

DESCRIPTION

Title of Invention: METHOD FOR PRODUCING THREE-DIMENSIONAL MUSCLE TISSUE

Technical Field

[0001]

The present invention relates to a method for producing a three-dimensional muscle tissue and a culture container for producing the three-dimensional muscle tissue.

Background Art

[0002]

With the increase in global population, economic development in newly developing countries and diversification of dietary habits, demand for meat has been accelerated worldwide. However, raising livestock is costly and livestock production is limited. It is predicted that the supply of edible meat will not keep up with the demand before long.

In this circumstance, a solution to stably supply edible meat is meat alternatives, which have been researched and developed.

[0003]

There are various alternatives to edible meat produced from livestock, and these alternatives are classified into, e.g., meat imitation, plant meat, cultured minced meat, and cultured steak meat. Of them, the cultured minced meat and cultured steak meat are called cultured meat. The cultured minced meat is an aggregate of disjointed myocytes and inferior in taste of meat, whereas the cultured steak meat is a meat alternative, in which a three-dimensional muscle tissue structure is reproduced, and from which a texture of meat can be given.

[0004]

To reproduce the three-dimensional structure of a muscle tissue, in other words, to construct the three-dimensional muscle tissue *in vitro*, it is necessary to culture cells three-dimensionally. However, for culturing cells three-dimensionally, there are many technical problems to be overcome.

[0005]

Cited Literature 1 discloses a method for producing a three-dimensional muscle tissue suitable for food. The method includes the steps of: preparing substantially rectangular first cell modules containing skeletal myoblasts in a hydrogel and having a plurality of

substantially rectangular holes mutually in parallel and substantially rectangular second cell modules containing skeletal myoblasts in a hydrogel and having a plurality of substantially rectangular holes mutually in parallel at a different position from the first cell modules in a vertical direction; alternately stacking the obtained first cell modules and second cell modules so as to obtain a stacked body; culturing skeletal myoblasts contained in the obtained stacked body to proliferate them; and inducing differentiation of the proliferated skeletal myoblasts into myotubes.

Citation List

Patent Literature

[0006]

PTL 1: JP 2020-141573 A

Summary of Invention

Technical Problem

[0007]

Based on the technology disclosed in Cited Literature 1, a method for producing a three-dimensional muscle tissue particularly suitable for food, more specifically, a three-dimensional muscle tissue having the same sarcomere structure as a muscle tissue derived from a living organism and expected to have a similar texture to farmed meat when it is subjected as a food.

Whereas, for simply producing a three-dimensional muscle tissue sufficiently large to eat, a further improvement is required.

An object of the present invention is to provide a method for simply producing a three-dimensional muscle tissue sufficiently large to eat, and a culture container for producing the three-dimensional muscle tissue.

Solution to Problem

[0008]

The present inventors conducted intensive studies in consideration of the above problems. As a result, they have found that the above problems can be solved by using a culture container including a pair of anchor sections facing each other on the edges of the container and a plurality of convex parts arranged over the space between the pair of anchor sections. The present invention has been accomplished by conducting further studies based on the finding.

[0009]

The present invention includes the following aspects.

<1> A method for producing a three-dimensional muscle tissue, including the following steps (A) and (B):

(A) supplying a myoblast-containing hydrogel to a culture container, wherein the culture container includes a pair of anchor sections facing each other on the edges of the container and a plurality of convex parts arranged over the space between the pair of anchor sections, and

(B) inducing differentiation of the myoblasts into myotubes in the culture container.

<2> The method for producing a three-dimensional muscle tissue according to <1>, wherein in the culture container, the in the longitudinal direction of the convex parts is 1 cm or more, and the length (Y) in the lateral direction of the convex parts is 1 cm or more.

<3> The method for producing a three-dimensional muscle tissue according to <1> or <2>, wherein in the culture container, a ratio $[(Y)/(X)]$ of the length (Y) in the lateral direction of the convex parts to the in the longitudinal direction of the convex parts is 0.7 or more.

<4> The method for producing a three-dimensional muscle tissue according to any one of <1> to <3>, wherein the convex parts have a height of 50 μm to 1 mm.

<5> The method for producing a three-dimensional muscle tissue according to any one of <1> to <4>, wherein the distance between the convex parts is 10 μm to 1 mm.

<6> A three-dimensional muscle tissue obtained by the production method according to any one of <1> to <5>.

<7> A culture container for producing a three-dimensional muscle tissue, wherein the culture container includes a pair of anchor sections facing each other on the edges of the container and a plurality of convex parts arranged over the space between the pair of anchor sections.

Advantageous Effects of Invention

[0010]

The present invention provides a method for producing a three-dimensional muscle tissue particularly suitable as food. The three-dimensional muscle tissue produced by the production method of the present has the same sarcomere structure as the muscle derived from a living organism. Furthermore, a three-dimensional muscle tissue sufficiently large to eat can be produced. Thus, the three-dimensional muscle tissue produced by the production

method of the present invention is expected to have a texture close to farmed meat, when it is subjected as food.

Brief Description of Drawings

[0011]

[Fig. 1] Fig. 1 is a perspective view of a culture container 100 according to an embodiment of the present invention.

[Fig. 2] Fig. 2 is a cross-sectional view of the container along the line A-A' of Fig. 1.

[Fig. 3] Fig. 3 is an enlarged view of the anchor section of Fig. 1.

[Fig. 4] Fig. 4 is a perspective view of a culture container 200 according to an embodiment of the present invention.

[Fig. 5] Fig. 5 shows (a) a plan view, (b) a front view, (c) a right-side view, and (d) a bottom view of the culture container 200 according to an embodiment of the present invention.

[Fig. 6] Fig. 6 is a perspective view of a culture container 300 according to an embodiment of the present invention.

[Fig. 7] Fig. 7 shows (a) a plan view, (b) a front view, (c) a right-side view, and (d) a bottom view of the culture container 300 according to an embodiment of the present invention.

[Fig. 8] Fig. 8 is a perspective view of a culture container 400 according to an embodiment of the present invention.

[Fig. 9] Fig. 9 shows (a) a plan view, (b) a front view, (c) a right-side view, and (d) a bottom view of the culture container 400 according to an embodiment of the present invention.

[Fig. 10] Fig. 10 shows an overview of the culture container 100 according to an embodiment of the present invention. The length (X) of the convex parts of the culture container 100 in the longitudinal direction is 4 cm, and the length (Y) of the convex parts of thereof is 3 cm. (i) Anchor section: members thereof are cylinders of 0.5 mm in diameter and 5 mm in height, and arranged at the interval of 1 mm in terms of distance between center points. (ii) Convex parts facilitating formation of orientation: members thereof have a height of 200 μ m and a width of 50 μ m and are disposed at a distance of 2 mm from the anchor.

[Fig. 11-1] Fig. 11-1 shows images of SAA immunostaining.

[Fig. 11-2] Fig. 11-2 shows images obtained by inverting the colors of the images of Fig. 11-1.

[Fig. 12] Fig. 12 is a graph showing a change in a shrinkage of a three-dimensional muscle tissue over time, more specifically, a change in the width of the tissue in the lateral direction. The vertical axis indicates the length (cm) of the tissue in the lateral direction.

[Fig. 13] Fig. 13 is a graph showing a change in movement (contraction amount) of a three-dimensional muscle tissue over time. (A) a case where the height of the convex parts is 100 μm , (B) a case where the height of convex parts is 300 μm , (C) a case where the height of convex parts is 500 μm .

Description of Embodiments

[0012]

[Method for Producing Three-Dimensional Muscle Tissue]

The method for producing a three-dimensional muscle tissue of the present invention includes the following steps (A) and (B):

(A) supplying a myoblast-containing hydrogel to a culture container, wherein the culture container includes a pair of anchor sections facing each other on the edges of the container and a plurality of convex parts arranged over the space between the pair of anchor sections, and

(B) inducing differentiation of the myoblasts into myotubes in the culture container.

[0013]

<Three-Dimensional Muscle Tissue>

In the present invention, the three-dimensional muscle tissue refers to principally muscle not derived from a living organism but artificially produced. The three-dimensional muscle tissue of the present invention is constituted of myocytes. The myocytes are preferably striated myocytes having a contractile ability, more specifically skeletal myocytes or cardiomyocytes. The myocytes are multinucleated myoblasts as precursor cells thereof and present in the form of myotubes or muscle fibers.

[0014]

Generally, the muscle fiber is constituted of myofibrils as structural units each constituted of a fiber of actin (actin filament), which is a protein constituting muscle and a fiber of myosin (myosin filament), which is a protein constituting muscle. Further, the myofibril has a structure where a plurality of sarcomere units is continuously arranged in the longitudinal axis. The muscle contracts and relaxes based on the interaction (sliding) between actin and myosin in a sarcomere.

[0015]

The three-dimensional muscle tissue of the present invention has a sarcomere structure. However, it does not matter whether sliding occurs or not in the sarcomere structure.

[0016]

Whether a three-dimensional muscle tissue has a sarcomere structure or not can be evaluated by a commonly known method. For example, the presence of sarcomeric α -actinin (SAA), which is a protein constituting Z membrane of a sarcomere structure, is evaluated by immunostaining of SAA. If SAA immunostaining is positive and SAA is regularly distributed like a stripe, the muscle tissue can be determined to have a sarcomere structure.

[0017]

The three-dimensional muscle tissue of the present invention is preferably edible. For example, in the case of an edible three-dimensional muscle tissue, myocytes constituting the muscle tissue are preferably skeletal myocytes. The edible three-dimensional muscle tissue can be rephrased as, e.g., "cultured meat" or "artificial meat".

[0018]

<Step (A)>

Step (A) is a step of supplying a myoblast-containing hydrogel to a culture container.

The culture container is a culture container having a plurality of approximately rectangular convex parts arranged mutually in parallel at the bottom and anchor sections at both edges of the convex parts in the longitudinal direction.

[0019]

<Myoblast-Containing Hydrogel>

(Myoblasts)

Myoblasts can be prepared by a commonly known method. For example, primary myoblasts obtained by treating a muscle tissue derived from a living organism with a degradative enzyme (for example, collagenase) can be used.

For example, in the case where the myocytes constituting a three-dimensional tissue are skeletal myocytes, the myoblasts are skeletal myoblasts.

Note that, a treatment by filtering can be made in order to remove impurities such as connective tissues from primary myoblasts. However, it is not essential to completely remove other cells except myoblasts and a mixture of myoblasts and other cells can be used as the myoblasts of the invention.

[0020]

Furthermore, stem cells having a pluripotency such as ES cells or iPS cells, or cells induced/differentiated from somatic stem cells having an ability to differentiate into myoblasts can be used as the myoblasts.

[0021]

The myoblasts are derived from a vertebrate such as mammals, birds, reptiles, amphibians or fish. Examples of the mammals include non-human mammals such as monkeys, cows, horses, pigs, sheep, goats, dogs, cats, guinea pigs, rats, and mice. Examples of the birds include ostriches, chickens, ducks and sparrows. Examples of the reptiles include snakes, crocodiles, lizards, and turtles. Examples of the amphibians include frogs, newts, and salamanders. Examples of fish include salmon, tunas, sharks, breams, and carps. In the case of using three-dimensional muscle tissue as food, myoblasts are preferably derived from mammals such as livestock animals including cows, pigs, sheep, goats, or horses, and more preferably derived from cows.

[0022]

As the myoblasts, myoblasts genetically modified by, e.g., homologous recombination, genome editing such as CRISPR/Cas9 method or myoblasts not genetically modified can be used. In an aspect of using a three-dimensional muscle tissue as food, myoblasts not genetically modified are preferably used in view of safety and consumers' preferences.

[0023]

(Hydrogel)

A hydrogel serves as a scaffold for culturing a three-dimensional muscle tissue.

As a preferable aspect of the hydrogel, it is possible to use a gel of a component constituting an extracellular basement membrane matrix, such as fibrin, fibronectin, laminin, collagen (for example, type I, type II, type III, type V, type XI), agar, agarose, glycosaminoglycans, hyaluronic acid, or proteoglycan. As the hydrogel, a commercially available product can be used. For example, components based on an extract from mouse EHS tumor (containing, e.g., type IV collagen, laminin, heparan sulfate proteoglycan), which is sold, for example, under the trade name "Matrigel", can be used.

[0024]

Note that, "collagen" used in the specification includes non-denatured collagen and denatured collagen. Examples of the denatured collagen include gelatin.

[0025]

As another preferable aspect of hydrogel, a gel containing blood-derived plasma and a coagulant is mentioned.

Animals from which blood is derived are mammals such as livestock animals such as cows, pigs, sheep, goats, or horses, and more preferably cows in view of availability.

The animals from which blood is derived may be antepartum fetuses or postpartum adults. The ages such as age in days, age in months and age in years of the animals from which blood is derived are not limited. In the case of adults, adults may be infants, young individuals, matured individuals, or old individuals, after delivered from female individuals.

In a preferable aspect, the animals from which blood is derived are adult cows. Of them, matured cows are more preferable in consideration of commercial availability and the quantity slaughtered.

Blood derived from adult cows can be collected as a specimen from cows slaughtered in meat markets and wholesale markets, or by other methods.

[0026]

In order to prevent coagulation during distribution and storage, an anticoagulant is sometimes added to blood and thereafter the blood is delivered. The blood containing an anticoagulant is centrifugally separated into erythrocytes and plasma containing fibrinogen. If an anticoagulant is added to blood, erythrocytes and plasma containing fibrinogen serving as a coagulation component are neither aggregated nor gelatinized.

[0027]

When a coagulant is added to a blood-derived plasma containing fibrinogen and centrifugally obtained, the plasma is gelatinized.

Note that, since the hydrogel of this aspect contains plasma, the supply of cell-culture components serving as nutrients during cell culture is expected.

[0028]

Furthermore, the plasma derived from adult cows containing fibrinogen may be platelet-rich plasma (PRP). The platelet-rich plasma is a plasma concentrate prepared by centrifuging plasma and rich in platelets. Not only platelets but also soluble proteins and growth factors are concentrated therein.

[0029]

The platelet-rich plasma can be collected by, e.g., centrifugation, double centrifugation, or selective filtration of blood containing an anticoagulant, more specifically, centrifugally collected from erythrocyte-free plasma. In the case of blood containing erythrocytes, the concentrated plasma layer present in the boundary with erythrocytes may be collected.

[0030]

In the case of using platelet-rich plasma, compared to the case of using whole plasma, gelatinization tends to quickly proceed. Because of this, in cell culture gel using platelet-rich plasma, precipitation of cells rarely occurs and cells can be uniformly dispersed.

[0031]

The type of anticoagulant is not limited as long as it prevents the coagulation of blood. Examples of the anticoagulant include substances binding to indispensable calcium ions for blood coagulation, such as sodium citrate, EDTA (ethylenediaminetetraacetate), and sodium fluoride.

In the case of sodium citrate, the addition amount thereof based on the total amount of blood and an anticoagulant is preferably 2 vol% to 4 vol% and more desirably around 3 vol%.

[0032]

The coagulant is not limited as long as it can gelatinize the blood plasma containing an anticoagulant. Examples of the coagulant include calcium chloride and DMEM (Dulbecco's Modified Eagle's Medium). A coagulant containing calcium ions is more preferable.

[0033]

In the case where the coagulant is calcium chloride, the coagulant can be added such that the concentration of calcium ions based on the total amount of plasma and a coagulant becomes preferably, for example, 5 mM to 70 mM, more preferably 10 mM to 65 mM, and further preferably 10 mM to 60 mM.

Note that, in the specification, "XX to YY" means "XX or more to YY or less".

[0034]

DMEM contains L-arginine, L-cystine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine, L-valine, calcium chloride, potassium chloride, magnesium sulfate, sodium chloride, sodium dihydrogen phosphate, D-glucose, folic acid, nicotinamide, riboflavin, vitamins B12, choline, inositol, pantothenic acid, pyridoxal phosphate, thiamine, and iron.

In the case where the coagulant is DMEM, DMEM can be added such that the amount of blood-derived plasma relative to the amount of DMEM becomes, preferably, for example, 0.1 vol% or more and 35 vol% or less, more preferably 0.1 vol% or more and 30 vol% or less, and further preferably 0.2 vol% or more and 30 vol% or less.

[0035]

The hydrogel may further contain components of a medium.

Examples of the components of a medium include DMEM (for example, manufactured by GIBCO), EMEM (for example, manufactured by GIBCO), MEMALPHA (for example,

manufactured by GIBCO), and RPMI-1640 (Roswell Park Memorial Institute 1640 culture; manufactured by GIBCO).

To the medium components, additives commonly used in a medium can be appropriately blended. Examples of components of the additives include antibiotic substances, vitamins, nucleic acids, amino acids, inorganic salts, sugars, polyamines, carbohydrates, proteins, fatty acids, lipids, pH-adjusting agents, zinc, copper, and selenium.
[0036]

The myoblasts in the hydrogel are added, for example, at a cell density of preferably about 1.0×10^6 cells/mL or more, more preferably about 1.0×10^7 cells/mL to about 1.0×10^8 cells/mL, and further preferably 5.0×10^7 cells/mL to about 1.0×10^8 cells/mL.
[0037]

(Culture Container)

The culture container to be used in the present invention is a culture container for producing a three-dimensional muscle tissue, wherein the culture container includes a pair of anchor sections facing each other on the edges of the container and a plurality of convex parts arranged over the space between the pair of anchor sections.

It is considered that the orientations of myotubes and a bundle of muscle fibers are efficiently formed by providing the convex parts.
[0038]

Now, preferred embodiments for carrying out the present invention will be described with reference to the accompanying drawings.

Fig. 1 shows a perspective view of the structure of a culture container 100 according to the present embodiment. Fig. 2 shows a cross-sectional view of the culture container 100.
[0039]

The material for constituting the culture container 100 is not limited. The material for constituting the culture container 100 is, for example, a thermoplastic resin or a thermosetting resin. Examples of the thermoplastic resin include polyolefin resins such as polypropylene; polyester resins such as polyethylene terephthalate; acrylic resins; thermoplastic elastomers; and silicone resins such as polydimethylsiloxane (PDMS).

The material for constituting the culture container 100 is preferably a non-adhesive material to cells or a material whose surface is treated to be non-adhesive. Examples of the non-adhesive surface treatment include parylene coating.

The material constituting the culture container 100 may be transparent or opaque. For convenience in observation, the material is preferably transparent.

The culture container 100 can be produced by, for example, a 3D printer.

[0040]

The culture container is preferably rectangular. In this case, the in the longitudinal direction of the convex parts is preferably 1 cm or more, and more preferably 1.5 cm or more, and the length (Y) in the lateral direction of the convex parts is preferably 1 cm or more, and more preferably 2 cm or more. The longitudinal direction of the convex parts is the direction along which a pair of anchor sections face each other direction, whereas the lateral direction of the convex parts is the direction along which convex parts are arranged.

The ratio of the length (Y) in the lateral direction of the convex parts to the in the longitudinal direction of the convex parts, $[(Y)/(X)]$ is 0.7 or more. The ratio $[(Y)/(X)]$ can be 1 or more, 1.5 or more, or 2 or more. The upper limit thereof is not particularly limited but, for example, 4 or less.

[0041]

The culture container 100 includes a plurality of convex parts arranged over the space between a pair of anchor sections 20 described later.

The shape of the anchor sections is not limited but, for example, approximately rectangular as viewed from the top of the culture container.

[0042]

The arrangement style of a plurality of convex parts is not limited. Preferably, a plurality of convex parts is provided on the bottom so as to be arranged mutually in parallel.

The convex part 10 has a height of preferably 50 μm to 1 mm, and more preferably 100 μm to 300 μm .

The interval between the adjacent convex parts is 10 μm to 5 mm, more preferably 50 μm to 2.5 mm, and further preferably 100 μm to 1 mm.

[0043]

The culture container to be used in the present invention has a pair of anchor sections facing each other on the edges of the container. The anchor section is not particularly limited as long as it is a means for immobilizing a hydrogel and a three-dimensional muscle tissue to be produced. For example, a member for immobilizing tissues by means of an adhesive component (for example, hydrogel such as fibrin) is mentioned.

In the culture container 100 shown in Fig. 1 as a preferable aspect, a plurality of cylindrical anchor sections 20 faces each other on the edges of the container. The cylindrical anchor sections 20 each have a diameter of about 100 μm to 2 mm and a height of about 3 mm to 10 mm.

[0044]

Figs. 4 to 9 show a culture container 200, a culture container 300, and a culture container 400 according to other embodiments each by way of a perspective view, plan view, front view, right-side view and bottom view.

[0045]

(Supply of Hydrogel)

A myoblast-containing hydrogel is supplied to the culture container as mentioned above. Preferably, the culture container is placed at the bottom and a formwork is provided on the side surface thereof. The myoblast-containing hydrogel is supplied to the culture container.

The amount of the myoblast-containing hydrogel to be supplied can be appropriately set, for example, can be set such that the thickness of the hydrogel becomes preferably 1 mm to 1 cm, and more preferably 2 to 5 mm.

[0046]

The hydrogel supplied to the culture container can be solidified by heating. The heating temperature range is preferably about 37°C. The heating time can be controlled depending on the progression of gelling, for example, about 5 minutes to 60 minutes, and more preferably about 10 minutes.

[0047]

Furthermore, a myoblast-containing hydrogel is cultured to proliferate myoblasts contained therein.

For example, in the case where a hydrogel contains a sufficient amount (for example, 1.0×10^8 cells/mL or more) of myoblasts, the hydrogel can be subjected to the following induction/differentiation step without culturing for proliferation. For example, in the case where myoblasts need to proliferate, the myoblasts are cultured for proliferation and then can be subjected to the following induction/differentiation step.

[0048]

The culture mentioned above can be performed, for example, in the aforementioned culture medium in accordance with a method commonly known to those skilled in the art. Examples of a suitable culture method include, but are not limited to, a culture method performed under the conditions of about 37°C and a carbon dioxide concentration of about 5 to 10% (v/v). The culture under the above conditions can be performed by, for example, using a CO₂ incubator commonly known.

[0049]

As the culture medium for proliferation, it is possible to use a liquid medium commonly used such as DMEM (Dulbecco's Modified Eagle's Medium), EMEM (Eagle's

minimal essential medium) or α MEM (alpha Modified Minimum Essential Medium) containing a serum component (for example, Horse Serum, Fetal Bovine Serum (FBS), Human Serum), a component such as a growth factor, and an antibiotic substance such as penicillin or streptomycin.

[0050]

In the case where a serum component is added to the culture medium, fetal bovine serum can be used as the serum component. The concentration of the serum component can be set at about 10% (v/v).

[0051]

The culture period can be set at, for example, one day to two weeks.

[0052]

If necessary, the medium can be exchanged. The culture conditions can be set in accordance with those used in a routine method.

[0053]

<Step (B)>

Step (B) is a step of inducing differentiation of myoblasts into myotubes in the culture container.

In the step, myoblasts are fused with surrounding cells and multi-nucleated to form myotubes. The myotubes are further matured into muscle fibers.

[0054]

The culture mentioned above can be performed in, for example, a medium for differentiation induction (medium for multinucleation) by a method commonly known to those skilled in the art. Examples of a suitable culture method include, but are not limited to, a culture method performed under the conditions of about 37°C and a carbon dioxide concentration of about 5 to 10% (v/v). The culture under the above conditions can be performed by, for example, using a CO₂ incubator commonly known.

[0055]

It is known that myoblasts start multinucleation by involving surrounding cells in poor nutrition. For this reason, induction of differentiation into myotubes can be performed in a medium lower in nutrition conditions than the medium for proliferation.

[0056]

In this manner, a three-dimensional muscle tissue is produced.

The present invention also relates to the three-dimensional muscle tissue obtained by the production method mentioned above.

Examples

[0057]

Now, the present invention will be further specifically described by way of Examples. However, the following Examples are not construed as limiting the range of the present invention.

[0058]

Example 1: Preparation of Large Contractible Muscle Tissue Using Bovine Myoblasts

Bovine myoblasts were embedded in a hydrogel having the following composition, fixed at both ends and cultured to prepare a muscle tissue of 7 mm in length. The conditions for forming and maturing the muscle tissue were examined by changing the hydrogel composition and cell density.

[0059]

(Preparation of Culture Container 100)

The culture container 100 shown in Fig. 10 was formed by a 3D printer (Formlabs 3B, manufactured by Formlabs, Inc.). In the culture container 100, the in the longitudinal direction of the convex parts was 4 cm, and the length (Y) in the lateral direction of the convex parts was 3 cm. A convex part 10 for facilitating orientation formation of myocytes has a height of 200 μ m and a width of 5 μ m and is provided at a distance of 2 mm from anchor sections. The length thereof in the longitudinal direction was 3 cm.

The anchor sections 20 each are a cylinder of 0.5 mm in diameter and 5 mm in height and were provided such that the distance between the center points of them became 1 mm.

The surface of the culture container formed was coated with parylene by using a parylene deposition device (Labcoater PDS2010, manufactured by Specialty Coating Systems). Then, the culture container was sterilized by an ozone sterilizer and anchor sections 20 were coated with fibronectin in order to facilitate cell adhesion.

[0060]

(Preparation of Culture Container as Control)

A culture container used as a control was formed in the same manner as in the culture container 100 except that convex parts were not formed.

[0061]

(Hydrogel Composition (1))

Fibrinogen (25 mg/mL) 480 μ L (final concentration 4 mg/mL) (F8630, manufactured by Sigma)

Matrigel 600 μ L (final concentration 20%) (356231 manufactured by Corning Inc.)

Thrombin (200 units/mL) 30 μ L (final concentration 2 units/mL) (T4648-10KU, manufactured by Sigma)

DMEM 1890 μ L (11965118, manufactured by Thermo Fisher Scientific, Inc.)
[0062]

(Preparation of Hydrogel)

Myoblasts (3.0×10^7 cells) were mixed with a hydrogel so as to obtain a cell density of 1.0×10^7 cells/mL to prepare the hydrogel having cells embedded therein. The culture container 100 or a culture container as a control was placed at the bottom and a formwork was provided on the side surface thereof. The obtained hydrogel having cells embedded therein was poured through the formwork and allowed to stand still at 37°C, for 30 minutes in a CO₂ incubator to solidify the hydrogel.

[0063]

(Culture)

Subsequently, a culture medium (10% FBS DMEM + 1% Penicillin/Streptomycin) was added. Culture was further performed at 37°C in 5% CO₂ for two days.

Thereafter, the medium was exchanged with a medium (2% HS DMEM + 1% Penicillin/Streptomycin + 100 μ M) for differentiation and culture was further continued for 5 days to obtain a three-dimensional muscle tissue. The medium was changed every other day.

With the passage of culture time, the three-dimensional muscle tissue contracted (shrank) in the lateral direction of the convex parts.

[0064]

(Electrical Stimulation)

The three-dimensional muscle tissues obtained on Day 5 and Day 7 after culture were electrically stimulated by an electrical stimulation culture system, C-Pace (manufactured by IonOptic) in the following conditions: frequency: 1 Hz, strength: 0.3 V/mm, time: 40 ms). The presence or absence of contraction movement in response to the electrical stimulation was observed by a microscope.

In the three-dimensional muscle tissue obtained by using the culture container 100, a clear contraction movement was observed. In contrast, in the three-dimensional muscle tissue obtained by using a culture container as a control, no contraction movement was observed.

[0065]

(SAA Immunostaining)

Seven days later, the obtained three-dimensional muscle tissue was fixed with 4% PFA. Immunostaining of Sarcomeric α -actinin (SAA), and cell-nucleus staining with Hoechst 33342 were performed.

The microscopic observation results are shown in Fig. 11-1.

In the three-dimensional muscle tissue (left figure) obtained by using the culture container 100, a large number of the myotube regions stained with SAA were observed and myotube regions are mutually oriented. In contrast, the number of the myotube regions observed in the three-dimensional muscle tissue (right figure) obtained by using the culture container used as a control was low, and the degree of orientation was low.

[0066]

From the above results, it was suggested that cell differentiation from myoblasts in a three-dimensional muscle tissue to myotubes and orientation formation of myotubes are facilitated by the convex structure of the culture container.

[0067]

Example 2: Examination of Size of Cultured Muscle Tissue

(Preparation of Culture Container 200, Culture Container 300, Culture Container 400)

A culture container 200 having X:Y of 4 cm:3 cm, a culture container 300 having X:Y of 4 cm:8 cm, and a culture container 400 having X:Y of 10 cm:10 cm where X is the length of convex parts in the longitudinal direction, and Y is the length in the lateral direction of the convex parts, were prepared in the same manner as in the culture container 100 in Example 1.

[0068]

(Preparation and Culture of Hydrogel)

A hydrogel was prepared in the same manner as in Example 1.

The obtained hydrogel having cells embedded therein was poured in amounts of 3 mL, 8 mL and 24 mL to the culture containers 200, 300 and 400, respectively and allowed to stand still at 37°C for 30 minutes in a CO₂ incubator to solidify the hydrogels.

Culture was performed in the same manner as in Example 1 for 7 days to obtain three-dimensional muscle tissues. With the passage of culture time, the three-dimensional muscle tissue contracted (shrank) in the lateral direction of the convex parts.

During culture from a hydrogel to a three-dimensional muscle tissue, the length of convex parts in the lateral direction was measured over time to observe the degree of contraction (shrinkage). The results are shown in Fig. 12.

Seven days after culture, the three-dimensional muscle tissues obtained by using the culture container 200 and the culture container 30 had a shrinkage of about 2 cm, whereas the

three-dimensional muscle tissue obtained by using the culture container 400 had a shrinkage of about 5 cm.

From this, it was suggested that the shrinkage of the three-dimensional muscle tissue is approximately 1/2 of the length of the convex parts of the culture container in the lateral direction; and found that any size of the three-dimensional muscle tissue can be prepared by using a culture container having a size determined on the presumption of the shrinkage.

[0069]

Example 3: Preparation of Hydrogel Using Edible-Pig Blood

(Preparation of Culture Container 500)

A culture container 500 having X:Y of 8 cm:12 cm where X is the length of convex parts in the longitudinal direction and Y is the length in the lateral direction of the convex parts was prepared in the same manner as in the culture container 100 in Example 1.

[0070]

(Hydrogel Composition (2))

Fibrinogen (25 mg/mL) 4000 μ L (final concentration 4 mg/mL) (F8630, manufactured by Sigma)

Edible-pig blood 5000 μ L (final concentration 20%)

Thrombin (200 units/mL) 250 μ L (final concentration 2 units/mL) (T4648-10KU manufactured by Sigma)

DMEM 15750 μ L (11965118, manufactured by Thermo Fisher Scientific)

[0071]

(Preparation and Culture of Hydrogel)

A hydrogel was prepared using the above composition except that edible pig blood was used in place of Matrigel in the hydrogel composition of Example 1.

Myoblasts 2.5×10^8 cells were mixed with the hydrogel so as to obtain a concentration of 1.0×10^7 cells/mL to prepare a hydrogel having cells embedded therein. The culture container 500 or a culture container used as a control was placed at the bottom and a formwork was provided on the side surface thereof. The obtained hydrogel having cells embedded therein was poured through the formwork and allowed to stand still at 37°C, for 30 minutes in a CO₂ incubator to solidify the hydrogel.

Subsequently, a three-dimensional muscle tissue was obtained in the same manner as in Example 1 except that the culture period was 14 days.

[0072]

Day 7 after culture, electrical stimulation was applied in the same manner as in Example 1 and the presence or absence of contraction movement in response to the electrical

stimulation was observed by a microscope. As a result, contraction movement was observed.

A three-dimensional muscle tissue of about 8 cm × 8 cm was obtained by 14-day culture. With the passage of culture time, the three-dimensional muscle tissue contracted (shrank) by about 4 cm in the lateral direction of the convex parts.

From the above results, it was found that a contractible tissue can be prepared even if edible pig blood was used in place of Matrigel, and that the shrinkage of the tissue is the same as that of Matrigel.

[0073]

Example 4: Examination of Height of Convex Part

(Preparation of Culture Container)

A culture container 600 having the convex part 10 of 100 μm in height, a culture container 700 having the convex part 10 of 300 μm and a culture container 800 having the convex part 10 of 500 μm were prepared in the same manner as in the culture container 100 of Example 1 except the height of the convex part 10.

(Preparation of Hydrogel and Culture)

A hydrogel was prepared and cultured in the same manner as in Example 1.

[0074]

(Electrical Stimulation)

On Day 14 after culture, electrical stimulation was performed in the same manner as in Example 3. The contraction movement of three-dimensional muscle tissue in response to the electrical stimulation was observed and recorded on video by a microscope. Based on the video recorded, a graph showing a change in moving distance over time was obtained by plotting the moving distances (Distance (μm) in the figure) of a three-dimensional muscle tissue by contraction as the contraction amount.

The results are shown in Fig. 13.

[0075]

In all cases, the contraction motion of a three-dimensional muscle tissue was observed. Of them, in the case where the height of the convex part 10 is 100 μm, the movement (contraction amount) was particularly large.

Reference Signs List

[0076]

100 Culture container

10 Convex parts

20	Anchor section
200	Culture container
300	Culture container
400	Culture container

CLAIMS

[Claim 1]

A method for producing a three-dimensional muscle tissue, comprising the following steps (A) and (B):

(A) supplying a myoblast-containing hydrogel to a culture container, wherein the culture container comprises a pair of anchor sections facing each other on the edges of the container and a plurality of convex parts arranged over the space between the pair of anchor sections, and

(B) inducing differentiation of the myoblasts into myotubes in the culture container.

[Claim 2]

The method for producing a three-dimensional muscle tissue according to claim 1, wherein in the culture container, a in the longitudinal direction of the convex parts is 1 cm or more, and a length (Y) in the lateral direction of the convex parts is 1 cm or more.

[Claim 3]

The method for producing a three-dimensional muscle tissue according to claim 1, wherein in the culture container, a ratio $[(Y)/(X)]$ of the length (Y) in the lateral direction of the convex parts to the in the longitudinal direction of the convex parts is 0.7 or more.

[Claim 4]

The method for producing a three-dimensional muscle tissue according to claim 1, wherein the convex parts have a height of 50 μm to 1 mm.

[Claim 5]

The method for producing a three-dimensional muscle tissue according to claim 1, wherein the distance between the convex parts is 10 μm to 1 mm.

[Claim 6]

A three-dimensional muscle tissue obtained by the production method according to any one of claims 1 to 5.

[Claim 7]

A culture container for producing a three-dimensional muscle tissue, wherein the culture container comprises a pair of anchor sections facing each other on the edges of the container and a plurality of convex parts arranged over the space between the pair of anchor sections.