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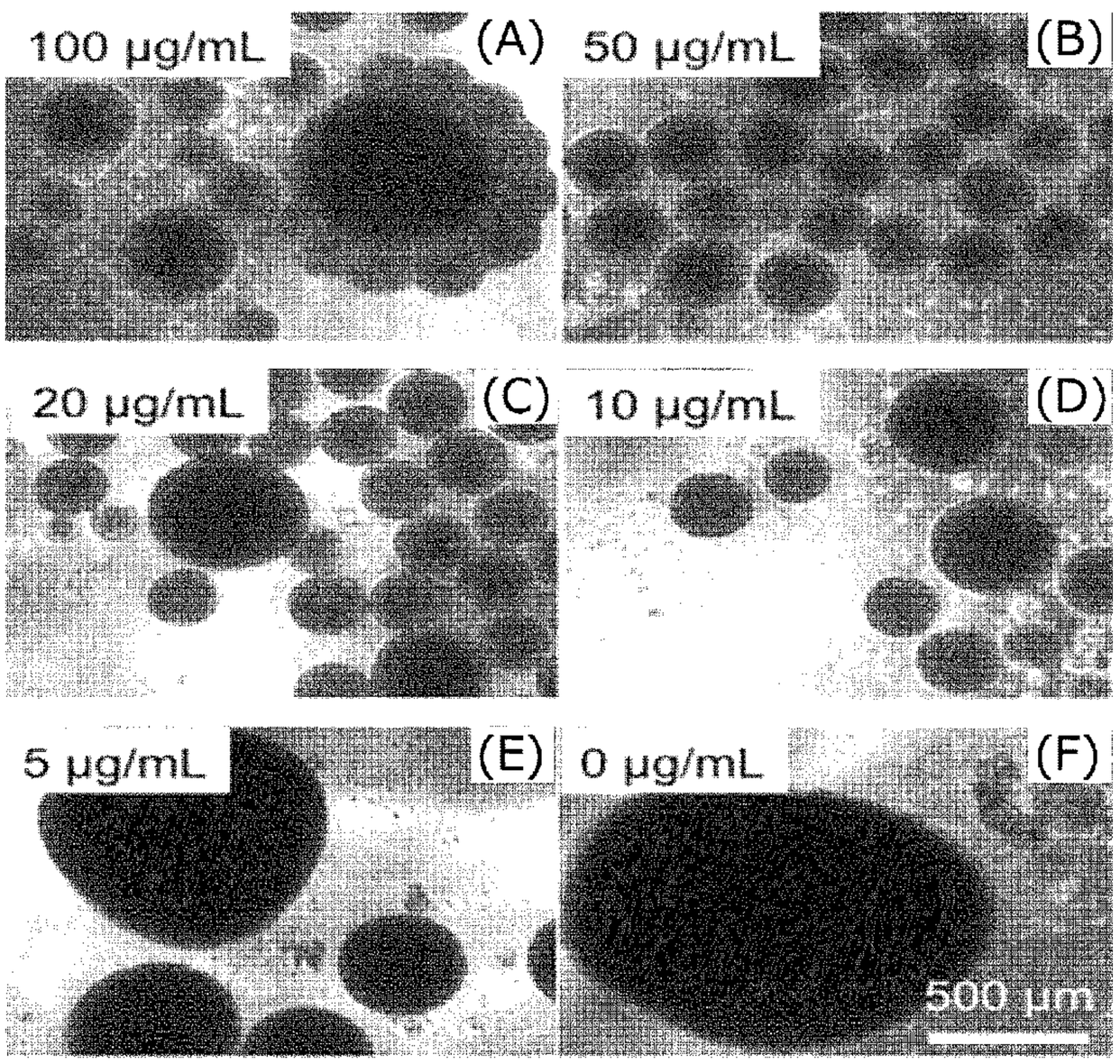
(54) Title: CELL CULTURE METHOD, CELL AGGREGATES, CELL AGGREGATION CONTROL AGENT, AND MEDIUM



(57) Abrégé/Abstract:
Provided are a cell culture method that makes it possible to control the diameter of aggregated cell masses and to obtain cells in large quantities, cell aggregates obtained by this method, a cell aggregation control agent, and a medium containing this cell aggregation control agent. A method for culturing cells using suspension culturing wherein the culturing method includes an aggregation control step for adding to the medium a substance that inhibits the cell adhesion molecules of these cells and for controlling the cell aggregation of these cells.

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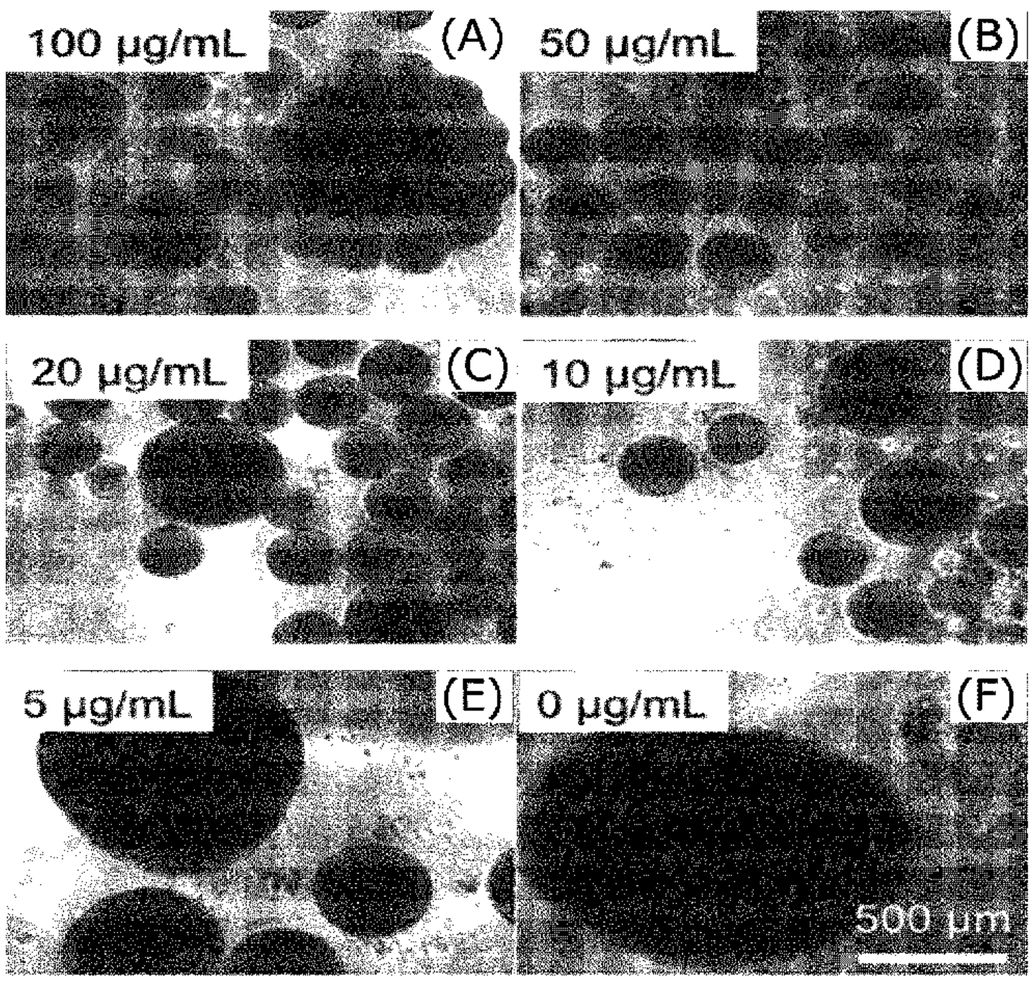
(54) **Title**: CELL CULTURE METHOD, CELL AGGREGATES, CELL AGGREGATION CONTROL AGENT, AND MEDIUM

(54) 発明の名称 : 細胞の培養方法、細胞の凝集体、細胞凝集制御剤、及び、培地



(57) **Abstract**: Provided are a cell culture method that makes it possible to control the diameter of aggregated cell masses and to obtain cells in large quantities, cell aggregates obtained by this method, a cell aggregation control agent, and a medium containing this cell aggregation control agent. A method for culturing cells using suspension culturing wherein the culturing method includes an aggregation control step for adding to the medium a substance that inhibits the cell adhesion molecules of these cells and for controlling the cell aggregation of these cells.

(57) 要約:　細胞の凝集塊の径を制御すること、及び、細胞を大量に得ることが可能な細胞の培養方法、該方法により得られる細胞の凝集体、細胞凝集制御剤、及び、該細胞凝集制御剤を含有する培地を提供する。浮遊培養による細胞の培養方法であって、前記細胞の細胞接着分子を阻害する物質を培地中に添加し、前記細胞の細胞凝集を制御する凝集制御工程を含む培養方法等である。

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CELL CULTURE METHOD, CELL AGGREGATES, CELL AGGREGATION CONTROL AGENT, AND MEDIUM

TECHNICAL FIELD

The present invention relates to a method for culturing cells, a cell aggregate, a cell aggregation control agent, and a medium, more specifically, to a method for culturing cells by which the diameter of cell aggregates can be controlled, and by which a large amount of cells can be obtained, a cell aggregate obtained by the method, a cell aggregation control agent, and a medium containing the cell aggregation control agent.

BACKGROUND ART

Human liver is usually constituted by about 250 billion cells. Based on the assumption that about 10% of the original liver is required for producing a liver tissue using pluripotent stem cells, the number of cells required is about 25 billion. Since tests in drug trials require cells having a uniform quality (hereinafter also referred to as "homogeneous" cells), a technique that enables culture of a large amount of uniform pluripotent stem cells is an indispensable process for industrial application and development. With such a background, suspension culture methods have been proposed since they enable simple high-density culture for the purpose of stably supplying a large amount of pluripotent stem cells. This culture method is a culture method which is expected to enable production of cells in a number that allows their application to regenerative medicine and the like, and this method is advantageous from the viewpoint of the facility and the cost.

However, since pluripotent stem cells easily form aggregates, various aggregates such as huge aggregates and aggregates having uneven diameters are formed. There is also a problem that such aggregates show low cell survival rates after subculture. In view of this, three-dimensional suspension culture, in which a stirring operation using a spinner flask or the like is carried out for preventing precipitation of cells and cell clusters in the culture vessel,

has been proposed (see Non-patent Document 1 and Non-patent Document 2). However, since pluripotent stem cells are sensitive to mechanical stresses caused by the stirring operation, the cells may be damaged during the culture, leading to deterioration of the quality due to the stresses, which is problematic.

Recently, in view of this, a process in which a medium, methyl cellulose, and gellan gum are contained in a culture bag, and pluripotent stem cells are cultured therein while pluripotent stem cell spheres are decomposed using a nylon mesh filter upon subculture has been proposed (see Non-patent Document 3). However, this method was not satisfactory from the viewpoint of the number of cells obtained. Further, since the viscosity of the culture liquid is increased with gellan gum for prevention of precipitation of cells and cell clusters, the stirring cannot even allow supply of nutrients and oxygen in the amounts at least required for the growth of the cells, so that, in cases where large cell clusters are formed, the cells in the inner side cannot survive, resulting in deterioration of the quality of the cells, which is problematic. Further, in cases where an increase in the size of cell clusters is inhibited in an attempt to allow survival of all cells in the resulting cell clusters, the number of cells obtained is small, which is problematic. Thus, the stem cells obtained by this culture method failed to satisfy both the cell quality and the number of cells obtained at the same time.

RELATED ART DOCUMENTS

NON PATENT DOCUMENTS

Non-patent Document 1: BIOTECHNOLOGY AND BIOENGINEERING, Vol. 92, NO. 7, 920 to 933, DECEMBER 30, 2005

Non-patent Document 2: TISSUE ENGINEERING: Part C Vol. 18, NO. 10, 1 to 13, 2012

Non-patent Document 3: Stem Cell Reports, Vol. 2, 734 to 745, May 6, 2014

SUMMARY OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

In view of this, an object of the present invention is to provide a method for culturing cells by which the diameter of cell aggregates can be controlled, and by which a large amount of cells can be obtained, a cell aggregate obtained by the method, a cell aggregation control agent, and a medium containing the cell aggregation control agent.

MEANS FOR SOLVING THE PROBLEMS

As a result of intensive study, the present inventors discovered that, by inclusion of a substance that inhibits a cell adhesion molecule(s) in the medium, the function of the cell adhesion molecule(s) involved in formation of aggregates can be controlled, and that, by controlling the diameter of the aggregates in suspension culture by this, the above problem can be solved, thereby completing the present invention.

That is, the present invention is the following [1] to [11], which relate to a method for culturing cells, a cell aggregate, a cell aggregation control agent, and a medium.

[1] A method for culturing cells by suspension culture, the method comprising an aggregation control step of adding a substance that inhibits a cell adhesion molecule(s) of the cells to a medium to control cell aggregation of the cells.

[2] The culture method according to [1], wherein, in the aggregation control step, the substance that inhibits a cell adhesion molecule(s) is added to the medium at a concentration of 10 to 50 μg/ml.

[3] The culture method according to [1] or [2], wherein the substance that inhibits a cell adhesion molecule(s) is at least one selected from cell adhesion molecules, proteins composed of partial regions of cell adhesion molecules, fusion proteins containing the whole or partial regions of cell adhesion molecules, neutralizing antibodies against cell adhesion molecules, peptides that bind to cell adhesion molecules, and derivatives thereof.

[4] The culture method according to any one of [1] to [3], wherein the substance that inhibits a cell adhesion molecule(s) contains at least one selected from E-cadherin, proteins composed

of partial regions of E-cadherin, fusion proteins containing the whole or a partial region of E-cadherin, neutralizing antibodies against E-cadherin, peptides that bind to E-cadherin, and derivatives thereof.

[5] The culture method according to any one of [1] to [4], wherein the cells are stem cells or epithelial cells.

[6] An aggregate of the cells obtained by the culture method according to any one of [1] to [5], having a uniform aggregation diameter.

[7] The aggregate according to [6], wherein the diameter of the aggregate after 48 hours of suspension culture is not less than 20 μm and less than 1 mm.

[8] A cell aggregation control agent comprising a substance that inhibits a cell adhesion molecule(s).

[9] The cell aggregation control agent according to [8], comprising, as the substance that inhibits a cell adhesion molecule(s), at least one selected from E-cadherin, proteins composed of partial regions of E-cadherin, fusion proteins containing the whole or a partial region of E-cadherin, neutralizing antibodies against E-cadherin, peptides that bind to E-cadherin, and derivatives thereof.

[10] The cell aggregation control agent according to [9], wherein the peptide that binds to E-cadherin is a peptide composed of the amino acid sequence of SEQ ID NO: 3.

[11] A medium containing the cell aggregation control agent according to any one of [8] to [10].

EFFECTS OF THE INVENTION

The present invention enables to provide a method for culturing cells by which the diameter of cell aggregates can be controlled, and by which a large amount of cells can be obtained, a cell aggregate obtained by the method, a cell aggregation control agent, and a medium containing the cell aggregation control agent.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is an explanatory diagram showing an embodiment of the culture method of the present invention.

FIG. 2 shows photograph diagrams ((A) to (F)) showing the shapes of aggregates obtained in Examples 1-1 to 1-5 and Comparative Example 1-1, wherein human iPS cells were treated with E-cadherin-Fc in an amount of 0, 5, 10, 20, 50, or 100 μg/ml and then subjected to rotation suspension culture for 5 days, which photographs were taken with a phase-contrast microscope. The scale bar represents 500 μm.

FIG. 3 is a graph diagram showing the glucose consumption of cells as measured for the culture media used in Examples 1-1 to 1-5 and Comparative Example 1-1.

FIG. 4 is a graph diagram showing the densities of cells per 1 ml of medium, which cells were collected after 5 days of culture in Examples 1-1 to 1-5 and Comparative Example 1-1.

FIG. 5 shows photograph diagrams ((A) to (D)) showing the shapes of aggregates obtained in Examples 2-1 to 2-3 and Comparative Example 2-1, wherein human iPS cells were treated with recombinant E-cadherin (10 μg/ml), E-cadherin antibody (16 μg/ml), or E-cadherin-Fc (10 μg/ml), or without addition of a substance that inhibits a cell adhesion molecule(s), and then subjected to rotation suspension culture for 1 day, which photographs were taken with a phase-contrast microscope. The scale bar represents 500 μm.

FIG. 6 shows photograph diagrams ((A) to (C)) showing the shapes of aggregates obtained in Examples 3-1 and 3-2, and Comparative Example 3-1, wherein human iPS cells were treated with E-cadherin-Fc in an amount of 0, 50, or 100 μg/ml and then subjected to rotation suspension culture for 2 days, which photographs were taken with a phase-contrast microscope. The scale bar represents 500 μm.

FIG. 7 shows photograph diagrams ((A) to (C)) showing the shapes of aggregates

obtained in Examples 3-1 and 3-2, and Comparative Example 3-1, wherein human iPS cells were treated with E-cadherin-Fc in an amount of 0, 50, or 100 μg/ml and then subjected to rotation suspension culture for 5 days, which photographs were taken with a phase-contrast microscope. The scale bar represents 500 μm. The shape of the aggregate in FIG. 7 (C) is thought to be due to deformation during pipetting.

FIG. 8 is a graph diagram showing the glucose consumption of cells as measured for the culture media used in Examples 3-1 and 3-2, and Comparative Example 3-1.

FIG. 9 is a graph diagram showing the densities of cells per 1 ml of medium, which cells were collected after 5 days of culture in Examples 3-1 and 3-2, and Comparative Example 3-1.

MODE FOR CARRYING OUT THE INVENTION

The culture method of the present invention is a method for culturing cells by suspension culture, and comprises an aggregation control step of adding a substance that inhibits a cell adhesion molecule(s) of the cells to a medium to control cell aggregation of the cells. By the culture method of the present invention, the diameter of the aggregates can be controlled to a uniform diameter, to thereby enable culture of a large amount of cells having uniform quality.

As described below in detail, the method for obtaining the cells to be used for the suspension culture is not limited. The cells are preferably cells having E-cadherin, more preferably stem cells or epithelial cells.

The substance that inhibits a cell adhesion molecule(s) of the cells is preferably at least one selected from cell adhesion molecules, proteins composed of partial regions of cell adhesion molecules, fusion proteins containing the whole or partial regions of cell adhesion molecules, neutralizing antibodies against cell adhesion molecules, peptides that bind to cell adhesion molecules, and derivatives thereof. The cell adhesion molecule is more preferably

a molecule belonging to the cadherin family. The molecule belonging to the cadherin family is preferably E-cadherin, or a molecule having structural similarity to this molecule, containing the EC1 domain and one or more of the EC2 domain, EC3 domain, EC4 domain, and EC5 domain related to E-cadherin, and having a homophilic binding capacity to the pluripotent stem cells.

The substance that inhibits a cell adhesion molecule(s) of the cells is more preferably at least one selected from E-cadherin, proteins composed of partial regions of E-cadherin, fusion proteins containing the whole or a partial region of E-cadherin, neutralizing antibodies against E-cadherin, peptides that bind to E-cadherin, and derivatives thereof.

After the aggregation control step, the obtained cell aggregates may be subjected to suspension culture in a medium that does not contain the substance that inhibits a cell adhesion molecule(s). This is because, by uniformly controlling the diameter of the aggregates in the early phase of the aggregation control step, the size of the aggregates can be uniformly controlled even in cases where the subsequent suspension culture is carried out in a medium that does not contain the substance that inhibits a cell adhesion molecule(s). Such subsequent suspension culture in a medium that does not contain the substance that inhibits a cell adhesion molecule(s) is preferred from the viewpoint of increasing the number of cells.

As a specific embodiment of the method for culturing cells of the present invention, an example using human iPS cells is described below with reference to FIG. 1, but the present invention is not limited thereto. First, in order to obtain the cells to be subjected to the suspension culture, human iPS cells adhering to Matrigel, which is a common culture substrate, are detached by enzyme treatment (FIG. 1 (A)), and collected (FIG. 1 (B)). In the aggregation control step, the collected iPS cells are mixed with E-cadherin-Fc fusion protein, which is a substance that inhibits a cell adhesion molecule(s) of iPS cells, and suspension culture is then carried out to perform treatment for inhibition of adhesion of the cells to each

other (FIG. 1 (C)). E-cadherin is a membrane protein involved in cell-cell adhesion of human iPS cells, and E-cadherin-Fc fusion protein is a recombinant protein in which an antibody Fc tag is attached to the adhesive domain of E-cadherin. By allowing adhesion of E-cadherin-Fc fusion protein to the E-cadherin-adhesive domain on human iPS cells, cell-cell adhesion using E-cadherin is inhibited. Examples of other substances that inhibit E-cadherin include recombinant E-cadherin, E-cadherin antibodies, and E-cadherin-inhibiting peptides. This treatment is carried out for a period of about 1 to 2 days, and rotation culture is carried out using a shaker during this period. After the treatment, the medium is replaced with a normal medium, and medium replacement is carried out every day thereafter (FIG. 1 (D)).

Modes for carrying out the present invention are described below in detail. The term "pluripotent stem cells" as used in the present description means cells having a capacity to grow permanently or for a long period in *in vitro* culture while maintaining the undifferentiated state, which cells show a normal karyotype (chromosomal type) and have a capacity to differentiate into cells belonging to the lineage of any of the three germ layers (ectoderm, mesoderm, and endoderm) under appropriate conditions. "Pluripotent stem cells" are ES cells, which are isolated from an early embryo, iPS cells, or other similar cells, and examples of the pluripotent stem cells include, but are not limited to, EG cells, which are isolated from primordial germ cells in the fetal period. In the present description, "ES cells" may also include "EG cells".

The term "undifferentiated" as used in the present description means a property that exhibits, in pluripotent stem cells, an undifferentiated state which can be confirmed based on expression of at least one undifferentiation marker, for example, ALP activity, expression of the Oct-3/4 gene(s) (product(s)), and/or expression of various antigen molecules. The undifferentiated state in pluripotent stem cells means a state where the cell growth of the pluripotent stem cells is possible permanently or for a long period, which cells show a normal

karyotype (chromosomal type) and have a capacity to differentiate into cells belonging to the lineage of any of the three germ layers under appropriate conditions.

The term "pluripotency of differentiation" as used in the present description means a capacity to differentiate into various types of cells. The differentiated cells are not limited as long as the cells are of a type which can be generally induced by differentiation of pluripotent stem cells. Specific examples of the differentiated cells include ectodermal cells, cells derived from ectodermal cells, mesodermal cells, cells derived from mesodermal cells, endodermal cells, and cells derived from endodermal cells.

The term "medium" as used in the present description includes all liquid media that can be applied to conventional methods for subculture of pluripotent stem cells.

The method for obtaining the cells to be used in the suspension culture in the present invention is not limited, and a culture method which has been conventionally used may be used. The method for culturing the pluripotent stem cells is described below.

As the method for culturing the pluripotent stem cells, any method used for culture of pluripotent stem cells may be used. Examples of such a method include plate culture methods using, for example, a culture dish, or a microplate or plate with 96 wells, 48 wells, or the like; and suspension culture methods using, for example, a flask, bag, reactor, or the like.

In cases where the plate culture is carried out, a cell-adhesive substrate needs to be provided on a surface of a container such as a dish or a plate. Examples of such a cell-adhesive substrate include agar, gelatin, Matrigel, laminin, vitronectin, and E-cadherin. The amount of the cell-adhesive substrate used is not limited as long as it is a concentration used for culture of pluripotent stem cells. The amount is about 2 to 50 μg/ml in terms of the concentration in the aqueous solution for the immobilization on the container.

Examples of methods applicable to immobilization of the adhesive substrate on the solid-phase surface of the container, or to coating of the solid-phase surface of the container

with the adhesive substrate, include physical methods such as adsorption and chemical methods such as covalent bonding. From the viewpoint of simplicity of operation, a method by adsorption is preferred. The adsorption can be achieved by bringing a solution containing the adhesive substrate into contact with the surface of the substrate for a predetermined period, preferably for several minutes to one day and night, more preferably for 20 minutes to 12 hours. An antigenic molecule may be artificially attached to/fused with an adhesive molecule in advance, and binding of a specific antibody to the antigenic molecule may be utilized.

The thus prepared culture vessel can be used as it is for a usual culture method for pluripotent stem cells. That is, an appropriate number of pluripotent stem cells may be suspended in a commonly used liquid medium or cell culture medium, and the resulting suspension may be added to the culture substrate. Replacement of the liquid medium and subculture may also be carried out thereafter in the same manner as in conventional methods.

By culturing pluripotent stem cells using the above material, pluripotent stem cells can be obtained in a number required for the suspension culture. Isolation of cells from the cell substrate and collection of the cells are usually carried out by enzyme treatment. Examples of the enzyme used therefor include trypsin, collagenase, hyaluronidase, elastase, and pronase.

[Substance That Inhibits Cell Adhesion Molecule(s)]

The substance that inhibits a cell adhesion molecule(s) used in the culture method of the present invention is a substance capable of inhibiting a cell adhesion molecule(s) involved in aggregability of cells. As described above, the substance is preferably a cell adhesion molecule or a derivative thereof, more preferably at least one selected from E-cadherin, proteins composed of partial regions of E-cadherin, fusion proteins containing the whole or a partial region of E-cadherin, neutralizing antibodies against E-cadherin, peptides that bind to E-cadherin, and derivatives thereof.

Cadherin is an adhesion molecule involved in Ca^{2+}-dependent cell-cell adhesion/bonding called adhesive bonding or adherens junction, and known representative examples of cadherin include the E (epithelial) type, N (neural) type, and P (placental) type. These cadherin molecules are membrane-bound glycoprotein molecules composed of 700 to 750 amino acid residues, and the extracellular domain of each molecule has five repeat structures each composed of about 110 amino acid residues, the so-called Extracellular Cadherin (EC) domains. For example, in the case of human E-cadherin (whose amino acid sequence is shown in SEQ ID NO: 1), the EC1, EC2, EC3, EC4, and EC5 domains correspond to 157 to 262, 265 to 375, 378 to 486, 487 to 595, and 596 to 700, respectively (each number represents the residue position in the amino acid sequence of SEQ ID NO: 1). In the case of mouse E-cadherin (whose amino acid sequence is shown in SEQ ID NO: 2), the EC1, EC2, EC3, EC4, and EC5 domains correspond to 159 to 264, 267 to 377, 380 to 488, 489 to 597, and 598 to 702, respectively (each number represents the residue position in the amino acid sequence of SEQ ID NO: 2). These EC domains have homologies among different cadherin molecular species, and domains positioned in the N-terminal side (EC1 and EC2) have especially high homologies. As cadherin molecules having such similar structures, not less than 50 types of molecules are known at present, and these molecules are collectively called the cadherin family. For reviews on cadherin, see, for example, Takeichi, Curr. Opin. Cell Biol. 7: 619, 1995; Marrs & Nelson, Int. Rev. Cytol. 165: 159, 1996; Yap et al., Annu. Rev. Cell Dev. Biol. 13: 119, 1997; Yagi & Takeichi, Genes Dev. 14: 1169, 2000; and Gumbiner, J. Cell Biol. 148: 399, 2000.

Expression of E-cadherin (another name, cadherin-1) is widely found in parenchymal cells of internal organs such as liver, kidney, and lung, and epithelial cells such as keratinocytes, and E-cadherin is known to be an important adhesion molecule responsible for their cell-cell adhesion (for reviews, see, for example, Mareel et al., Int. J. Dev. Biol. 37: 227,

1993; Mays et al., Cord Spring Harb. Symp. Quant. Biol. 60: 763, 1995; El-Bahrawy & Pignatelli, Microsc. Res. Tech. 43: 224, 1998; Nollet et al., Mol. Cell. Biol. Res. Commun. 2: 77, 1999).

The method for preparing a protein belonging to E-cadherin is not limited. It is preferred to use a recombinant protein prepared and purified using molecular biological methods. Besides this, any method may be used as long as it is a method which exhibits a similar effect. For example, a protein belonging to E-cadherin of pluripotent stem cells may be extracted and purified from a biological tissue/cells, or the peptide may be chemically synthesized.

For example, the E-cadherin gene has been isolated and identified from animals such as human (SEQ ID NO: 1), mouse (SEQ ID NO: 2), and rat, and their base sequences are available in public DNA databases by NCBI and the like (accession numbers: (human) NM_004360; (mouse) NM_009864; (rat) NM_031334). Thus, those skilled in the art can obtain and use cDNA of the E-cadherin gene by designing a primer(s) and/or a probe(s) specific to the E-cadherin gene, and using common molecular biological methods. cDNA of the E-cadherin gene can also be purchased from RIKEN Gene Bank (Tsukuba, Japan), American Type Culture Collection (ATCC), Invitrogen/ResGen, and the like. The gene encoding the protein belonging to the cadherin family used is preferably one derived from the same animal species as the species from which the pluripotent stem cells are derived. For example, in cases where mouse ES cells are used to carry out the present invention, mouse E-cadherin cDNA is preferably used. However, E-cadherin cDNA derived from a different animal species, that is, E-cadherin cDNA derived from human, monkey, cow, horse, pig, sheep, or a bird (for example, chicken), or an amphibian (for example, *Xenopus laevis*) may also be used.

A preferred example of the method for preparation of the recombinant protein of a

protein belonging to E-cadherin is characterized in that a gene encoding the molecule is introduced into mammalian cells such as COS cells, 293 cells, or CHO cells, and allowing expression of the gene. The gene is preferably linked to a nucleic acid sequence that enables transcription and expression of the gene in a wide variety of mammalian cells, that is, the so-called promoter sequence, such that the transcription and expression are possible under the control of the promoter. The gene to be transcribed and expressed is preferably further linked to a poly(A) addition signal. Preferred examples of the promoter include promoters derived from viruses such as SV (Simian Virus) 40 virus, Cytomegaro Virus (CMV), and Rous sarcoma virus; β-actin promoter; and EF (Elongation Factor) 1α promoter.

The gene used for the preparation of the recombinant protein does not necessarily need to contain the entire region of a gene encoding the molecule, and may be a partial gene sequence as long as the level of adhesion activity of the protein or peptide molecule encoded by the partial sequence is almost the same as, or higher than, that of the original molecule. For example, in cases of E-cadherin, which is used for preferred cases in the present invention, a recombinant protein prepared from a partial sequence encoding the extracellular domain containing 690 to 710 amino acid residues from the N-terminus, that is, a protein containing the EC1 to EC5 domains, may be used. In general, in cadherin molecules, the domain positioned closest to the N-terminus (EC1) defines the binding specificity, that is, homophilicity, of the molecule (Nose et al., Cell 61: 147, 1990). Therefore, a protein molecule which contains at least EC1, but does not contain one or several other domains may be prepared and used. Further, a protein which exhibits an amino acid homology of not less than 80%, preferably not less than 85%, more preferably not less than 90%, most preferably not less than 95% to the protein molecule described above, and which has the adhesion activity, may also be used.

The recombinant protein may also be prepared as a fusion protein with another protein

or peptide. For example, the recombinant protein may be prepared as a fusion protein with the Fc region of an immunoglobulin, with GST (Glutathione-S-Transferase) protein, with MBP (Mannnose-Binding Protein) protein , with avidin protein, with a His (oligohistidine) tag, with an HA (HemAgglutinin) tag, with a Myc tag, with a VSV-G (Vesicular Stromatitis Virus Glycoprotein) tag, or the like, and the prepared fusion protein may be applied to a protein A/G column, specific antibody column, or the like to allow easy and efficient purification of the recombinant protein. Fc fusion proteins are especially preferred for carrying out the present invention since they form dimers and hence have stable activity as proteins.

Genes encoding the Fc regions of immunoglobulins have already been isolated and identified in mammals including human. A large number of their base sequences have also been reported. For example, sequence information on base sequences containing the Fc regions of human IgG1, IgG2, IgG3, and IgG4 is available in public DNA databases in NCBI and the like, wherein those sequences are deposited under accession numbers: AJ294730, AJ294731, AJ294732, and AJ294733, respectively. Thus, those skilled in the art can obtain and use cDNA encoding an Fc region portion by designing a primer(s) and/or a probe(s) specific to the Fc region, and using common molecular biological methods. In such cases, the animal species and the subtype of the gene encoding the Fc region used are not limited. Genes encoding Fc regions having a strong binding capacity to protein A/G, such as human IgG1 and IgG2, and mouse IgG2a and IgG2b, are preferred. A method in which the binding capacity to protein A is enhanced by introduction of a mutation(s) to the Fc region is also known (see Nagaoka et al., Protein Eng. 16: 243, 2003). An Fc protein prepared by gene modification by this method may also be used.

In cases of E-cadherin, which is preferably used for carrying out the present invention, an example of the production method for the recombinant protein has been reported (see

Nagaoka et al., Biotechnol. Lett. 24: 1857, 2002; Protein Eng. 16: 243, 2003).

A purified recombinant protein prepared by introducing a fusion gene prepared by linking cDNA of a sequence encoding the Fc region portion of human IgG and a His tag sequence to cDNA encoding the extracellular domain of mouse or human E-cadherin into mouse cells and allowing expression of the fusion gene (Recombinant Human/Mouse E-cadherin-Fc Chimera; R&D systems; Genzyme Techne) is commercially available, and this protein may also be used as a mouse- or human-derived E-cadherin protein (E-Cad-Fc protein).

The method for preparing the peptide that binds to a cell adhesion molecule(s) such as E-cadherin is not limited. For example, using a cell adhesion molecule such as E-cadherin as a target molecule, affinity selection which is conventionally carried out such as phage display may be used to perform screening (for example, Devemy E, Blaschuk O. Identification of a novel N-cadherin antagonist. Peptides 2008; 29: 1853-61). One specific example of the method is as follows. First, peptides selected from a peptide library are placed in wells of a plate that are preliminarily coated with E-cadherin-Fc fusion protein, and the plate is then incubated. Subsequently, the wells are washed for removal of unbound phages. Bound phages are eluted in two steps. The first elution step is carried out using TBS supplemented with 2 mM EDTA, and elution in the second elution step is carried out using 0.2 M glycine-HCl (pH 2.2). The eluate at pH 2.2 is neutralized using 1 M Tris-HCl (pH 9.1). By infection of ER2738 cells with each eluted phage fraction, the phages are amplified. The amplified phages are purified by polyethylene glycol precipitation. The phages amplified from the EDTA fraction were used for the second biopanning operation, and only the EDTA eluate was used for the third biopanning operation. The phages amplified from the acid fraction were used for the second biopanning operation, and only the acid eluate was amplified and used for the final biopanning operation. At the end of the screening, the

phages are plated, and isolated clones are randomly collected and amplified. Single-stranded DNA is extracted from each phage clone, and its amino acid sequence is deduced by DNA sequencing. Examples of peptides that bind to E-cadherin include SWELYYPLRANL, which is shown in the amino acid sequence of SEQ ID NO: 3, and the peptides shown in the amino acid sequences of SEQ ID Nos: 4 to 30. These peptides may have a conventionally known modification(s) (for example, PEGylation and/or C-terminal amidation) as long as binding to the target molecule is not lost. These peptides may be used individually, or two or more of these may be used in combination.

The method for preparing the neutralizing antibody against a cell adhesion molecule such as E-cadherin is not limited, and known methods which have been conventionally carried out may be employed. A peptide that binds to the cell adhesion molecule such as E-cadherin may be prepared as an antibody and used. Examples of such a method include a method in which an antibody is prepared by immunizing an animal using as an antigen a cell adhesion molecule or a peptide that binds to a cell adhesion molecule, and a method in which the gene of the protein of interest incorporated in an expression vector is introduced to an animal, and the gene is expressed in the body of the animal, to prepare an antibody using the protein of interest as an antigen, followed by collecting the antibody (DNA immunization method).

In the present description, the protein composed of a partial region of a cell adhesion molecule or E-cadherin means a protein in which one or more molecules are absent compared to the parent molecule of the cell adhesion molecule or E-cadherin, which protein has cell adhesiveness.

In the present description, the derivative means a molecule showing one or more differences in its sequence compared to the parent molecule, which molecule has cell adhesiveness. Examples of the derivative include reaction products between the whole or a

part of a cell adhesion molecule or E-cadherin, and another chemical substance or a low-molecular-weight polymer component or oligomer component. The homology of the derivative to the parent molecule is preferably not less than 75%, more preferably not less than 85%, still more preferably not less than 90%.

[Aggregation Control Step]

The aggregation control step in the culture method of the present invention is a step of adding a substance that inhibits a cell adhesion molecule(s) of the cells to be cultured to a medium to control cell aggregation of the cells. In the aggregation control step, a desired medium may be placed in a container, and the cells and the substance that inhibits a cell adhesion molecule(s) may be included in the medium, followed by performing suspension culture while leaving the mixture to stand or carrying out stirring or the like. By this, the substance that inhibits a cell adhesion molecule(s) can be allowed to act on the cell surface, to thereby control cell-cell adhesiveness such that aggregates having a desired size can be obtained.

The amount of the substance that inhibits a cell adhesion molecule(s) added in this step is appropriately selected depending on the amount of the cells and components in the medium. In particular, the amount of the substance is appropriately selected such that the size (diameter) of the aggregates 48 hours after the beginning of the suspension culture by addition of the substance that inhibits a cell adhesion molecule(s) is not less than 20 μm and less than 1 mm. For example, in cases where a protein belonging to E-cadherin or a neutralizing antibody is used, it is preferably added to a concentration of 5 to 100 μg/ml, and, in cases where a peptide that binds to a cell adhesion molecule(s) such as E-cadherin is used, it is preferably added to a concentration of 0.01 to 1000 μM. In cases where the amount of the substance added is smaller than the range described above, the size of the aggregates obtained may be too large, so that survival of the cells in the inner side of the aggregates may

be impossible, or, when stem cells are used, the cells may lose undifferentiation. In cases where the amount exceeds the range described above, aggregates having remarkably uneven sizes may be obtained, or the surfaces of large aggregates may be covered with small aggregates, so that survival of the cells in the large aggregates may be impossible. From the viewpoint of obtaining a large amount of homogeneous cells, when a protein belonging to E-cadherin or a neutralizing antibody is used, its concentration is preferably 7 to 80 μg/ml, more preferably within the range of 8 to 65 μg/ml, still more preferably within the range of 10 to 50 μg/ml. When a peptide that binds to a cell adhesion molecule(s) such as E-cadherin is used, its concentration is preferably 0.1 to 900 μM, more preferably within the range of 0.5 to 800 μM, still more preferably within the range of 1 to 600 μM. In cases where the concentration is within the above-described ranges, the size of the aggregates after 48 hours can be controlled to not less than 20 μm and less than 1 mm. By controlling the size of the aggregates to this size, the size of the aggregates finally obtained can be made uniform, and, as a result, the cell survival rate and the number of cells can be increased. This is thought to be because, unlike conventional methods which simply make cells less adhesive to each other, the substance that inhibits a cell adhesion molecule(s) in the present invention directly acts on an adhesion factor(s) to enable cellular-level control of the level of adhesion between cells.

When a protein belonging to E-cadherin or a neutralizing antibody is used, the amount of the substance that inhibits a cell adhesion molecule(s) is, taking into account the protein adsorption property depending on the protein content in the medium, within the range of 25 to 100 μg/ml, 30 to 100 μg/ml, preferably 40 to 100 μg/ml, more preferably 50 to 100 μg/ml in cases where a medium with a low protein content (for example, with a protein content of not more than 1 mg/ml) is used, or within the range of 7 to 80 μg/ml, preferably 8 to 65 μg/ml, more preferably 10 to 50 μg/ml in cases where a medium with a high protein content (for example, with a protein content of not less than 10 mg/ml) is used. By adding the substance

that inhibits a cell adhesion molecule(s) in such an amount, secure blocking of cell membrane cadherin is possible since the substance that inhibits a cell adhesion molecule(s) is adsorbed to another cell membrane or culture substrate before blocking of the cell membrane cadherin of interest.

The length of time of the treatment of the cells after addition of the substance that inhibits a cell adhesion molecule(s) may be appropriately determined depending on the size of the aggregates. The length of time is usually 24 to 48 hours. In cases where the treatment time is less than this length, formation of the aggregates may be difficult in some cases, while in cases where the length of time is longer than this range, the aggregates hardly grow, leading to a small number of cells obtained.

As described above, in the culture method of the present invention, from the viewpoint of increasing the number of cells, the cell aggregates obtained in the aggregation control step are preferably subjected to normal suspension culture, that is, suspension culture in a medium that does not contain the substance that inhibits a cell adhesion molecule(s). In this process, the culture may be stopped when the sizes of all aggregates are not less than 250 μm and less than 1 mm, preferably within the range of about 250 to 950 μm, more preferably within the range of about 300 to 750 μm, still more preferably within the range of about 300 to 600 μm.

In the present invention, the method of suspension culture is not limited as long as it is a method in which cells are subjected to suspension culture in a container such as a bag, flask, or reactor. The suspension culture may be static culture, but, from the viewpoint of giving nutrients and oxygen to the cells, the culture is preferably carried out under conditions where these can be given, for example, under conditions accompanied by stirring, flow, or the like.

Preferred examples of conditions for the stirring include stirring at about 20 to 150 rpm. Examples of methods, other than stirring, for giving nutrients and oxygen include the bubbling method, in which a gas is included in the culture liquid, and a method in which the

culture liquid is circulated. Examples of methods for giving oxygen other than the methods described above include a method in which hemoglobin is included in the culture liquid to enable efficient supply of oxygen.

The medium used in the suspension culture of the present invention is not limited, and a medium which is used for cell culture and suitable for the type of the cells may be used.

The medium for culture of pluripotent stem cells is described below in detail. The liquid medium for culture of pluripotent stem cells is not limited as long as it is applicable to a conventional method for subculturing pluripotent stem cells. Specific examples of the liquid medium include Dulbecco's Modified Eagle's Medium (DMEM), Glasgow Minimum Essential Medium (GMEM), and RPMI1640 medium. These media are usually used after addition of about 2 mM glutamine and/or about 100 μM 2-mercaptoethanol. KnockOut® DMEM (Invitrogen), ES cell-qualified DMEM (Cell & Molecular Technologies), TX-WES (Thromb-X), and the like which are commercially available as media for culturing ES cells may also be used. These media are preferably supplemented with 5 to 25% FBS, but serum-free culture may also be carried out by using, for example, 15 to 20% KnockOut® Serum Replacement (Invitrogen) instead. A culture supernatant of MEF cells, or a medium supplemented with bFGF/FGF-2, SCF, and/or the like may also be used. Details of such a method are known (Xu et al., Nature Biotech. 19: 971, 2001; WO 01/51616; WO 03/020920; Amit et al., Biol. Reprod., 70: 837, 2004). Examples of the liquid medium used for culture of pluripotent stem cells include, besides those described above, conventionally used known media such as Dulbecco's Modified Eagles's Medium (DMEM), DMEM/F12 medium, McCoy's 5A medium, Ham's Nutrient Mixture F12, MEM medium (Minimum Essential Medium), αMEM medium (alpha Modified Eagles's Minimum Essential Medium; αMEM) Eagles's MEM medium (Eagles's Minimum Essential Medium; EMEM), RPMI 1640 medium, IPL 41 medium, Iscove's Modified Dulbecco's Medium (IMDM), William's

medium E, MCDB 131 medium, Fischer's medium, StemPro® 34 (manufactured by Invitrogen), StemPro® hESC SFM (manufactured by Invitrogen), Sf-900® II (manufactured by Invitrogen), Opti-Pro (manufactured by Invitrogen), X-VIVO® 10 (manufactured by Cambrex Corporation), X-VIVO® 15 (manufactured by Cambrex Corporation), HPGM® (manufactured by Cambrex Corporation), StemSpan® H3000 (manufactured by StemCell Technologies Inc.), StemSpan® SFEM (manufactured by StemCell Technologies Inc.), mTeSR®1 or 2 medium (manufactured by StemCell Technologies Inc.), Stemline® II (manufactured by Sigma Aldrich), QBSF®-60 (manufactured by Quality Biological, Inc.), Essential 8® medium (manufactured by Gibco), MesenPRO RS® medium (manufactured by Gibco), Repro FF or Repro FF2 (manufactured by ReproCELL Inc.), PSGro hESC/iPSC medium(manufactured by System Biosciences, Inc.), NutriStem® medium (manufactured by Biological Industries), CSTI-7 medium (manufactured by Cell Science & Technology Institute, Inc.), and MF-Medium mesenchymal stem cell growth medium (manufactured by Toyobo Co., Ltd.).

In cases where a medium containing a large amount of protein such as albumin (for example, a medium whose protein content is not less than 10 mg/ml) is used, adsorption of protein to cell membrane surfaces, the culture substrate, and the like tends to be suppressed due to the protein in the medium. In cases where a medium containing a small amount of protein (for example, a medium whose protein content is not more than 1 mg/ml) is used, adsorption of protein to cell membrane surfaces, the culture substrate, and the like is more likely to occur compared to cases where a medium containing a large amount of protein is used.

Further, the medium may also contain a component conventionally used for culture of pluripotent stem cells, such as sodium, potassium, calcium, magnesium, phosphorus, chlorine, amino acid, vitamin, antibiotic, serum, fatty acid, or sugar, depending on the purpose. In

culture of cells and/or a tissue derived from an animal, the medium may also contain one or more kinds of other chemical components and biological components in combination depending on the purpose.

Examples of the component(s) added to the medium for cells and/or a tissue derived from an animal include fetal bovine serum, human serum, horse serum, insulin, transferrin, lactoferrin, cholesterol, ethanolamine, bovine serum albumin, sodium selenite, monothioglycerol, 2-mercaptoethanol, polyethylene glycol, sodium pyruvate, vitamins, amino acids, agar, agarose, collagen, methylcellulose, cytokines, hormones, growth factors, extracellular matrices, and cell adhesion molecules.

Examples of the cytokines include interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-11 (IL-11), interleukin-12 (IL-12), interleukin-13 (IL-13), interleukin-14 (IL-14), interleukin-15 (IL-15), interleukin-18 (IL-18), interleukin-21 (IL-21), interferon-α (IFN-α), interferon-β (IFN-β), interferon-γ (IFN-γ), granulocyte colony-stimulating factor (G-CSF), monocyte colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), leukemia cell inhibitory factor (LIF), flk2/flt3 ligands (FL), oncostatin M (OM), erythropoietin (EPO), and thrombopoietin (TPO).

Examples of the hormones include melatonin, serotonin, thyroxine, triiodothyronine, epinephrine, norepinephrine, dopamine, adiponectin, anti-Mullerian hormone, adrenocorticotropic hormone, angiotensinogen, angiotensin, antidiuretic hormone, atrial natriuretic peptide, calcitonin, cholecystokinin, corticotropin releasing hormone, erythropoietin, follicle-stimulating hormone, gastrin, ghrelin, glucagon, gonadotropin releasing hormone, growth hormone releasing hormone, human chorionic gonadotropin, human placental lactogen, growth hormone, insulin, insulin-like growth factor, inhibin, leptin,

luteinizing hormone, melanocyte-stimulating hormone, parathyroid hormone, thyroid stimulating hormone, thyrotropin releasing hormone, oxytocin, secretin, somatostatin, thrombopoietin, prolactin, cortisol, aldosterone, testosterone, dehydroepiandrosterone, androstenedione, dihydrotestosterone, estradiol, estrone, estriol, progesterone, calcitriol, calcidiol, prostaglandin, prostacyclin, leukotrien, thromboxane, prolactin-releasing hormone, lipotropin, brain natriuretic peptide, neuropeptide Y, histamine, endothelin, pancreatic polypeptide, renin, and enkephalin.

Examples of the growth factors include, but are not limited to, transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β), macrophage inflammatory protein-1α (MIP-1α), epidermal growth factor (EGF), fibroblast growth factor-1, 2, 3, 4, 5, 6, 7, 8, and 9 (FGF-1, 2, 3, 4, 5, 6, 7, 8, and 9), nerve cell growth factor (NGF), leukemia inhibitory factor (LIF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), protease nexin I, protease nexin II, cholinergic differentiation factor (CDF), chemokine, Notch ligands (Delta1 and the like), Wnt protein, angiopoietin-like proteins 2, 3, 5, and 7 (Angpt 2, 3, 5, and 7), insulin-like growth factor (IGF), insulin-like growth factor binding protein (IGFBP), and Pleiotrophin.

The medium may also contain a product produced by artificially modifying an amino acid sequence of a cytokine or a growth factor described above by genetic recombination. Examples of such a product include IL-6/soluble IL-6 receptor complex and Hyper IL-6 (fusion protein of IL-6 and soluble IL-6 receptor).

Examples of the extracellular matrices and cell adhesion molecules include collagen I to XIX, fibronectin, vitronectin, laminin-1 to 12, nitogen, tenascin, thrombospondin, von Willebrand factor, osteopontin, fibrinogen, elastins, proteoglycans, cadherins, integrins, desmocollin, desmoglein, E-selectin, P-selectin, L- selectin, immunoglobulin superfamily, poly-D-lysine, poly-L-lysine, chitin, chitosan, sepharose, hyaluronic acid, Matrigel, alginate

gel, and hydrogels, as well as cleaved fragments thereof.

Examples of the antibiotics include sulfa preparations, penicillin, ampicillin, phenethicillin, methicillin, nafcillin, oxacillin, cloxacillin, dicloxacillin, flucloxacillin, amoxicillin, cyclacillin, carbenicillin, ticarcillin, piperacillin, azlocillin, meczlocillin, amdinocillin, mecillinam, cephalosporin and derivatives thereof, oxolinic acid, amifloxacin, temafloxacin, nalidixic acid, piromidic acid, pipemidic acid, clavulanic acid, β-bromopenicillanic acid, β-chloropenicillanic acid, 6-acetylmethylene-penicillanic acid, ciprofloxacin, cinoxacin, norfloxacin, perfloxacin, rosaxacin, ofloxacin, enoxacin, sulbactam, cefoxazole, sultampicillin, formaldehyde-hydrate ester of adinocillin and sulbactam, tazobactam, aztreonam, sulfazethin, isosulfazethin, norcardicin, m-carboxyphenyl, phenylacetamide methyl phosphonate, chlortetracycline, oxytetracycline, tetracycline, demeclocycline, doxycycline, methacycline, and minocycline.

The aggregate of the present invention is a cell aggregate obtained by the culture method of the present invention, and has a uniform aggregation diameter. A large number of cells can be obtained from the aggregate of the present invention, and all of the obtained cells maintain undifferentiation and pluripotency of differentiation. From the viewpoint of the growth ability and pluripotency of differentiation, the diameters of all aggregates after 48 hours in the suspension culture are not less than 20 μm and less than 1 mm, preferably within the range of 20 to 950 μm, more preferably within the range of 20 to 750 μm, still more preferably within the range of 50 to 500 μm, especially preferably within the range of 100 to 300 μm.

The cell aggregation control agent of the present invention comprises a substance that inhibits a cell adhesion molecule(s). The cell aggregation control agent of the present invention is preferably used for control of the particle size of aggregates in suspension culture of cells. The substance that inhibits a cell adhesion molecule(s) is not limited, and

preferably at least one selected from E-cadherin, proteins composed of partial regions of E-cadherin, fusion proteins containing the whole or a partial region of E-cadherin, neutralizing antibodies against E-cadherin, peptides that bind to E-cadherin, and derivatives thereof.

The culture method of the present invention is very useful as a method for culturing stem cells, which method enables production of a large amount of cells, especially pluripotent stem cells such as iPS cells and ES cells, having uniform quality. The culture, cell aggregate, cell aggregation control agent, and medium of the present invention are very useful in the fields of regenerative medicine and drug discovery.

EXAMPLES

The present invention is described below in more detail by way of Examples. However, the present invention is not limited by these Examples.

<Providing of Pluripotent Stem Cells >

An aqueous Matrigel solution (50-fold dilution in DMEM) was placed in an untreated tissue culture 6-well plate (manufactured by Iwaki & Co., Ltd.) at 1 mL/well, and incubated at 37°C for 2 hours. Thereafter, the coating agent was removed to provide a human iPS cell culture plate.

As pluripotent stem cells, hiPS cells (TkDN4-M, Stem Cell Bank, The University of Tokyo) were used. These cells were plated on the above culture plate at a density of 100,000 to 400,000 cells/well, and cultured for four days. The culture was carried out using, as a culture medium, 2 mL/well of mTeSR®1 (manufactured by StemCell Technologies). During the culture, medium replacement was carried out every day except for the day after the plating.

For detachment and collection of cells, TrypLE® select (manufactured by Life Technologies), which is a trypsin-like enzyme, was used. After removal of the culture liquid, TrypLE® select was added to the wells at 1 mL/well, and the plate was incubated at 37°C for

2 to 5 minutes. Thereafter, TrypLE® select was removed, and mTeSR®1 supplemented with 10 μM Y-27632, which is a ROCK inhibitor, was added to the wells at 2 mL/well, followed by pipetting using a 1000-μL micropipette to detach the cells. The collected cell suspension was passed through a 40-μm cell strainer (BD) to collect the cells as single cells and/or microaggregates, to thereby obtain cells to be applied to suspension culture.

<Expression and Purification of E-cadherin-Fc Fusion Protein>

Expression and purification of E-cadherin-Fc fusion protein were carried out according to Nagaoka M, Akaike T., ProteinEng. 2003; 16: 243-245. In the present Example, extracellular domain cDNA obtained from mouse full-length E-cadherin (RIKEN BRC DNA Bank, code 1184) and mutated IgG1-Fc domain cDNA (T252M/T254S) were ligated, and E-cad-Fc fusion protein was expressed.

<Measurement of Diameters of Cell Aggregates >

In the following Examples and Comparative Examples, morphology and sizes of cell aggregates during the culture period were observed and measured using a phase-contrast microscope (product name, DM IRB; manufactured by LEICA).

<Example 1>

[Example 1-1]

To a 12-well plate subjected to low-cell-adhesion treatment, 1 mL/ml of mTeSR®1 was added, and hiPS cells (2×10^5 cells/ml) provided in advance and, as a substance that inhibits a cell adhesion molecule(s) of the hiPS cells, E-cadherin-Fc (5 μg/ml) were added at the same time, followed by performing rotation culture at a speed of 90 to 120 rpm for 48 hours. The sizes of the aggregates 48 hours after the E-cadherin-Fc treatment were within the range of 300 to 700 μm. Subsequently, medium replacement was carried out, and culture was carried out for three days under the same conditions as described above except that E-cadherin-Fc was not added, to obtain aggregates. The sizes of the aggregates were within

the range of 500 to 1000 μm.

[Example 1-2]

Aggregates were obtained in the same manner as in Example 1-1 except that the concentration of E-cadherin-Fc in Example 1-2 was 10 μg/ml. The sizes of the aggregates 48 hours after the E-cadherin-Fc treatment were within the range of 200 to 400 μm. The sizes of the aggregates obtained by the medium replacement and the three days of culture without addition of E-cadherin-Fc were within the range of 300 to 1000 μm.

[Example 1-3]

Aggregates were obtained in the same manner as in Example 1-1 except that the concentration of E-cadherin-Fc in Example 1-3 was 20 μg/ml. The sizes of the aggregates 48 hours after the E-cadherin-Fc treatment were within the range of 100 to 500 μm. The sizes of the aggregates obtained by the medium replacement and the three days of culture without addition of E-cadherin-Fc were within the range of 300 to 600 μm.

[Example 1-4]

Aggregates were obtained in the same manner as in Example 1-1 except that the concentration of E-cadherin-Fc in Example 1-4 was 50 μg/ml. The sizes of the aggregates 48 hours after the E-cadherin-Fc treatment were within the range of 100 to 300 μm. The sizes of the aggregates obtained by the medium replacement and the three days of culture without addition of E-cadherin-Fc were within the range of 300 to 600 μm.

[Example 1-5]

Aggregates were obtained in the same manner as in Example 1-1 except that the concentration of E-cadherin-Fc in Example 1-5 was 100 μg/ml. The sizes of the aggregates 48 hours after the E-cadherin-Fc treatment were within the range of 150 to 700 μm. The sizes of the aggregates obtained by the medium replacement and the three days of culture without addition of E-cadherin-Fc were within the range of 300 to 1000 μm.

[Comparative Example 1-1]

Aggregates were obtained in the same manner as in Example 1-1 except that E-cadherin-Fc was not used in Comparative Example 1-1. The sizes of the aggregates 48 hours after the E-cadherin-Fc treatment were within the range of 1000 to 3000 μm. The sizes of the aggregates obtained by the three days of culture were also within the range of 1000 to 3000 μm.

<Observation of Cell Aggregates>

In Examples 1-1 to 1-5 and Comparative Example 1-1, morphology of cell aggregates on Day 2 of the culture (after 48 hours of treatment with E-cadherin-Fc) was observed using a phase-contrast microscope (product name, DM IRB; manufactured by LEICA). The obtained micrographs are shown in FIG. 2 (A) to (F). From FIG. 2 (A) to (F), it can be seen that the aggregates obtained in Examples 1-1 to 1-5 (E-cadherin-Fc concentrations = 5, 10, 20, 50, and 100 μg/ml, respectively) have better-controlled sizes as compared to the aggregates obtained in Comparative Example 1-1 (E-cadherin-Fc concentration = 0 μg/ml). It can be seen, in particular, that use of E-cadherin-Fc at a concentration within the range of 10 to 50 μg/ml was effective for suppression of excessive aggregation and achievement of formation of uniform aggregates even by simple rotation culture.

<Measurement of Glucose Consumption>

During the whole culture period, 300 μL of the culture liquid was collected upon replacement of the medium, and the glucose concentration was analyzed using an enzyme-electrode type bioanalyzer (YSI2950) to calculate changes in the glucose consumption. The results are shown in FIG. 3.

<Measurement of Cell Number>

After completion of the culture, the cells were dispersed into single cells using TrypLE® select, and the number of live cells was counted using trypan blue (manufactured

by Life Technologies) and an eosinophil counter (manufactured by Tatai). The results are shown in FIG. 4.

Based on the graphs shown in FIGs. 3 and 4, the glucose consumption can be used as an index of the number of living cells. From the results shown in FIGs. 3 and 4, it can be seen that the aggregates obtained in Examples 1-1 to 1-5 (E-cadherin-Fc concentrations = 5, 10, 20, 50, and 100 μg/ml, respectively) have better effects in both the glucose consumption and the number of cells as compared to the aggregates obtained in Comparative Example 1-1 (E-cadherin-Fc concentration = 0 μg/ml). In particular, on Day 5 of the culture, the glucose consumption was about twice higher, and the number of cells was about 1.5 to 1.75 times larger in Example 1-1 compared to those in Comparative Example 1-1. Such a tendency was even stronger in Examples 1-2 to 1-5, wherein the glucose consumption was 2 times higher in Examples 1-2 and 1-5, and 2.5 times higher in Examples 1-3 and 1-4; and the number of cells was about 1.8 times larger in Example 1-2, about 2.5 times larger in Example 1-3, about 3 times larger in Example 1-4, and about 2.3 times larger in Example 1-5, so that it can be seen that a large amount of pluripotent stem cells were obtained. Further, since better results were obtained in Examples 1-2 to 1-4, wherein the diameters of aggregates were more uniformly controlled as shown in FIG. 2, it can be seen that the growth efficiency can be increased by suppressing excessive aggregation of cells to allow formation of more aggregates having appropriate particle sizes. From these results, it can be seen that the obtained cells are stem cells maintaining undifferentiation and pluripotency of differentiation. In general, taking into account the fact that there is a limit in the size to which a cell aggregate can grow depending on dispersion of nutrient substrates and oxygen, control of the diameter of the aggregates is assumed to significantly contribute to simplification of the process of large-scale culture of human iPS cells.

<Example 2>

[Example 2-1]

To a 12-well plate subjected to low-cell-adhesion treatment, 1 mL of mTeSR®1 was added, and hiPS cells (TkDN4-M, Stem Cell Bank, The University of Tokyo) (5×10^5 cells/ml) provided in advance and, as a substance that inhibits a cell adhesion molecule(s) of the hiPS cells, recombinant E-cadherin (hRP-0339, manufactured by LD Biopharma) (10 μg/ml) were added at the same time, followed by performing rotation culture at a speed of 90 rpm for 1 day.

[Example 2-2]

Aggregates were obtained in the same manner as in Example 2-1 except that a neutralizing antibody against E-cadherin (E-cadherin antibody MAB3199Z, manufactured by Millipore) (16 μg/ml) was used as the substance that inhibits a cell adhesion molecule(s) of the hiPS cells in Example 2-2.

[Example 2-3]

Aggregates were obtained in the same manner as in Example 2-1 except that E-cadherin-Fc (10 μg/ml) was used as the substance that inhibits a cell adhesion molecule(s) of the hiPS cells in Example 2-3.

[Comparative Example 2-1]

Aggregates were obtained in the same manner as in Example 2-1 except that the recombinant E-cadherin was not used in Comparative Example 2-1.

<Observation of Cell Aggregates>

Morphology of cell aggregates in Examples 2-1 to 2-3 and Comparative Example 2-1 was observed using a phase-contrast microscope (product name, DM IRB; manufactured by LEICA). The obtained micrographs are shown in FIG. 5 (A) to (D). From FIG. 5 (A) to (D), it can be seen that the aggregates obtained using any of the recombinant E-cadherin, neutralizing antibody against E-cadherin, and E-cadherin-Fc can have more uniformly

controlled diameters than the aggregates obtained in Comparative Example 2-1 (without addition of a substance that inhibits a cell adhesion molecule(s)). Moreover, since E-cadherin-Fc produces larger aggregates having more uniform shapes than the recombinant E-cadherin or the neutralizing antibody against E-cadherin, it is assumed that E-cadherin-Fc allows production of aggregates all of which have diameters of not less than 20 μm and less than 1 mm after 48 hours of the culture that are larger than those obtained using the recombinant E-cadherin or the neutralizing antibody against E-cadherin, and also allows production of a larger number of cells.

<Example 3>

[Example 3-1]

To a 12-well plate subjected to low-cell-adhesion treatment, 1 mL of Essential 8® was added, and hiPS cells (TkDN4-M, Stem Cell Bank, The University of Tokyo) (2×10^5 cells/ml) provided in advance and, as a substance that inhibits a cell adhesion molecule(s) of the hiPS cells, E-cadherin-Fc (50 μg/ml) were added at the same time, followed by performing rotation culture at a speed of 100 rpm for 5 days. On Day 2 of the culture and thereafter (from 48 hours after the beginning of the culture), medium replacement was carried out every day with a medium to which E-cadherin-Fc was not added.

[Example 3-2]

Aggregates were obtained in the same manner as in Example 3-2 except that the concentration of E-cadherin-Fc in Example 3-1 was 100 μg/ml.

[Comparative Example 3-1]

Aggregates were obtained in the same manner as in Example 3-1 except that E-cadherin-Fc was not used in Comparative Example 3-1.

<Observation of Cell Aggregates>

Morphology of cell aggregates in Examples 3-1 and 3-2, and Comparative Example 3-

1 on Day 2 and Day 5 of the culture was observed using a phase-contrast microscope (product name, DM IRB; manufactured by LEICA). The obtained micrographs are shown in FIGs. 6 and 7. From the results shown in FIGs. 6 and 7, it can be seen that the size of the aggregates can be controlled also in cases where Essential 8® is used as the medium. On Day 5 of the culture, in the cases where E-cadherin-Fc was added, the size of the aggregates was larger than that on Day 2 of the culture, and the diameter of the aggregates was controlled at about 500 μm.

Here, taking into account Examples 1-1 to 1-5, wherein mTeSR®1 was used as the medium (FIG. 2), it can be seen that use of mTeSR®1, whose protein content is high (protein content, about 18 mg/ml), as the medium produces an effect equivalent to that produced by use of Essential 8®, whose protein content is low (protein content, about 9 μg/ml), even with a smaller amount of the substance that inhibits a cell adhesion molecule(s). This is assumed to be because, when the medium with low protein content was used, adsorption of the substance that inhibits a cell adhesion molecule(s) to other cell membranes and the culture substrate occurred before blocking of the cell membrane cadherin of interest.

<Measurement of Glucose Consumption and Number of Cells>

As a result of measurement of the glucose consumption and the number of cells for Examples 3-1 and 3-2, and Comparative Example 3-1 in the same manner as described above (FIG. 8 and FIG. 9), it could be confirmed that addition of E-cad-Fc exhibits an excellent effect in both the glucose consumption and the number of cells.

CLAIMS

1. A method for culturing cells by suspension culture, said method comprising an aggregation control step of adding a substance that inhibits a cell adhesion molecule(s) of said cells to a medium to control cell aggregation of said cells.

2. The culture method according to claim 1, wherein, in said aggregation control step, said substance that inhibits a cell adhesion molecule(s) is added to said medium at a concentration of 10 to 50 μg/ml.

3. The culture method according to claim 1, wherein said substance that inhibits a cell adhesion molecule(s) is at least one selected from cell adhesion molecules, proteins composed of partial regions of cell adhesion molecules, fusion proteins containing the whole or partial regions of cell adhesion molecules, neutralizing antibodies against cell adhesion molecules, peptides that bind to cell adhesion molecules, and derivatives thereof.

4. The culture method according to claim 1, wherein said substance that inhibits a cell adhesion molecule(s) contains at least one selected from E-cadherin, proteins composed of partial regions of E-cadherin, fusion proteins containing the whole or a partial region of E-cadherin, neutralizing antibodies against E-cadherin, peptides that bind to E-cadherin, and derivatives thereof.

5. The culture method according to any one of claims 1 to 4, wherein said cells are stem cells or epithelial cells.

6. An aggregate of said cells obtained by the culture method according to claim 1, having a uniform aggregation diameter.

7. The aggregate according to claim 6, wherein the diameter of said aggregate after 48 hours of suspension culture is not less than 20 μm and less than 1 mm.

8. A cell aggregation control agent comprising a substance that inhibits a cell adhesion

molecule(s).

9. The cell aggregation control agent according to claim 8, comprising, as said substance that inhibits a cell adhesion molecule(s), at least one selected from E-cadherin, proteins composed of partial regions of E-cadherin, fusion proteins containing the whole or a partial region of E-cadherin, neutralizing antibodies against E-cadherin, peptides that bind to E-cadherin, and derivatives thereof.

10. The cell aggregation control agent according to claim 9, wherein said peptide that binds to E-cadherin is a peptide composed of the amino acid sequence of SEQ ID NO: 3.

11. A medium containing the cell aggregation control agent according to any one of claims 8 to 10.

FIG. 1

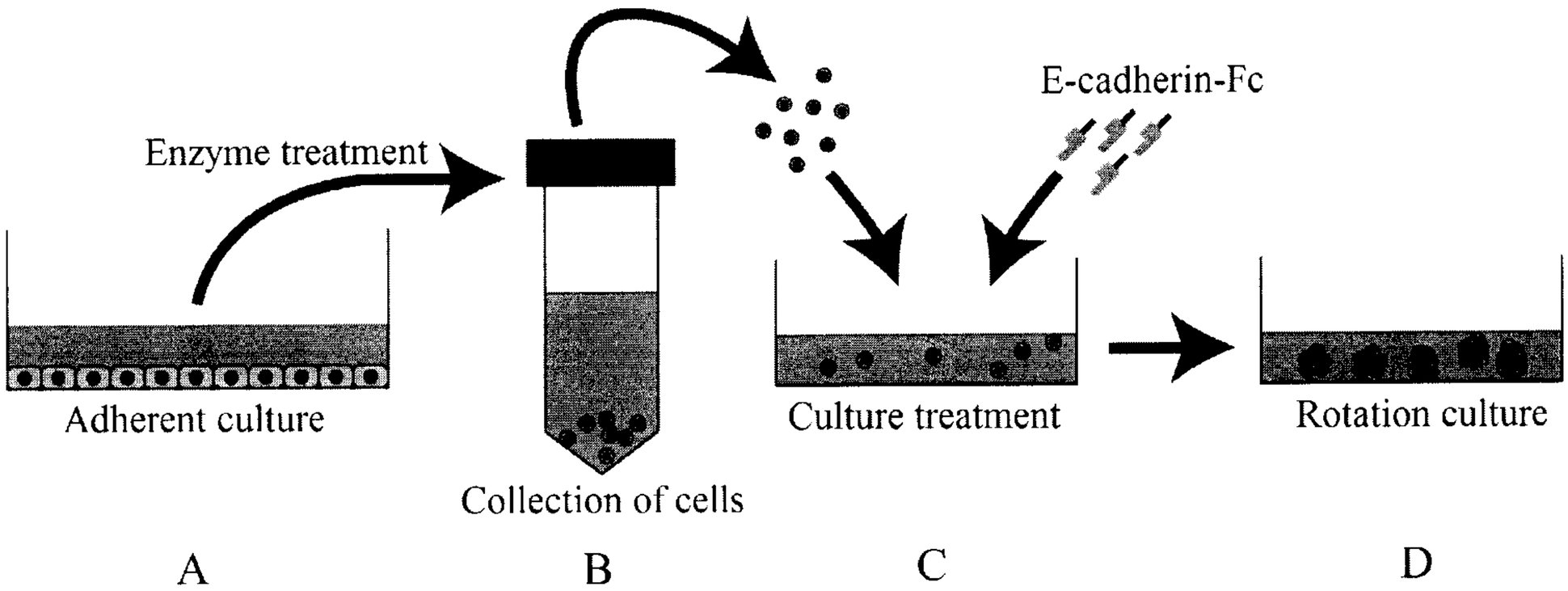

Enzyme treatment
E-cadherin-Fc
Adherent culture
Collection of cells
Culture treatment
Rotation culture
A
B
C
D

FIG. 2

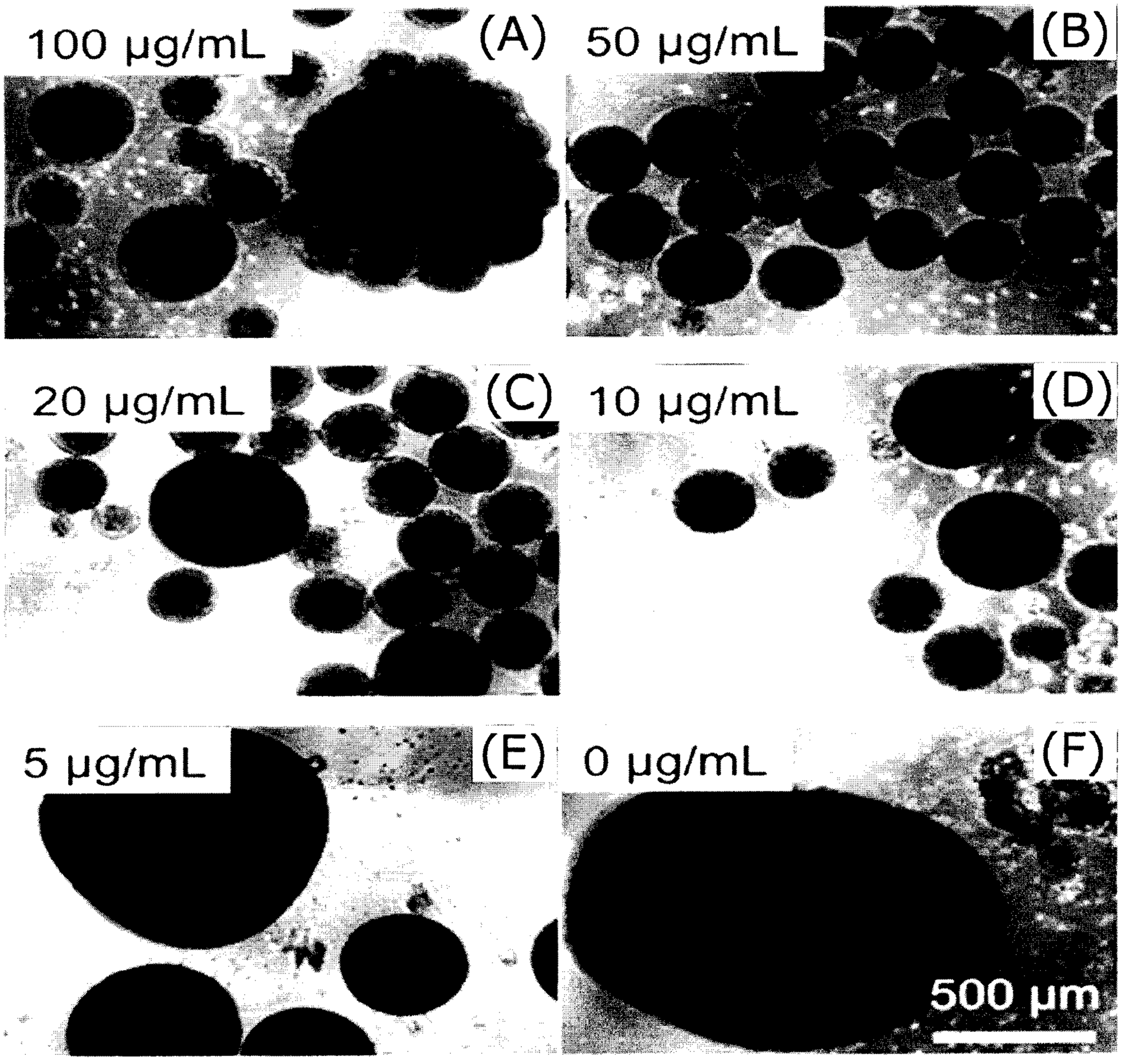


FIG. 3

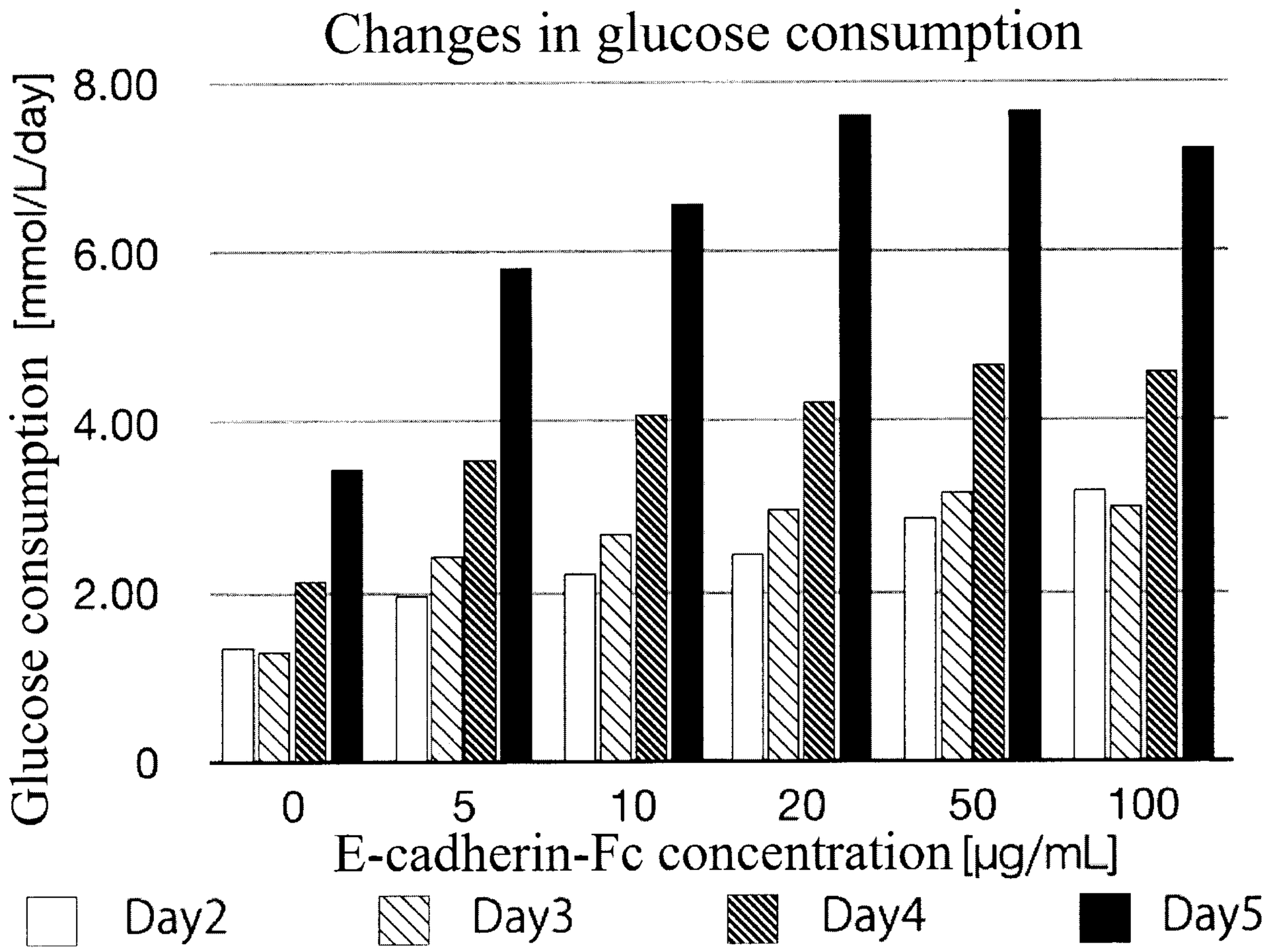

Changes in glucose consumption
Glucose consumption [mmol/L/day]
8.00
6.00
4.00
2.00
0
0
5
10
20
50
100
E-cadherin-Fc concentration [μg/mL]
Day2
Day3
Day4
Day5

FIG. 4

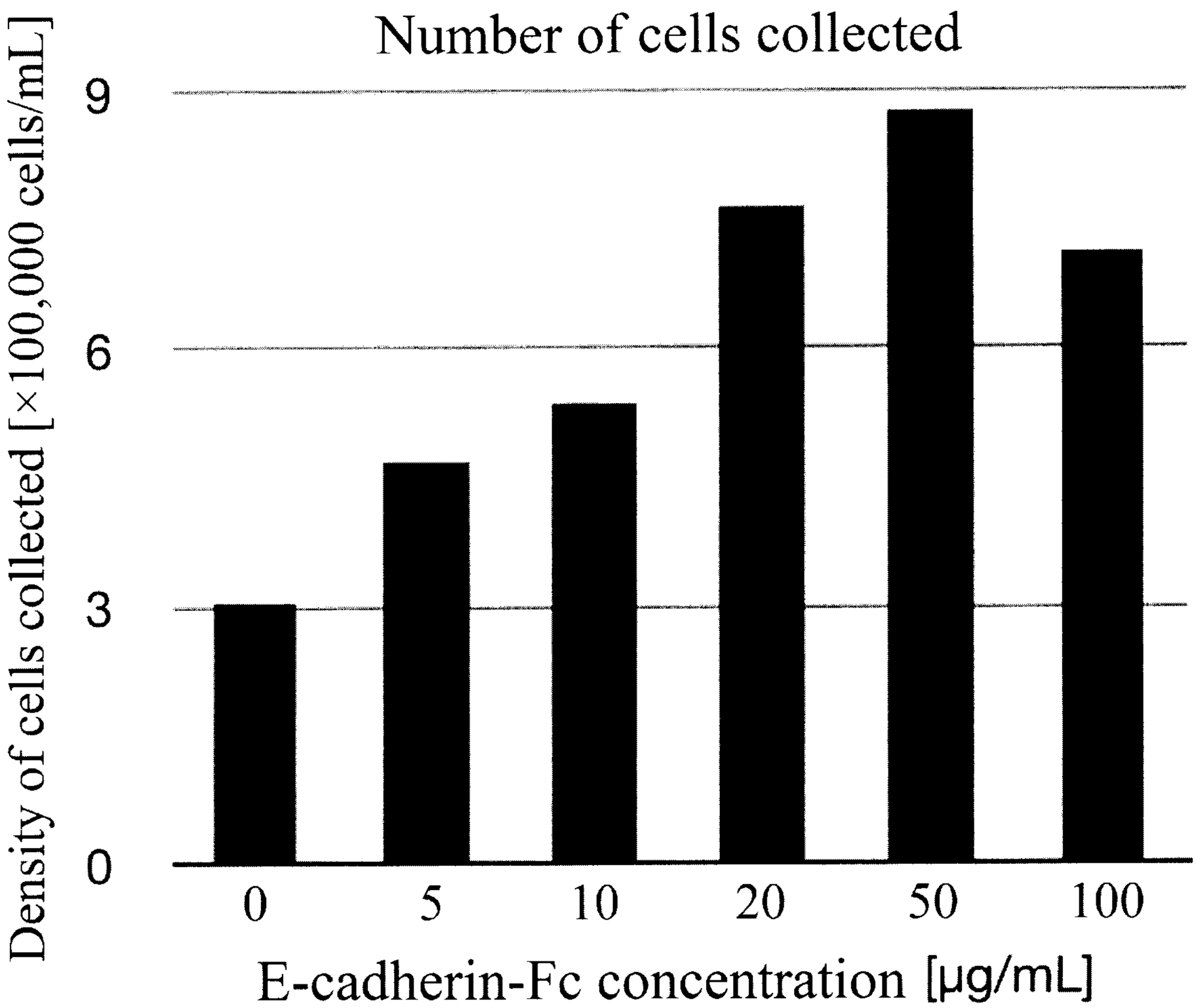

Number of cells collected
Density of cells collected [×100,000 cells/mL]
9
6
3
0
0
5
10
20
50
100
E-cadherin-Fc concentration [μg/mL]

FIG. 5

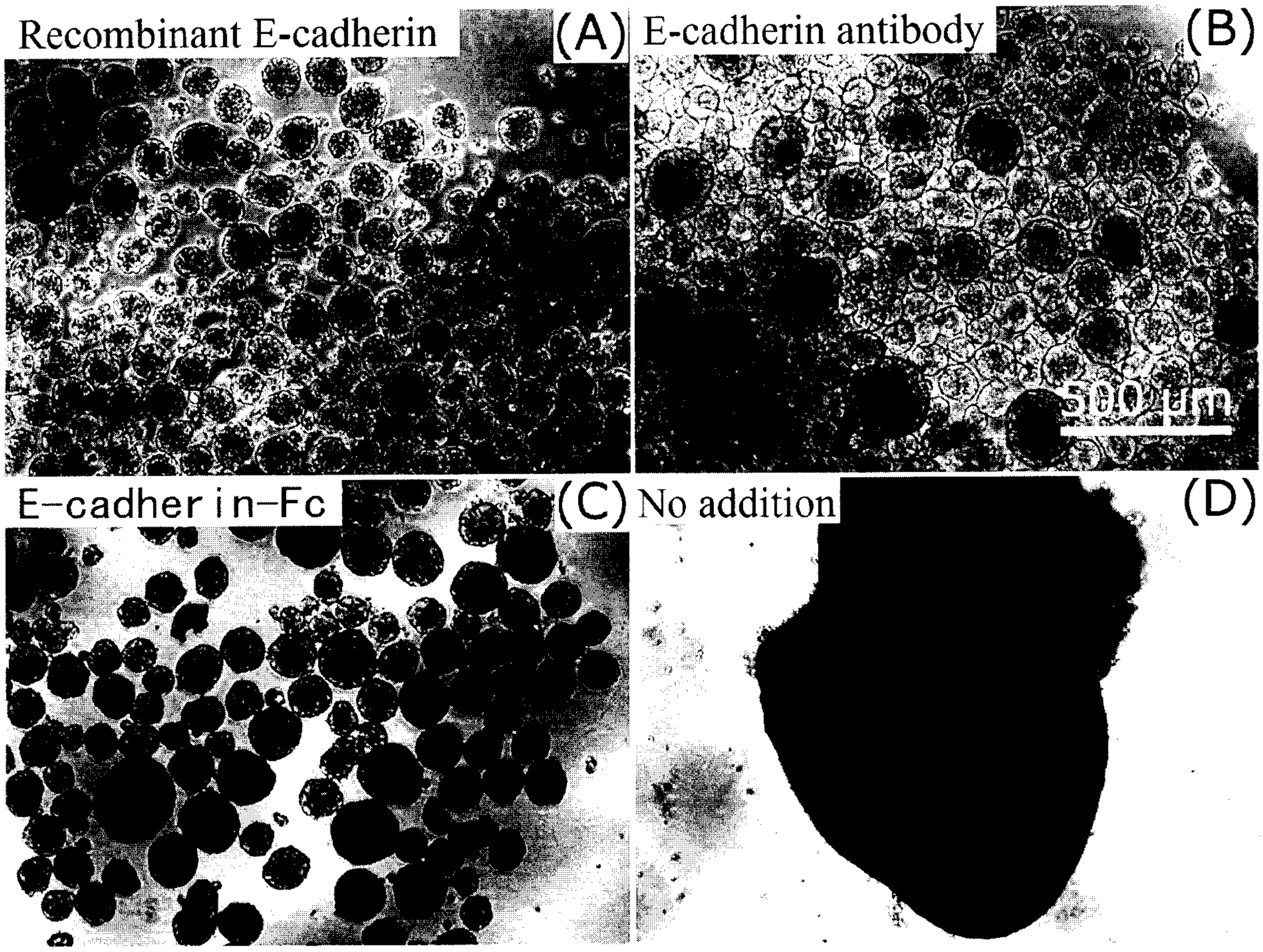

Recombinant E-cadherin
(A)
E-cadherin antibody
(B)
500 μm
E-cadherin-Fc
(C)
No addition
(D)

FIG. 6

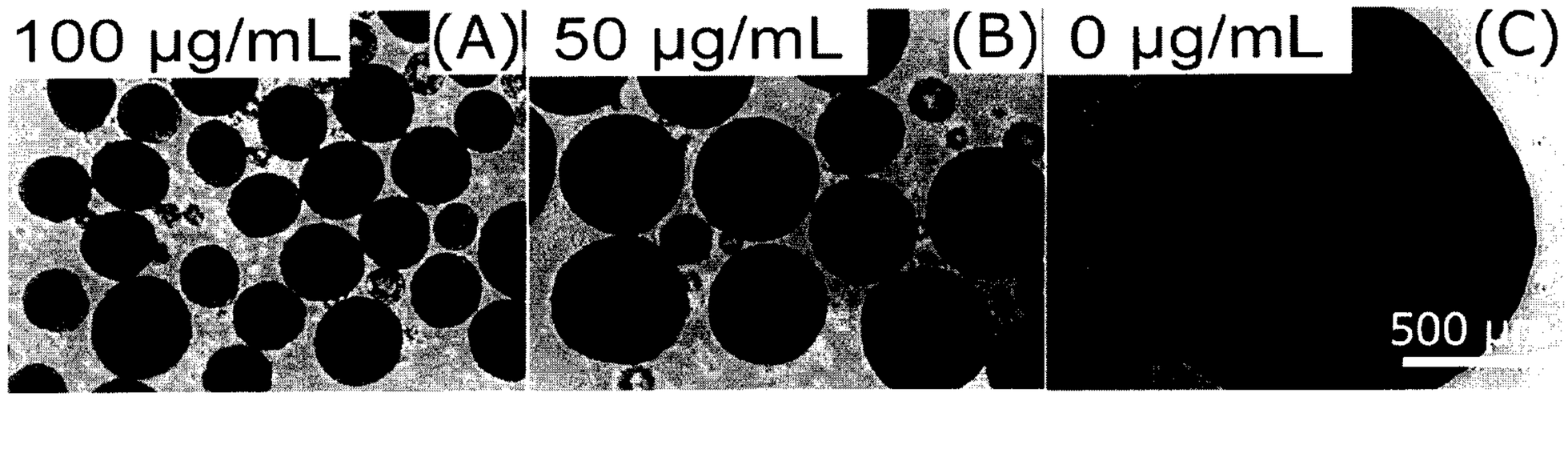

100 μg/mL
(A)
50 μg/mL
(B)
0 μg/mL
(C)
500 μ

FIG. 7

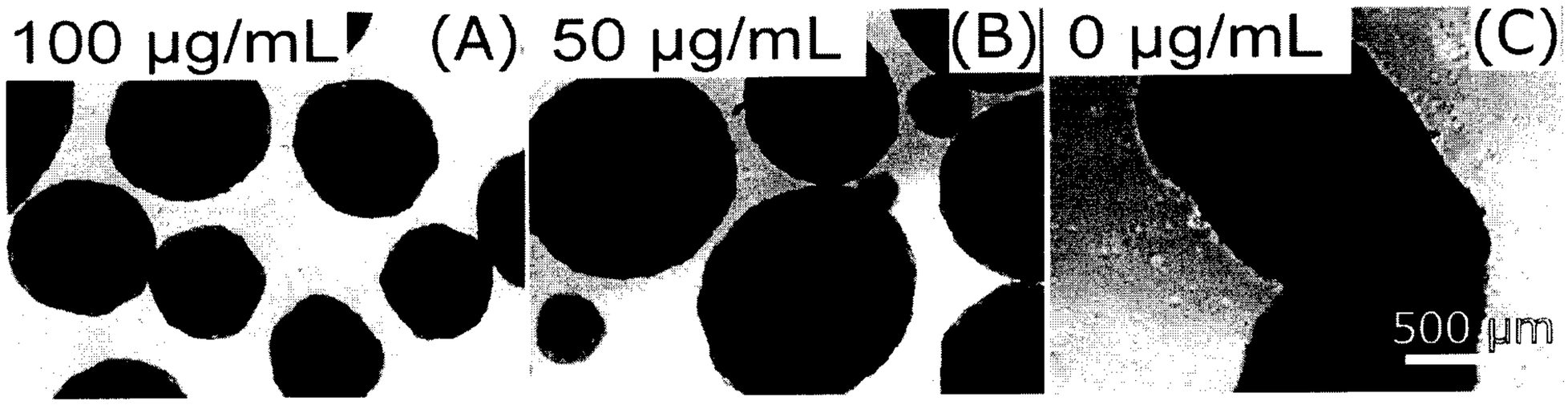

100 µg/mL
(A)
50 µg/mL
(B)
0 µg/mL
(C)
500 µm

FIG. 8

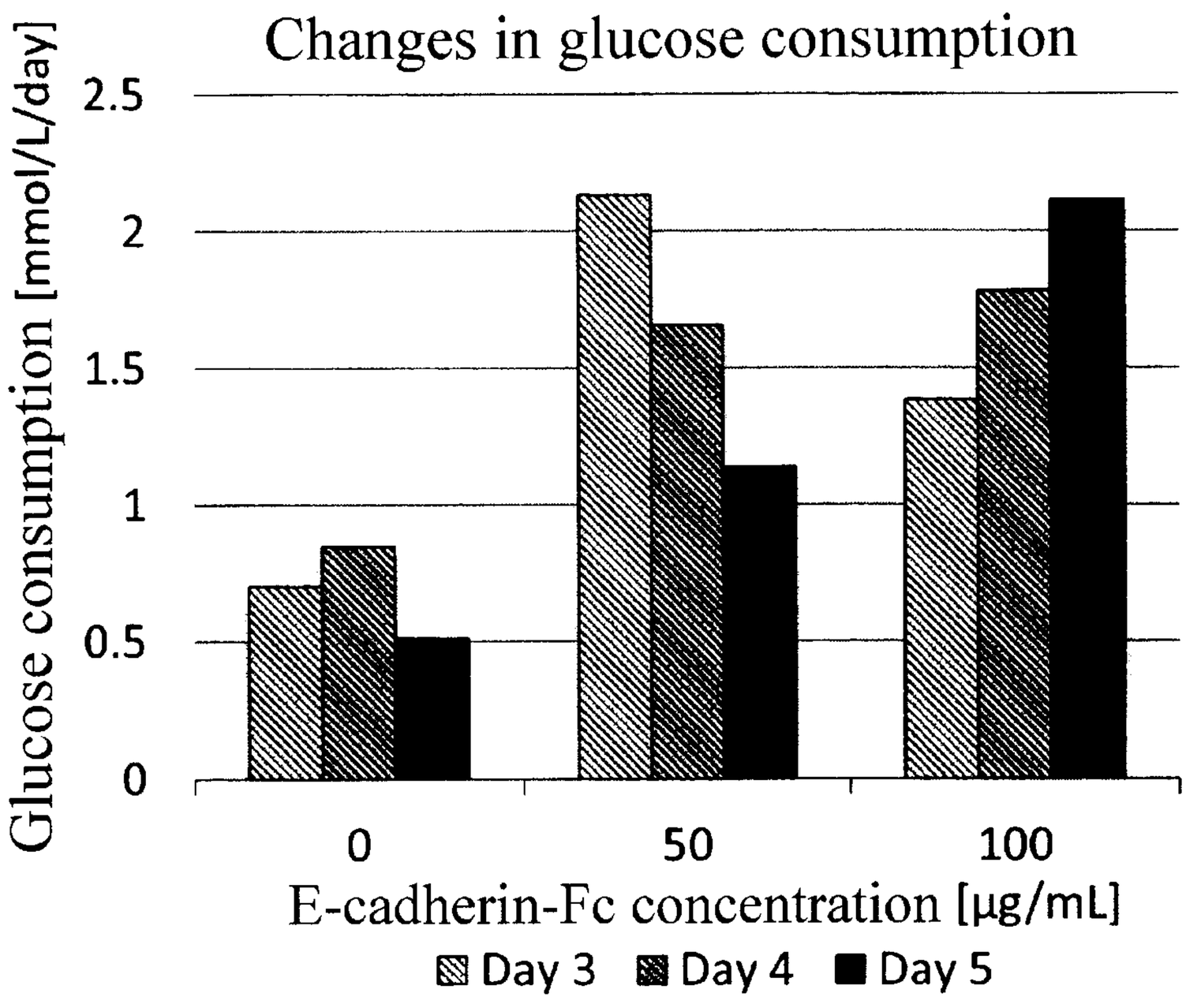
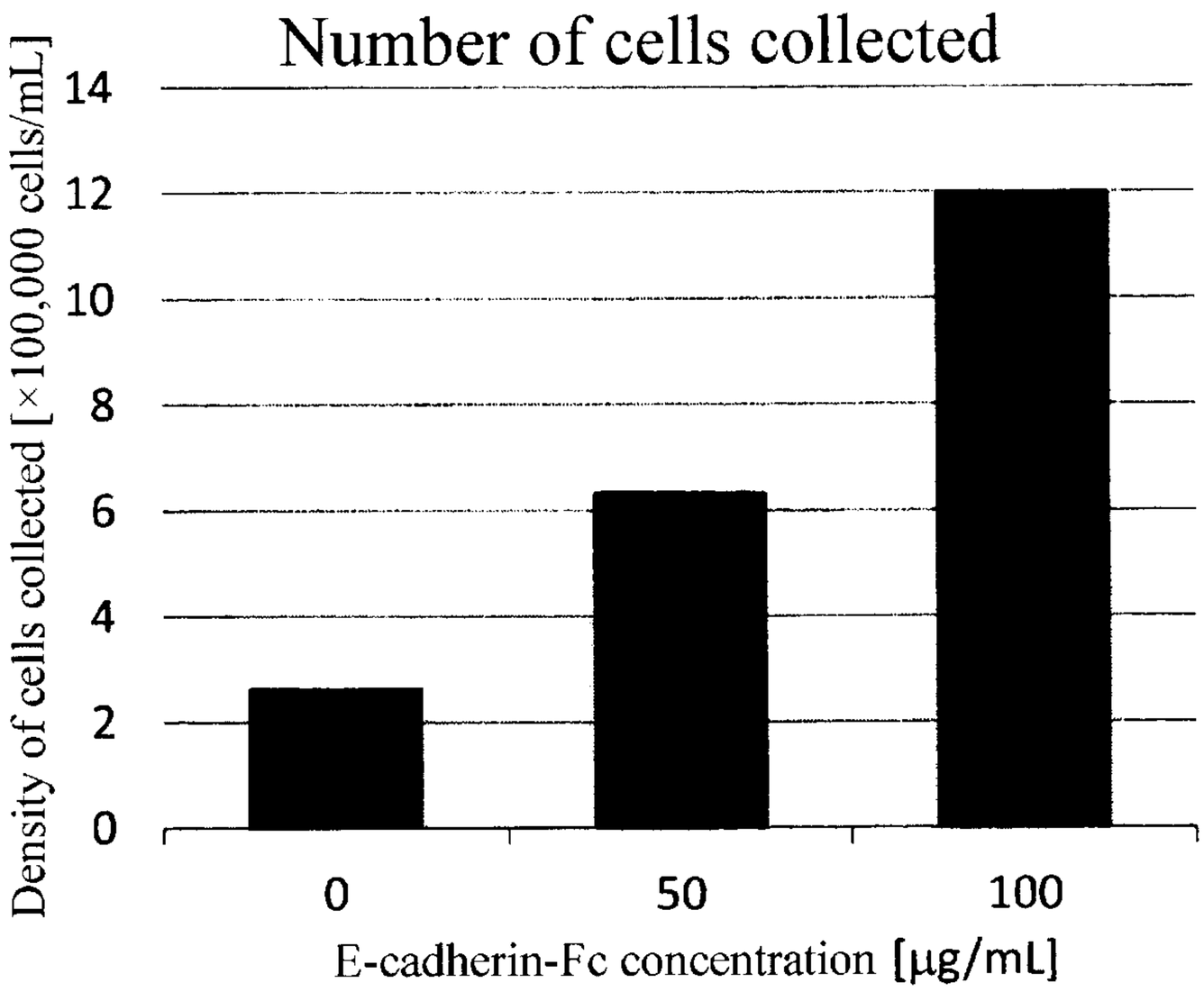
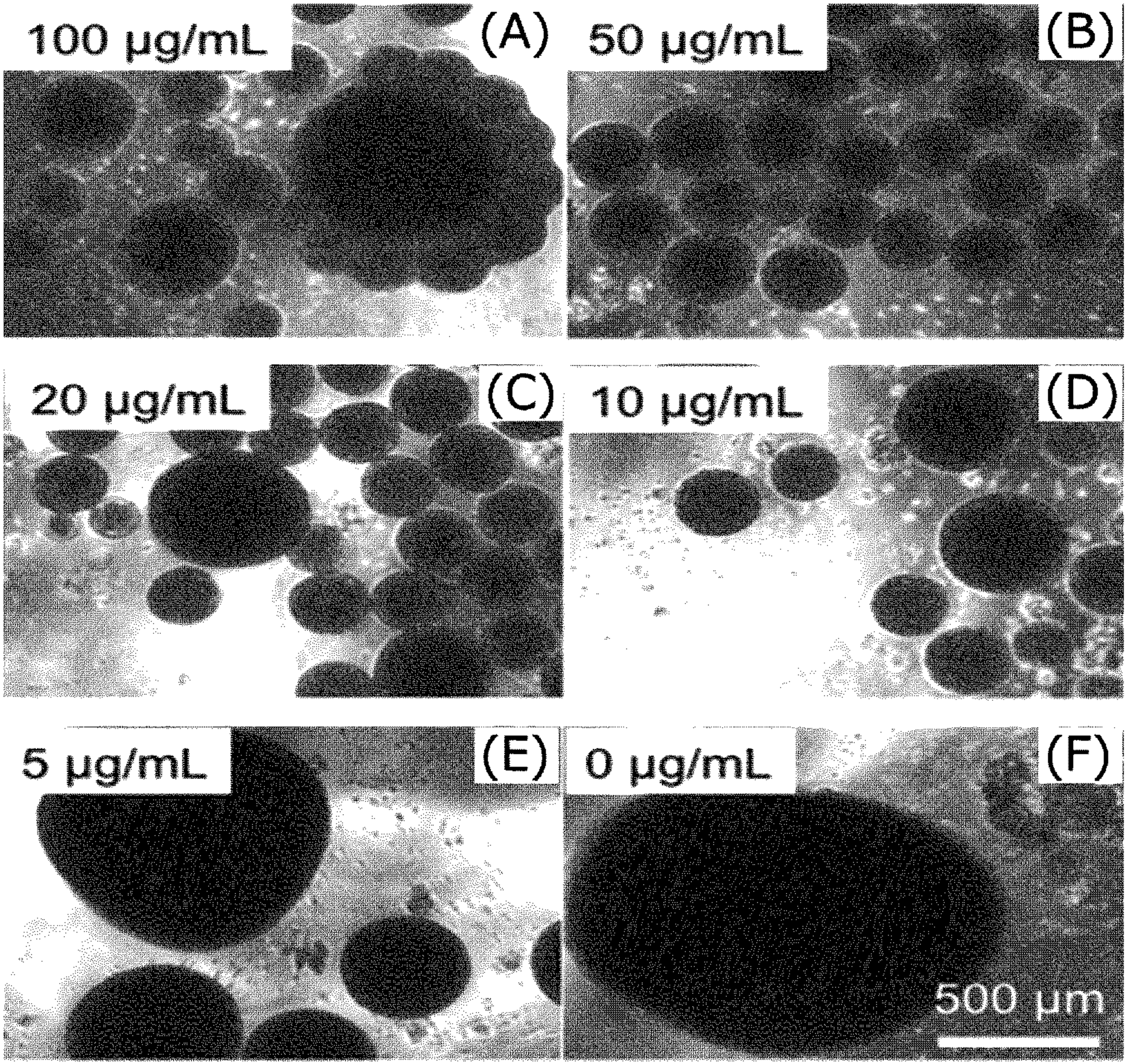

Changes in glucose consumption
Glucose consumption [mmol/L/day]
2.5
2
1.5
1
0.5
0
0
50
100
E-cadherin-Fc concentration [µg/mL]
Day 3
Day 4
Day 5

FIG. 9


Number of cells collected
Density of cells collected [×100,000 cells/mL]
14
12
10
8
6
4
2
0
0
50
100
E-cadherin-Fc concentration [μg/mL]


100 µg/mL
(A)
50 µg/mL
(B)
20 µg/mL
(C)
10 µg/mL
(D)
5 µg/mL
(E)
0 µg/mL
(F)
500 µm