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(54) Titre : COMPOSITION POUR LA CULTURE D'EMBRYONS

(54) Title: COMPOSITION FOR EMBRYO CULTURE

Components	mM
L-Alanine	0.297 ± 0.089
L-Asparagine	0.015 ± 0.005
L-Aspartic acid	0.120 ± 0.036
L-Glutamic acid	0.550 ± 0.165
Glycine	0.979 ± 0.294
L(-)-Proline	0.105 ± 0.032
L-Serine	0.176 ± 0.053
L(+)-Arginine	0.108 ± 0.032
L(-)-Cystine	0.048 ± 0.014
L-Histidine	0.053 ± 0.016
L(+)-Isoleucine	0.036 ± 0.011
L-Leucine	0.081 ± 0.024
L(+)-Lysine	0.176 ± 0.053
L-Methionine	0.022 ± 0.007
L(-)-Phenylalanine	0.045 ± 0.013
L(-)-Threonine	0.109 ± 0.033
L-Tryptophan	0.018 ± 0.005
L-Tyrosine	0.048 ± 0.014
L-Valine	0.108 ± 0.032
L-Glutamine or glutamine derivative	0.398 ± 0.119
Taurine	1.412 ± 0.424

(57) Abrégé/Abstract:

Provided is a composition for embryo culture, which contains a constitution suitable for embryo culture. Provided is a composition for embryo culture, which contains (a) a constitution shown in Table A below.

ABSTRACT

Provided is a composition for embryo culture, which contains a constitution suitable for embryo culture. Provided is a composition for embryo culture, which contains (a) a constitution shown in Table A below.

Table A

Components	mM
L-Alanine	0.297 $\pm$ 0.089
L-Asparagine	0.015 $\pm$ 0.005
L-Aspartic acid	0.120 $\pm$ 0.036
L-Glutamic acid	0.550 $\pm$ 0.165
Glycine	0.979 $\pm$ 0.294
L(-)-Proline	0.105 $\pm$ 0.032
L-Serine	0.176 $\pm$ 0.053
L(+)-Arginine	0.108 $\pm$ 0.032
L(-)-Cystine	0.048 $\pm$ 0.014
L-Histidine	0.053 $\pm$ 0.016
L(+)-Isoleucine	0.036 $\pm$ 0.011
L-Leucine	0.081 $\pm$ 0.024
L(+)-Lysine	0.176 $\pm$ 0.053
L-Methionine	0.022 $\pm$ 0.007
L(-)-Phenylalanine	0.045 $\pm$ 0.013
L(-)-Threonine	0.109 $\pm$ 0.033
L-Tryptophan	0.018 $\pm$ 0.005
L-Tyrosine	0.048 $\pm$ 0.014
L-Valine	0.108 $\pm$ 0.032
L-Glutamine or glutamine derivative	0.398 $\pm$ 0.119
Taurine	1.412 $\pm$ 0.424

COMPOSITION FOR EMBRYO CULTURE

Technical Field

[0001]

5 The present invention relates to a culture composition.
More particularly, the present invention relates to a
composition for embryo culture.

Background Art

10 [0002]

In vitro fertilization, embryo culture and embryo
transplantation and the like are important techniques in a
wide range of fields such as the improvement and production
of animals with a high economic value, regenerative
15 medicine and reproductive medicine. In *in vitro*
fertilization and embryo culture, gametes or embryos are
treated *in vitro*. Until now, various media for embryo
culture have been developed, such as sequential culture
media which are made to imitate changes in the environment
20 of peripheral body fluids during embryonic development,
serum media which are supplemented with serum and serum-
free simple media called "single medium" which can be
easily treated. When using a single medium, medium
exchange in accordance with the developmental stage of
25 embryos as a sequential culture media is not required.

When using a serum-free medium, risks present in serum media can be reduced, such as quality variation and virus contamination because of products derived from organisms.
[0003]

5 As one of serum-free single media, KSOM medium is known (Proc Natl Acad Sci USA 2007; 104(36): 14289-14293 and Manipulating the Mouse Embryo, THIRD EDITION 2005; 149-193.). In embryos cultured in the KSOM medium, the number of cells in blastocysts is increased as compared to
10 that in embryos cultured in a medium before the appearance of KSOM (Fertil Steril 2006; 86: 1252-1265.), however, the number does not reach the number of cells in blastocysts developed *in vivo* (Biol Reprod 1994; 50: 1027-1033 and Reproduction 2009; 137: 271-283.).

15 [0004]

 It is known that amino acids act as biosynthetic precursors, energy sources, organic osmolytes, intracellular pH buffer substances, antioxidants, chelators and the like, and the addition of amino acids to media for
20 embryo culture effectively acts on embryonic development (Semin Reprod Med 2000; 18(2): 205-218). The KSOM medium was a medium which contained only glutamine as an amino acid. It has been revealed, however, that the blastocyst development rate, the hatching rate and the number of cells
25 in blastocysts are improved by adding 20 types of amino

acids to the KSOM medium (Mol Reprod Dev 1995; 41(2):
232-238).

[0005]

When developing a medium for culturing human pre-
5 implantation embryos, researches using mouse embryos are
recommended (Human Reproduction 1998; 13(4): 173-183 and
Human Reproduction Update 2003; 9(6): 557-582). The KSOM
medium or KSOMaa medium to which 20 types of amino acids
are added has been researched using mouse embryos (Biol
10 Reprod 1994; 50: 1027-1033 and Mol Reprod Dev 1995; 41(2):
232-238). It has been verified, however, that the media
are suitable for culturing not only human embryos but also
various animal embryos such as bovine, rabbit, rhesus
monkey, swine and rat, and the media are now widely used.

15

Disclosure of Invention

Problems to be solved by the Invention

[0006]

The effect of each component in a culture medium
20 depends on the concentrations of other components (Human
Reproduction Update 2003; 9(6): 557-582), and thus a vast
number of examinations are required in order to set
concentrations suitable for embryonic development by
individually combining 20 types of amino acids. Besides,
25 in the research of embryos in the pre-implantation phase,

such extensive analysis has been difficult due to a
scarcity of samples.

[0007]

Because of this, most concentrations of amino acids
5 added to an embryo culture medium such as KSOMaa medium are
concentrations decided using the proliferation ability of
somatic cells as an index and are not optimized for
embryonic development.

[0008]

10 The concentrations of amino acids added to the KSOMaa
medium, for example, are just set to one-half of each
specific concentration of 13 amino acids belonging to the
essential amino acid group (Science 1959; 130, 432-437) and
other 7 amino acids belonging to the non-essential amino
15 acid group, which specific concentration has been decided
using the auxotrophy of somatic cells as an index by Eagle
and others. In addition, in a culture fluid G1 or G2
developed for human embryo culture, the concentrations
decided using auxotrophy of somatic cells as an index by
20 Eagle are directly used as the concentrations of amino
acids added to the culture media.

Therefore, the development of culture media having
concentrations of amino acids suitable for *in vitro* embryo
culture has been still demanded.

Means for Solving the Problems

[0009]

In view of such demand, the present inventors repeated intensive research and consequently found amino acid concentrations suitable for embryo culture.

[0010]

By a mode of the present invention, there is provided a composition for embryo culture, which contains a constitution shown in Table A below.

10 [Table 1]

Table A

Component	mM
L-Alanine	0.297 $\pm$ 0.089
L-Asparagine	0.015 $\pm$ 0.005
L-Aspartic acid	0.120 $\pm$ 0.036
L-Glutamic acid	0.550 $\pm$ 0.165
Glycine	0.979 $\pm$ 0.294
L(-)-Proline	0.105 $\pm$ 0.032
L-Serine	0.176 $\pm$ 0.053
L(+)-Arginine	0.108 $\pm$ 0.032
L(-)-Cystine	0.048 $\pm$ 0.014
L-Histidine	0.053 $\pm$ 0.016
L(+)-Isoleucine	0.036 $\pm$ 0.011
L-Leucine	0.081 $\pm$ 0.024
L(+)-Lysine	0.176 $\pm$ 0.053
L-Methionine	0.022 $\pm$ 0.007
L(-)-Phenylalanine	0.045 $\pm$ 0.013
L(-)-Threonine	0.109 $\pm$ 0.033
L-Tryptophan	0.018 $\pm$ 0.005
L-Tyrosine	0.048 $\pm$ 0.014
L-Valine	0.108 $\pm$ 0.032
L-Glutamine or glutamine derivative	0.398 $\pm$ 0.119
Taurine	1.412 $\pm$ 0.424

[0011]

In an embodiment of the present invention, there is provided a composition for embryo culture, which further contains at least one component selected from the group consisting of electrolytes, organic acids, carbohydrates, pH indicators, pH adjusters, pH buffers, antibiotics, vitamins, trace metal elements, chelators, hormones, growth factors, lipids or constituents thereof, carrier proteins, extracellular matrix components, reducing substances and polymers in addition to the constitution shown in Table A above.

[0012]

In an embodiment of the present invention, there is provided a composition for embryo culture, which contains the constitution shown in Table A above and electrolytes. In another embodiment of the present invention, there is provided a composition for embryo culture, which contains the constitution shown in Table A above, electrolytes, and organic acids and/or carbohydrates. In yet another embodiment of the present invention, there is also provided a composition for embryo culture, which contains the constitution shown in Table A above, electrolytes, and organic acids and/or carbohydrates, and further contains at least one component selected from the group consisting of pH indicators, pH adjusters, pH buffers, antibiotics,

vitamins, trace metal elements, chelators, hormones, growth factors, lipids or constituents thereof, carrier proteins, extracellular matrix components, reducing substances and polymers.

5

Mode for Carrying Out the Invention

[0013]

As used herein, the term "composition for embryo culture" means a composition for treating gametes or embryos. In an embodiment of the present invention, using a composition for embryo culture, fertilized eggs or blastomeres can be cultured until blastocysts or until hatching of blastocysts. The term "can be cultured until blastocysts" contains, for example, the culture of fertilized eggs or blastomeres until two to eight-cell embryos or morulae, but not limited thereto.

10
15

[0014]

The composition for embryo culture involved in an embodiment of the present invention can be also used when sperm or egg cells are collected, matured, maintained or washed, or egg cells are fertilized in vitro. The composition for embryo culture involved in an embodiment of the present invention can be exchanged with a fresh composition for embryo culture involved in an embodiment of

20

the present invention during embryo culture, but not limited thereto.

[0015]

The "gamete" means a sperm or an ovum. In the present
5 description, the "embryo" contains reconstructed embryos
such as a fertilized egg, an early embryo and a nuclear
transplant embryo, and contains embryos derived from
mammals such as human, mouse, bovine, rabbit, rhesus monkey,
swine and rat but not limited thereto. The "mammals" can
10 be human, mouse, bovine, rabbit, rhesus monkey, swine or
rat, but not limited thereto.

[0016]

The blastocyst indicates an embryo after the cleavage
stage in the early development of mammals. The blastocyst
15 consists of an inner cell mass, a blastocoel and a
trophectoderm surrounded by a zona pellucida. Hatching of
blastocysts indicates a step of escape of embryos from the
zona pellucida and is an essential process for reaching
implantation of embryos.

20 [0017]

It is known that in cultured embryos with
abnormalities in the structure and physiological functions
of embryos, hatching is delayed or hatching does not occur
(Reprod Biomed Online 2003; 7: 228-234). Meanwhile, it is
25 known that, for example, a blastocyst which hatches within

a fixed period of time, has a high implantation rate as compared to a blastocyst which does not hatch within a fixed period of time (Fertil Steril 2000; 74: 163-165).

It is known that the number of cells in blastocysts is positively correlated with the fetal development rate (J Reprod Fertil 1997; 109: 153-164) and positively correlated with the normal rate of chromosome (Hum Reprod; 2010: 1916-1926).

[0018]

As methods for evaluating the quality of cultured embryos, there are a method for evaluating the morphological features of embryos using a microscope, such as a development speed, a blastocyst development rate and a hatching rate from a zona pellucida, an evaluation method by measuring the number of cells in blastocysts by nuclear staining, and the like.

Among these evaluation methods, the evaluation method based on hatching is one of the favorable evaluation methods for selecting cultured embryos with high quality. The number of cells in blastocysts is also one of the important evaluation methods for evaluating the quality of cultured embryos.

[0019]

The amino acids contained in a composition for embryo culture involved in an embodiment of the present invention

can be in the free form or in the form of a
pharmaceutically acceptable salt. The amino acids
contained in a composition for embryo culture involved in
another embodiment of the present invention can be also
5 those which can be decomposed by hydrolysis and the like
and converted into free amino acids. Such amino acids can
be, for example, in the ester form, the *N*-acyl form, an
oligopeptide and the like.

[0020]

10 Glutamine, for example, can be a glutamine derivative
such as glycyl-L-glutamine, L-alanyl-L-glutamine, L-leucyl-
L-glutamine, L-valyl-L-glutamine or L-isoleucyl-L-glutamine.
These glutamine derivatives can be used alone or two or
more glutamine derivatives can be used in combination. In
15 an embodiment of the present invention, the glutamine
derivative is, for example, glycyl-L-glutamine or L-alanyl-
L-glutamine. In another embodiment of the present
invention, the glutamine derivative is glycyl-L-glutamine.
In another embodiment of the present invention, the
20 glutamine derivative is L-alanyl-L-glutamine.

 In addition, for example, in cystine, a portion or all
thereof can be cysteine.

[0021]

 In an embodiment of the present invention, taurine
25 contained in a composition for embryo culture can be in the

free form or in the form of a pharmaceutically acceptable salt. In another embodiment of the present invention, taurine contained in a composition for embryo culture can be that which can be converted into taurine by
5 dehydrogenation and the like. Such taurine, for example, can be hypotaurine.

[0022]

The composition for embryo culture involved in an embodiment of the present invention can contain at least
10 one component selected from the group consisting of electrolytes, organic acids, carbohydrates, pH indicators, pH adjusters, pH buffers, antibiotics, vitamins, trace metal elements, chelators, hormones, growth factors, lipids or constituents thereof, carrier proteins,
15 extracellular matrix components, reducing substances and polymers and the like, as needed.

[0023]

The electrolytes are not limited, and include sodium chloride, potassium chloride, sodium dihydrogen phosphate, calcium chloride dihydrate, magnesium sulfate heptahydrate,
20 sodium hydrogen carbonate, calcium chloride, calcium gluconate, magnesium chloride, magnesium sulfate and dipotassium hydrogen phosphate and the like. These electrolytes can be used alone or two or more electrolytes
25 can be used in combination.

[0024]

The organic acids are not limited, and include pyruvic acid, acetic acid, citric acid, succinic acid, malic acid, α -ketoglutaric acid, fumaric acid, oxaloacetic acid, isocitric acid, oxalosuccinic acid, tartaric acid, adipic acid, lactic acid and salts thereof. These organic acids can be used alone or two or more organic acids can be used in combination.

[0025]

The carbohydrates are not limited, and include glucose, maltose, fructose, xylitol, sorbitol and trehalose and the like. These carbohydrates can be used alone or two or more carbohydrates can be used in combination.

[0026]

The pH indicators are not limited, and include phenol red and the like.

[0027]

The pH adjusters are not limited, and include hydrochloric acid, acetic acid, sodium hydroxide and the like.

[0028]

The pH buffers are not limited, and include HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), MOPS (3-morpholinopropane sulfonic acid), tris[hydroxymethyl]aminomethane, *N*-

tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid and the like. These pH buffers can be used alone or two or more pH buffers can be used in combination.

[0029]

5 The antibiotics are not limited, and include penicillin, streptomycin, kanamycin, gentamicin, erythromycin, amphotericin B, nystatin and the like. These antibiotics can be used alone or two or more antibiotics can be used in combination.

10 [0030]

 The vitamins are not limited, and include vitamin A, vitamin B group, vitamin C, vitamin D group, vitamin E, nicotinic acid, biotin, folic acid and the like. These vitamins can be used alone or two or more vitamins can be
15 used in combination.

[0031]

 The trace metal elements are not limited, and include zinc, iron, manganese, copper, iodine, selenium and cobalt. These trace metal elements are not limited, and can be used
20 in the free form or can be used as pharmaceutically acceptable compounds containing these trace metal elements. These trace metal elements can be used alone or two or more trace metal elements can be used in combination.

[0032]

The chelators are not limited, and include EGTA (ethyleneglycol bis tetraacetic acid), EDTA (ethylene diamine tetraacetic acid), EDDA (ethylene diamine diacetic acid) and DTPA (diethylene triamine pentaacetic acid) and the like. These chelators can be used alone or two or more chelators can be used in combination.

[0033]

The hormones are not limited, and include insulin, hydrocortisone, dexamethasone, triiodothyronine, gonadotropin, estrogen, progesterone and the like. These hormones can be used alone or two or more hormones can be used in combination.

[0034]

The growth factors are not limited, and include epidermal growth factor, fibroblast growth factors, platelet-derived growth factors, insulin-like growth factors, growth hormone and the like. These growth factors can be used alone or two or more growth factors can be used in combination.

[0035]

The lipids or constituents thereof are not limited, and include fatty acids such as oleic acid, linoleic acid, linolenic acid, arachidonic acid, palmitic acid, oleic acid, palmitoleic acid, stearic acid, myristic acid, and salts

thereof, or cholesterol, ethanolamine, choline, sphingomyelin, cardiolipin and the like. These lipids or constituents thereof can be used alone or two or more thereof can be used in combination.

5 [0036]

The carrier proteins are not limited, and include albumin, transferrin, ceruloplasmin and the like. These carrier proteins can be used alone or two or more carrier proteins can be used in combination. Albumin is not
10 limited, and can be bovine serum albumin, human serum albumin, recombinant bovine serum albumin or recombinant human serum albumin, or a mixture thereof.

[0037]

The extracellular matrix components are not limited,
15 and include fibronectin, collagen, gelatin, hyaluronan and the like. These extracellular matrix components can be used alone or two or more extracellular matrix components can be used in combination.

[0038]

20 The reducing substances are not limited, and include 2-mercaptoethanol, dithiothreitol, reduced glutathione and the like. These reducing substances can be used alone or two or more reducing substances can be used in combination.

[0039]

The polymers are not limited, and include PVP (polyvinyl pyrrolidone), PVA (polyvinyl alcohol), dextran and the like. These polymers can be used alone or two or
5 more polymers can be used in combination.

[0040]

The composition for embryo culture involved in an embodiment of the present invention can be produced by combining constituents by a conventional method. A
10 composition for embryo culture, for example, can be produced or supplied in the form of a sterile solution, in the form of a dilution type sterile concentrated solution, or in the form of a dissolution type sterile lyophilized product by a conventional method.

15 Therefore, the composition for embryo culture involved in an embodiment of the present invention can be in the form of a sterile solution, in the form of a sterile concentrated solution, or in the form of a sterile lyophilized product. When the composition for embryo
20 culture involved in an embodiment of the present invention is in the form of a sterile concentrated solution or in the form of a sterile lyophilized product as described above, by diluting or dissolving the composition with sterile water before use, a composition for embryo culture in the

form of a sterile solution can be obtained, but not limited thereto.

Examples

5 [0041]

Example 1

Medium constitution

Table 2 shows, in the constitution of the KSOMaa medium (Comparative Example 1) as a control and the
10 constitution of the medium in Example 1, component names contained in the media, molecular weights (M.W.) and contents thereof from the left column.

[0042]

Table 2

Component Name	M.W.	Comparative Example 1		Example 1	
		mM	g/L	mM	g/L
L-Alanine	89.09	0.050	0.004	0.297	0.026
L-Asparagine monohydrate	150.13	0.050	0.008	0.015	0.002
L-Aspartic acid	133.10	0.050	0.007	0.120	0.016
L-Glutamic acid	147.13	0.050	0.007	0.550	0.081
Glycine	75.07	0.050	0.004	0.979	0.073
L(-)-Proline	115.13	0.050	0.006	0.105	0.012
L-Serine	105.09	0.050	0.005	0.176	0.018
L(+)-Arginine hydrochloride	210.66	0.300	0.063	0.108	0.023
L(-)-Cystine	240.30	0.050	0.012	0.048	0.012
L-Histidine hydrochloride monohydrate	209.63	0.100	0.021	0.053	0.011
L(+)-Isoleucine	131.17	0.200	0.026	0.036	0.005
L-Leucine	131.17	0.200	0.026	0.081	0.011

L(+)-Lysine hydrochloride	182.65	0.200	0.037	0.176	0.032
L-Methionine	149.21	0.050	0.007	0.022	0.003
L(-)-Phenylalanine	165.19	0.100	0.017	0.045	0.007
L(-)-Threonine	119.12	0.200	0.024	0.109	0.013
L-Tryptophan	204.23	0.025	0.005	0.018	0.004
L-Tyrosine	181.19	0.100	0.018	0.048	0.009
L-Valine	117.15	0.200	0.023	0.108	0.013
Glycyl-L-glutamine monohydrate	221.21	1.000	0.221	0.398	0.088
Taurine	125.15	-	-	1.412	0.177
Sodium chloride	58.44	95.000	5.552	114.718	6.704
Potassium chloride	74.55	2.500	0.186	5.200	0.388
Potassium dihydrogen phosphate	136.09	0.350	0.048	0.300	0.041
Calcium chloride dihydrate	147.01	1.710	0.251	1.117	0.164
Magnesium sulfate heptahydrate	246.48	0.200	0.049	0.467	0.115
Sodium hydrogen carbonate	84.01	25.000	2.100	25.000	2.100
D-Glucose	180.16	0.200	0.036	2.998	0.540
Sodium pyruvate	110.04	0.200	0.022	0.183	0.020
Sodium L-lactate	112.06	10.000	2.186	4.540	0.992
Trisodium citrate dihydrate	294.10	-	-	0.127	0.037
EDTA·2Na	372.24	0.010	0.004	-	-
Phenol red	354.38	0.003	0.001	0.003	0.001
Gentamicin sulfate	-	-	0.010	-	0.010

[0043]

Collection of sperm

After sacrificing ICR male mice (Japan SLC, Inc.), the cauda epididymis was cut out. A sperm mass obtained by

5 incising the epididymal duct in the middle of the cauda epididymis was collected in 300 μ L of TYH medium supplemented with 0.4% bovine serum albumin prepared under

mineral oil. The collected sperm mass was precultured under conditions of 37°C and 6% CO₂ for an hour.

[0044]

Collection of ova

5 To ICR female mice (Japan SLC, Inc.), 7.5 international unit of PMSG (pregnant mare serum gonadotropin, ASKA Pharmaceutical Co., Ltd., SEROTROPIN (Trade Mark)) was intraperitoneally administered. After 48 hours, 7.5 international unit of hCG (human chorionic gonadotropin, ASKA Pharmaceutical CO., Ltd., GONATROPIN 10 (Trade Mark)) was intraperitoneally administered to induce superovulation, and such mice were sacrificed 15 hours after administration of hCG. A uterine tube was cut out immediately after sacrificing. From the ampulla of the 15 uterine tube, cumulus oocyte complexes were collected in 300 µL of TYH medium supplemented with 0.4% bovine serum albumin prepared with mineral oil. The collected cumulus oocyte complexes were cultured under conditions of 37°C and 6% CO₂ until *in vitro* fertilization.

20 [0045]

In vitro fertilization

 To the TYH medium containing the collected cumulus oocyte complexes, the precultured sperm were added to be 150 sperm/µL. After *in vitro* fertilization under 25 conditions of 37°C and 6% CO₂ for 6 hours, an ovum in which

the second polar body and two pronuclei could be observed was designated as a fertilized egg and was directly used for the following experiment.

[0046]

5 Embryo culture test

Using the medium in Example 1 and the medium in Comparative Example 1, a 100 μ L drop of each medium supplemented with 0.1% bovine serum albumin was prepared in a culture dish with a diameter of 60 mm under mineral oil,
10 and was left to stand under conditions of 37°C, 5% O₂ and 6% CO₂ overnight for equilibration.

[0047]

To the drop of each medium after equilibration, a fertilized egg was transferred. The fertilized egg was
15 washed by moving into several drops. The washed fertilized egg was transferred to a fresh drop of each medium and cultured under conditions of 37°C, 5% O₂ and 6% CO₂ for 4 days. The embryo on the 4th day of culture was observed with a microscope and the percentage of embryos reaching
20 from fertilized eggs to blastocysts or the hatching stage was calculated. The obtained results are shown in Table 3.

[0048]

[Table 3]

Medium	Number of cultured fertilized eggs (Repetition frequency)	Blastocyst development rate (%)	
		Total	Hatching
Comparative Example 1	104 (6)	95.8±2.2	72.8±3.0
Example 1	104 (6)	98.1±1.3	87.8±2.9*

Mean value ± Standard error

* Significant difference compared with the medium in

5 Comparative Example 1 (p = 0.0065, Wilcoxon test)

[0049]

The blastocysts obtained on the 4th day of culture were fluorescently stained using DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride) (Roche Diagnostics K.K.) and the number of cells in the blastocysts was measured. The obtained results are shown in Table 4.

[0050]

[Table 4]

Medium	Number of analyzed blastocysts	Total number of cells
Comparative Example 1	98	80.2±1.5
Example 1	94	90.2±2.0**

Mean value ± Standard error

15 ** Significant difference compared with the medium in Comparative Example 1 (p = 0.0003, Wilcoxon test)

[0051]

As shown in Table 3 and Table 4, in the group cultured in the medium in Example 1, the hatching rate was significantly improved and the number of cells in blastocysts was significantly increased as compared to those in the group cultured in the medium in Comparative Example 1. This result shows that the medium in Example 1 involved in an embodiment of the present invention is excellent in terms of being capable of causing the stimulatory action and quality improvement on embryonic development compared to the medium in Comparative Example 1.

[0052]

Example 2

A medium (Example 2) was prepared by replacing the constitution of amino acids contained in the medium in Comparative Example 1 shown in Table 2 above with the constitution of amino acids including taurine contained in the medium in Example 1 shown in Table 2 (Table 5).

[0053]

[Table 5]

Component Name	M.W.	Comparative Example 1		Example 2	
		mM	g/L	mM	g/L
L-Alanine	89.09	0.050	0.004	0.297	0.026
L-Asparagine monohydrate	150.13	0.050	0.008	0.015	0.002
L-Aspartic acid	133.10	0.050	0.007	0.120	0.016
L-Glutamic acid	147.13	0.050	0.007	0.550	0.081

Glycine	75.07	0.050	0.004	0.979	0.073
L(-)-Proline	115.13	0.050	0.006	0.105	0.012
L-Serine	105.09	0.050	0.005	0.176	0.018
L(+)-Arginine hydrochloride	210.66	0.300	0.063	0.108	0.023
L(-)-Cystine	240.30	0.050	0.012	0.048	0.012
L-Histidine hydrochloride monohydrate	209.63	0.100	0.021	0.053	0.011
L(+)-Isoleucine	131.17	0.200	0.026	0.036	0.005
L-Leucine	131.17	0.200	0.026	0.081	0.011
L(+)-Lysine hydrochloride	182.65	0.200	0.037	0.176	0.032
L-Methionine	149.21	0.050	0.007	0.022	0.003
L(-)-Phenylalanine	165.19	0.100	0.017	0.045	0.007
L(-)-Threonine	119.12	0.200	0.024	0.109	0.013
L-Tryptophan	204.23	0.025	0.005	0.018	0.004
L-Tyrosine	181.19	0.100	0.018	0.048	0.009
L-Valine	117.15	0.200	0.023	0.108	0.013
Glycyl-L-glutamine monohydrate	221.21	1.000	0.221	0.398	0.088
Taurine	125.15	-	-	1.412	0.177
Sodium chloride	58.44	95.000	5.552	95.000	5.552
Potassium chloride	74.55	2.500	0.186	2.500	0.186
Potassium dihydrogen phosphate	136.09	0.350	0.048	0.350	0.048
Calcium chloride dihydrate	147.01	1.710	0.251	1.710	0.251
Magnesium sulfate heptahydrate	246.48	0.200	0.049	0.200	0.049
Sodium hydrogen carbonate	84.01	25.000	2.100	25.000	2.100
D-Glucose	180.16	0.200	0.036	0.200	0.036
Sodium pyruvate	110.04	0.200	0.022	0.200	0.022
Sodium L-lactate	112.06	10.000	2.186	10.000	2.186
Trisodium citrate dihydrate	294.10	-	-	-	-
EDTA·2Na	372.24	0.010	0.004	0.010	0.004
Phenol red	354.38	0.003	0.001	0.003	0.001
Gentamicin sulfate	-	-	0.010	-	0.010

[0054]

Using the medium in Comparative Example 1 and the medium in Example 2, the embryo culture test was carried out in the same conditions as in the embryo culture test described in Example 1. The obtained results are shown in Table 6.

[0055]

[Table 6]

Medium	Number of analyzed blastocysts	Total number of cells
Comparative Example 1	131	86.2±1.4
Example 2	131	90.2±1.6*

Mean value ± Standard error

* Significant difference compared with the medium in Comparative Example 1 (p = 0.0467, Wilcoxon test)

[0056]

As shown in Table 6, in the group cultured in the medium in Example 2 involved in an embodiment of the present invention, the number of cells in blastocysts was significantly increased as compared to that in the group cultured in Comparative Example 1. As described above, the constitution of amino acids including taurine contained in a composition for embryo culture involved in an embodiment of the present invention has action to stimulate the

development of embryos regardless of medium constitutions other than the above constitution.

[0057]

Examples 3 and 4

5 A medium having a constitution with 0.7 times the concentration of the constitution of amino acids including taurine contained in the medium in Example 1 shown in Table 2 (Example 3) and a medium having a constitution with 1.3 times the concentration thereof (Example 4) were
10 prepared (Table 7).

[0058]

[Table 7]

Component Name	Example 1	Example 3	Example 4
	mM	mM	mM
L-Alanine	0.297	0.208	0.386
L-Asparagine monohydrate	0.015	0.011	0.020
L-Aspartic acid	0.120	0.084	0.156
L-Glutamic acid	0.550	0.385	0.715
Glycine	0.979	0.685	1.273
L(-)-Proline	0.105	0.074	0.137
L-Serine	0.176	0.123	0.229
L(+)-Arginine hydrochloride	0.108	0.076	0.140
L(-)-Cystine	0.048	0.034	0.062
L-Histidine hydrochloride monohydrate	0.053	0.037	0.069
L(+)-Isoleucine	0.036	0.025	0.047
L-Leucine	0.081	0.057	0.105
L(+)-Lysine hydrochloride	0.176	0.123	0.229
L-Methionine	0.022	0.015	0.029
L(-)-Phenylalanine	0.045	0.032	0.059
L(-)-Threonine	0.109	0.076	0.142
L-Tryptophan	0.018	0.013	0.023
L-Tyrosine	0.048	0.034	0.062

L-Valine	0.108	0.076	0.140
Glycyl-L-glutamine monohydrate	0.398	0.279	0.517
Taurine	1.412	0.988	1.836
Sodium chloride	114.718	114.718	114.718
Potassium chloride	5.200	5.200	5.200
Potassium dihydrogen phosphate	0.300	0.300	0.300
Calcium chloride dihydrate	1.117	1.117	1.117
Magnesium sulfate heptahydrate	0.467	0.467	0.467
Sodium hydrogen carbonate	25.000	25.000	25.000
D-Glucose	2.998	2.998	2.998
Sodium pyruvate	0.183	0.183	0.183
Sodium L-lactate	4.540	4.540	4.540
Trisodium citrate dihydrate	0.127	0.127	0.127
EDTA·2Na	-	-	-
Phenol red	0.003	0.003	0.003
Gentamicin sulfate	0.010 (g/L)	0.010 (g/L)	0.010 (g/L)

In Table 7, the unit of concentration of each component is mM, unless otherwise described.

[0059]

Using the medium in Example 1, the medium in Example 3
5 and the medium in Example 4, the embryo culture test was carried out in the same conditions as in the embryo culture test described in Example 1. The obtained results are shown in Table 8 and Table 9.

[0060]

[Table 8]

Medium	Number of cultured fertilized eggs (Repetition frequency)	Blastocyst development rate (%)	
		Total	Hatching
Example 1	150 (15)	92.7±3.0	72.7±3.6
Example 3	150 (15)	94.0±2.4	74.7±4.1
Example 4	150 (15)	94.0±2.4	72.0±3.9

Mean value ± Standard error

No significant differences between groups (Steel-Dwass
test)

5

[0061]

[Table 9]

Medium	Number of analyzed blastocysts	Total number of cells
Example 1	124	102.1±2.0
Example 3	142	99.4±1.4
Example 4	137	101.4±1.6

Mean value ± Standard error

No significant differences between groups (Steel-Dwass
test)

10

[0062]

As shown in Table 8 and Table 9, even with the medium
having the constitution with 0.7 times the concentration of
the constitution of amino acids including taurine contained
in the medium in Example 1 (Example 3) and the medium
having the constitution with 1.3 times the concentration
thereof (Example 4), significant differences in validity

15

were not observed. This reveals that the validity of the constitution of amino acids including taurine contained in the medium in Example 1, which is the composition for embryo culture involved in the present embodiment, is not
 5 lost within a concentration range of at least 0.7 to 1.3-times.

[0063]

Example 5

Human embryo culture test

10 Table 10 shows, in the constitution of a medium in Example 5, component names contained in the medium, molecular weights (M.W.) and contents thereof from the left column.

[0064]

15 [Table 10]

Component Name	M.W.	Example 5	
		mM	g/L
L-Alanine	89.09	0.297	0.026
L-Asparagine monohydrate	150.13	0.015	0.002
L-Aspartic acid	133.10	0.120	0.016
L-Glutamic acid	147.13	0.550	0.081
Glycine	75.07	0.979	0.073
L(-)-Proline	115.13	0.105	0.012
L-Serine	105.09	0.176	0.018
L(+)-Arginine hydrochloride	210.66	0.108	0.023
L(-)-Cystine	240.30	0.048	0.012
L-Histidine hydrochloride monohydrate	209.63	0.053	0.011
L(+)-Isoleucine	131.17	0.036	0.005
L-Leucine	131.17	0.081	0.011

L(+)-Lysine hydrochloride	182.65	0.176	0.032
L-Methionine	149.21	0.022	0.003
L(-)-Phenylalanine	165.19	0.045	0.007
L(-)-Threonine	119.12	0.109	0.013
L-Tryptophan	204.23	0.018	0.004
L-Tyrosine	181.19	0.048	0.009
L-Valine	117.15	0.108	0.013
Glycyl-L-glutamine monohydrate	221.21	0.398	0.088
Taurine	125.15	1.412	0.177
Sodium chloride	58.44	114.718	6.704
Potassium chloride	74.55	5.200	0.388
Potassium dihydrogen phosphate	136.09	0.300	0.041
Calcium chloride dihydrate	147.01	1.117	0.164
Magnesium sulfate heptahydrate	246.48	0.467	0.115
Sodium hydrogen carbonate	84.01	25.000	2.100
D-Glucose	180.16	2.998	0.540
Sodium pyruvate	110.04	0.183	0.020
Sodium L-lactate	112.06	4.540	0.992
Trisodium citrate dihydrate	294.10	0.127	0.037
EDTA·2Na	372.24	0.010	0.004
Phenol red	354.38	0.003	0.001
Gentamicin sulfate	-	-	0.010

[0065]

A 20 μ L drop of the medium in Example 5 to which 0.05% recombinant human albumin was added was prepared in a culture dish with a diameter of 35 mm under mineral oil and was left to stand under conditions of 37°C, 4%O₂ and 6%CO₂ overnight for equilibration.

[0066]

As human fertilized eggs, fertilized eggs after freezing and thawing were used, which fertilized eggs were not intended for subsequent use for treatment and were

5 agreed to be used for research. Each one of human fertilized eggs after freeze-thawing was transferred to each medium after equilibration. After that, the human fertilized egg was washed by moving into several drops. The washed fertilized egg was transferred to a fresh drop

10 of each medium and cultured under conditions of 37°C, 4%O₂ and 6%CO₂ for 5 to 6 days. The embryo was observed with a microscope every day from the second day after the onset of culture and the developmental stage of the embryo was observed. The obtained typical results are shown in

15 Table 11.

[0067]

[Table 11]

Embryo medium	No.	Developmental stage			
		Day2	Day3	Day4	Day5-6
Example 5	1	4 cell stage	8 cell stage	Morula stage	Blastocyst stage
	2	4 cell stage	6 cell stage	8 cell stage	Morula stage
	3	2 cell stage	6 cell stage	9 cell stage	Blastocyst stage
	4	2 cell stage	4 cell stage	5 cell stage	Morula stage

As shown in Table 11, it is clear that the medium in Example 5 according to an embodiment of the present invention is an excellent culture medium for human embryos.

CLAIMS

1. A mammalian composition for embryo culture,
comprising the following components and concentrations:

Components	mM
L-Alanine	0.297 ± 0.089
L-Asparagine	0.015 ± 0.005
L-Aspartic acid	0.120 ± 0.036
L-Glutamic acid	0.550 ± 0.165
Glycine	0.979 ± 0.294
L(-)-Proline	0.105 ± 0.032
L-Serine	0.176 ± 0.053
L(+)-Arginine	0.108 ± 0.032
L(-)-Cystine	0.048 ± 0.014
L-Histidine	0.053 ± 0.016
L(+)-Isoleucine	0.036 ± 0.011
L-Leucine	0.081 ± 0.024
L(+)-Lysine	0.176 ± 0.053
L-Methionine	0.022 ± 0.007
L(-)-Phenylalanine	0.045 ± 0.013
L(-)-Threonine	0.109 ± 0.033
L-Tryptophan	0.018 ± 0.005
L-Tyrosine	0.048 ± 0.014
L-Valine	0.108 ± 0.032
L-Glutamine or glutamine derivative	0.398 ± 0.119
Taurine	1.412 ± 0.424

wherein the glutamine derivative is L-alanyl-L-glutamine,
5 L-asparaginyl-L-glutamine, L-aspartyl-L-glutamine,
L-glutamyl-L-glutamine, glycyl-L-glutamine, L(-)-prolyl-L-
glutamine, L-seryl-L-glutamine, L(+)-arginyl-L-glutamine,
L(-)-cysteinyl-L-glutamine, L-histidyl-L-glutamine, L(+)-
isoleucyl-L-glutamine, L-leucyl-L-glutamine, L(+)-lysyl-L-
10 glutamine, L-methionyl-L-glutamine, L(-)-phenylalanyl-L-

glutamine, L(-)-threonyl-L-glutamine, L-tryptophyl-L-glutamine, L-tyrosyl-L-glutamine, L-valyl-L-glutamine, L-glutaminy-L-glutamine or tauryl-L-glutamine.

5 2. The mammalian composition for embryo culture according to claim 1, which further contains electrolytes.

 3. The mammalian composition for embryo culture according to claim 2, which further contains organic acids
10 and/or carbohydrates.

 4. The mammalian composition for embryo culture according to claim 3, which further contains at least one of pH indicators, pH adjusters, pH buffers, antibiotics,
15 vitamins, trace metal elements, chelators, hormones, growth factors, lipids or constituents thereof, carrier proteins, extracellular matrix components, reducing substances or polymers.

20 5. The mammalian composition for embryo culture according to any one of claims 1 to 4, wherein the glutamine derivative is glycyl-L-glutamine, L-alanyl-L-glutamine, L-leucyl-L-glutamine, L-valyl-L-glutamine or L-isoleucyl-L-glutamine.

Components	mM
L-Alanine	0.297 ± 0.089
L-Asparagine	0.015 ± 0.005
L-Aspartic acid	0.120 ± 0.036
L-Glutamic acid	0.550 ± 0.165
Glycine	0.979 ± 0.294
L(-)-Proline	0.105 ± 0.032
L-Serine	0.176 ± 0.053
L(+)-Arginine	0.108 ± 0.032
L(-)-Cystine	0.048 ± 0.014
L-Histidine	0.053 ± 0.016
L(+)-Isoleucine	0.036 ± 0.011
L-Leucine	0.081 ± 0.024
L(+)-Lysine	0.176 ± 0.053
L-Methionine	0.022 ± 0.007
L(-)-Phenylalanine	0.045 ± 0.013
L(-)-Threonine	0.109 ± 0.033
L-Tryptophan	0.018 ± 0.005
L-Tyrosine	0.048 ± 0.014
L-Valine	0.108 ± 0.032
L-Glutamine or glutamine derivative	0.398 ± 0.119
Taurine	1.412 ± 0.424