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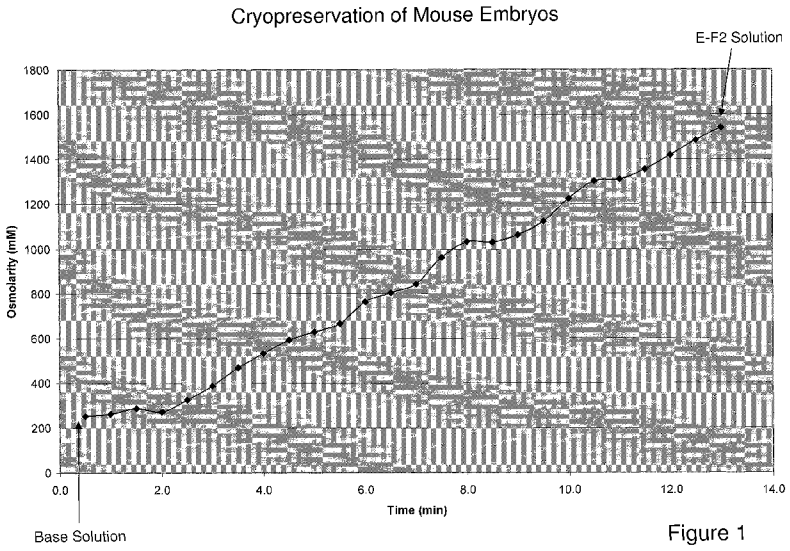


Figure 1

(57) Abstract: Disclosed are methods and compositions useful for maintaining substantially spherical, oocytes, embryos or blastocysts during cryopreservation and reanimation.

POPULATIONS OF SUBSTANTIALLY SPHERICAL, REDUCED VOLUME OOCYTES

FIELD OF THE INVENTION

[0001] The present invention relates generally to the field of cryopreservation. In particular, the present invention relates to methods and compositions for the cryopreservation and/or reanimation of substantially spherical oocytes, embryos or blastocysts.

BACKGROUND OF THE INVENTION

[0002] Throughout this disclosure, various technical and patent publications are referenced to more fully describe the state of the art to which this invention pertains. These publications are incorporated by reference, in their entirety, into this application.

[0003] The ability to cryopreserve and then reanimate oocytes, embryos or blastocysts is desirable for many reasons. However, conventional techniques have proven difficult to reproduce in an effective, efficient and consistent manner. Such conventional techniques are typically labor-intensive, requiring substantial handling of the oocytes, embryos or blastocysts by a highly skilled human technician. For example, conventional cryopreservation of an oocyte requires that the technician manually move the oocytes from one location to another in the cryopreservation process, such as from incubation to washing solution to a cryoprotectant solution. Further, oocytes frequently incur structural damage during conventional cryopreservation techniques. For example, conventional manual movement of oocytes among cryopreservation solution baths can impart osmotic and thermal shock. For instance, formation of ice crystals within the oocyte can cause intracellular damage in the oocyte. Oocytes undergoing conventional cryopreservation techniques can also experience a loss of sphericity and undesirable changes in volume. Such effects may result in structural damage in addition to toxicity, thereby significantly diminishing the viability of the oocyte and ultimately reducing the probability of successful fertilization.

[0004] Human involvement and conventional preservation techniques greatly contribute to the lack of consistency in cryopreservation of oocytes, embryos or blastocysts and results in an undesirably low fertilization success rate. Therefore, it is desirable to provide a method for the repeatable and efficient cryopreservation and reanimation of oocytes, embryos or blastocysts that mitigate effects harmful to the viability of the oocyte, embryo or blastocyst, and thereby increasing the rate of successful fertilization.

SUMMARY OF THE INVENTION

[0005] Disclosed are methods and compositions useful for maintaining a population of substantially spherical oocytes, embryos or blastocysts during cryopreservation or reanimation.

[0006] This invention provides a method for producing a substantially spherical, partially cryopreserved oocyte, embryo or blastocyst. The substantially spherical shape of the partially cryopreserved oocyte, embryo or blastocyst is provided by contacting an oocyte, embryo or blastocyst with a cryoprotecting solution having an initial osmolarity under continuous process conditions. In one aspect, the continuous process conditions comprise gradually increasing the osmolarity of the cryoprotecting solution over a predetermined period of time such that a portion of cytoplasmic water of the oocyte, embryo or blastocyst is replaced with cryoprotectant from the solution, thereby producing a substantially spherical, partially cryopreserved oocyte, embryo or blastocyst. During cryopreservation, the substantially spherical shape of the oocyte, embryo or blastocyst reduces or alternatively substantially eliminates the stress on the cell walls and the interior proteins that are key to maintaining viability of the oocyte, embryo or blastocyst after reanimation.

[0007] This invention also provides a method for producing a partially reanimated oocyte, embryo or blastocyst comprising contacting a cryopreserved oocyte, embryo or blastocyst with a reanimating solution having an initial osmolarity under continuous process conditions. In one aspect the continuous process conditions comprise gradually decreasing the osmolarity of the reanimating solution over a predetermined period of time such that a portion of cytoplasmic cryoprotectant of the oocyte, embryo or blastocyst is

replaced with water from the solution, thereby producing a substantially spherical partially reanimated oocyte, embryo or blastocyst.

[0008] In one embodiment of this invention, there is provided a population of substantially spherical, partially cryopreserved oocytes, embryos or blastocysts characterized in that a portion of their cytoplasmic water has been replaced with a cryoprotectant while maintaining their substantially spherical shape. In one aspect the population provides for a viable pregnancy at a ratio of less than about 10:1, or alternatively less than 8:1 or alternatively less than about 5:1. The ratio is a number of cryopreserved and reanimated oocytes, embryos or blastocysts used to produce a single viable pregnancy.

[0009] In yet another embodiment of this invention, there is provided a population of partially reanimated oocytes, embryos or blastocysts characterized in that a portion of their cytoplasmic cryoprotectant has been replaced with water. In one aspect, the population provides for a viable pregnancy at a ratio of less than about 10:1, or alternatively less than 8:1 or alternatively less than about 5:1. The ratio is a number of cryopreserved and reanimated oocytes, embryos or blastocysts used to produce a single viable pregnancy.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Figure 1 shows a graphic representation of the gradual increase in osmolarity of the cryoprotecting solution during the cryopreservation of mouse embryos using the methods of Example 1. The X-axis represents time (min), whereas the Y-axis represents the osmolarity (mM) of the cryoprotecting solution.

[0011] Figure 2 shows a graphic representation of the gradual decrease in osmolarity of the reanimating solution during the reanimation of mouse embryos using the methods of Example 2. The X-axis represents time (min), whereas the Y-axis represents the osmolarity (mM) of the reanimating solution.

[0012] Figure 3 shows a photograph of mouse embryos following cryopreservation and reanimation of mouse embryos using the methods of Example 1.

[0013] Figure 4 shows a photograph of the mouse embryos of Figure 3 following 1 day of growth.

[0014] Figure 5 shows a photograph of the mouse embryos of Figure 3 following 2 days of growth.

DETAILED DESCRIPTION OF VARIOUS EMBODIMENTS

[0015] Before the compositions and methods are described, it is to be understood that the invention is not limited to the particular methodologies, protocols, assays, and reagents described, as these may vary. It is also to be understood that the terminology used herein is intended to describe particular embodiments of the present invention, and is in no way intended to limit the scope of the present invention as set forth in the appended claims.

Definitions

[0016] All numerical designations, e.g., pH, temperature, time, concentration, and molecular weight, including ranges, are approximations which are varied (+) or (-) by increments of 0.1 or 1 where appropriate. It is to be understood, although not always explicitly stated that all numerical designations are preceded by the term "about". The term "about" also includes the exact value "X" in addition to minor increments of "X" such as "X + 0.1 or 1" or "X – 0.1 or 1," where appropriate. It also is to be understood, although not always explicitly stated, that the reagents described herein are merely exemplary and that equivalents of such are known in the art.

[0017] As used herein, certain terms may have the following defined meanings. As used in the specification and claims, the singular form "a," "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a oocyte" includes a plurality of oocytes, including populations thereof.

[0018] As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the composition or method. "Consisting of" shall mean excluding more than trace elements of other ingredients for claimed compositions and substantial method steps. Embodiments defined by each of these transition terms are within the scope of this invention. Accordingly, it is intended that the methods and compositions can include additional steps and components (comprising) or alternatively including steps and compositions of no significance (consisting essentially

of) or alternatively, intending only the stated methods steps or compositions (consisting of).

[0019] The term "population" refers to a composition of at least two individual oocytes, embryos, blastocysts or equivalents thereof. In another aspect, a "population" refers to at least three, or alternatively at least four, or alternatively at least five, or alternatively at least six, or alternatively at least seven, or alternatively at least eight, or alternatively, at least nine, or alternatively at least ten individual oocytes, embryos, blastocysts.

[0020] The term, "substantially spherical," refers to an oocyte, embryo or blastocyst which has no more than ± 40% change in its surface area as compared to the oocyte prior to introduction of a cryoprotectant during freezing or cryopreserving or prior to introduction of water during thawing or reanimating. The change in the surface area of the oocyte, embryo or blastocyst can be a decrease in surface area due to shrinkage or an increase in surface area due to the introduction of undulations or other surface deformities which arise during shrinkage. In another embodiment, the oocyte, embryo or blastocyst has no more than about ± 30% change, no more than about ± 20% change, no more than about ± 15% change, no more than about ± 10% change, or no more than about ± 5% change in its surface area during shrinkage.

[0021] The term, "substantially non-spherical," refers to an oocyte, embryo or blastocyst which has more than ± 40% change in its surface area as compared to the oocyte prior to introduction of a cryoprotectant during freezing or cryopreserving or prior to introduction of water during thawing or reanimating. The change in the surface area of the oocyte, embryo or blastocyst can be a decrease in surface area due to shrinkage or an increase in surface area due to the introduction of undulations or other surface deformities which arise during shrinkage.

[0022] The term "partially cryopreserved" refers to an oocyte, embryo or blastocyst having a portion of its cytoplasmic water replaced with cryoprotectant prior to freezing. In one aspect of the invention, the portion of cytoplasmic water that has been replaced with water is more than about 1 v/v%, or alternatively more than about 10 v/v%, or alternatively more than about 50 v/v%, or alternatively more than about 60 v/v%, or alternatively more than about 70 v/v%, or alternatively more than about 80 v/v%, or

alternatively more than about 90 v/v%. In one embodiment, substantially all of the cytoplasmic water of the oocyte, embryo or blastocyst (90-100 v/v%) is replaced with the cryoprotectant. In yet another embodiment, the portion of cytoplasmic water of the oocyte, embryo or blastocyst that has been replaced with cryoprotectant is sufficient to protect the oocyte, embryo or blastocyst. It is understood that one of skill in the art will be able to readily ascertain the amount of cryoprotectant necessary to protect the oocyte, embryo or blastocyst.

[0023] In another embodiment, "cryopreserved oocytes, embryos or blastocysts" refers to frozen oocytes, embryos or blastocysts, comprising a cryoprotectant, which are preserved by cooling to low sub-zero temperatures, such as, but not limited to, 77 K or −196 °C (the boiling point of liquid nitrogen).

[0024] The term "partially reanimated" refers to an oocyte, embryo or blastocyst having at least a portion of the cytoplasmic cryoprotectant that is replaced with water after being thawed. In one aspect of the invention, the portion of the cytoplasmic cryoprotectant that is replaced with water is more than about 1 v/v%, or alternatively more than about 10 v/v%, or alternatively more than about 50 v/v%, or alternatively more than about 60 v/v%, or alternatively more than about 70 v/v%, or alternatively more than about 80 v/v%, or alternatively more than about 90 v/v%. In one embodiment, all of the cytoplasmic cryoprotect of the oocyte, embryo or blastocyst (100 v/v%) is replaced with the water. In yet another embodiment, the cytoplasmic cryoprotectant of the "partially reanimated" oocyte, embryo or blastocyst has been replaced with water that is sufficient to reanimate the oocyte, embryo or blastocyst. It is understood that one of skill in the art will be able to readily ascertain the amount of water necessary to reanimate the oocyte, embryo or blastocyst.

[0025] In another embodiment, the term "reanimated oocytes, embryos or blastocysts" refers to thawed oocytes, embryos or blastocysts which are capable of fertilization and/or embryo development.

[0026] The term "oocyte" refers to an unfertilized freshly harvested or mature oocyte. The term "freshly harvested oocyte" means that the oocyte was harvested from the animal donor within 8 hours of initiation of the stabilization/cryopreservation process, or

alternatively within about 4 hours of initiation of the stabilization/cryopreservation process, or alternatively within about 1 hour of initiation of the stabilization/cryopreservation process, or alternatively within about 0.1 hour of initiation of the stabilization/cryopreservation process. The mature oocytes mean harvested oocytes which are graded on a maturation scale as "mature stage -MII." This scale further identifies harvested oocytes as "intermediate stage - (MI)" or "immature stage - (GV)". The term "egg" as used herein is meant to be synonymous with the term "oocyte".

[0027] The term "stabilized oocytes" refer to mature oocytes still retaining the cumulus mass (granulosis cells) which permit maturation of the oocyte by nutrient intake through gap junctions in the cumulas mass. The mature oocyte is characterized by formation of the meiotic spindle in conjunction with extrusion of the first polar body while maintaining the integrity/activity of the intracellular proteins.

[0028] The term "blastocyst" refers to a fertilized egg freshly harvested from about 5 days after fertilization up to implantation in the uterus. The term "freshly harvested blastocyst" means that the blastocysts were harvested from the animal donor within about 8 hours of initiation of the stabilization/cryopreservation process, or alternatively within about 4 hours of initiation of the stabilization/cryopreservation process, or alternatively within about 1 hour of initiation of the stabilization/cryopreservation process, and alternatively within about 0.1 hour of initiation of the stabilization/cryopreservation process.

[0029] The term "embryo" refers to a fertilized egg freshly harvested from between the time of the first division to two cells to about 5 days after fertilization. The term "freshly harvested embryo" means that the embryos were harvested from the animal donor within about 8 hours of initiation of the stabilization/cryopreservation process, preferably within about 4 hours of initiation of the stabilization/cryopreservation process, more preferably within about 1 hour of initiation of the stabilization/cryopreservation process, and even more preferably within about 0.1 hour of initiation of the stabilization/cryopreservation process.

[0030] The term "stabilization process" refers to the incubation of the oocytes, embryos or blastocyst in a stabilization solution, which provides the oocytes, embryos or blastocyst

an opportunity to stabilize in a solution of low to intermediate osmolality prior to incubation in a cryoprotecting solution having gradually increasing osmolality.

[0031] The term "cryoprotecting solution" refers to a solution having at least one cryoprotectant. A "cryoprotectant" permeates across the cell wall of the oocyte, embryo or blastocyst typically by osmotic methods and promotes survival and retention of viability of the oocyte, embryo or blastocyst during the process of cryopreserving as well as in the cryopreserved state and/or during reanimation. Suitable cryoprotectants are well known in the art and include, by way of example only, dimethyl sulfoxide (DMSO), ethylene glycol, propylene glycol (1,2-propanediol), glycerol, as well as mixtures of 2 or more of such cryoprotectants, and the like. In one aspect, the "cryoprotecting solution" has an initial osmolarity. In another aspect, the "cryoprotecting solution" comprises a dehydrating agent. In yet a another aspect, the "cryoprotecting solution" has a cryoprotectant concentration, osmolarity and/or a dehydrating agent concentration as described in Tables 1 or 2.

[0032] "Osmolarity" refers to the amount of solute (dissolved chemical) per liter of total solution and is typically measured in milliosmoles per liter (mOsmol/L).

[0033] In another embodiment, the cryoprotecting solutions useful in methods of the invention further comprise at least one or more components such as, but not limited to, sterile water, HEPES, sodium bicarbonate, sodium hydroxide, sodium chloride, potassium chloride, calcium chloride, potassium phosphate, magnesium sulfate, dextrose, sodium lactate solution, sodium pyruvate, gentamicin sulfate, and human serum albumin. In yet another aspect, the cryoprotecting solution of the invention does not comprise alpha globulin or beta globulin. In yet a further aspect, the cryoprotecting solution of the invention comprises component concentrations as described in Tables 1 or 2.

[0034] The term "dehydrating agent" refers to an agent that facilitates dehydration of the intra-cytoplasmic water in the oocyte, embryo or blastocyst during cryopreservation. In one aspect, such agents do not osmotically traverse the cellular wall of the oocyte. Dehydrating agents include, sucrose, dextrose, trehalose, lactose, raffinose, and the like.

[0035] The term "reanimating solution" refers to a solution having at least one cryoprotectant and water. A "reanimating solution" allows water to permeate across the

cell wall of the oocyte, embryo or blastocyst, typically by osmotic methods and promotes survival and retention of viability of the oocyte, embryo or blasocycst during the process of reanimating. In one aspect, the "reanimating solution" has an initial osmolarity. In another aspect, the "reanimating solution" comprises a dehydrating agent. In yet another aspect, the "reanimating solution" has a cryoprotectant concentration, osmolarity and/or dehydrating agent concentration as described in Tables 1 or 2.

[0036] By way of example, reanimating solutions of the invention further comprise at least one or more components such as, but not limited to, sterile water, HEPES, sodium bicarbonate, sodium hydroxide, sodium chloride, potassium chloride, calcium chloride, potassium phosphate, magnesium sulfate, dextrose, sodium lactate solution, sodium pyruvate, gentamicin sulfate and human serum albumin. In yet another aspect, the reanimating solution of the invention does not comprise alpha globulin or beta globulin. In yet a further aspect, the reanimating solution of the invention comprises component concentrations as described in Tables 1 or 2.

[0037] The term "gradually" refers to proceeding by fine or incremental steps or degrees. In one embodiment of the invention, the phrase "gradually increasing" refers to increasing the amount of a component in a solution by no more than about 0.001%, or alternatively no more than about 0.01%, or alternatively no more than about 0.1%, or alternatively no more than about 1%, or alternatively no more than about 5%, or alternatively no more than about 10%. In another aspect of the invention, the osmolarity of a solution is "gradually increased" at a given rate, for example, from about 90 mOsmol/L per 1 minute to about 110 mOsmol/L per 1 minute. In another embodiment of the of the invention, the phrase "gradually decreasing" refers to decreasing the amount of a component in a solution by no more than about 0.001%, or alternatively no more than about 0.01%, or alternatively no more than about 0.1%, or alternatively no more than about 1%, or alternatively no more than about 5%, or alternatively no more than about 10%. In another aspect of the invention, the osmolarity of a solution is "gradually decreased" at a given rate, for example, from about 30 mOsmol/L per 1 minute to about 50 mOsmol/L per 1 minute. In yet another aspect of the invention, the temperature of a solution is "gradually" increased or decreased from one temperature to another

temperature over a predetermined period of time. In one aspect, the above gradual changes occurs under continuous process conditions.

[0038] The term "continuous process conditions" refers to the uninterrupted conditions used in a process of making a product. In one embodiment of the invention, the "continuous process conditions" comprise contacting of an oocyte, embryo or blastocyst or a population thereof with one or more solutions described herein. In one embodiment of the invention, the "continuous process conditions" comprise gradually increasing the osmolarity of a solution. In another aspect, the "continuous process conditions" comprise gradually decreasing the osmolarity of the solution. In another aspect of the invention, the "continuous process conditions" comprise controlling one or more characteristic of the solution, such as temperature, concentration of a component, pH level, pressure or flow rate of the solution contacting the oocyte, embryo or blastocyst. Examples of "continuous process conditions" are described in U.S. Provisional Application 61/116,255, filed November 19, 2008 entitled, "Systems And Methods For Automated Change In Solution Environment For Cryopreservation Of Oocytes, Embryos Or Blastocysts", which is herein incorporated by reference in its entirety.

[0039] The term "viable pregnancy" refers to the implantation of an embryo into the uterine wall of a subject. Methods for identifying a viable pregnancy include, but are not limited to, detection of fetal cardiac activity and/or fetal development using ultrasound or an equivalent thereof or detection of β-human chorionic gonadotropin (β-hCG) in the subject's blood and/or urine. In another aspect, the viability of a pregnancy is determined by serial quantitative blood tests done about 2 to 3 days apart, wherein if the blood serum β-hCG level of a subject is below 1,200 mIU/ml and increases by about 50% to about double every 48 to 72 hours, or alternatively, if the blood serum β-hCG levels of a subject is between 1,200 and 6,000 mIU/ml and doubles about every 72–96 hours, or alternatively if the blood serum β-hCG level of a subject is above 6,000 mIU/ml and the β-hCG takes more than about four days to double, all of the above are an indication of a viable pregnancy. In yet another aspect, a "successive viable pregnancy" is a viable pregnancy in a subject which is subsequent to a preceding viable pregnancy in the same subject following termination of the preceding pregnancy. Termination of a pregnancy

includes, but is not limited to, successful delivery of a neonate, miscarriage of a fetus, or any other action with removes a developing neonate or fetus from the subject.

[0040] The term "predetermined period of time" refers to the amount of time in which the oocyctes, embryos or blastocysts are contacted with the solutions described herein to obtain the desired portion of a cryoprotectant or water in the oocyctes, embryos or blastocysts thereby producing a population of substantially spherical, partially cryopreserved or partially reanimated oocyctes, embryos or blastocysts, respectively.

[0041] The term "progressive rate" refers to advancing a measured quantity with respect to another measured quantity. In one embodiment of the invention, the osmolarity of a solution is increased at a "progressive rate" such as, but not limited to, mOsmol/L per minute, or alternatively from an initial osmolarity of a solution to a final osmolarity of a solution over a predetermined amount of time. In another embodiment, the osmolarity of solution is decreased at a "progressive rate" such as, but not limited to, mOsmol/L per minute, or alternatively from an initial osmolarity of a solution to a final osmolarity of a solution over a predetermined amount of time. In one aspect the "progressive rate" is substantially linear, wherein the advancing measured quantity is equal throughout the other measured quantity. In this aspect, the "substantially linear progressive rate" will vary by no more than about ± 1%, or alternatively no more than about ± 5%, or alternatively no more than about ± 10%, or alternatively no more than about ± 15%, or alternatively no more than about ± 20%, or alternatively no more than about ± 25%, or alternatively no more than about ± 30%, or alternatively no more than about ± 40% from a desired rate. In another aspect, the "progressive rate" is substantially non-linear, wherein the advancing measured quantity is not equal throughout the other measured quantity. A non-limiting example of this substantially non-linear progressive rate is an initial increase in osmolarity of a solution that is lower than the later rate of increasing osmolarity or vise-versa, all within a predetermined period of time.

Methodology

[0042] This invention provides a method for producing a substantially spherical, partially cryopreserved oocyte, embryo or blastocyst comprising contacting an oocyte, embryo or blastocyst with a cryoprotecting solution having an initial osmolarity under

continuous process conditions, wherein the conditions comprise gradually increasing the osmolarity over a predetermined period of time, such that a portion of cytoplasmic water of the oocyte, embryo or blastocyst is replaced with cryoprotectant from said solution, thereby producing a substantially spherical, partially cryopreserved oocyte, embryo or blastocyst. In one aspect, the portion of cytoplasmic water replaced with cryoprotect from the solution is sufficient to protect the oocyte, embryo or blastocyst. It is understood that one of skill in the art will be able to readily ascertain the amount of cryoprotectant necessary to protect the oocyte, embryo or blastocyst. In a further aspect, more than about 10% of the cytoplasmic water is replaced with cryoprotectant.

[0043] In one aspect of the above methods, the oocyte, embryo or blastocyst is freshly harvested and stabilized to *in vitro* conditions prior to the initiation of cryopreservation. In one aspect, the oocyte, embryo or blastocyst is incubated or stored in one or more stabilization solutions for at least about 1 hour, or alternatively at least 2 hours, or alternatively at least 3 hours after harvesting. In one aspect, the stabilization solutions are intended to permit further maturation of the oocytes *in vitro*. Specifically, the stabilization solutions are designed to mimic the environment within the follicle from which the eggs were removed. The follicle supports ongoing processes of the oocyte maturation *in vivo* which in one aspect of the invention are duplicated when the oocyte is removed from the follicle and placed into the stabilization solution. The stabilization solution also provides nutrients for further meiotic development and supports metabolic processes continuing within the oocyte, embryo or blastocyst as well as supports the cell membrane. A further function of the stabilization solution is to remove biological wastes occurring from metabolism within the oocyte, embryo or blastocyst and to stabilize the intra-oocyte or intra-cellular pH. In another aspect, the stabilization solution maintains the integrity and activity of these proteins/polypeptides so that after cryopreserving and subsequent reanimating, these proteins/polypeptides can be employed within the oocyte, embryo or blasocycst for their intended use. The stabilization solution does not include any cryoprotectant, as described herein. One of ordinary skill in the art will recognize that there are several alternative stabilization solutions and methods available to perform this step.

[0044] Stabilization solutions useful in this invention include, by way of the example, Global® media (available from Life Global, IVF Online), Global® media supplemented with SSS (available from Irvine Scientific, Santa Ana, California, USA), human tubal fluid (HTF – available from Irvine Scientific, Santa Ana, California, USA) optionally supplemented with SSS and/or an antibiotic (e.g., gentamicin) and modified HTF (HTF with HEPES (mHTF) – available from Life Global, IVF Online) optionally supplemented with SSS and/or an antibiotic (e.g., gentamicin), phosphate buffered saline (PBS), sodium depleted PBS (e.g., sodium hydrogen phosphate (H_2NaPO_4)) or equivalents thereof.

[0045] One such stabilization method includes incubation or storage of the oocyte, embryo or blastocyst in Global media supplemented with about 5% to about 20% Synthetic Serum Substitute (SSS), or alternatively Global media supplemented with about 10% human serum albumin (HSA), or alternatively human tubal fluid (HTF), or alternatively modified human tubal fluid (mHTF), which includes HTF and HEPES buffer. In another aspect, the stabilization of the oocyte, embryo or blastocyst will include the incubation or storage of the oocyte, embryo or blastocyst in two or more stabilization solutions. In a further aspect, the stabilization solutions are maintained at a temperature of from about 33°C to about 38°C, or alternatively from about 35°C to about 38°C, or alternatively from about 36.5°C to 37.5°C for a sufficient period to permit the oocyte, embryo or blastocyst to be stabilized to its *in vitro* conditions.

[0046] In another aspect of the above methods, the osmolarity of the cryoprotecting solution is increased at a progressive rate throughout the predetermined period of time. In a further aspect, the progressive rate is substantially linear or substantially non-linear. In yet a further aspect, the substantially linear progressive rate is from about 50 mOsmol/L per 1 minute to about 200 mOsmol/L per 1 minute, or alternatively from about 75 mOsmol/L per 1 minute to about 150 mOsmol/L per 1 minute, or alternatively from about 90 mOsmol/L per 1 minute to about 110 mOsmol/L per 1 minute, or alternatively about 100 mOsmol/L per 1 minute.

[0047] In another aspect of the above methods, the osmolarity of the cryoprotecting solution is increased from the initial osmolarity of about 300 mOsmol/L to a final osmolarity of at least about 1300 mOsmol/L within the predetermined period of time. In a further aspect of the above methods, the cryoprotectant concentration of the

cryoprotecting solution is gradually increased from substantially no cryoprotectant to at least 1.5 M cryoprotectant within the predetermined period of time. In yet a further aspect, the predetermined period of time is from about 5 minutes to about 20 minutes, or alternatively from about 8 minutes to about 18 minutes, or alternatively from about 12 minutes to about 16 minutes, or alternatively about 13 minutes.

[0048] In another aspect of the above methods, the increase in the cryoprotectant concentration and/or the osmolarity of the cryoprotecting solution comprises mixing of at least a first and a second solution, wherein the first solution comprises substantially no cryoprotectant and an osmolarity lower than the second solution and the second solution comprises more cryoprotectant than the first solution and has an osmolarity higher than the first solution. In another aspect, the cryoprotecting solution does not contain alpha globulin or beta globulin. In yet another aspect, the cryoprotecting solution further comprises a dehydrating agent. In a further aspect, the concentration of the dehydrating agent is gradually increased from substantially no dehydrating agent to at least 0.1 M dehydrating agent within the predetermined period of time.

[0049] In yet another aspect of the above methods, the continuous process conditions comprise maintaining the cryoprotecting solution at a temperature of about 22°C to about 26°C, or alternatively the cryoprotecting solution is gradually decreasing the temperature of the cryoprotecting solution from about 38°C to about 22°C within the predetermined period of time.

[0050] In another embodiment of the invention, following the above methods the oocytes, embryos or blastocysts are transferred into a sealed container and cryopreserved. Cryopreservation of the prepared oocyte proceeds via conventional techniques including slow freezing of the oocytes in a manner similar to that described by Fabbri, et al., U.S. Patent No. 7,011,937, which patent is incorporated herein by reference in its entirety. In one aspect, the oocytes, embryos or blastocysts are loaded into a suitable container such as a 0.25 mL plastic straw. The straws are sealed at both ends and placed in an automated biological vertical freezer such as the Kryo 10 Series III (Planer 10/1.7 GB).

[0051] The straws are chilled at a controlled cooling rate to about -7°C whereupon "seeding" is performed to induce the water molecules to undergo crystallization. In one

embodiment, the straws are cooled at a rate of from about -0.5°C per minute to about -4°C; or alternatively the straws are cooled at a rate of from about -1°C per minute to about -3°C; or alternatively the straws are cooled at a rate of about -2°C per minute. After a holding period at -7°C, cooling is resumed at a controlled rate until the straw reaches about -33°C which allows diffusion of the cryoprotectant into the oocytes, embryos or blastocysts. In one embodiment, the straws are cooled at a rate of from about -0.1°C per minute to about -1°C; or alternatively the straws are cooled at a rate of from about -0.2°C per minute to about -0.5°C; or alternatively the straws are cooled at a rate of about -0.3°C per minute. The straws are then plunged into liquid nitrogen for storage.

[0052] In another embodiment, this invention provides a method for producing a partially reanimated oocyte, embryo or blastocyst comprising contacting a cryopreserved oocyte, embryo or blastocyst with a reanimating solution having an initial osmolarity under continuous process conditions, wherein said conditions comprise gradually decreasing the osmolarity over a predetermined period of time, thereby producing a partially reanimated oocyte, embryo or blastocyst. In one aspect, the portion of the cytoplasmic cryoprotect replaced with water is sufficient to reanimate the oocyte, embryo or blastocyst. It is understood that one of skill in the art will be able to readily ascertain the amount of water necessary to reanimate the oocyte, embryo or blastocyst. In a further aspect, more than about 10% of the cytoplasmic cryoprotectant has been replaced with water.

[0053] In another aspect of the above methods, the osmolarity of the reanimating solution is increased at a progressive rate throughout the predetermined period of time. In a further aspect, the progressive rate is substantially linear or substantially non-linear. In yet a further aspect the substantially linear progressive rate of the osmolarity of the reanimating solution is from about 10 mOsmol/L per 1 minute to about 70 mOsmol/L per 1 minute, or alternatively from about 20 mOsmol/L per 1 minute to about 60 mOsmol/L per 1 minute, or alternatively from about 30 mOsmol/L per 1 minute to about 50 mOsmol/L per 1 minute, or alternatively about 40 mOsmol/L per 1 minute.

[0054] In another aspect of the above methods, the osmolarity of the cryoprotecting solution is gradually decreased from an initial osmolarity of at least about 1100 mOsmol/L to a final osmolarity of about 300 mOsmol/L within the predetermined period

of time. In a further aspect of the above methods, the concentration of cryoprotectant in the reanimating solution is gradually decreased from at least 1.0 M cryoprotectant to substantially no cryoprotectant within the predetermined period of time. In yet a further aspect, the predetermined period of time is from about 15 minutes to about 60 minutes, or alternatively from about 30 minutes to about 50 minutes, or alternatively from about 40 minutes to about 45 minutes, or alternatively about 42 minutes.

[0055] In another aspect of the above methods, the decrease in the cryoprotectant concentration and/or the osmolarity of the reanimating solution comprises mixing of at least two solutions, wherein the first solution comprises substantially no cryoprotectant and an osmolarity lower than the second solution and the second solution comprises more cryoprotectant than the first solution and has an osmolarity higher than the first solution. In another aspect, the reanimating solution does not contain alpha globulin or beta globulin. In yet another aspect, the reanimating solution further comprises a dehydrating agent. In a further aspect, the dehydrating agent concentration of the reanimating solution is gradually decreased from at least 0.1 M dehydrating agent to substantially no dehydrating agent within the predetermined period of time.

[0056] In yet another aspect of the above method, the continuous process conditions comprise gradually increasing the temperature of the reanimating solution from about 30°C to about 38°C within the predetermined period of time.

[0057] In another aspect of the above methods, upon completion of the reanimation, the oocyte, embryo or blastocyst is placed into a stabilization solution prior to fertilization and/or implantation. The stabilization solutions useful in this invention include, by way of the example, Global® media (available from Life Global, IVF Online), Global® media supplemented with SSS (available from Irvine Scientific, Santa Ana, California, USA), human tubal fluid (HTF – available from Irvine Scientific, Inc., Santa Ana, California) optionally supplemented with SSS and/or an antibiotic (e.g., gentamicin) and modified HTF (HTF with HEPES (mHTF) – available from Irvine Scientific, Inc., Santa Ana, California) optionally supplemented with SSS and/or an antibiotic (e.g., gentamicin), phosphate buffered saline (PBS), sodium depleted PBS (e.g., sodium hydrogen phosphate (H_2NaPO_4)) and the like. This solution stabilizes the oocyte, embryo or blastocyst relative to physiological temperature while providing a nutrient rich environment. In

another aspect, a reanimated oocyte is immersed in a reanimation stabilization solution to reestablish the processes relating to formation of meiotic spindle required for fertilization as well as enhancing mitochondria energy production and reestablishing the mRNA processes and protein activity. In one aspect, the oocyte, embryo or blastocys is maintained in this solution until stabilized. In a further aspect, stabilization occurs within about 2 hours. At this point the oocytes are ready for fertilization and/or implantation.

[0058] It is understood to a person of skill in the art that the methods described herein are applicable to an oocyte, embryo or blastocyst or a population of oocytes, embryos or blastocysts.

Methods for Measuring a Substantially Spherical Shape

[0059] In one embodiment, a cross-sectional area of the oocyte, embryo or blastocyst is measured during the process of cryopreservation and/or reanimation. In one embodiment, the measuring method is, by way of example only, birefringence imaging and NADH measurement. In one embodiment, the oocyte, embryo or blastocyst is observed under a microscope to determine a substantially spherical shape of the oocyte, embryo or blastocyst.

[0060] In one embodiment, a sample of an oocyte, embryo or blastocyst or a population of oocytes, embryos or blastocysts is imaged under magnification to investigate structural elements within the oocyte, embryo or blastocyst. Images obtained under magnification can be recorded while the sample is mounted in a microscope. A variety of different imaging techniques can be used to obtain the images. For example, broad- or narrow-bandwidth light sources can be used to illuminate the sample, and light transmitted through the sample can be viewed by an operator through a microscope eyepiece and/or can be directed to a detector such as a CCD camera.

[0061] In one embodiment, the oocytes can be graded as immature, mature, and post mature before *in vitro* fertilization. Embryos and blastocysts are typically graded by symmetry of the cells and sphericity. Damaged embryos or blastocysts will typically be aspherical or in the case of embryos, composed of a odd number of cells. Grading of the oocytes can be performed at the time of oocyte pickup under a stereomicroscope fitted with a warm stage and at, for example, x15 and x 40 magnification. The oocytes can be

graded based on the status of a first polar body, size of the perivitelline space, and/or presence of cytoplasmic inclusions. The evaluation of the morphology of the oocyte includes, but not limited to, variations such as shape of the oocyte, color, granularity, and homogeneity of the cytoplasm, size of perivitelline space, debris in the perivitelline space, vacuolization, inclusions and abnormalities of the first polar body or zona pellucida. For example, the grading of the oocytes as described in P. Xia, *Human Reproduction*, 1997, 12(8), 1750-1755, is incorporated herein by reference in its entirety.

[0062] In one embodiment, birefringence imaging techniques can be used to measure the substantially spherical shape of the oocyte, embryo or blastocyst. The birefringence imaging techniques can provide contrast between structural elements of interest and the rest of the sample of an oocyte, embryo or blastocyst or a population of oocytes, embryos or blastocysts. For example, birefringence imaging techniques will provide contrast even when the elements appear approximately transparent in images recorded using other techniques, e.g., broadband illumination. In birefringent imaging systems, input light is incident on the sample with a selected polarization, which is then altered by the presence of birefringence in the sample. Output polarization analyzers can then be used to create contrast (e.g., by polarization-dependent attenuation) in an image of the sample. For example, different regions of the sample through which corresponding portions of the input light pass exhibit different amounts of retardation, and thereby changes to the state of polarization of the different input light portions being imaged. Birefringence imaging systems are disclosed, for example, in U.S. Pat. Publ. No. 2007-0231784, entitled "Quantitation of Oocytes and Biological Samples Using Birefringent Imaging," by Clifford C. Hoyt and Cathy Boutin, filed on April 4, 2006; U.S. Pat. No. 5,521,705 entitled "Polarizied Light Microscopy" by Rudolf Oldenbourg and Guang Mei, filed on May 12, 1994; and U.S. Pat. No. 6,924,893 entitled "Enhancing Polarized Light Microscopy" by Rudolf Oldenbourg et al., filed on May 12, 2003, the entire contents of all of which are incorporated herein by reference.

[0063] In one embodiment, the substantially spherical shape of the oocyte, embryo or blastocyst is measured using a method of NADH measurement. The method involves reducing the endogenous NADH concentration of the oocyte, embryo or blastocyst by placing the oocyte, embryo or blastocyst in a control medium and obtaining at least one

control NADH fluorescence measurement for the nutrient-deprived oocyte. Thereafter, the oocyte is contacted with a nutrient for a period of time sufficient for the oocyte to acquire (i.e., take up and metabolically process) the nutrient. At least one post-nutrient NADH fluorescence measurement is obtained. The quality of the oocyte, embryo or blastocyst is assessed by comparing the control NADH fluorescence measurement(s) to the post-nutrient NADH fluorescence measurement(s). Higher quality oocytes, embryos or blastocysts are those for which the post-nutrient NADH fluorescence measurement is significantly (i.e., at least 2%) greater than the control NADH fluorescence measurement. The NADH measurement is disclosed, for example, in U.S. Pat. No. 5,541,081, entitled, "Process for Assessing Oocyte and Embryo Quality", by R. Ian Hardy, David E. Golan, and John D. Biggers, filed March 22, 1994, the entire content of which is incorporated herein by reference. The NADH measurement method can be used for evaluating membrane integrity and function (and obtaining metabolite transfer rates). The method involves measuring the time of onset of NADH fluorescence increase following nutrient perfusion.

[0064] Other methods that can be used for evaluating the oocyte, embryo or blastocyst include, by way of example only, a development rating system (Cummins, J. et al, (1986) *J. IVF-ET* 3(5), 284), fluorescein diacetate fluorescence measurement (Mohr, L. et al, (1980) *J. Reprod. Fertil.* 58, 189), determination of immunosuppressive activity in pre-implantation culture media (Clark, D. et al, (1989) *J. IVF-ET* 6(1), 51-58), and measurement of immunoactive factors in culture media including progesterone (Hardy, R. et al, (1993) *Soc. Gyn. Invest. Annu. Mtg.*, abstract), interleukin-1-alpha (Hardy, R. et al, (1993) *Am. Fertility Soc. Annu. Mtg.*, abstract) and interleukin-1-beta (Baranao, R. et al, (1992) *Am. Fertility Soc. Annu. Mtg.*, abstract). Ultramicrofluorometric technology is used for non-invasive measurement of nutrient uptake (Leese, H. et al, (1984) *Anal. Biochem.* 140(2), 443-448; Gardner, D. et al, (1986) *Hum. Reprod.* 1, 25). Glucose and pyruvate uptake, lactate production, and purine utilization have been indirectly quantified using traditional enzymatic analysis based on changes in the concentration of fluorescent NADH in culture media (O'Fallon, J. et al, (1986) *Biol. Reprod.* 34, 58; Gardner, D. et al, (1987) *J. Exp. Zoology* 242, 103).

Compositions

[0065] In one embodiment of this invention, there is provided a population of substantially spherical, partially cryopreserved oocytes, embryos or blastocysts characterized in that a portion of their cytoplasmic water has been replaced with a cryoprotectant while maintaining their substantially spherical shape. This population provides for a viable pregnancy at a ratio of less than about 10:1, or alternatively less than 8:1, or alternatively less than 5:1, wherein said ratio is a number of cryopreserved and reanimated oocytes, embryos or blastocysts used to produce a single viable pregnancy. In another aspect of the above population, the viable pregnancy is at least a second, or alternatively at least a third, or alternatively at least a fourth successive viable pregnancy resulting from the same population of cryopreserved oocytes, embryos or blastocysts.

[0066] In another aspect of the above population, the oocytes, embryos or blastocysts are animal, including a human, or non-human animal including, for example, a rodent, such as a rat and mouse, a canine, such as a dog, a leporid, such as a rabbit, livestock, sport animal or pet.

[0067] In yet another aspect of the above population, the portion of cytoplasmic water replaced with cryoprotectant is sufficient to protect the oocytes, embryos or blastocycts. In a further aspect, more than about 10 % of the cytoplasmic water is replaced with cryoprotectant.

[0068] In yet another embodiment of this invention, there is provided a population of partially reanimated oocytes, embryos or blastocysts characterized in that a portion of their cytoplasmic cryoprotectant has been replaced with water. This population provides for a viable pregnancy at a ratio of less than about 10:1, or alternatively less than 8:1, or alternatively less than 5:1, wherein said ratio is a number of cryopreserved and reanimated oocytes, embryos or blastocysts used to produce a single viable pregnancy. In another aspect of the above population, the viable pregnancy is at least a second, or alternatively at least a third, or alternatively at least a fourth successive viable pregnancy resulting from the same population of cryopreserved oocytes, embryos or blastocysts.

[0069] In another aspect of the above population, the oocytes, embryos or blastocysts are animal, including a human, or non-human animal including, for example, a rodent,

such as a rat and mouse, a canine, such as a dog, a leporid, such as a rabbit, livestock, sport animal or pet.

[0070] In yet another aspect of the above population, the portion of their cytoplasmic cryoprotectant that has been replaced water is sufficient to reanimate the oocytes, embryos or blastocycts. In a further aspect, more than about 10% of the cytoplasmic cryoprotectant has been replaced with water.

[0071] The following examples are provided to illustrate certain aspects of the present invention and to aid those of skill in the art in practicing the invention. These examples are in no way to be considered to limit the scope of the invention.

EXAMPLES

[0072] The following compositions are described and are available commercially as stated below:

[0073] Global Media, IVF Online, p/n LGGG;

[0074] Synthetic Serum Substitute (SSS), Irvine Scientific, p/n 99193;

[0075] Modified Human Tubular Fluid (mHTF), Irvine Scientific, p/n 90126;

[0076] Propylene Glycol, Sigma Chemicals, p/n 241229; and

[0077] Sucrose, Sigma Chemicals, p/n S1888.

[0078] In the Examples below, all percents are percents by volume unless otherwise specified. Likewise, all temperatures are reported in degrees Celsius unless otherwise stated.

[0079] In the Examples, the following abbreviations have the following meanings:

M	=	Molar
mM	=	Millimolar
g	=	gram
L	=	liter
mL	=	Milliliter
Min	=	Minute
mOsmol/L	=	milliosmoles per liter

mHTF	=	modified human tubal fluid
SSS	=	Synthetic Serum Substitute

[0080] The methods and solutions disclosed herein produce a substantially spherical oocyte or embryo. The benefit of this spherical entity is to minimize stress on the cell walls and the interior proteins that are key to further development of the oocyte or embryo.

EXAMPLE 1

Embryo Cryopreservation and Reanimation

Solutions:

Table 1: Embryo Cryoprotecting and Reanimating Solutions

Components	Base Solution	E-F2 Solution	E-T1 Solution
Sterile Water for Irrigation	A/R	A/R	A/R
HEPES	5.000 g/L	5.000 g/L	5.000 g/L
Sodium Bicarbonate	4.000 mM	4.000 mM	4.000 mM
Sodium Hydroxide	±7.2 mM	±7.2 mM	±7.2 mM
Sodium Chloride	97.800 mM	97.800 mM	97.800 mM
Potassium Chloride	4.690 mM	4.690 mM	4.690 mM
Calcium Chloride	2.040 mM	2.040 mM	2.040 mM
Potassium Phosphate	0.370 mM	0.370 mM	0.370 mM
Magnesium Sulfate	0.200 mM	0.200 mM	0.200 mM
Dextrose	2.780 mM	2.780 mM	2.780 mM
Sodium Lactate Solution	21.400 mM	21.400 mM	21.400 mM
Sodium Pyruvate	0.330 mM	0.330 mM	0.330 mM
Gentamicin Sulfate	0.017 mM	0.017 mM	0.017 mM
Human Serum Albumin	12.000 g/L	12.000 g/L	12.000 g/L
Propylene Glycol	N/A	1.5 M	1.0 M
Sucrose	N/A	0.1 M	0.2 M
Theoretical Osmolarity	292	1892	1492
Measured Osmolarity:	273	1325	1169

[0081] Table 1 lists a theoretical osmolality and a measured osmolality as measured with a Wescor, model 5500, vapor pressure osmometer.

[0082] The rate of change in osmolarity in the herein described method was controlled by mixing two separate solutions to blend the osmolality from the start to the end point

(Table 1). As shown in **Figure 1** and **Figure 2**, a relatively straight line change in osmolality was successfully demonstrated. However, a more aggressive and faster change is possible by increasing the slope of the osmolality curve or driving the fluid exchange in a varying manner.

[0083] The images in **Figure 3**, **Figure 4** and **Figure 5** show the results obtained from a continuously changing osmolality solution in the cryopreservation and subsequent return to normal osmolality of a batch of mouse embryos. These embryos were processed on an automated instrument and held within an egg/embryo holder.

Methods:

[0084] Normally, embryos are stored and grown in a media such as Global media with 10% human serum albumin (HSA).

[0085] The cryopreservation process was initiated on an automated system and proceeded from an initial osmolality of approximately 300 mOsmol/L in the Base Solution (Table 1) to a final osmolality of about 1325 mOsmol/L (measured) in the E-F2 solution (Table 1). An example of the osmolality curve for this cryopreservation program is shown in **Figure 1**. As the E-F2 solution was increased in its volumetric flow rate the Base solution was decreased. This program used a total volumetric flow rate of about 70 microliters per minute and proceeded at a temperature of 23.5°C.

[0086] At the end of the process, the embryo holders were removed and the ends were sealed. The sealed embryo holders were placed into an automated freezer and cooled to -7°C where upon "seeding" was performed to induce crystallization. After holding at -7°C for approximately 15 minutes, the cooling resumed to -33°C at -0.3°C/minute. Once the -33°C was achieved the holders were plunged into liquid nitrogen for storage.

[0087] The reanimation process was initiated by removing the frozen embryo holder from liquid nitrogen and warming in room temperature air for 30 seconds followed by 37°C water for 30 seconds. The ends were cut and then the embryo holder was installed on the automated instrument. The solutions used in this process were the Base solution and the E-T1 solution of Table 1. The initial fluid introduced was the E-T1 solution at an approximate osmolality of 1169 mOsmol/L (measured) and then proceeded by mixing the

two solutions to a final osmolality of 300 mOsmol/L for the Base solution. An example of the osmolality curve for this reanimation program is shown in **Figure 2**. This program used a total volumetric flow rate of about 70 microliters per minute. The temperature of the solutions was initially 23.5°C and proceeded for 31.5 minutes at this temperature and then rised to 37.0°C in about 1 minute for the remainder of the time.

Results:

[0088] The results of the cryopreservation and reanimation process are shown in the sequence of images shown in **Figure 3**, **Figure 4** and **Figure 5**. **Figure 3** was the embryos immediately after reanimation and being placed in Global media with 10% HSA. The subsequent **Figure 4** and **Figure 5** were of the embyos on successive days 1 and 2, respectively. These photos demonstrate the 100% grow out of this batch of embryos.

EXAMPLE 2

Oocyte Cryopreservation and Reanimation

Solutions:

Table 2: Oocyte Cryoprotecting and Reanimating Solutions

Components	Base Solution	O-F2 Solution	O-T1 Solution
Sterile Water for Irrigation	A/R	A/R	A/R
HEPES	5.000 g/L	5.000 g/L	5.000 g/L
Sodium Bicarbonate	4.000 mM	4.000 mM	4.000 mM
Sodium Hydroxide (pH tuning)	±7.2 mM	±7.2 mM	±7.2 mM
Sodium Chloride	97.800 mM	97.800 mM	97.800 mM
Potassium Chloride	4.690 mM	4.690 mM	4.690 mM
Calcium Chloride	2.040 mM	2.040 mM	2.040 mM
Potassium Phosphate	0.370 mM	0.370 mM	0.370 mM
Magnesium Sulfate	0.200 mM	0.200 mM	0.200 mM
Dextrose	2.780 mM	2.780 mM	2.780 mM
Sodium Lactate Solution	21.400 mM	21.400 mM	21.400 mM
Sodium Pyruvate	0.330 mM	0.330 mM	0.330 mM
Gentamicin Sulfate	0.017 mM	0.017 mM	0.017 mM
Human Serum Albumin	12.000 g/L	12.000 g/L	12.000 g/L
Propylene Glycol	N/A	1.5 M	1.0 M
Sucrose	N/A	0.3 M	0.3 M

Components	Base Solution	O-F2 Solution	O-T1 Solution
Theoretical Osmolarity:	292	2092	1592
Measured Osmolarity:	273	1638	1362

[0089] Table 2 lists a theoretical osmolality and a measured osmolality as measured with a Wescor, model 5500, vapor pressure osmometer.

Methods:

[0090] In this method the oocytes were harvested and stabilized to *in vitro* conditions by storage in a stabilization solution for at least about 1 hour after harvesting from the ovaries. Several alternative solutions and methods are available to perform this step. One such method is Global media supplemented with 5 to 20% Synthetic Serum Substitute (SSS). This is so common that one might consider it industry practice.

[0091] The cryopreservation process for oocytes was initiated on an automated system and proceeded from an initial osmolality of approximately 300 mOsmol/L in the Base Solution (Table 2) to a final osmolality of about 1638 mOsmol/L (measured) in the O-F2 solution (Table 2). The osmolality curve for this cryopreservation program was similar to **Figure 1**, except for the final osmolality exceeding 1600 mOsmol/L and the time interval being about 20 minutes. This program used volumetric flow rate of about 70 microliters per minute. The temperature was controlled during this process and ranged from 37°C at the start of the process and decreased to 25°C over the course of the fluid exchange time period. The combination of temperature and osmolality were used to control the fluid exchange from the solution into the oocyte and vice versa.

[0092] At the end of the process, the oocyte holders were removed and the ends were sealed. The sealed oocyte holders were placed into an automated freezer and cooled to -7°C where upon "seeding" was performed to induce crystallization. After holding at -7°C for approximately 15 minutes, the cooling resumed to -33°C at -0.3°C/minute. Once the -33°C was achieved the holders were plunged into liquid nitrogen for storage.

[0093] The reanimation process was initiated by removing the frozen oocyte holder from liquid nitrogen and warming in room temperature air for 30 seconds followed by 37°C water for 30 seconds. The ends were cut and then the oocyte holder was installed

on the automated instrument. The solutions used in this process were the Base solution and the O-T1 solution of Table 2. The initial fluid introduced was the O-T1 solution at an approximate osmolality of 1362 mOsmol/L and then proceeded by mixing the two solutions to a final osmolality of 300 mOsmol/L for the Base solution. The temperature during this thawing and reanimation process started around 30°C and raised slowly to 37°C over the course of approximately 15 minutes. This program used volumetric flow rate of about 70 microliters per minute. This flow rate can range from 35 to 150 microliters per minute, but is preferred to be in the 50 to 100 micoliters per minute.

Results:

[0094] Here, again, the temperature and the osmolality of the solution were used to drive the cryoprotectants out of the oocyte and water from the solutions into the oocyte. The infinitesimal changes in osmolality from second to second create a nearly constant driving force for fluid exchange and mass flow rate from the oocyte to the solution and vice versa. This minute and constant osmotic change drives the exchange of fluid constituents of the cell and the solution. This also prevents over driving the exchange which leads to drastic changes in the sphericity of the oocyte. The osmolality curve for this reanimation program looked very similar to **Figure 2** and had a time period of approximately 15 minutes.

[0095] In one aspect of the methods described in Examples 1 or 2, the automated instrument used 10 ml syringes and a syringe pump to drive the individual solutions into a manifold where they were mixed and temperature controlled. The embryo or oocyte holder of either Example 1 or 2 were constructed from a Surlyn® extrusion which is drawn to narrow it in an area and had a polycarbonate membrane filter which had approximately 10 micron pores to allow fluid through, but retain the embryos or oocytes.

[0096] One difference in the solutions described in Tables 1 and 2 versus prior publications, is that they did not contain alpha or beta globulins. Testing has confirmed these components are un-necessary due to the biochemical processes being suspended during the transition from *in vitro* growth to cryopreservation and subsequent reanimation in the thawing process. These components were not required during the freeze/thaw

process, while many publications have demonstrated that these components are needed for supporting the growth of the cells.

[0097] In another aspect of the solutions described in Tables 1 and 2, the solutions include removing all or substantially all of the sodium ions from the solutions by substituting choline chloride for sodium chloride as there are examples of sodium toxicity during the cryopreservation process as the wave front of solidification proceeds through the fluid during the "seeding" process. This sodium toxicity is thought to cause changes in the membrane due to "sodium pump" mechanism in the cell wall. Other chemicals could be exchanged such as potassium hydroxide for sodium hydroxide and also potassium bicarbonate for sodium bicarbonate. This would essentially remove substantially all sodium ions from the solution.

EXAMPLE 3

Viable Pregnancy Following Fertilization and/or Insemination

[0098] The population of cryopreserved and/or reanimated embryos and oocytes described in Examples 1 and 2 above will result in an improved viable pregnancy ratio.

[0099] If insemination of the oocytes is planned, conventional cytoplasmic sperm injection technique(ICSI) is employed because the zona pellucida will have become hardened as a by-product of the freezing process. Failure to recognize this important step will lead to very low fertilization rates accounting for the disappointing results with other protocols.

[0100] Thus, using the embodiments of the invention for cryopreserving and/or reanimating oocytes, embryos or blastocysts, excellent survival rates, improved fertilization and embryo cleavage rates can be achieved.

[0101] The table below provides a comparison of contemplated viable pregnancy ratios relative to the reported literature:

Table 3: Summary of Viable Pregnancy Ratios

Article	Ratio	No. of Oocytes Cryopreserved & Reanimated	No. of Viable Pregnancies
Porcu et al. (1992) *Fertil. Steril.* 72(3)(Suppl. 1).S2: Abstract O-004	100:1	1502	16
Tucker et al. (1998) *Human Prod.* 13(11):3156-3159	62:1	311	5
Yang et al. (1999), *Fertil. Steril.* 72(3)(Suppl. 1)S86: Abstract O-224	17:1	120	7
Fosas et al. (2003) *Hum. Reprod.* 18(7):1417-01421	22:1	88	4
Barritt et al. (2007) *Fert.& Ster.* 87(1):189.e13-189-e17	13:1	79	6 (fetal heartbeats)[1]
This Invention	≤ 10:1		

[1] Barritt et al. used fetal heartbeats as a measure of pregnancy. The other literature citations used β-hCG levels.

[0102] The above contemplated results show that the cryopreservation methods of this invention, and/or the reanimation methods of this invention, provide for a high success rate for producing a viable pregnancy.

WHAT IS CLAIMED IS:

1. A population of substantially spherical, partially cryopreserved oocytes, embryos or blastocysts characterized in that a portion of their cytoplasmic water has been replaced with a cryoprotectant while maintaining their substantially spherical shape, wherein said population provides for a viable pregnancy at a ratio of less than about 10:1, wherein said ratio is a number of cryopreserved and reanimated oocytes, embryos or blastocysts used to produce a single viable pregnancy.

2. The population of substantially spherical, partially cryopreserved oocytes, embryos or blastocysts of claim 1, wherein the ratio is less than about 8:1.

3. The population of substantially spherical, partially cryopreserved oocytes, embryos or blastocysts of claim 1, wherein the ratio is less than about 5:1.

4. The population of substantially spherical, partially cryopreserved oocytes, embryos or blastocysts of claim 1, wherein the viable pregnancy is at least a second successive viable pregnancy resulting from the same population of cryopreserved oocytes, embryos or blastocysts.

5. The population of substantially spherical, partially cryopreserved oocytes, embryos or blastocysts of claim 1, wherein the viable pregnancy is at least a third successive viable pregnancy resulting from the same population of cryopreserved oocytes, embryos or blastocysts.

6. The population of substantially spherical, partially cryopreserved oocytes, embryos or blastocysts of claim 1, wherein the viable pregnancy is at least a fourth successive viable pregnancy resulting from the same population of cryopreserved oocytes, embryos or blastocysts.

7. The population of substantially spherical, partially cryopreserved oocytes, embryos or blastocysts of claim 1, wherein the oocytes are human oocytes.

8. The population of substantially spherical, partially cryopreserved oocytes, embryos or blastocysts of claim 1, wherein the embryos are human embryos.

9. The population of substantially spherical, partially cryopreserved oocytes, embryos or blastocysts of claim 1, wherein the blastocysts are human blastocysts.

10. The population of substantially spherical, partially cryopreserved oocytes, embryos or blastocysts of claim 1, wherein the portion of cytoplasmic water replaced with cryoprotectant is sufficient to protect the oocytes, embryos or blastocysts.

11. The population of substantially spherical, partially cryopreserved oocytes, embryos or blastocysts of claim 10, wherein more than about 10% of the cytoplasmic water is replaced with cryoprotectant .

12. A population of partially reanimated oocytes, embryos or blastocysts characterized in that a portion of their cytoplasmic cryoprotectant has been replaced with water, wherein said population provides for a viable pregnancy at a ratio of less than about 10:1, wherein said ratio is a number of cryopreserved and reanimated oocytes, embryos or blastocysts used to produce a single viable pregnancy.

13. The population of partially reanimated oocytes, embryos or blastocysts of claim 12, wherein the ratio is less than about 8:1.

14. The population of partially reanimated oocytes, embryos or blastocysts of claim 12, wherein the ratio is less than about 5:1.

15. The population of partially reanimated oocytes, embryos or blastocysts of claim 12, wherein the viable pregnancy is at least a second successive viable pregnancy resulting from the same population of cryopreserved oocytes, embryos or blastocysts.

16. The population of partially reanimated oocytes, embryos or blastocysts of claim 12, wherein the viable pregnancy is at least a third successive viable pregnancy resulting from the same population of cryopreserved oocytes, embryos or blastocysts.

17. The population of partially reanimated oocytes, embryos or blastocysts of claim 12, wherein the viable pregnancy is at least a fourth successive viable pregnancy resulting from the same population of cryopreserved oocytes, embryos or blastocysts.

18. The population of partially reanimated oocytes, embryos or blastocysts of claim 12, wherein the oocytes are human oocytes.

19. The population of partially reanimated oocytes, embryos or blastocysts of claim 12, wherein the embryos are human embryos.

20. The population of partially reanimated oocytes, embryos or blastocysts of claim 12, wherein the blastocysts are human blastocysts.

21. The population of partially reanimated oocytes, embryos or blastocysts of claim 12, wherein the portion of their cytoplasmic cryoprotectant that has been replaced with water is sufficient to reanimate the oocytes, embryos or blastocysts.

22. The population of partially reanimated oocytes, embryos or blastocysts of claim 21, wherein more than about 10% of the cytoplasmic cryoprotectant has been replaced with water.

23. A method of producing a substantially spherical, partially cryopreserved oocyte, embryo or blastocyst, the method comprising:

contacting an oocyte, embryo or blastocyst with a cryoprotecting solution having an initial osmolarity under continuous process conditions, wherein said conditions comprise gradually increasing the osmolarity over a predetermined period of time, such that a portion of cytoplasmic water of the oocyte, embryo or blastocyst is replaced with cryoprotectant from said solution, thereby producing a substantially spherical, partially cryopreserved oocyte, embryo or blastocyst.

24. The method of claim 23, wherein the portion of cytoplasmic water replaced with cryoprotectant is sufficient to protect the oocyte, embryo or blastocyst.

25. The method of claim 24, wherein more than about 10% of the cytoplasmic water is replaced with cryoprotectant.

26. The method of claim 23, wherein the osmolarity is increased at a progressive rate throughout the predetermined period of time.

27. The method of claim 26, wherein the progressive rate is substantially linear or substantially non-linear.

28. The method of claim 27, wherein the substantially linear progressive rate is from about 90 mOsmol/L per 1 minute to about 110 mOsmol/L per 1 minute.

29. The method of claim 23, wherein the osmolarity is increased from an initial osmolarity of about 300 mOsmol/L to a final osmolarity of at least about 1300 mOsmol/L within the predetermined period of time.

30. The method of claim 29, wherein the predetermined period of time is from about 5 to about 20 minutes.

31. The method of claim 29, wherein the predetermined period of time is from about 8 to about 18 minutes.

32. The method of claim 29, wherein the predetermined period of time is from about 12 to about 16 minutes.

33. The method of claim 23, wherein the cryoprotectant is selected from the group consisting of DMSO, ethylene glycol, propylene glycol and glycerol.

34. The method of claim 23, wherein the cryoprotecting solution comprises one or more components selected from the group consisting of sterile water, HEPES, sodium bicarbonate, sodium hydroxide, sodium chloride, potassium chloride, calcium chloride, potassium phosphate, magnesium sulfate, dextrose, sodium lactate solution, sodium pyruvate, gentamicin sulfate and human serum albumin.

35. The method of claim 23, wherein the cryoprotecting solution does not contain alpha globulin or beta globulin.

36. The method of claim 23, wherein the cryoprotecting solution comprises a dehydrating agent.

37. The method of claim 36, wherein said conditions comprise gradually increasing the concentration of the dehydrating agent from substantially no dehydrating agent to at least 0.1 M dehydrating agent within the predetermined period of time.

38. The method of claim 23, wherein said conditions comprise maintaining the cryoprotecting solution at a temperature of about 22°C to about 26°C.

39. The method of claim 23, wherein said conditions comprise gradually decreasing the temperature of the cryoprotecting solution from about 38°C to about 22°C within the predetermined period of time.

40. A method of producing a partially reanimated oocyte, embryo or blastocyst, the method comprising:

contacting a cryopreserved oocyte, embryo or blastocyst with a reanimating solution having an initial osmolarity under continuous process conditions, wherein said conditions comprise gradually decreasing the osmolarity over a predetermined period of time, such that a portion of cytoplasmic cryoprotectant of the oocyte, embryo or blastocyst is replaced with water from said solution, thereby producing a partially reanimated oocyte, embryo or blastocyst.

41. The method of claim 40, wherein the portion of cytoplasmic cryoprotectant replaced with water is sufficient to reanimate the oocyte, embryo or blastocyst.

42. The method of claim 41, wherein more than 10% of the cytoplasmic cryoprotectant has been replaced with water.

43. The method of claim 40, wherein the osmolarity is decreased at a progressive rate throughout the predetermined period of time.

44. The method of claim 43, wherein the progressive rate is substantially linear or substantially non-linear.

45. The method of claim 44, wherein the substantially linear progressive rate is from about 30 mOsmol/L per 1 minute to about 50 mOsmol/L per 1 minute.

46. The method of claim 39, wherein the osmolarity of the reanimating solution is gradually decreased from an initial osmolarity of at least about 1100 mOsmol/L to a final osmolarity of about 300 mOsmol/L within the predetermined period of time.

47. The method of claim 46, wherein the predetermined period of time is from about 15 to about 60 minutes.

48. The method of claim 46, wherein the predetermined period of time is from about 30 to about 50 minutes.

49. The method of claim 46, wherein the predetermined period of time is from about 40 to about 45 minutes.

50. The method of claim 40, wherein the cryoprotectant is selected from the group consisting of DMSO, Ethylene glycol, propylene glycol and glycerol.

51. The method of claim 40, wherein the reanimating solution comprises one or more components selected from the group consisting of sterile water, HEPES, sodium bicarbonate, sodium hydroxide, sodium chloride, potassium chloride, calcium chloride, potassium phosphate, magnesium sulfate, dextrose, sodium lactate solution, sodium pyruvate, gentamicin sulfate and human serum albumin.

52. The method of claim 40, wherein the reanimating solution does not contain alpha globulin or beta globulin.

53. The method of claim 40, wherein the reanimating solution comprises a dehydrating agent.

54. The method of claim 53, wherein said conditions comprise gradually decreasing the concentration of the dehydrating agent from at least 0.1 M dehydrating agent to substantially no dehydrating agent within the predetermined period of time.

55. The method of claim 40, wherein said conditions comprise gradually increasing the temperature of the reanimating solution from about 30°C to about 38°C within the predetermined period of time.

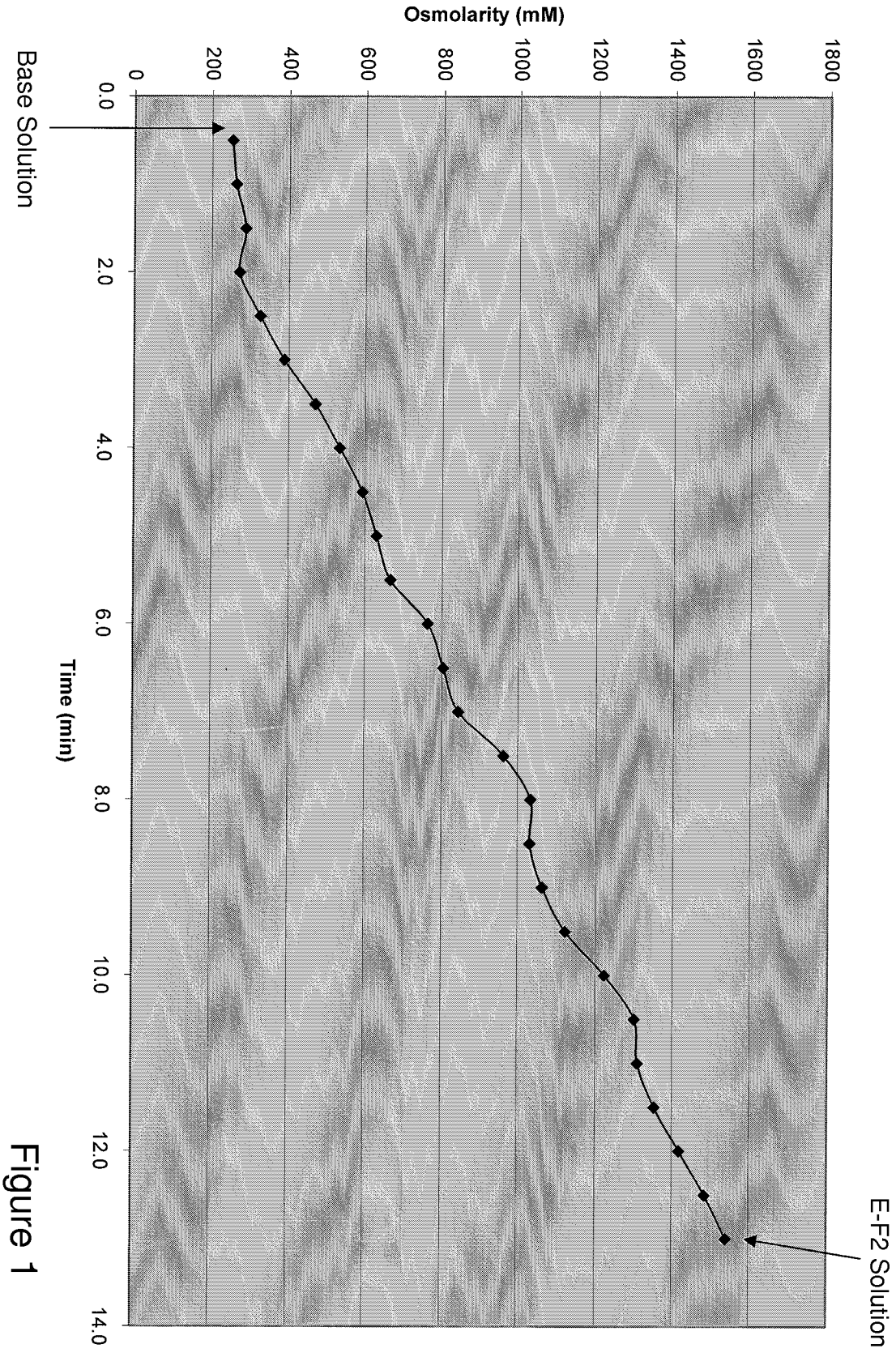

Cryopreservation of Mouse Embryos
Osmolarity (mM)
0
200
400
600
800
1000
1200
1400
1600
1800
Time (min)
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
Base Solution
E-F2 Solution

Figure 1

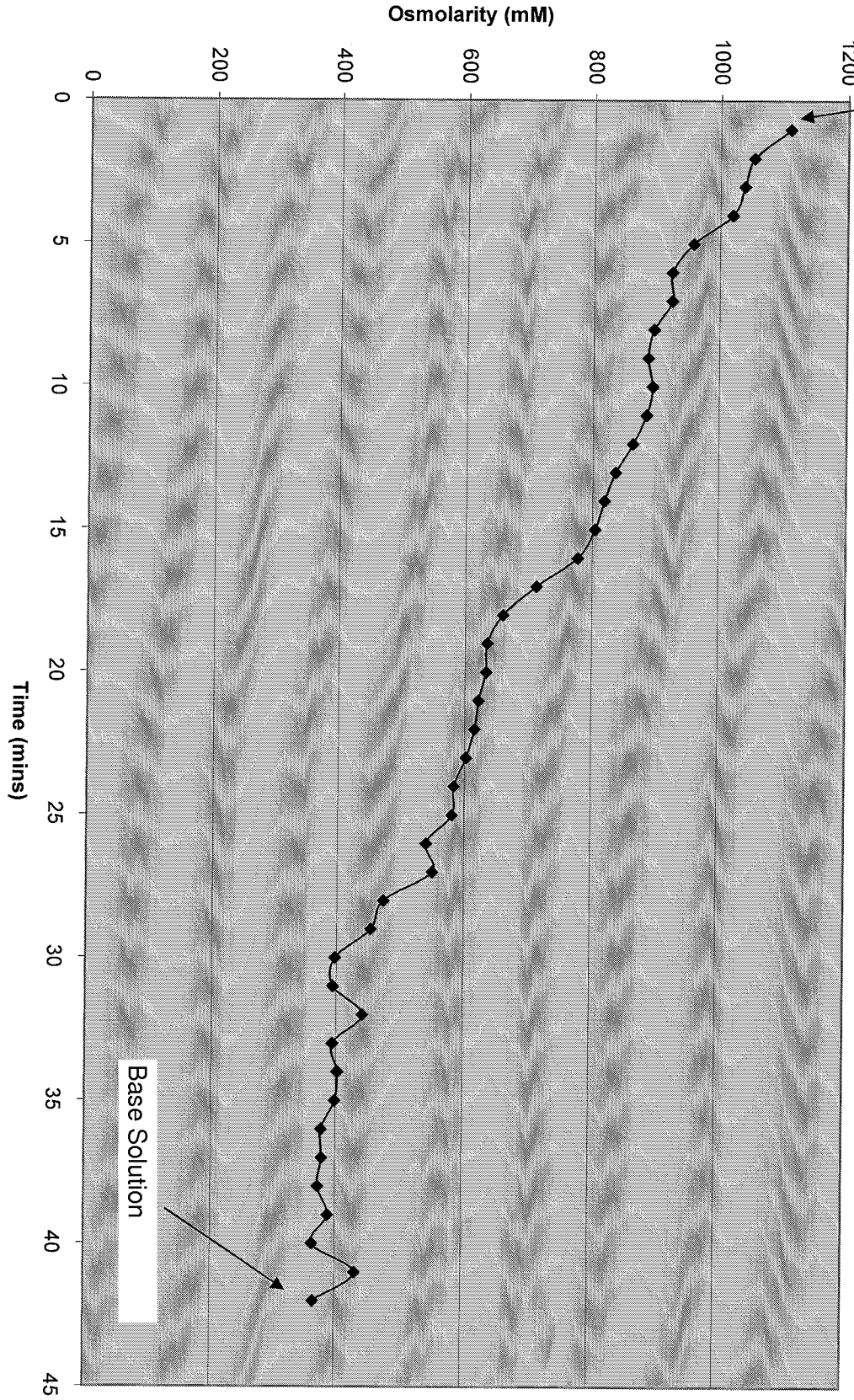

Reanimation of Mouse Embryos
Osmolarity (mM)
0
200
400
600
800
1000
1200
E-T1 Solution
Base Solution
Time (mins)
0
5
10
15
20
25
30
35
40
45

Figure 2

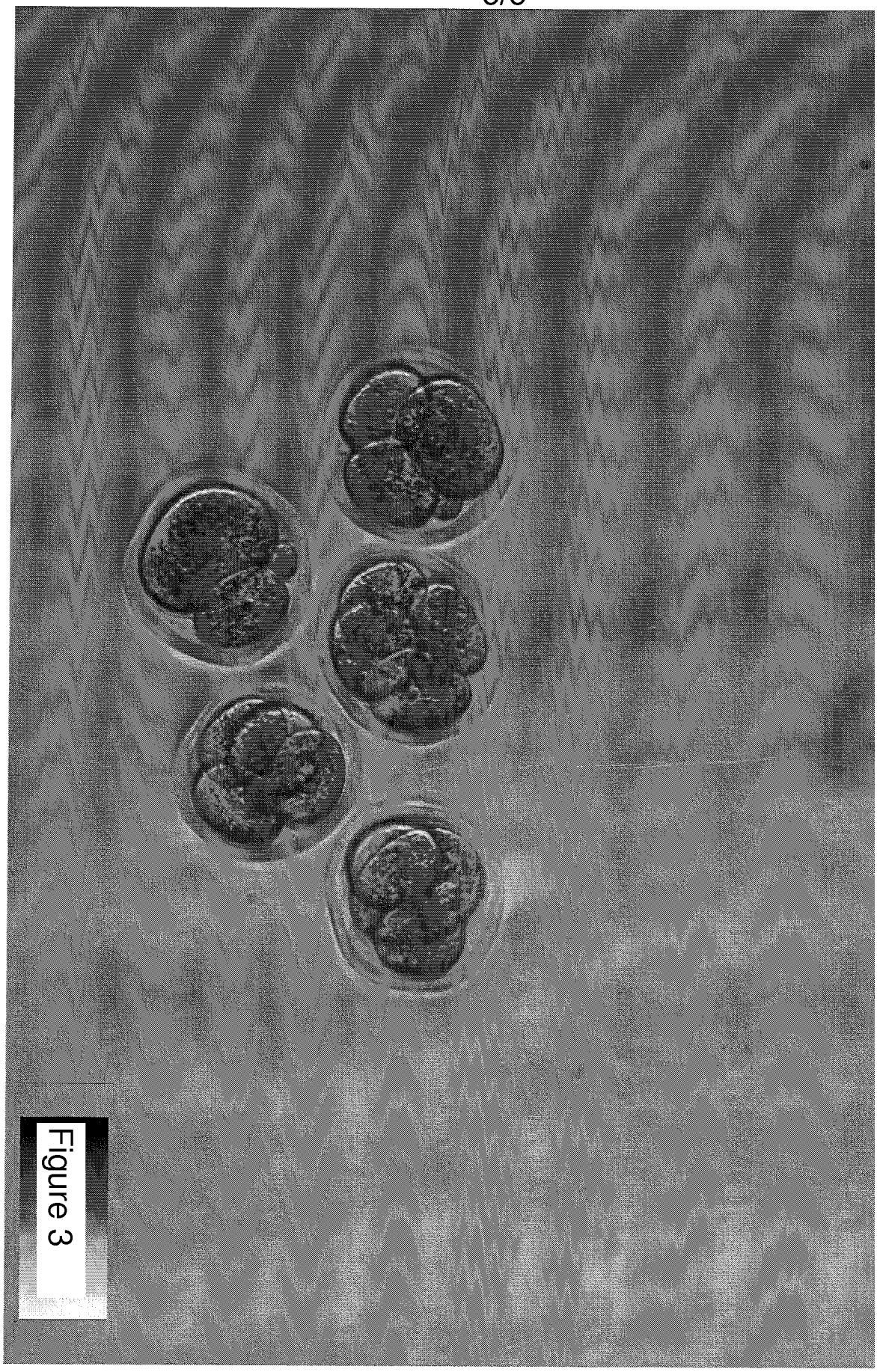


Figure 3

Mouse Embryos Following Cryopreservation and Reanimation

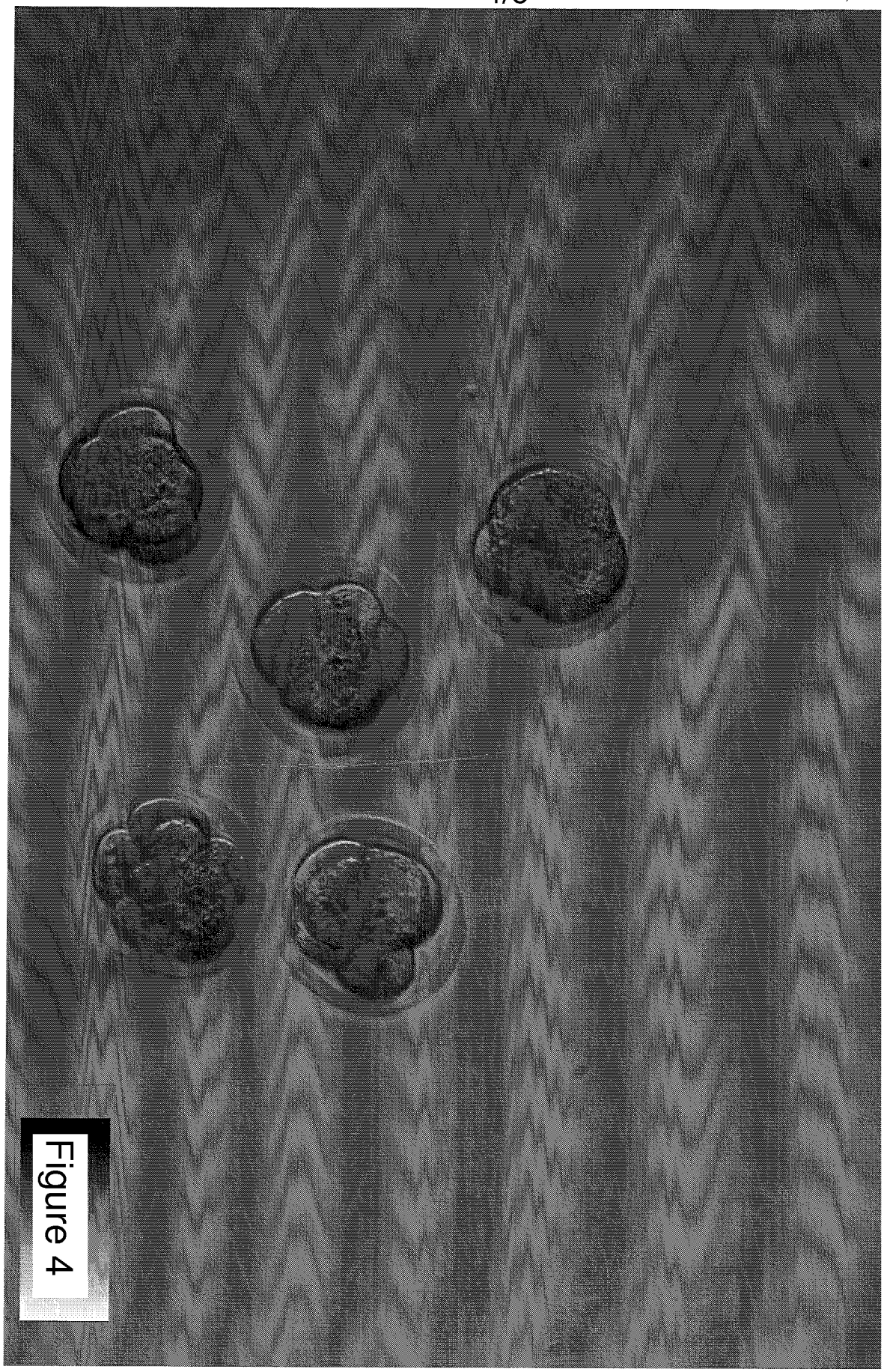
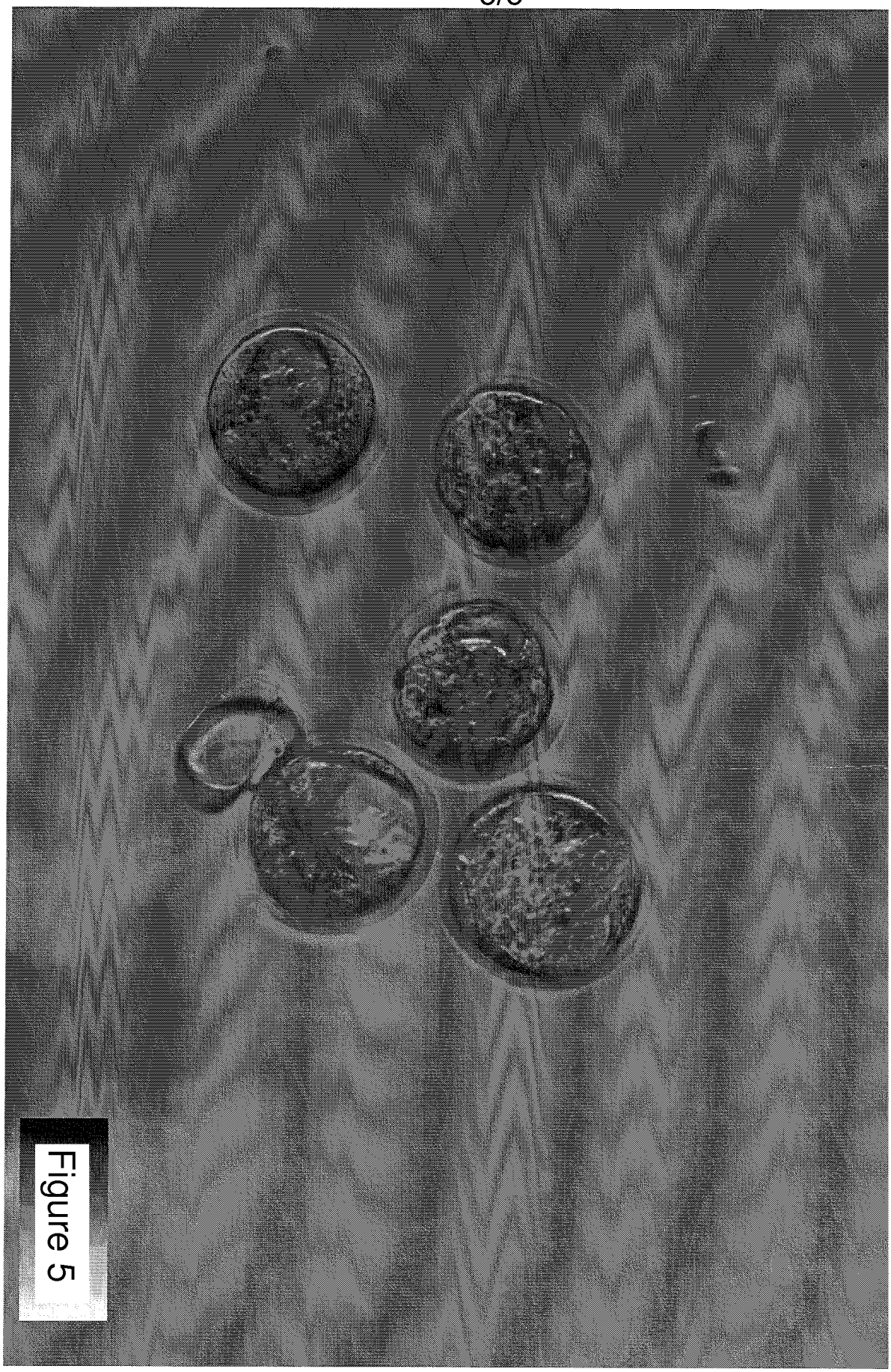


Figure 4

Mouse Embryos Following Cryopreservation and Reanimation - 1 Day Growth



Mouse Embryos Following Cryopreservation and Reanimation - 2 Days Growth

Figure 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/36390

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 5/02 (2010.01)
USPC - 435/325
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)
USPC- 435/325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 800/14, 800/18, 800/24; 435/377, 435/366, 435/325, 435/2, 435/366, 435/404, 435/405, 435/406 (keywords below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB,USPT,USOC,EPAB,JPAB); Google; PubMed: cryopreservation, cryoprotectant, oocyte, embryo, blastocyst, continuous process, globulin, spherical, osmolarity, reanimation, pregnancy, viable, dehydrating agent, cytoplasmic water

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2008/0213887 A1 (DIAZ) 4 September 2008 (04.09.2008) abstract; para [0011]-[0018], [0023], [0033]-[0034], [0048]-[0049], [0055]-[0056], [0098], [0117], [0122]; Table 3	1-3, 7-11, 23-34, 36-39 --- 4-6, 35
X --- Y	US 2008/0145930 A1 (DIAZ) 15 May 2008 (15.05.2008) abstract; para [0012]-[0017], [0025], [0041], [0049]-[0050], [0056], [0060], [0096], [0100], [0103]	12-14, 18-22, 40-51, 53-55 --- 15-17, 52
Y	SILLS et al. Ovarian hyperstimulation syndrome and prophylactic human embryo cryopreservation: analysis of reproductive outcome following thawed embryo transfer. J. Ovarian Res. 2008, 1(1): Article 7, pages 1-6; abstract	4-6, 15-17
Y	MAGLI et al. Cryopreservation of biopsied embryos at the blastocyst stage. Hum. Reprod. 2006, 21(10):2656-2660; pg 2659, para 3	35, 52

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 July 2010 (02.07.2010)	19 JUL 2010
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)