the seeded cells form the sheet-like cell culture product when they are cultured in a non-proliferating culture
20 solution which is substantially free of growth factor. For example, it is necessary for skeletal muscle blast cells to be seeded onto the culture substrate at a density of about 6,500 cells/cm² if the seeded cells are to form a sheet-like cell culture product by using a

culture solution containing a growth factor (see Patent Document 1, for example). The cells seeded at such a cell density do not form any sheet-like cell culture product as desired even though they are cultured in a culture solution free of growth factor. The cell density for seeding in the embodiment of the present invention is higher than that which is used for the culture solution containing growth factor. Specifically, such a cell density is typically equal to or higher than approximately 1.0×10^5 cells/cm² for skeletal muscle blast cells. The upper limit of the cell density for seeding is lower than approximately 3.4×10^6 cells/cm² for skeletal muscle blast cells. It is not specifically restricted so long as the seeded cells form the sheet-like cell culture product smoothly without differentiation.

[0045]

The step of making the seeded cells into a sheet may also be accomplished by any known technique under any conditions. Some of such known techniques are found in Patent Documents 1 to 3, for example. Presumably, conversion of cells into a sheet takes places adhesion between cells through adhesive molecules or extracellular matrices which function as the cell-to-cell adhesion

mechanism. Consequently, the step of making the seeded cells into a sheet can be achieved by culturing cells under the condition which permits cells to adhere to one another.

5 [0046]

The producing method according to the present invention may employ any culture solution, which is not specifically restricted so long as it keeps cells alive. Typical examples of the culture solution include amino
10 acids, vitamins, and those which are composed mainly of electrolytes. According to one embodiment of the present invention, the culture solution is one which is based on the minimal essential medium for cell culture. Its examples unrestrictedly include DMEM, minimum essential
15 medium (MEM), F12, DMEM/F12, DME, Roswell park memorial institute (RPMI) 1640, molecular, cellular and developmental biology (MCDB) (MCDB102, 104, 107, 120, 131, 153, and 199), Leibovitz 15 (L15), skeletal muscle cell basal medium (SkBM), and RITC80-7. Most of them are
20 commercially available and their compositions are known. When they are employed for the producing method of the present invention, their composition may be properly altered according to the species and conditions of cells.
[0047]

In one embodiment of the producing method according to the present invention, the step of freezing cells may be followed by the step of forming the sheet-like cell culture product without substantial cell proliferation.

5 This procedure helps further increase the activity of the sheet-like cell culture product.

The term "without substantial cell proliferation" means a situation in which cells do not proliferate beyond the limit of measurement errors. The cell proliferation may be evaluated from difference between
10 the number of cells counted at the time of seeding and the number of cells counted after the sheet-like cell culture product has been formed. According to the present invention, the number of cells counted after the
15 formation of the sheet-like cell culture product should typically be equal to or less than approximately 300% of the cell counted at the time of seeding, preferably equal to or less than approximately 200%, more preferably equal to or less than approximately 150%, much more preferably
20 equal to or less than approximately 125%, and most desirably equal to or less than about 100%.

[0048]

Since the proliferation of cells depends on various conditions, such as the number of cells seeded (or the

density of cells seeded), the environment of culture (or the culture period and culture temperature), and the composition of the culture medium, it is possible to substantially prevent the proliferation of cells by

5 controlling the above-mentioned conditions. If the density of cells seeded is increased, it is possible to obtain the sheet-like cell culture product within a comparatively short period of time while preventing the proliferation of cells. Consequently, according to the

10 present invention, it is desirable to control the proliferation of cells by adjusting the density of cells seeded among the above-mentioned conditions. The foregoing has described the density of cells which permits the sheet-like cell culture product to be formed

15 without substantial proliferation of cells. Therefore, according to one preferred embodiment, the step of thawing cells is followed by the step of making the thawed cells into a sheet, with the step of cell proliferation skipped, under the condition which

20 substantially prevents the cell proliferation.

[0049]

Another aspect of the present invention relates to a kit used to produce the above-mentioned sheet-like cell culture product. The kit partly or entirely includes the

components for production of the sheet-like cell culture product which does not experience the step of cell proliferation.

5 The kit according to the present invention is not specifically restricted; it may be composed of cells which form the sheet-like cell culture product, culture solution, culture dish, instruments, and instructions for the method of producing the sheet-like cell culture product. The cells may be cryopreserved cells or cells
10 recovered by the recovery method according to the present invention. The instruments include pipette, dropper, tweezers, etc. The instructions include user's manual and any medium which contains information about the method for production and the method for recovery of
15 cryopreserved cells defined in the present invention. The medium includes flexible disk, compact disc (CD), digital versatile disc (DVD), Blu-ray disc, memory card, universal serial bus (USB) memory, etc.

[0050]

20 The present invention will be described in more detail with reference to the embodiments thereof, with no intention to limit the scope thereof.

EXAMPLES

[0051]

Example 1. Interrelation between the rate of addition of
diluent and the survival ratio of cells

The following procedure was carried out to thaw
5 cryopreserved cells and recover living cells. First, a
cryotube (1.8 mL) containing skeletal muscle blast cells,
which had been cryopreserved therein, was placed in a
water bath kept at 37°C for three to four minutes, so
that the cryopreserved cells were thawed. The resulting
10 suspension of the thawed cells was transferred from the
cryotube to a conical tube (225 mL). The cryotube was
rinsed with a cleaning liquid (1 mL) composed of HBSS and
albumin, for recovery of cells remaining in the cryotube.
The resulting rinsing liquid was added to the cell
15 suspension at a different rate of addition. The cleaning
liquid (30 mL) was added to the conical tube at a
different rate of addition in the same way as the above-
mentioned rinsing liquid. The content of the conical tube
was centrifuged at 4°C for seven minutes with a
20 centrifugal force of $240 \times g$, and the supernatant was
discarded. The cleaning liquid (30 mL) was added again,
followed by centrifugation at 4°C for seven minutes with
a centrifugal force of $240 \times g$, and the supernatant was
discarded. The cleaning liquid (5 mL) was added to give

the cell suspension as desired.

A portion of the thus obtained cell suspension was extracted and mixed with trypan blue. The number of cells was counted. The result of the cell count was used to

5 calculate the number of living cells remaining after thawing. The survival ratio of living cells was calculated according to the following formula.

Cell survival ratio (%) = Number of living cells remaining after thawing/Number of total cells counted

10 after thawing $\times 100$

[0052]

The rinsing liquid was added at a rate equal to or greater than 1.2 mL/minute, 4.0 mL/minute, 15.0 mL/minute, and 360 mL/minute. The cleaning liquid was added at a

15 rate four times as large as that of the rinsing liquid in the case where it was added dropwise. The cleaning liquid was added at a rate twice as large as that of the rinsing liquid in the case where it was added non-dropwise.

The maximum load of osmotic pressure was calculated

20 according to the following formula. Incidentally, the initial osmotic pressure of the cell suspension was assumed to be 1400 mOsm, and the osmotic pressure of the diluent was assumed to be 300 mOsm.

[Formula 1]

Maximum load of osmotic pressure (mOsm/second) =

$$\left[1400 \text{ mOsm} - \frac{1400 \text{ mOsm} \times \text{liquid amount (mL)} + 300 \text{ mOsm} \times \text{rate of addition (mL/second)} \times 1 \text{ second}}{\text{liquid amount (mL)} + \text{rate of addition (mL/ second)} \times 1 \text{ second}} \right] \\ / 1 \text{ second}$$

The results are shown in the table below.

- 5 Incidentally, in the table, the amount at the time of start of dilution indicates the amount of cell suspension at the time of start of dilution.

[0053]

[Table 1]

Condition	Amount at start of dilution (mL)	Diluent	Rate of addition (mL/minute)	Maximum load of osmotic pressure (mOsm/second)	Cell survival ratio (%)
Dropping 1	1.0	Rinsing liquid	0.2	ca. 4	91.0 $\pm$ 3.6
		Cleaning liquid	0.8		
Dropping 2	1.0	Rinsing liquid	0.67	ca. 12	90.9 $\pm$ 2.2
		Cleaning liquid	2.67		
Dropping 3	1.0	Rinsing liquid	2.5	ca. 44	91.5 $\pm$ 1.4
		Cleaning liquid	10.0		
Dropping 4	1.0	Rinsing liquid	15.0	ca. 220	90.0 $\pm$ 2.3
		Cleaning liquid	60.0		
Dropping 5	0.2	Rinsing liquid	15.0	ca. 610	83.0 $\pm$ 1.0
No-dropping	1.0	Rinsing liquid	60 or more	ca. 550	87.1 $\pm$ 0.9
		Cleaning liquid	120 or more		

[0054]

In the case of dropping patterns 1 to 4, the ratio of cell survival was greater than 90% on average. By contrast, it was approximately 87% in the case of no-dropping. In the case where dropping is combined with dilution, the maximum load of osmotic pressure was approximately 610 mOsm/second under the condition (Dropping 5) in which the amount of cell suspension was decreased. In this case, the ratio of cell survival was approximately 83% on average. The highest ratio of cell

survival was obtained in the case of Dropping 3 where the maximum load of osmotic pressure was approximately 44 mOsm/second among Droppings.

Claims

1. A method for recovering living cells from cryopreserved cells, comprising
thawing cryopreserved cells; and
5 diluting the thawed cell suspension with a diluent,
wherein the dilution is performed in such a way
that the maximum load of osmotic pressure at the time of
dilution is equal to or lower than 250 mOsm/second.
2. The method as defined in claim 1, wherein the
10 diluent is added in such a way that the maximum load of
osmotic pressure at the time of dilution is equal to or
lower than 50 mOsm/second.
3. The method as defined in claim 1 or 2, wherein
the diluent is added in such a way that the maximum load
15 of osmotic pressure at the time of dilution ranges from
40 mOsm/second to 50 mOsm/second.
4. The method as defined in any one of claims 1 to
3, wherein the cells are skeletal muscle blast cells.
5. The method as defined in any one of claims 1 to
20 4, wherein the diluent contains rinsing liquid resulting
from rinsing of a container for cryopreservation from
which the thawed cell suspension has been transferred to
another container.
6. A system for thawing cryopreserved cells,

comprising:

(i) an actuating unit configured to inject a
diluent; and

(ii) an arithmetic control unit configured to
5 determine the rate at which the actuating unit injects
the diluent.

7. The system as defined in claim 6, further
comprising

(iii) a measuring unit configured to measure
10 osmotic pressure of liquid.

8. A kit comprising:

cells; and

a culture substrate used for a method of producing
a sheet-like cell culture product without necessity for
15 proliferation culture, the method including a step of
seeding at a density suitable to form the sheet-like cell
culture product without substantial cell proliferation,

wherein cells are those which have been recovered
by any one method defined in claims 1 to 5 above.