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(54) USE OF THE BIO-FREQUENCY SPECTRUM IN ANIMAL EMBRYO-ENGINEERING

(57) The invention discloses the use of bio-frequency spectrum in embryogenesis and development of animal embryo, and the use of bio-frequency spectrum in promoting propagation and development of animals. The mature rate of oocyte, the fertility of fertilized egg, and the quality of embryo can be improved by exposure oocyte, egg cell, sperm and embryo to the minic bio-frequency spectrum irradiation in the course of production of embryo *in vitro*.

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Description

The present invention relates to biological engineering, particularly to the application of bio-spectrum embryonic engineering.

The fundamental researches and technical development on animal embryonic engineering have achieved great progress in recent years. The researches on embryonic engineering are moving quickly from the stage of laboratory test to practicalization and commercialization. The animal embryonic engineering mainly includes production of embryos in vitro, cryo-preservation of embryos and micro-manipulation of embryos, etc.

The development of the technique for production of embryos in vitro can make full use of animal genetic resources, accelerate improvement of animal gene. The technique can overcome infertility of some rare animals and preserve resources of rare animals. The technique can also supply embryos for production of gene transfer in animals and embryos' sexing. However, the production efficiency and the qualities of embryos produced in vitro are lower. The rate of pregnancy is also lower by embryo transfer.

Cryo-preservation of animal embryos is the key technique, which affect whether or not the technique of embryo transfer can be put into practical use. Through the cryo-preserving technique, the animal embryos reservoir can be established to facilitate the transportation and exchange of animal breed resources internationally. Currently there are two internationally acceptable methods of cryo-preserving embryos. One is the slow freezing method, the other is the cryo-preservation. However, the survival rate of frozen/thawed embryos produced in vitro is about 65%, and the pregnant rate of the frozen/thawed embryos is decreased 20% than the fresh embryos. Micro-manipulation of embryos consists of sexing of embryos, embryos'cloning including embryo bisection and nuclear transfer, and gene transfer. The aim of sexing of embryos is to produce defined sexual offspring. Cloning of embryos can produce many identical offspring from an outstanding animal embryo, improving the reproduction efficiency and speeding the animal breeding. Gene transfer animals can be produced by micro-injection and sperm mediated method. The aim of gene transfer technique is to speed up the growth rate and increase resistance to diseases of animals, improve quality of animal production, and therefore supply many valuable medicines for human beings. However, the efficiency of micro-manipulation is very low because the embryos will definitely be injured.

The invention is based on the following understandings. Very few people have conducted research on the regulation of reproductive and growth abilities of animals with physical method, especially application of physical method to embryos' engineering for resolving technical problems The inventor recognized that all living matter has chemical and physical characteristics at the same time. The living matter has special physical characteristics such as electric charge in cells, etc. The interactions between the substances in living matter which have electric charges and the electromagnetic field in environment can be produced when electromagnetic field in environment and some main physical characteristics of living matter have the same characteristics. The interactions can influence the molecules, atoms and electrons at the same time to produce significant biologic effects. For example, tissues and cells can grow and develop normally in the chemical and physical environment of a living body. The growth and development of tissues and cells are decreased significantly when they are separated from the living body, although many kinds of chemical protecting materials and nutritive materials are used for improving the culture conditions. The inventor further recognized that the growth ability of tissues and cells in culture condition will be improved by applying a simulated bio-spectrum which is a weak electromagnetic field. So, it has significant meanings to apply the simulated bio-spectrum to embryos' engineering. The bio-spectrum will help improve the reproductive ability, growth speed and resistance to diseases of the animals.

The object of the invention is to induce irradiation bio-effects by applying the bio-spectrum to animal embryos engineering and animals, which includes:

improving the rate of maturing oocytes, fertility in vitro and embryos by bio-spectrum irradiation in production of embryos in vitro;

improving the survival and pregnancy rate of frozen/thawed embryos by bio-spectrum irradiation in the process of cryo-preservation of embryos;

improving the efficiency of micro-manipulation and repairing the injury of embryos by bio-spectrum irradiation in the process of micro-manipulation;

improving the rate of embryos survival, ovulation, fertilization and the development of uterus by bio-spectrum irradiation to live female animals;

improving qualities of sperms by bio-spectrum irradiation to live male animals.

The simulated bio-spectrum mentioned above is described in Chinese patent application No. 91109014.2 which is a wide band synthesized physical field. Its wavelength is 0.2 μm to 10cm. The irradiation signal is very weak in the wave band of 30 μm - 10cm. Some parts of the physical field can produce certain effects.

The object of the present invention is realized by the following technical solutions.

Methods of applying bio-spectrum to in vitro production of animal embryos.

1. Collection of oocytes.

2. In vitro maturation of oocytes: oocytes deposited in a standard or defined medium are irradiated with bio-spectrum generator for 3 to 20 minutes. In this period, the medium temperature is kept not higher than 40 °C.

3. Capacitation of spermatozoa: semen diluted in a standard or defined medium are irradiated with BIO-SPECTRUM generator for 3 to 20 minutes. In this period, the medium temperature is kept not higher than 40 °C.

4. In vitro fertilization of oocytes: both matured oocytes and capacitated sperms are co-cultured in one test tube containing a standard or defined medium and irradiated with bio-spectrum generator for 1-3 times, 3-25 minutes each time. During irradiation, the medium temperature is not higher than 40 °C.

5. In vitro culture of embryos: zygotes transferred in a standard or defined medium are irradiated with bio-spectrum generator for 3-30 minutes. During irradiation the medium temperature is not higher than 40 °C.

Methods of applying bio-spectrum to cryo-preservaton of animal embryos

Thawed embryos deposited in a culture medium are irradiated with bio-spectrum generator for 3 20 minutes. During irradiation the medium temperature is not higher than 40 °C.

Methods of applying bio-spectrum to micro-manipulation of animal embryos

After micro-manipulation embryos are deposited in a culture medium and are irradiated with bio-spectrum generator for 3-25 minutes. In order to get stronger effects, embryos are irradiated with bio-spectrum generator for 3-25 minutes before micro-manipulation. During irradiation the medium temperature is not higher that 40 °C.

Methods of applying bio-spectrum to animal reproduction, development and growth.

Animals are irradiated with bio-spectrum generator once or twice every day, for 30-60 minutes each time. During irradiation, the surface temperature of the animals is kept not higher than 45 °C. The results will be better when conventional techniques of animal reproduction, development and growth, are used together with the irradiation such as all kinds of gonadotrophin.

Methods of applying bio-spectrum to prevention and cure animal diseases

Parts or whole bodies of animals are irradiated with bio-spectrum generator for 1-3 times every day, 6-60 minutes each time. During irradiation the temperature of the part irradiated is not higher than 45 °C.

By using irradiation with bio-spectrum in micro-manipulation of embryos in vitro, the development rate of hemi-morulae and hemi-blastocysts and the rate of embryos stained success could be significantly improved.

By using irradiation with bio-spectrum in cryop-reservation of embryos, the survival rate of embryos after frozen/thawed increases by at least 16% and the pregnant rate of transferred embryos could be also significantly improved.

By using irradiation with bio-spectrum in micro-manipulation of embryos, in vitro the development rate of demi-morulae and demi-blastocysts and the rate of embryos stained success could be significantly improved.

By using irradiation with bio-spectrum in reproduction of animal, the uterus development, ovulation rate, fertilization rate of oocytes, survival and developmental rate of fertilized egg in female animals and the sperm quality in male animals could be improved.

By using irradiation with bio-spectrum in animal disease control, the diseases could be alleviated and cured.

EXAMPLE 1

The oocytes are collected from donor cows by ordinary methods, and than put in common medium or special medium for maturation. During maturation, oocytes are irradiated with bio-spectrum device (model WS-101D) for 15 minutes (at weak level). The temperature should be kept at 38-40 °C by adjusting the distance between embryo container and irradiation device. The special medium is made according to the procedure of Brackett et al. The receipt of the special medium is as follows:

components	g/l
NaCl	6.55
KCl	0.30
$CaCl_2 \cdot 2H_2O$	0.33
$NaH_2PO_4 \cdot H_2O$	0.11
$MgCl_2 \cdot 6H_2O$	0.11

(continued)

components	g/l
$NaHCO_3$	3.10
Glucose	2.25
Bovine Serum Albumin	3.00
Sodium Pyruvate (or Pyruvate)	0.14(or 0.11)
Na-Penicillin	0.031(50 IU/ml)

The medium needs to be sterilized by filtration afier the components have dissolved completely in 1000 ml water. This medium is required to equilibrate in the incubator at 38 °C in 59% CO_2 in the air. The osmolality of this medium is about 300 mOsm/kg H_2O. The special medium is made by addition of 84 mg $NaHCO_3$ to 100 ml so-prepared sulotion. The m-HIS is made by addition of 34 mg NaCl to 10 ml medium.

Sperm capacitation

The fresh semen is firstly treated following the procedure of Bracktt et al. Semen is pre-diluted with special medium (350g). After centrifugation, the sperm pellet is re-suspended with m-HIS medium and put in water bath of 38 °C for 15 minutes. After another centrifugation, the sperm pellet is re-suspended with special medium in a tube for irradiation with bio-spectrum device at weak level for 10 minutes. During irradiation, the temperature of medium should be kept at 38-40 °C by adjusting the distance between the tube and irradiation device

In vitro fertilization of oocytes

The in-vitro matured oocytes and in-vitro capacitated sperm are put in the special medium in a tube for irradiation with bio-spectrum device for 20 minutes. The tube is put in CO_2 incubator for one hour culture at 38 °C. Afterwards, the tube is irradiated with bio-spectrum again for 18 minutes and again put in incubator for 5-hour culture. In irradiation, weak level is used and the temperature of medium is kept at 38-40 °C by adjusting the distance between the tube and irradiation device.

In vitro culture of embryos

Before culture in incubator, the in-vitro fertilized eggs are put in the special medium for irradiation with BIO-SPECTRUM for 25 minutes. Weak level is used. During irradiation the temperature of medium is kept at 38-40 °C by adjusting the distance between the medium container and irradiation device.

EXAMPLE 2

Bovine embryos are put in special medium for irradiation with bio-spectrum for 15 minutes. The weak level is used. During irradiation the temperature of medium is kept at 38-40 °C by adjusting the distance between the medium container and irradiation device. The cryo-protectant is added following the procedure of the Japanese Journal of Animal Reproduction (28:150-153,1982). The embryos are transferred step by step in PBS plus 20% CS and 0.18,0.33,0.75,0.88,1.0M Glycerol, respectively, each step for 5 minutes. The embryos are put in freezing medium (PBS plus 20% CS and 1.0M Glycerol) for 30 minutes and then placed in 0.5 ml straw. The straws are cooled in a freezer to -7 °C at a speed of 1 °C /min., then artificially seeded, slowly cooled at -0.3 °C/min. to -35 °C and cooled at -0.1 °C/min. to -36 °C, then plunged into liquid nitrogen (-196 °C) and stored.

Embryo thawing: the straws are took out from nitrogen and inserted into 21 °C water bath, slightly shaken until the ice is melted. The speed of thawing is 360 °C/min. After thawing the cryo-protectant is removed by holding embryos each step in reverse of adding cryo-protectant. Then embryos are placed in the special medium for irradiation with bio-spectrum for 20 minutes. Weak level is used. During irradiation the temperature of medium is kept at 38-40 °C by adjusting the distance between the medium container and the irradiation device.

EXAMPLE 3

Cryo-preservation of embryos by vitrification (Kasai et.al. J. Reproduction & Fert. 89:91-97). The bovine embryos are put in special medium for irradiation with bio-spectrum for 15 minutes. The weak level is used. During irradiation the temperature of medium is kept at 38-40 °C by adjusting the distance between the medium container and irradiation device. The 0.25 ml straw is used to take in 100μl S-PBS medium, 20μl air, 6μl EFS medium, 6μl air, 40μl EFS medium with embryos after 3 minutes equilibration in EFS medium at the room temperature, 6μl air, 6μl EFS medium, 15μl air and 20μl S-PBS medium in turn. Then the end of straws is sealed using hot forceps.

Thawing:

The straws are took out from nitrogen and inserted into 20 °C water bath, slightly shaken until the ice melted. Then embryos are quickly flushed out from straws with 0.5ml S-PBS medium, then transferred to S-PBS medium, 5 minutes later, embryos are transferred to m-PBS medium. After washing three times in the special medium, the embryos are irradiated with BIO-SPECTRUM for 10 minutes. Weak level is used. The temperature of medium is kept at 38-40 °C by adjusting the distance between the medium container and the irradiation device.

The receipt of S-PBS medium is as follows:

Components	g/l
NaCl	8
KCl	0.2
NaH_2PO_4	1.15
KH_2PO_4	0.2
$CaCl_2$	0.1
$MgCl \cdot 6H_2O$	0.1
Sodium pyruvate	0.036
Glucose	1
Penicillin	100IU/ml
Streptomycin	0.05 g/l
Glycerol	0.5M

EPS medium is 0.5M sucrose solution (EF medium) which contains 30% polysucrose. EFS medium is made by mixture of ethylene (40%) and EF medium (60%).

EXAMPLE 4

Embryo splitting: The rat, goat and bovine embryo Is split by metal knife following the procedure of matsu Moto Katsu Ya et al. (Japanese Journal of Animal Reproduction). The micro-surgical blade made from razor is fixed with a micro-manipulator. Before splitting, the embryos are put in the culture medium for irradiation with bio-spectrum for 15 minutes. After irradiation, the embryos are held in a droplet of 0.5ml PBS plus 20% FCS on the center of the plastic dish (diameter 8cm, height 1 cm). The micro-surgical bisection is performed using a micro-manipulation unit consisting of an inverted microscope.

After splitting and treatment, hemi-embryos are put in the culture medium for irradiation with BIO-SPECTRUM for 30 minutes. Weak level is used. During irradiation the temperature of medium is kept at 38-40 °C by adjusting the distance between the medium container and the irradiation device.

EXAMPLE 5

Sex Identification: The sex of the embryo can be accurately determined through sampling several cells from the embryo. After sampling, the embryo is partly damaged, and the viability decreases. The viability of the embryo can be raised with the treatment of bio-spectrum for 20 minutes (Model WS-101D).

EXAMPLE 6

Reproduction, Development and Growth of Animal

Mice are used as experimental animals. Female mice are randomly divided into 2 groups, A and B, 20 in each group. Group A is irradiated with bio-spectrum, group B is used as control group without irradiation of bio-spectrum. Both group A and group B are under the same experimental conditions, each treatment had 2 replicates, 10 mice per replicate. The mice in group A are irradiated with bio-spectrum for 20 minutes once a day at fixed time, the model of bio-spectrum machine is WS-101, made by Zhoulin Bio-Spectrum Company, high level is used. The temperature over the mouse back is controlled under 38 °C, the mice are irradiated for 10 times in 10 days. On 4th day, every mouse in group A and group B is injected with PMSG101U, on 6th day , further injected with HCG101U, after treatment, one male mouse is used for mating in each cage. On 7th day, fertilized eggs are collected from the oviducts of 10 mice in each group, the comparative results showed that bio-spectrum irradiation could significantly protect the fertilized eggs, on day 10, blastocysts are collected from the remaining 10 mice in each group. The results showed that bio-spectrum could improve the ovulation and fertilization ability of egg, and significantly improve the development of blastocyst. The comparison between group A and group B also shows that bio-spectrum irradiation can strongly stimulate the development of uterus in female mouse. This experiment shows that bio-spectrum irradiation can improve the reproduction, development and growth of animal.

EXAMPLE 7

WS-101 Bio-Spectrum Health Care Device (BIO-SPECTRUM Device) made in Beijing Zhoulin-Bio-Spectrum Company is used for irradiating the abdomen of lambs with diarrhea once a day, 20 minutes each time. Totally for 2-4 days, while high level is used and the surface temperature of the part being irradiated is not more than 45 °C, the diarrhea could be controlled and the results are obvious.

The inventor believes the bio-spectrum would apply to many objects in bio-engineering besides the above examples according to main aspects of the invention. It could not only solve many buffing problems in areas of both embryonic engineering and bio-engineering, but also simplify the complex and difficult techniques in the areas. So there will be other embodiments in the area of bio-engineering and these embodiments will fall into the protection scope of this invention.

Claims

1. A method of applying bio-frequency spectrum irradiation to animal embryonic engineering which

includes :

(1) collection of oocytes ;
(2) *in vitro* maturation of oocytes ;
(3) capacitation of spermatozoa ;
(4) *in vitro* culture of embryos

wherein during the *in vitro* maturation, oocytes cultured in a standard or defined medium are irradiated with a bio-spectrum generator for 3 to 20 minutes, the temperature of the medium during irradiation being kept not higher than 40°C ; during capacitation, semen diluted with a standard or defined medium is irradiated with bio-spectrum generator for 3 to 20 minutes, the temperature of the medium during irradiation being not higher than 40°C ;

during *in vitro* fertilization, both oocytes and spermatozoa capacited *in vitro* co-cultured in a test tube containing with bio-spectrum generator for 1 to 3 times, from 3 to 25 minutes each time ; the medium temperature during irradiation being not higher than 40°C ;
during *in vitro* culture of embryos, potential zygotes are transferred into a standard or defined medium and irradiated with bio-spectrum generator for 3 to 20 minutes ; the medium temperature in this period being not higher than 40°C.

2. A method according to claim 1 wherein the components of said defined medium are NaCl 6.55 g/l, KCl 0.30 g/l, $CaCl_2 \cdot 2H_2O$ 0.33 g/l, $NaH_2PO_4 \cdot H_2O$ 0.11 g/l, $MgCl_2 \cdot 6H_2O$ 0.11 g/l, $NaHCO_3$ 3.10 g/l, glucose 2.5 g/l, sodium pyruvate 0.14 g/l (Pyruvate 0.11 g/l), bovine serum albumin 3.00 g/l, sodium salt penicillin 0.031 g/l (50 IU/ml) and 84 mg $NaHCO_3$ is added to 100 ml of said defined medium.

3. A method of applying bio-frequency spectrum irradiation to cryo-preservation of animal embryos wherein embryos are put in culture medium and irradiated with bio-spectrum generator from 3 to 35 minutes before or after cryo-preservation, or embryos are irradiated for 3-35 minutes, after being thawed ; the medium temperature during irradiation is not higher than 40°C.

4. A method of applying bio-frequency spectrum irradiation to animal embryo micro-manipulation wherein embryos are placed in culture medium and irradiated with bio-spectrum generator for 3 to 35 minutes before or after micro-manipulation ; the medium temperature during irradiation being kept not higher than 40°C.

5. A method of applying bio-frequency spectrum irradiation to animal reproduction, development and growth wherein animals are irradiated with bio-spectrum once or twice everyday for 3 to 60 minutes per time, the surface temperature of animal during irradiation being kept not higher than 45°C.

6. A method of applying bio-frequency spectrum irradiation to the prevention and cure of animal diseases wherein parts or whole bodies of animals are irradiated with bio-spectrum one to three times everyday, 6-60 minutes per time ; the surface temperature during irradiation being kept at 45°C.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN 95/00087

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6] A61D 19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6] A61D 19/00, C12N 5/06, A61M 36/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SU, A, 1724204(Гродненский государжевский медицинский институт) 07.04.1992, Whole document	1,2
Y	SU, A, 731976(Ленинградский научно-исследовательский институт) 05.05.1980, Whole document	5
Y	SU, A, 1757676(Гроский сельскохозяйственный институт) 03.08.1992, Whole document	6
A	SU, A, 1152583(Красюго знамени сельскохозяйственный институт) 30.04.1985, Whole document	5

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN 95/00087

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SU, A, 1402343(Кубамский селвскохозяйственный институт) 15. 06. 1988, Whole document	1,2
A	EP, A, 86113792(HELMUT K. PINSCH GMBH & CO.) 13. 08. 1988, Whole document	5

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