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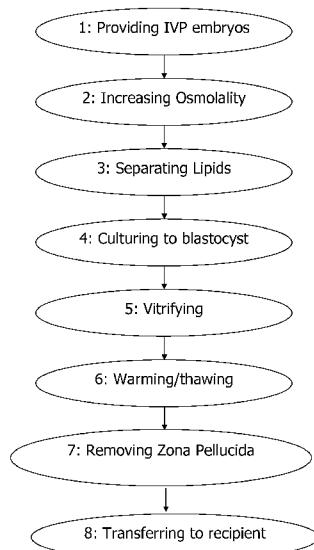
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*[Continued on next page]*

(54) Title: HIGH-THROUGHPUT AND NON-INVASIVE METHOD TO VITRIFY PORCINE EMBRYOS

(57) **Abstract:** The present invention provides a practical, non-invasive, and efficient method for cryopreservation of an in-vitro-produced porcine embryo. The inventive method treats the IVP (such as IVF- or NT-derived) embryo at the one-cell or cleavage stage prior to compaction with high osmolality followed by high speed centrifugation. The high osmolality treatment enlarges the perivitelline space, and with centrifugation, enables the separation of the lipids from the cytoplasm. The lipid-separated embryos after high osmolality treatment have been successfully cryopreserved and later recovered and transferred to produce live offspring.

FIG. 1





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ML, MR, NE, SN, TD, TG).

**HIGH-THROUGHPUT AND NON-INVASIVE METHOD  
TO VITRIFY PORCINE EMBRYOS**

**GRANT STATEMENT**

**[0001]** The invention was made in part from government support under Grant No. R01 RR013438 and Grant No.U42 RR018877 from the National Institutes of Health. The Government has certain rights in the invention.

**FIELD OF INVENTION**

**[0002]** The present invention relates to a method of porcine embryo preservation, more particularly to a new and improved method to preserve in-vitro produced porcine embryos.

**BACKGROUND OF INVENTION**

**[0003]** Successful cryopreservation of early mammalian embryos provides opportunities for the preservation of germplasm as well as the movement of genetics nationally and internationally. Unfortunately, the pig embryo has been more difficult than many mammalian embryos to cryopreserve. Significant advances have been made towards the successful cryopreservation of pig embryos based on the observation that pig embryos are very sensitive to hypothermic conditions and that removal of intracellular lipids (delipation) appears to alleviate this sensitivity [1-4]. Most studies have focused on in vivo produced embryos, as they are considered to be more developmentally competent than in vitro produced embryos. Alternatives to mechanical delipation include destabilizing the cytoskeleton [5] or altering the vitrification conditions [6-8].

**[0004]** According to prior studies on cryopreservation of in vivo produced embryos, after centrifugation of the pig oocyte or embryo with an intact zona pellucida, the polarized lipid droplets tend to remain connected with the cytoplasm of the oocyte or blastomere of the embryo via a bridge-like structure [11]. The polarized lipid droplets can redistribute back into the oocyte

or blastomere during subsequent culture or cryopreservation procedures. If the perivitelline space is enlarged, the bridge-like structure will break after centrifugation and the lipid droplets will not redistribute into the cytoplasm of the oocyte or the blastomere of the embryo, but will stay within the intact zona pellucida. Thus in vivo-derived embryos need to be cryopreserved immediately after centrifugation in order to prevent lipid redistribution prior to cryopreservation [12].

**[0005]** Prior studies also found that the lipid droplets are abundant and large in the early stage porcine embryo and gradually decline in size and abundance as the embryo advances to and beyond the blastocyst stage [15, 16]. Interestingly, the large lipid droplets in the early stage embryos are easier to remove by centrifugation than the smaller droplets in the later stage embryos.

**[0006]** In-vitro production of pig embryos, such as embryos derived from in-vitro fertilization (IVF) or by nuclear transfer (NT), has been used to create disease models or potential organ donors for xenotransplantation. As a result, the demand for effective cryopreservation of in vitro produced embryos has dramatically increased. However, in-vitro produced (IVP) embryos are even more sensitive to cryopreservation, thus more difficult to cryopreserve, than their in vivo produced counterparts [9].

**[0007]** So far, very limited success has been achieved to cryopreserve IVP embryos. In 2006, the inventors' lab reported two litters of transgenic piglets produced from cryopreserved NT embryos [9]. Subsequently, Nagashima et al. [10] reported piglets produced from cryopreserved IVF-derived embryos. However both of these successful reports of the cryopreservation of IVF- or NT-derived embryos used mechanical delipation through centrifugation and micromanipulation [9, 10]. Mechanical delipation substantially increases the potential of pathogen transmission because of the damage inflicted upon the zona pellucida during micromanipulation. It is also labor-intensive and time-consuming.

**[0008]** Two other groups [13, 14] have reported the attempts to employ partial enzymatic digestion and subsequent centrifugation to improve the cryopreservation survival of pig parthenogenetic embryos and hand-made cloned embryos. Specifically, when the zona pellucida is partially digested by trypsin, pronase, or another enzyme, it swells in size, which results in an increase in the amount of space between the oocyte plasma membrane and the zona pellucida. Thus when the oocyte or embryo is centrifuged sufficient space is present for the lipids to completely separate. However, the partial enzymatic digestion treatment has some disadvantages when used for lipid separation. For example, the enzyme (such as Trypsin or Pronase) can elicit parthenogenetic activation of oocytes. Additionally, the enzymatic digestion treatment may not work consistently and needs to be observed and monitored closely in small groups, since the effect of the enzyme treatment is heavily dependent on the individual batch of enzyme. Furthermore, neither group reported any piglet produced from the cryopreserved embryos using the combination of enzymatic digestion and centrifugation method.

**[0009]** Therefore, there is a need to develop a practical and non-invasive method for lipid separation and cryopreservation of IVP (such as IVF-derived or NT-derived) porcine embryos, which is suitable for research and commercial purposes.

#### **SUMMARY OF INVENTION**

**[0010]** In one aspect of the invention, a new and improved method to separate or remove the lipids from the cytoplasm of the in-vitro-produced (IVP) (in-vitro-fertilization (IVF) derived or nuclear transfer (NT) produced) porcine embryo is described. The inventive lipid removal method comprises the steps of (1) producing IVP porcine embryos at the one-cell or cleavage stage (prior to compaction), (2) condensing the embryos to produce condensed embryos, and (3) centrifuging the condensed embryos to separate the lipids from the cytoplasm to produce lipid-separated embryos.

[0011] According to one embodiment of the inventive method, the volume of embryos may be condensed through high osmolality treatment. Particularly, the IVF- or NT-derived embryos at the one-cell or cleavage stage prior to compaction may be exposed to a medium with a pre-selected osmolality greater than the previous culture medium for a pre-determined short time period. The osmolality of a medium may be adjusted by addition of salt, such as NaCl, sugar, such as Sucrose, raffinose, fructose, mannitol or trehalose, or other organic reagents, such as DMSO, or ethylene glycol, to the medium according to any standard procedure.

[0012] In another aspect of the invention, a new and improved method for cryopreservation and later transfer of the lipid-separated IVF- or NT-derived porcine embryos is described. The lipid-separated porcine embryos may be cryopreserved after further embryo development to the blastocyst stage and subjecting such to vitrification. The vitrified embryos may be warmed, have their zonae pellucidae removed, and transferred into a recipient (such as a surrogate pig).

#### **BRIEF DESCRIPTION OF DRAWINGS**

[0013] FIG. 1 is a flow diagram of the inventive cryopreservation and recovery process.

[0014] FIGs. 2(a) to (f) are photos of the development of the in-vitro-fertilization derived embryos after high osmolality treatment.

[0015] FIGs. 3(a) to (f) are photos of the development of the NT-derived embryos after high osmolality treatment.

[0016] FIG. 4 includes the photos of embryos treated with different osmolarities (adjusted by NaCl or sucrose) (Row 1) and their corresponding photos immediately after centrifugation (Row 2).

#### **DETAILED DESCRIPTION OF INVENTION**

[0017] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this

invention belongs. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

**[0018]** The present invention, building on the prior studies, teaches that the lipid removal, or separation, at an early embryo developmental stage is critical for cryopreservation of an IVP (IVF- or NT-derived) porcine embryo, and that besides swelling the zona pellucid through partial enzymatic digestion, the perivitelline space of an IVP porcine embryo may be enlarged by condensing the volume of the embryo to enable easy lipid removal/separation. The invention also discloses that exposing an IVP embryo to a high osmolality treatment may condense the embryo but preserve the vitality of the embryo. Furthermore, the inventive lipid-separation methods may be employed to treat multiple embryos at once, in contrast to the current delipidation procedures that require micromanipulation of each individual oocyte or embryo.

**[0019]** Referring to FIG. 1, which is a flow diagram of the inventive cryopreservation and recovery process via the inventive embryo-condensing method. Step 1 in FIG. 1 is to provide IVP embryos at a pre-selected early developmental stage, specifically the one-cell or cleavage stage prior to compaction. Any standard IVP procedure, such as IVF or NT, may be adapted.

**[0020]** According to one embodiment of the inventive method, the in-vitro-fertilization process may start with the oocytes aspirated from the antral follicles of one or multiple pig ovaries. The oocytes may be cultured to maturity in a maturation medium for a period of time and denuded. An exemplary maturation medium may contain TCM 199 (Gibco, 31100035, Grand Island, NY) with 0.1% PVA, 3.05 mmol/L glucose, 0.91 mmol/L sodium pyruvate, 0.57 mmol/L cysteine, 0.5 µg/mL LH, 0.5 µg/mL FSH, 10 ng/mL epidermal growth factor, 75 µg/mL penicillin and 50 µg/mL streptomycin. The oocytes may be cultured in the maturation medium for about 40-44 h at 38.5°C, 5% CO<sub>2</sub> in humidified air. After the maturation, the oocytes may

be denuded by removing the cumulus cells via vortexing for a short period of time, such as about 4 minutes, in TL-HEPES [20] supplemented with 0.1% PVA and 0.1% hyaluronidase. The denuded oocytes may be stored in many different media before insemination. An excellent medium may contain TCM199 with 0.6 mmol/L NaHCO<sub>3</sub>, 2.9 mmol/L Hepes, 50 µg/ml penicillin, 60 µg/ml streptomycin, 30 mmol/L NaCl and 3 mg/mL BSA [26].

**[0021]** Any standard insemination procedure may be followed to produce the IVF embryos. According to one embodiment, the denuded oocytes with a polar body may be first transferred to a suitable IVF medium to be combined with a sperm suspension. An exemplary IVF medium may contain a modified Tris-buffered medium with 113.1 mmol/L NaCl, 3 mmol/L KCl, 7.5 mmol/L CaCl<sub>2</sub>, 5 mmol/L sodium pyruvate, 11 mmol/L glucose, 20 mmol/L Tris, 2 mmol/L caffeine, and 2 mg/mL BSA.

**[0022]** According to another embodiment of the invention, NT-derived embryos may start with nuclear transfer donor cells and commercial oocytes. The nuclear transfer donor cells may be collected from a transgenic piglet or produced through genetic modification of wild type cells. After the oocytes are allowed to mature, the cumulus cells are removed from the oocytes by vortexing for about 4 min in TL-HEPES supplemented with 0.1% PVA and 0.1% hyaluronidase. The first polar body and the adjacent cytoplasm from these oocytes are then aspirated while in manipulation medium with 7.0 µg/ml cytochalasin B. A donor cell is then transferred into the perivitelline space. Fusion and activation can be accomplished simultaneously with two 30 µs pulses of 1.2 kV/cm in fusion/activation medium (such as 0.3 M mannitol, 1.0 mM CaCl<sub>2</sub>, 0.1 mM MgCl<sub>2</sub>, and 0.5 mM HEPES). Alternatively fusion and activation may be accomplished stepwise, first exposing in a fusion only medium with a lower concentration of calcium (such as 0.3 M mannitol, 0.1mM CaCl<sub>2</sub>, 0.1 mM MgCl<sub>2</sub> and 0.5 mM HEPES), then exposing to 200 µM Thimerosal for about 10 min in the dark and then 8 mM DTT for 30 min to activation [21] or any other suitable method.

**[0023]** After insemination or NT, the IVP embryos are cultured to the one-cell or cleavage stage (zygote, 2-cell or 4-cell stage, prior to compaction). Any suitable culture procedure may be adapted. According to one embodiment, the IVP embryos (derived from in-vitro-fertilization or NT) may be cultured in a variety of different culture media at 38.5°C, 5% CO<sub>2</sub> in air for 28 to 30 hours to select 2-cell stage embryos. An exemplary culture medium, PZM3 [27], may contain NaCl 108.0 mmol/L, KCl 10.0 mmol/L, KH<sub>2</sub>PO<sub>4</sub> 0.35 mmol/L, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.4 mmol/L, NaHCO<sub>3</sub> 25.07 mmol/L, Na-pyruvate 0.2 mmol/L, Ca(Lactate) 2·5H<sub>2</sub>O 2.0 mmol/L, Glutamine 1.0 mmol/L, Hypotaurine 5.0 mmol/L, BME amino acid solution 20 ml/L, MEM amino acid solution 10 ml/L, Gentamicin 0.05 mg/mL, BSA 3 mg/mL, with Osmolarity at 288±2, and pH at 7.3±2.

**[0024]** Step 2 in FIG. 1 is to increase the osmolality of the embryos to condense the embryo. Several different media formulations may be employed to increase the osmolality in order to condense the volume of an oocyte or embryo. The invention provides examples using NaCl or sucrose at different concentrations (resulting different osmolalities), but other formulations that result in a higher osmolality and subsequent shrinkage of the volume of the cell(s) should work.

**[0025]** According to one embodiment of the inventive method, the osmolality may be increased by adjusting the osmolality of a medium where the embryos are submerged. For example, NaCl or sucrose may be added to a stock medium with about 300 mOsmo (such as a stock solution with 300-310 mOsmo with 7.0 µg/mL cytochalasin B and 0.1 mg/mL BSA) to result a medium with various osmolalities, such as 350, 400, 500, 600 and 800-850 mOsmo. The IVP-derived embryos may be exposed to a pre-selected high osmolality medium for a short period of time, about 5 to 10 min, before centrifugation.

**[0026]** Step 3 in FIG. 1 is to separate the lipid from the condensed embryos, normally through centrifugation. For example, the condensed embryos in the high osmolality medium can

be centrifuged at 13,400 x g for about 6 to 20 min. The centrifugation condition (force or duration) may likely be varied to a large range of centrifugation force and time as long as it is sufficient to achieve full lipid separation while preserve the vitality of the embryos. A shorter duration may work especially if the force is increased. Likewise a longer duration may be necessary if a lower force is used. Pro-longed exposure to high osmolality may affect the vitality of an embryo.

**[0027]** The lipid separation may be checked after about 12 hours of culturing; in some cases (especially for NT-derived embryos, since they are relatively more valuable) a second round of high osmolality treatment and subsequent centrifugation may be applied to achieve a relatively complete lipid separation. The second high osmolality treatment may be an optional as long as the embryos remain at the cleavage stage prior to compaction.

**[0028]** The lipid-separated embryos are then allowed to develop further to the blastocyst stage Step 4, in FIG. 1. Specifically, the lipid-separated embryos may be cultured in PZM3 for 3 to 6 days for the embryo to attain the blastocyst stage.

**[0029]** Step 5 in FIG. 1 is the vitrification of the further-developed embryos. The embryos may be vitrified via any standard method/procedure. According to one embodiment, the further-developed embryos may be vitrified at the blastocyst stage by using a modified open pulled straw ('OPS') method. Specifically, the further-developed embryos may be placed in an equilibration solution for a short period of time (such as about 2 min) followed by exposure to a vitrification solution, then loaded into an OPS straw and immediately plunged into liquid nitrogen. An exemplary equilibration solution may contain 10% ethylene glycol, 10% dimethyl sulfoxide ('DMSO'), while an exemplary vitrification solution may contain 20% ethylene glycol, and 20% DMSO. The process before plunging into nitrogen may be conducted on a 38.5 °C warm stage. The duration from exposure to the vitrification solution to plunging into nitrogen is generally short ranging between about 25 to 30 seconds.

**[0030]** Steps 6 to 8 in FIG. 1 are steps to recover the preserved embryos and transfer such into a recipient. Specifically, the vitrified embryos may be thawed by immersing into a buffer solution (such as sucrose) for a period of time at a slightly elevated temperature (such as about 38.5 °C). The thawed embryos may be treated with 0.5% pronase to soften and remove the zona pellucida. The lipid-separated and zona-removed embryos may then be transferred to the oviduct or uterus of a recipient or surrogate.

**[0031]** FIGs. 2(a)-(f) are the photos of IVF-derived embryos after high osmolality treatment (at 400 mOsm with NaCl). FIG. 2(a) shows the embryos cultured for several hours after high osmolality treatment and centrifugation. FIGs. 2(b) and 2(c) show the embryos at blastocyst stage. FIG. 2(d) shows the embryos after vitrification and warming. FIG. 2(e) shows the embryos after removal of their zona pellucida. FIG. 2(f) shows the re-expanded embryos after in vitro culture in BRL cell conditioned medium.

**[0032]** FIGs. 3(a)-(f) are the photos of the development of the NT-derived embryos after high osmolality treatment with NaCl or sucrose. FIGs. 3(a) and 3(b) show the NT-derived embryos cultured for several hours after high osmolality treatment and centrifugation; FIGs. 3(c) and 3(d) show the embryos cultured further to the blastocyst stage; FIG. 3(e) shows the embryo warmed after vitrification; and FIG. 3(f) shows the re-expanded embryos after in vitro culture in BRL cell conditional medium.

**[0033]** The invention further studied the effects of different reagents (adjusting osmolality), different osmolality and centrifugation time on the rate of lipid separation. The invention finds that different reagents, such as NaCl or sucrose, have similar effects on lipid separation; the ideal osmolality for lipid separation ranges from about 350 to about 500 mOsm; and centrifugation duration ranging from about 6 minutes to about 20 minutes at a suitable centrifugation force/speed also has a positive effect on the lipid separation rate.

**[0034]** FIG. 4 shows the photos of the in-vitro-fertilized embryos treated with different osmolalities (adjusted with NaCl or sucrose) before and after centrifugation. Row 1 shows the photos of embryos exposed at different osmolalities, 300 mOsm (the control), 400 mOsm (adjusted with NaCl), 600 mOsm (adjusted with sucrose), and 800 mOsm (adjusted with sucrose), with condensation clearly shown at the elevated osmolalities (compared to the control). Row 2 lists the corresponding photos of embryos after centrifugation. The bridge-like structure after centrifugation (indicating incomplete lipid separation) can be seen in the control; complete lipid separation can be observed in the embryo exposed at 400 mOsm, while large bridge-like structures are present in the embryos treated with 600 or 800 mOsm. FIG. 2 indicates that osmolality at about 400 mOsm provides the most complete lipid separation, when osmolality increased to 600 and above, the lipid separation is hindered.

**[0035]** The invention further quantitatively evaluated the impacts of the different osmolalities and centrifugation conditions on the lipid separation rate, the embryos' development, and the hatching ability. Based on the data included in Tables 1 (IVF-derived embryos, osmolality adjusted with NaCl), 2 (IVF-derived embryos, osmolality adjusted with sucrose), and 3 (NT-derived embryos, osmolality adjusted with both NaCl and sucrose, all three tables attached), the preferred condition for lipid separation is to expose the IVF embryos to osmolality ranging from above 300 to about 500 mOsm, preferably from about 350 to about 450 mOsm, followed by centrifugation for about 6 to about 20 minutes at 13,400 x g speed. The centrifugation time may be shortened or extended depending upon the centrifugation speed. However, extending centrifugation time may subject the embryos to prolonged exposure to high osmolality, which may have adverse impact on the vitality of the embryos. Furthermore, in Table 3, the second high osmolality treatment is elected for the NT-derived embryos that failed to condense upon the first round of treatment, which increases the total lipid separation rate.

The second high osmolality treatment may be elected as long as the embryos are still at the cleavage stage prior to compaction.

Table 1. The lipid separation and development of IVP embryos after treatment with different osmolalities and different centrifugation time at 18-20 hrs after the beginning of IVF\*

Treatment	Osmolality	Centrifugation time (min)	Total No. of Embryos Treated		Lipid separated embryo		Development to the blastocyst stage	
			No.	%	Mean ± SEM	No.	/Lipid Separated Mean ± SEM	%
300	6	216	106	49.1 ± 6.0 <sup>f</sup>	15	14.2 ± 5.8 <sup>abc</sup>	6.9 ± 3.5 <sup>cd</sup>	
		221	166	75.1 ± 5.3 <sup>de</sup>	23	13.9 ± 2.3 <sup>abc</sup>	10.4 ± 1.6 <sup>bcd</sup>	
		217	182	83.9 ± 4.2 <sup>cd</sup>	36	19.8 ± 3.7 <sup>a</sup>	16.6 ± 3.2 <sup>ab</sup>	
400	223	200	89.7 ± 2.4 <sup>ab</sup>	29	14.5 ± 3.8 <sup>ab</sup>	13.0 ± 3.5 <sup>abc</sup>		
		228	195	85.5 ± 2.2 <sup>bc</sup>	35	17.9 ± 3.7 <sup>ab</sup>	15.4 ± 3.4 <sup>ab</sup>	
		300	12	200	133	66.5 ± 3.3 <sup>dc</sup>	24	18.4 ± 3.5 <sup>ab</sup>
350	217	182	83.9 ± 3.0 <sup>cd</sup>	33	18.1 ± 3.6 <sup>ab</sup>	12.0 ± 2.6 <sup>abcd</sup>		15.2 ± 3.2 <sup>ab</sup>
		216	187	86.6 ± 3.9 <sup>bc</sup>	21	11.3 ± 1.8 <sup>abc</sup>		9.7 ± 1.5 <sup>bcd</sup>
		213	192	90.1 ± 3.4 <sup>abc</sup>	36	18.8 ± 2.8 <sup>ab</sup>		16.9 ± 2.9 <sup>ab</sup>
450	211	200	94.8 ± 1.2 <sup>ab</sup>	29	14.5 ± 2.9 <sup>ab</sup>			13.7 ± 2.7 <sup>abcd</sup>
		300	20	216	153	70.8 ± 5.2 <sup>dc</sup>	27	17.6 ± 5.0 <sup>ab</sup>
		350	212	193	91.0 ± 1.9 <sup>abc</sup>	29	15.0 ± 2.9 <sup>ab</sup>	12.5 ± 3.9 <sup>abcd</sup>
500	219	213	97.3 ± 1.6 <sup>a</sup>	27	12.7 ± 1.9 <sup>abc</sup>			13.7 ± 2.7 <sup>abc</sup>
		400	209	195	93.3 ± 2.2 <sup>abc</sup>	21	10.8 ± 2.5 <sup>bc</sup>	12.3 ± 1.9 <sup>abcd</sup>
		450	223	206	92.4 ± 1.5 <sup>abc</sup>	12	5.8 ± 1.9 <sup>c</sup>	10.0 ± 2.5 <sup>bcd</sup>
Control		262	-	-	47	17.9 ± 2.1 <sup>ab</sup>	5.4 ± 1.7 <sup>d</sup>	17.9 ± 2.1 <sup>a</sup>

a,b,c,d,e,f

Different superscripts within a column are different P&lt;0.05.

\*Summary of six replicates.

Table 2: The lipid separation and development of IVP embryos after treatment with different osmolalities and centrifugation for 6 min at 18-20 hrs after IVF\*

Osmolality	Chemical	Treatment	Total embryos	Lipid separated embryos		Development to the blastocyst stage		
				No.	%, lipid separated embryos/ Total embryos Mean ± SEM	No.	%	/Lipid separated embryos Mean ± SEM
400	NaCl	133	117	88.0±9.1 <sup>a</sup>	14	12.0±6.1 <sup>ab</sup>	10.5±3.5 <sup>ab</sup>	
400	Sucrose	92	83	90.2±0.6 <sup>ab</sup>	14	16.9±8.1 <sup>a</sup>	15.2±7.2 <sup>a</sup>	
500		133	97	73.0±8.1 <sup>b</sup>	7	7.2±1.2 <sup>ab</sup>	5.3±0.4 <sup>abc</sup>	
600		94	23	24.5±13.0 <sup>c</sup>	1	4.3±12.5 <sup>ab</sup>	1.1±1.2 <sup>bc</sup>	
800		125	1	0.8±0.8 <sup>c</sup>	0	0 <sup>b</sup>	0 <sup>c</sup>	
Control		144	-	-	20	3.9±3.4 <sup>ab</sup>	13.9±3.4 <sup>a</sup>	

\*Summary of three replications

<sup>a,b,c</sup> Different superscripts within a column are different P<0.05.

Table 3. Lipid separation of NT embryos after treatment with different osmolalities and centrifugation times at 14-18 hrs after fusion\*

Treatments			Total No. of embryos	Lipid separated embryos		Lipid separated blastocysts		
Osmo	Chemical used to make high osmolarity medium	Centrifugation time (min)		1 <sup>st</sup> high osmolarity treatment and centrifugation	1 <sup>st</sup> +2 <sup>nd</sup> osmolarity treatment and centrifugation	No.	% (Mean ± SEM)	
			No.	% (Mean±SEM)	No.	% (Mean±SEM)	/Lipid separated embryos	/Total embryos
400	Sucrose	6	Low Calcium +Thi+DTT	531	336	63.3±2.5 <sup>d</sup>	444	83.6±0.9 <sup>c</sup>
400	Sucrose	6	Electrical	818	546	66.7±1.7 <sup>bcd</sup>	671	82.0±1.4 <sup>c</sup>
400	NaCl	6	Electrical	109	67	61.5±0.4 <sup>cd</sup>	89	81.7±0.2 <sup>c</sup>
400	NaCl	12	Electrical	118	88	74.6±5.1 <sup>abc</sup>	100	84.7±0.7 <sup>bc</sup>
400	NaCl	20	Electrical	550	415	75.5±2.3 <sup>ab</sup>	491	89.3±1.7 <sup>ab</sup>
350	NaCl	20	Electrical	712	570	80.1±2.9 <sup>a</sup>	653	91.7±0.7 <sup>a</sup>
Control				-	-	-	8	13.3±3.3 <sup>b</sup>
								13.3±3.3

\*Summary of two to six replicates.

<sup>a,b,c,d</sup> Different superscripts within a column are different P<0.05.

[0036] Table 4 evaluates the pregnancy and offspring data on the IVF-derived embryos preserved by high osmolality treatment, centrifugation and vitrification. Among the data included in Table 4, three surrogates out of nine established pregnancies and produced normal offspring. One surrogate received the embryos treated with osmolality at 350 mOsm and produced five piglets, three males and two females; one surrogate received embryos treated with 400 mOsm and produced four piglets, two males and two females; and one surrogate received the embryos treated with 450 mOsm and produced three piglets, one male and two females.

Table 4. Transfer of IVP embryos derived from high osmolality treatment and centrifugation after vitrification and warming.

Date of ET	Osmo. (mOsm)	Chemical	Centrifuge (min)	Number of embryos transferred	Zona removal after vitrification and warming	Recipient	Pregnancy	No. of Piglets	Note
6/14/2007	350	NaCl	6	25	+	O089	+	5	3 males 2 females
6/14/2007	350	NaCl	6	50	-	O090	-		Returned to estrus on day 19
6/28/2007	350	NaCl	6	50	-	O105	-		Returned to estrus on day 27
7/5/2007	400	NaCl	6	25	+	O082	-		Returned to estrus on day 21
7/13/2007	400	NaCl	6	25	+	O112	+	4	2 females 2 males
11/1/2007	450	NaCl	20	25	+	O174	-		Returned to estrus on day 25
11/8/2007	450	NaCl	20	25	+	O188	+	3	2 females 1 male
11/9/2007	450	NaCl	12	25	+	O128	-		Returned to estrus on day 24
12/12/2007	450	NaCl	12	25	+	O185	+		Returned to estrus on day 20

[0037] Table 5 lists the transfer, pregnancy, and offspring data of the NT-derived embryos after high osmolality treatment, centrifugation, and vitrification. Three embryo transfers were performed and recorded. For each transfer, 80 to 90 embryos with the zona pellucida softened or removed by pronase treatment were transferred into the surrogates. Two of the three surrogates receiving the embryos treated with 400 mOsmo with 6 min centrifugation and the one receiving embryos treated with 350 mOsmo with 20 min centrifugation resulted in pregnancy, with the former a single male piglet was produced. The data in Tables 4 and 5 demonstrates that the inventive method, especially when applying the preferred range of osmolality (from about 350 to about 450 mOsm), is a successfully cryopreservation method for the IVP embryos.

Table 5. Transfer of NT embryos after vitrification and warming \*

Date of embryo transfer	High osmolality treatment			Number of embryos transferred	Zona removal after vitrification and warming	Recipient		No. of piglets	Note
	mOsm	Chemical	Centrifuge (mins)			No.	Pregnant		
8/24/2007	400	Sucrose	6	83	+	0141	+	1 (male)	
11/16/2007	400	NaCl	6-20	80	+	0184	-	-	Returned on day 22 of the cycle
12/6/2007	350	NaCl	20	90	+	0214	+	-	Returned on day 19 of the cycle

\*One gilt was not included in the above data as it developed a reproductive tract infection.

**[0038]** While the invention has been described in connection with specific embodiments thereof, it will be understood that the inventive methodology is capable of further modifications. This patent application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features herein before set forth and as follows in scope of the appended claims.

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**CLAIMS**

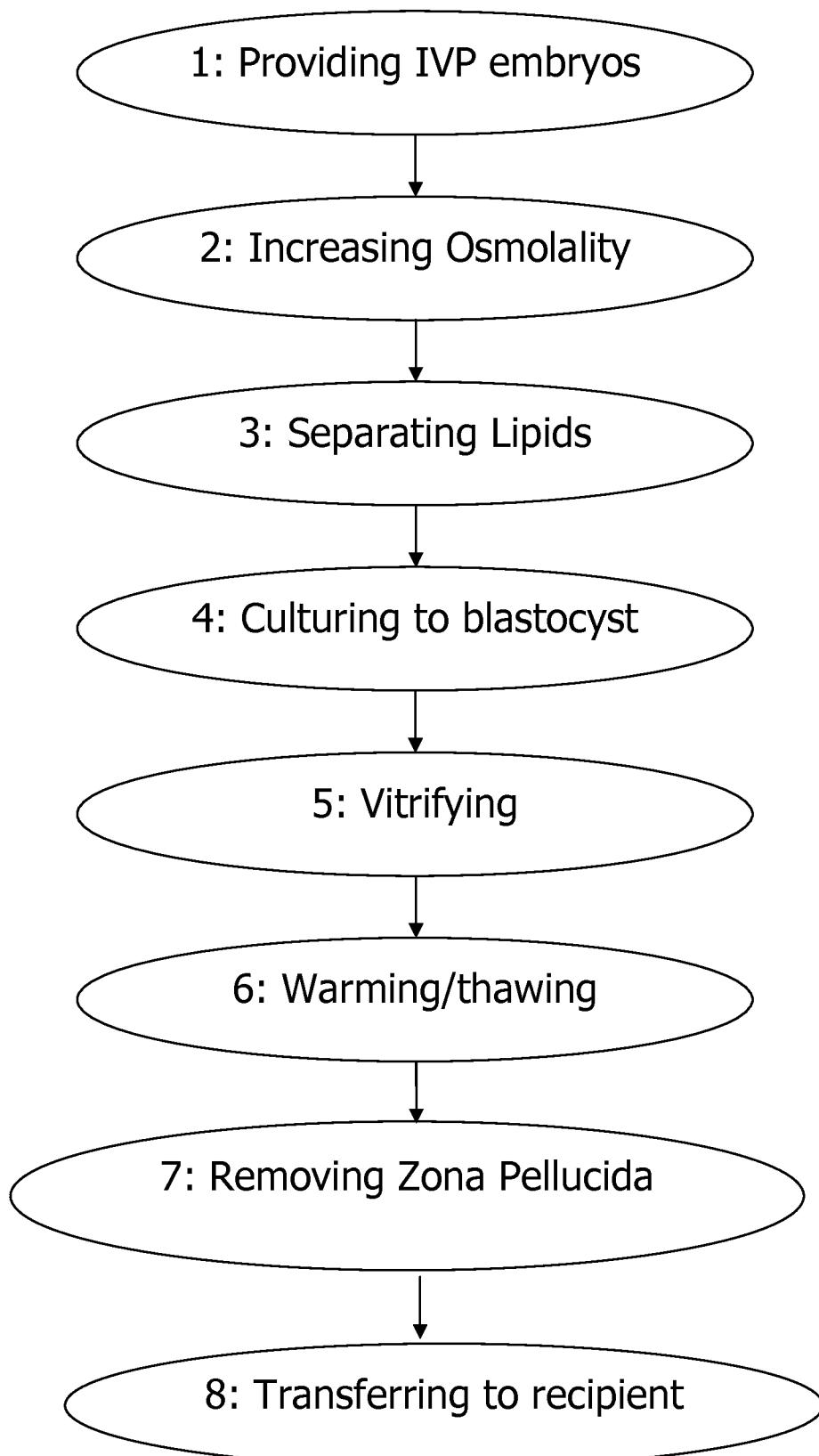
What is claimed is:

1. A method for lipid separation of an IVP porcine embryo comprising
  - (a) producing an IVP porcine embryo at the one-cell or cleavage stage prior to compaction,
  - (b) exposing such embryo to a medium with a pre-selected osmolality for a short period of time to produce a condensed embryo, and
  - (c) centrifuging such condensed embryo at a pre-selected speed and for a pre-selected duration to produce a lipid-separated embryo.
2. The method of Claim 1, wherein said IVP porcine embryo is produced through in-vitro fertilization, nuclear transfer, or other in-vitro method.
3. The method of Claim 1, wherein osmolality of said medium ranges from about 300 mOsmo to about 500 mOsmo.
4. The method of Claim 4, wherein said centrifugation condition ranges from about 6 to about 20 minutes at 13,400 x g speed.
5. A method of cryopreservation and recovery of in-vitro-produced porcine embryos comprising:
  - (a) producing in-vitro-produced porcine embryos at the one-cell or cleavage stage prior to compaction,
  - (b) exposing said embryos to a pre-selected osmolality for a short period of time to produce condensed embryos,
  - (c) centrifuging said condensed embryos to produce lipid-separated embryos,

- (d) culturing said lipid-separated embryos to the blastocyst stage to produce lipid-separated blastocysts,
- 10 (e) cryopreserving said blastocysts by vitrification or freezing,
- (f) recovering said blastocysts by warming, rehydrating, and removing the zona pellucida, and
- (g) transferring said zona-removed blastocysts into a recipient.

1/4

FIG. 1



2/4

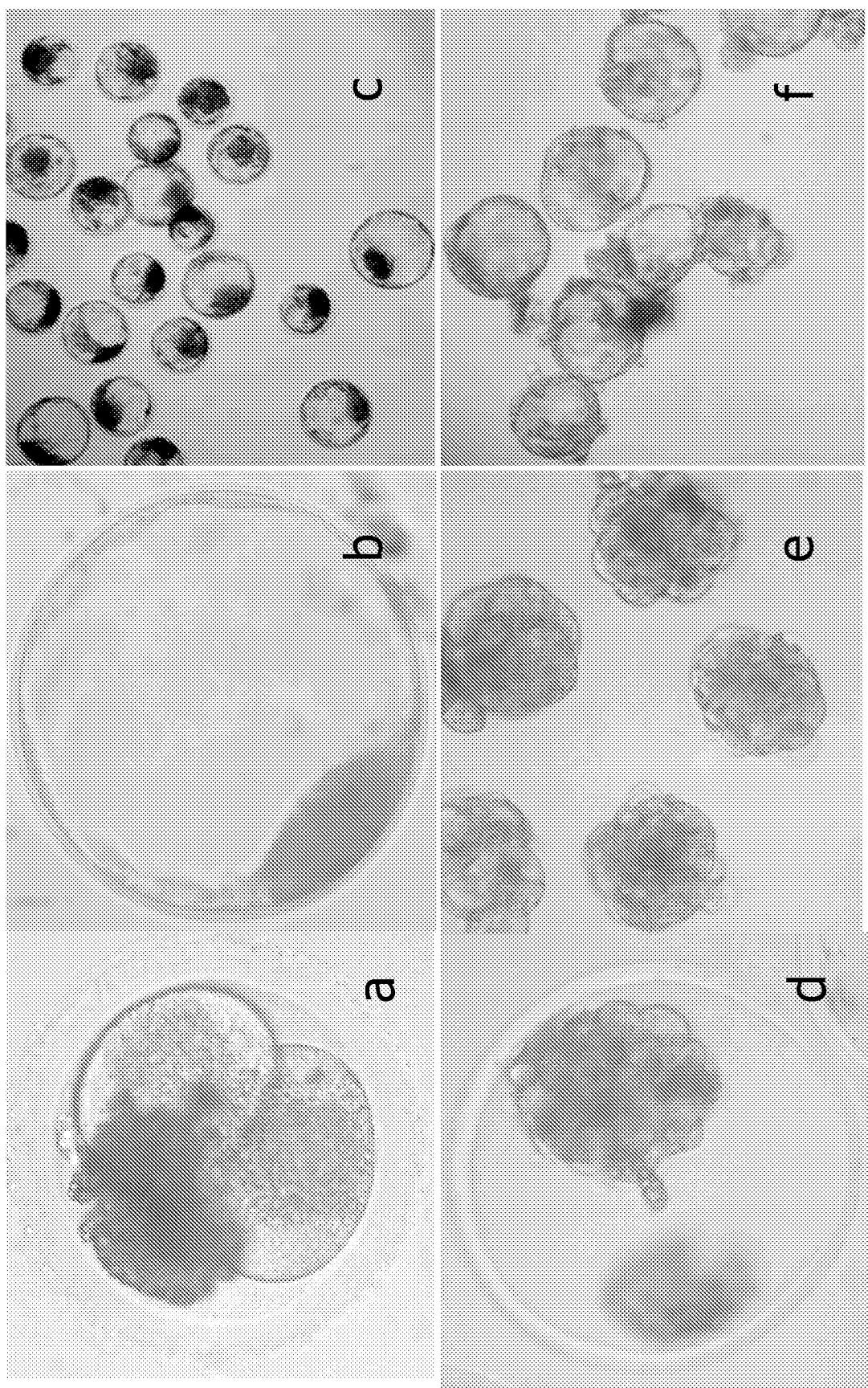
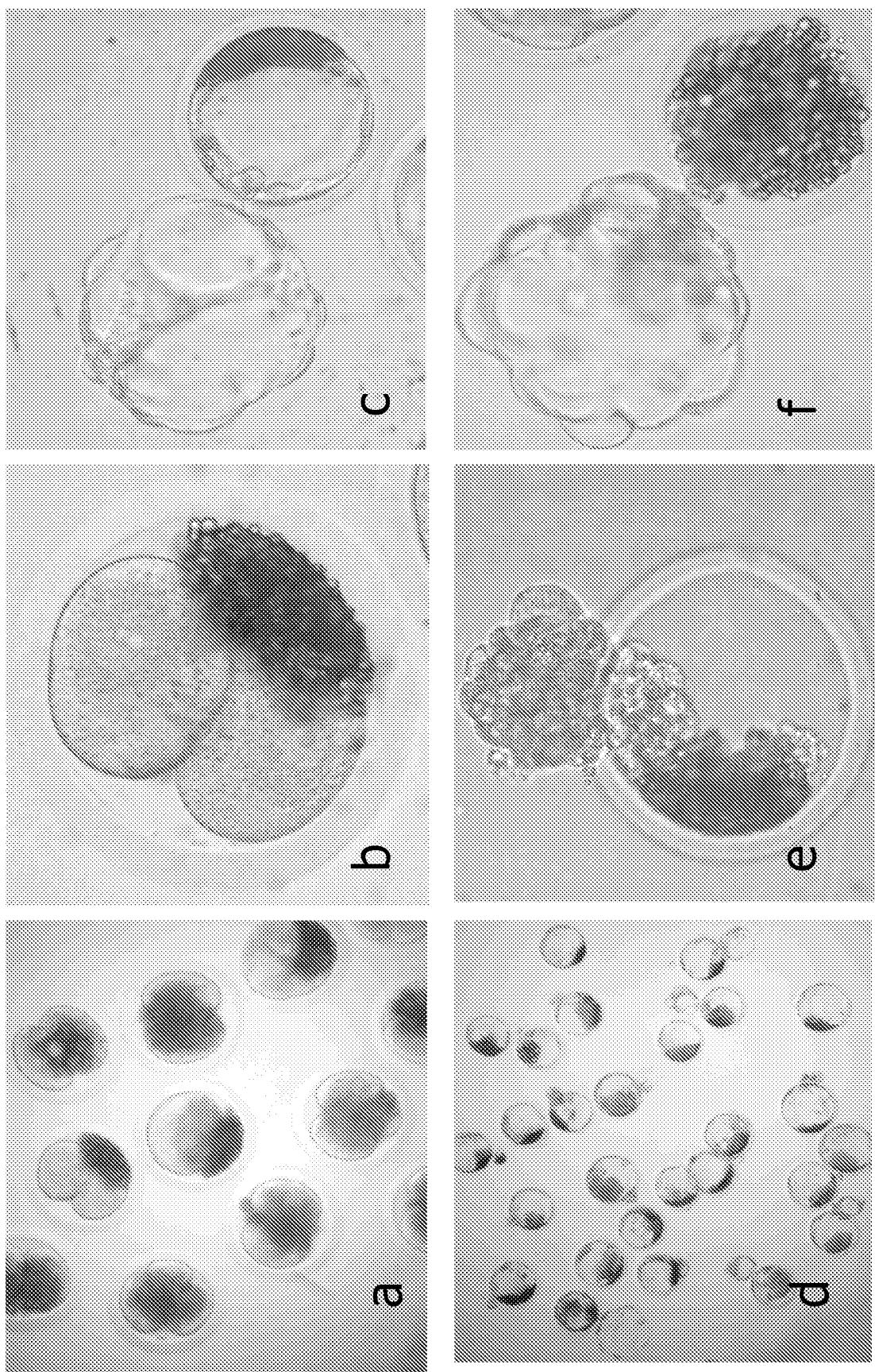


FIG. 2

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FIG. 3



4/4

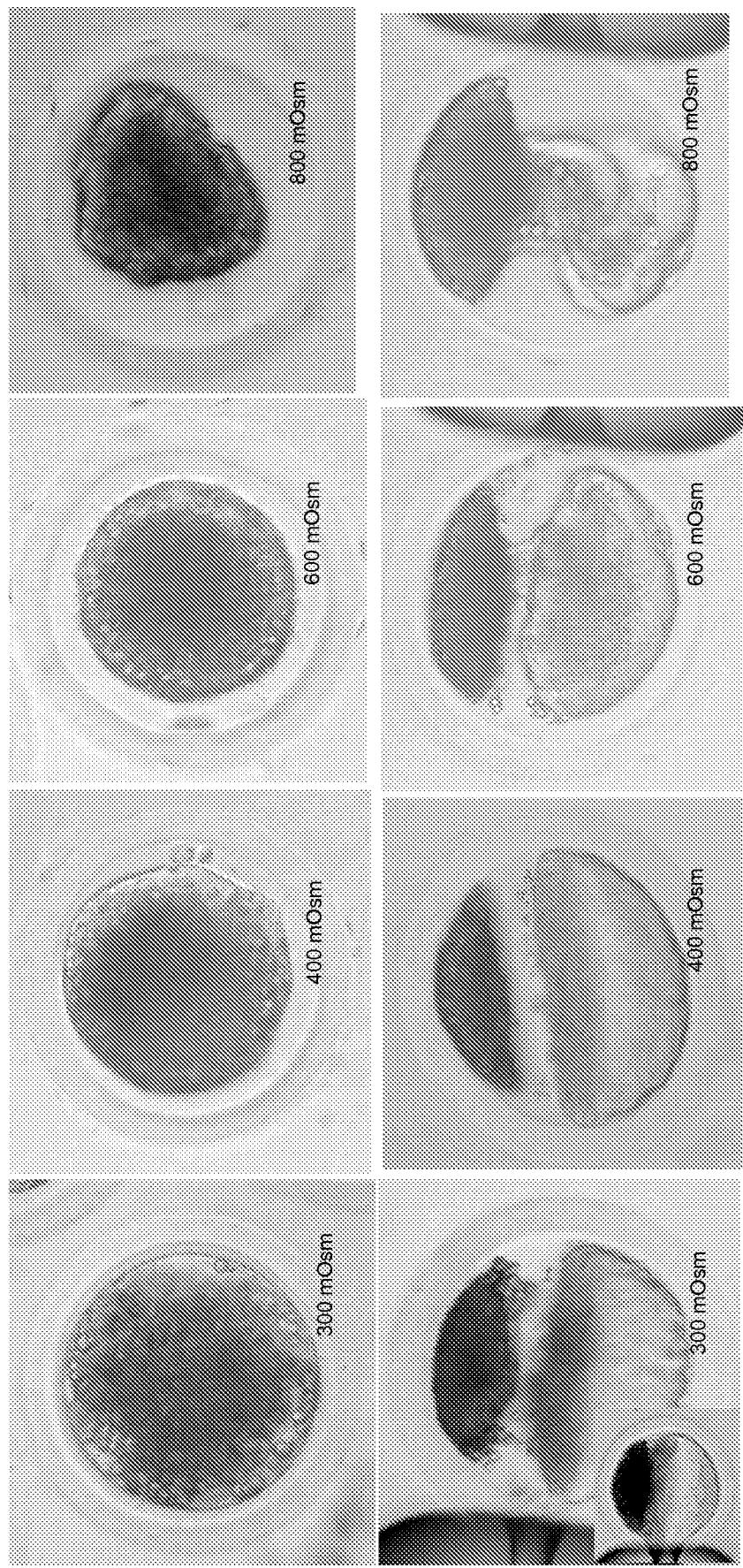


FIG. 4

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US 09/55424

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(8) - C12N 5/02 ; A61D 19/04; A01K 67/00 (2009.01)

USPC - 435/374, 435/404, 600/33, 800/8

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: 435/374, 435/404, 600/33, 800/8

IPC(8): C12N 5/02; A61D 19/04; A01K 67/00 (2009.01)

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

USPC: 435/325, 378, 424/582

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

Electronic Databases Searched: PubWEST DB=PGPB,USPT,USOC,EPAB,JPAB; PLUR=NO; OP=ADJ, Google Scholar, Google Patent

Search Terms Used: porcine, porcine embryo, in vitro, cryopres\$, vitrif\$, lipid separated

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6503698 B1 (Dobrinsky et al.) 7 January 2003 (07.01.2003) entire document, esp: abstract, col 5 ln 13-27, col 5 ln 34-col 6 ln 16, col 8 ln 60- col 9 ln 25, col 9 ln 46-54, col 13 ln 14-34, col 13 ln 66- col 14 ln 30,	1-5

Further documents are listed in the continuation of Box C.

\* Special categories of cited documents:

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“P” document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search	Date of mailing of the international search report
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