Non-patent Document 1: Gage, F.H. Science 287: 1433-1438 (2000)

Non-patent Document 2: Morrison, S.J. et al., Cell 96: 737-749 (1999)

Non-patent Document 3: Batle, E. et al., Cell 111: 251-263 (2002)

Non-patent Document 4: Chem, F. et al., Yonsei Med. J. 45: 1107-1114 (2004)

Summary ~~of the Invention~~

Object to be Solved by the ~~Invention~~Disclosure

[0007]

An object of the present ~~invention~~disclosure is to provide a method of washing an adherent cell, capable of effectively suppressing cell death due to proteolytic enzyme treatment for detaching the adherent cell from a culture vessel and subsequent cell treatment; a cell-washing solution used for the washing method; a method of producing a cell suspension for transplantation using the cell-washing solution; and a kit comprising the cell-washing solution.

Means to Solve the Object

[0008]

The present inventors found that the washing of mesenchymal stem cell detached from a cell culture vessel by proteolytic enzyme treatment with a trehalose-containing physiological aqueous solution suppressed the death of the mesenchymal stem cells (Japanese Patent Application No. 2011-072068). Because the damage of cells by proteolytic enzyme treatment induced a pathway of cell death (apoptosis or the like), such an effect by trehalose

was considered to be due to the suppression of an apoptosis pathway or the promotion of the function repairing the cell damage. However, in intensive studies for solving the above problems, it has been found that when cells happened to be washed with a trehalose-containing lactated Ringer's solution (hereinafter abbreviated as "LRT") before proteolytic enzyme treatment, cell death could be effectively suppressed compared to the case of using lactated Ringer's solution (hereinafter abbreviated as "LR") or phosphate buffered saline (PBS) as a control or the case of washing cells with LRT after proteolytic enzyme treatment, thereby accomplishing the present invention.

In one aspect, there is provided a method of washing an adherent cell, comprising washing the adherent cell using a cell-washing solution which is a physiological aqueous solution comprising trehalose or its derivative or a salt thereof, before detaching the adherent cell in a culture vessel from the culture vessel by proteolytic enzyme treatment.

[0009]

Specifically, the present ~~invention~~disclosure relates to (1) a method of washing an adherent cell, comprising washing the adherent cell using a cell-washing solution comprising a physiological aqueous solution added with trehalose or its derivative or a salt thereof, before detaching the adherent cell from a culture vessel by proteolytic enzyme treatment, (2) the method according to (1), wherein the adherent cell is a mesenchymal stem cell, and (3) the method according to (1) or (2), wherein the cell-washing

solution comprises trehalose or its derivative or a salt thereof in a concentration of 1 to 15 (w/v)%.

In one aspect, there is provided a cell-washing solution when used to wash an adherent cell before detaching the adherent cell in a culture vessel from the culture vessel by proteolytic enzyme treatment, wherein the cell-washing solution is a physiological aqueous solution comprising trehalose or its derivative or a salt thereof.

[0010]

The present ~~invention~~disclosure also relates to (4) a cell-washing solution for washing an adherent cell before detaching the adherent cell from a culture vessel by proteolytic enzyme treatment, comprising a physiological

aqueous solution added with trehalose or its derivative or a salt thereof, (5) the cell-washing solution according to (4), wherein the adherent cell is a mesenchymal stem cell, and (6) the cell-washing solution according to (4) or (5), comprising trehalose or its derivative or a salt thereof in a concentration of 1 to 15 (w/v)%.

In one aspect, there is provided a method of producing a cell suspension for transplantation, comprising the steps of:

(a) removing a medium in a culture vessel to which an adherent cell is adhered and washing the adherent cell with a cell-washing solution which is a physiological aqueous solution comprising trehalose or its derivative or a salt thereof;

(b) detaching the adherent cell in the culture vessel from the culture vessel by proteolytic enzyme treatment;

(c) suspending the adherent cell detached from the culture vessel in a protein-containing physiological aqueous solution to make a cell suspension;

and

(d) removing the solution in the cell suspension and washing the adherent cell with the physiological aqueous solution.

[0011]

The present ~~invention~~disclosure also relates to (7) a method of producing a cell suspension for transplantation, comprising the steps of (a) removing a medium in a culture vessel to which an adherent cell is adhered and washing

the adherent cell with a cell-washing solution comprising a physiological aqueous solution added with trehalose or its derivative or a salt thereof; (b) detaching the adherent cell from the culture vessel by proteolytic enzyme treatment; (c) suspending the adherent cell detached from the culture vessel in a protein-containing physiological aqueous solution to make a cell suspension; and (d) removing the solution in the cell suspension and washing the adherent cell with the physiological aqueous solution, (8) the production method according to (7), wherein the adherent cell is a mesenchymal stem cell, (9) the production method according to (7) or (8), wherein the cell-washing solution of the step (a) comprises trehalose or its derivative or a salt thereof in a concentration of 1 to 15 (w/v)%, (10) the production method according to any one of (7) to (9), wherein the proteolytic enzyme of the step (b) is trypsin, (11) the production method according to any one of (7) to (10), wherein the protein-containing physiological aqueous solution of the step (c)

further comprises trehalose or its derivative or a salt thereof, (12) the production method according to any one of (7) to (11), wherein the protein-containing physiological aqueous solution of the step (c) is a serum-containing basal medium for animal cell culture, and (13) the production method according to any one of (7) to (12), wherein the physiological aqueous solution of the step (d) further comprises trehalose or its derivative or a salt thereof.

[0012]

The present ~~invention~~disclosure also relates to (14) a kit for producing a cell suspension for transplantation, comprising the cell-washing solution according to any one of (4) to (6), and a package insert describing an effect of suppressing cell death due to proteolytic enzyme treatment by the cell-washing solution.

[0013]

In one aspect, there is provided a trehalose or its derivative or a salt thereof when used to wash an adherent cell in a culture vessel before detaching the adherent cell from the culture vessel by proteolytic enzyme treatment.

The present ~~invention~~disclosure also relates to (15) use of trehalose or its derivative or a salt thereof in the production of a cell-washing solution for washing an adherent cell before detaching the adherent cell from a culture vessel by proteolytic enzyme treatment.

[0014]

The present ~~invention~~disclosure further relates to (16)

trehalose or its derivative or a salt thereof for use to wash an adherent cell before detaching the adherent cell from a culture vessel by proteolytic enzyme treatment.

[0015]

Other embodiments of the present ~~invention~~disclosure can include a combination of a physiological aqueous solution

and trehalose or its derivative or a salt thereof for washing an adherent cell before detaching the adherent cell from a culture vessel by proteolytic enzyme treatment, and trehalose or its derivative or a salt thereof for use by adding to a physiological aqueous solution to wash an adherent cell before detaching the adherent cell from a culture vessel by proteolytic enzyme treatment. In addition, other embodiments of the present ~~invention~~disclosure can include an in vitro subculture method for an adherent cell, comprising washing the adherent cell by using a physiological aqueous solution comprising trehalose or its derivative or a salt thereof, and a kit for in vitro subculture of an adherent cell, comprising trehalose or its derivative or a salt thereof and a package insert describing an effect of suppressing cell death due to proteolytic enzyme treatment.

In one aspect, there is provided an *in vitro* subculture method for subculturing an adherent cell, comprising the following sequential steps (a) to (d):

(a) removing a medium in a culture vessel to which an adherent cell is adhered and washing the adherent cell with a cell-washing solution which is a physiological aqueous solution comprising trehalose or a derivative or a salt thereof;

(b) detaching the adherent cell from the culture vessel by proteolytic enzyme treatment;

(c) suspending the adherent cell detached from the culture vessel in a protein-containing physiological aqueous solution to stop the proteolytic enzyme treatment; and

(d) subculturing a portion of the cell suspension using a fresh culture vessel.

Effect of the Invention

[0016]

According to the present invention, the rate of dead cells can be decreased in a cell suspension for transplantation containing stem cells such as mesenchymal stem cells, and a good-quality cell suspension for transplantation in regenerative medicine can be provided.

Brief Description of Drawings

[0017] [Figure 1] Figure 1 is a graph showing the results of analyzing the effect of suppressing cell death when adherent cells were washed using LRT before trypsin treatment (in the figure, "washing solution before enzyme

CLAIMS

1. A method of washing an adherent cell, comprising washing the adherent cell using a cell-washing solution comprising a physiological aqueous solution added with trehalose or its derivative or a salt thereof, before detaching the adherent cell from a culture vessel by proteolytic enzyme treatment.

2. The method according to claim 1, wherein the adherent cell is a mesenchymal stem cell.

3. The method according to claim 1 or 2, wherein the cell-washing solution comprises trehalose or its derivative or a salt thereof in a concentration of 1 to 15 (w/v)%.

4. A cell-washing solution for washing an adherent cell before detaching the adherent cell from a culture vessel by proteolytic enzyme treatment, comprising a physiological aqueous solution added with trehalose or its derivative or a salt thereof.

5. The cell-washing solution according to claim 4, wherein the adherent cell is a mesenchymal stem cell.

6. The cell-washing solution according to claim 4 or 5, comprising trehalose or its derivative or a salt thereof in a concentration of 1 to 15 (w/v)%.

7. A method of producing a cell suspension for transplantation, comprising the steps of:

(a) removing a medium in a culture vessel to which an adherent cell is adhered and washing the adherent cell with a cell-washing solution comprising a physiological aqueous solution added with trehalose or its derivative or a salt thereof;

(b) detaching the adherent cell from the culture vessel by proteolytic enzyme treatment;

(c) suspending the adherent cell detached from the culture vessel in a protein-containing physiological aqueous solution to make a cell suspension; and

(d) removing the solution in the cell suspension and washing the adherent cell with the physiological aqueous solution.

8. The production method according to claim 7, wherein the adherent cell is a mesenchymal stem cell.

9. The production method according to claim 7 or 8, wherein the cell-washing solution of the step (a) comprises trehalose or its derivative or a salt thereof in a concentration of 1 to 15 (w/v)%.

10. The production method according to any one of claims 7 to 9, wherein the proteolytic enzyme of the step (b) is trypsin.

11. The production method according to any one of claims 7 to 10, wherein the protein-containing physiological

aqueous solution of the step (c) further comprises trehalose or its derivative or a salt thereof.

12. The production method according to any one of claims 7 to 11, wherein the protein-containing physiological aqueous solution of the step (c) is a serum-containing basal medium for animal cell culture.

13. The production method according to any one of claims 7 to 12, wherein the physiological aqueous solution of the step (d) further comprises trehalose or its derivative or a salt thereof.

14. A kit for producing a cell suspension for transplantation, comprising the cell-washing solution according to any one of claims 4 to 6 and a package insert describing an effect of suppressing cell death due to proteolytic enzyme treatment by the cell-washing solution.

15. Use of trehalose or its derivative or a salt thereof in the production of a cell-washing solution for washing an adherent cell before detaching the adherent cell from a culture vessel by proteolytic enzyme treatment.

16. Trehalose or its derivative or a salt thereof for use to wash an adherent cell before detaching the adherent cell from a culture vessel by proteolytic enzyme treatment.

17. An in vitro subculture method for an adherent cell, sequentially comprising the following steps (a) to (d):

(a) removing a medium in a culture vessel to which an adherent cell is adhered and washing the adherent cell with a cell-washing solution comprising a physiological aqueous solution added with trehalose or its derivative or a salt thereof;

(b) detaching the adherent cell from the culture vessel by proteolytic enzyme treatment;

(c) suspending the adherent cell detached from the culture vessel in a protein-containing physiological aqueous solution to stop the proteolytic enzyme treatment; and

(d) subculturing a portion of the cell suspension using a fresh culture vessel.

18. The in vitro subculture method according to claim 17, wherein the adherent cell is a mesenchymal stem cell.

19. The in vitro subculture method according to claim 17 or 18, wherein the cell-washing solution of the step (a) comprises trehalose or its derivative or a salt thereof in a concentration of 1 to 15 (w/v)%.

20. The in vitro subculture method according to any one of claims 17 to 19, wherein the proteolytic enzyme of the step (b) is trypsin.

21. The in vitro subculture method according to any one of claims 17 to 20, wherein the protein-containing physiological aqueous solution of the step (c) further comprises trehalose or its derivative or a salt thereof.

22. The in vitro subculture method according to any one of claims 17 to 21, wherein the protein-containing physiological aqueous solution of the step (c) is a serum-containing basal medium for animal cell culture.

23. The in vitro subculture method according to any one of claims 17 to 22, further comprising the step of (p) removing the solution in the cell suspension and washing the adherent cells with a physiological aqueous solution or protein-containing physiological aqueous solution, between the steps (c) and (d).

24. The in vitro subculture method according to claim 23, wherein the physiological aqueous solution or protein-containing physiological aqueous solution of the step (p) further comprises trehalose or its derivative or a salt thereof.

25. A kit for in vitro subculture of an adherent cell, comprising:
a cell-washing solution for washing an adherent cell before detaching the adherent cell from a culture vessel by proteolytic enzyme treatment, comprising a physiological aqueous solution added with trehalose or its derivative or a salt thereof; and
a package insert describing an effect of suppressing cell death due to proteolytic enzyme treatment.

CLAIMS

1. A method of washing an adherent cell, comprising washing the adherent cell using a cell-washing solution which is a physiological aqueous solution comprising trehalose or its derivative or a salt thereof, before detaching the adherent cell in a culture vessel from the culture vessel by proteolytic enzyme treatment.

2. The method according to claim 1, wherein the adherent cell is a mesenchymal stem cell.

3. The method according to claim 1 or 2, wherein the cell-washing solution comprises trehalose or its derivative or a salt thereof in a concentration of 1 to 15 (w/v)%.

4. A cell-washing solution when used to wash an adherent cell before detaching the adherent cell in a culture vessel from the culture vessel by proteolytic enzyme treatment, wherein the cell-washing solution is a physiological aqueous solution comprising trehalose or its derivative or a salt thereof.

5. The cell-washing solution according to claim 4, wherein the adherent cell is a mesenchymal stem cell.

6. The cell-washing solution according to claim 4 or 5, comprising trehalose or its derivative or a salt thereof in a concentration of 1 to 15 (w/v)%.

7. A method of producing a cell suspension for transplantation, comprising the steps of:

(a) removing a medium in a culture vessel to which an adherent cell is adhered and washing the adherent cell with a cell-washing solution which is a physiological aqueous solution comprising trehalose or its derivative or a salt thereof;

(b) detaching the adherent cell in the culture vessel from the culture vessel by proteolytic enzyme treatment;

(c) suspending the adherent cell detached from the culture vessel in a protein-containing physiological aqueous solution to make a cell suspension; and

(d) removing the solution in the cell suspension and washing the adherent cell with the physiological aqueous solution.

8. The production method according to claim 7, wherein the adherent cell is a mesenchymal stem cell.

9. The production method according to claim 7 or 8, wherein the cell-washing solution of the step (a) comprises trehalose or its derivative or a salt thereof in a concentration of 1 to 15 (w/v)%.

10. The production method according to any one of claims 7 to 9, wherein the proteolytic enzyme of the step (b) is trypsin.

11. The production method according to any one of claims 7 to 10, wherein the protein-containing physiological aqueous solution of the step (c) further comprises trehalose or its derivative or a salt thereof.

12. The production method according to any one of claims 7 to 11, wherein the protein-containing physiological aqueous solution of the step (c) is a serum-containing basal medium for animal cell culture.

13. The production method according to any one of claims 7 to 12, wherein the physiological aqueous solution of the step (d) further comprises trehalose or its derivative or a salt thereof.

14. Trehalose or its derivative or a salt thereof when used to wash an adherent cell in a culture vessel before detaching the adherent cell from the culture vessel by proteolytic enzyme treatment.

15. An *in vitro* subculture method for subculturing an adherent cell, comprising the following sequential steps (a) to (d):

(a) removing a medium in a culture vessel to which an adherent cell is adhered and washing the adherent cell with a cell-washing solution which is a physiological aqueous solution comprising trehalose or a derivative or a salt thereof;

(b) detaching the adherent cell from the culture vessel by proteolytic enzyme treatment;

(c) suspending the adherent cell detached from the culture vessel in a protein-containing physiological aqueous solution to stop the proteolytic enzyme treatment; and

(d) subculturing a portion of the cell suspension using a fresh culture vessel.

16. The *in vitro* subculture method according to claim 15, wherein the adherent cell is a mesenchymal stem cell.

17. The *in vitro* subculture method according to claim 15 or 16, wherein the cell-washing solution of the step (a) comprises trehalose or its derivative or a salt thereof in a concentration of 1 to 15 (w/v)%.

18. The *in vitro* subculture method according to any one of claims 15 to 17, wherein the proteolytic enzyme of the step (b) is trypsin.

19. The *in vitro* subculture method according to any one of claims 15 to 18, wherein the protein-containing physiological aqueous solution of the step (c) further comprises trehalose or its derivative or a salt thereof.

20. The *in vitro* subculture method according to any one of claims 15 to 19, wherein the protein-containing physiological aqueous solution of the step (c) is a serum-containing basal medium for animal cell culture.

21. The *in vitro* subculture method according to any one of claims 15 to 20, further comprising between steps (c) and (d) the step of (c)(i) comprising removing the solution in the cell suspension and washing the adherent cells with a physiological aqueous solution or protein-containing physiological aqueous solution.

22. The *in vitro* subculture method according to claim 21, wherein the physiological aqueous solution or protein-containing physiological aqueous solution of the step (c)(i) further comprises trehalose or its derivative or a salt thereof.