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Conventional slow freezing, vitrification and open pulled straw (OPS) vitrification of rabbit embryos

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Abstract

Three different methods of cryopreservation viz., conventional slow freezing, vitrification and open pulled straw vitrification were compared for their ability to support post thaw in vitro and in vivo development of rabbit embryos. Morula stage rabbit embryos were collected from super-ovulated donor does. They were randomly allocated to different freezing methods and stored up to 3 months in liquid nitrogen. After thawing and removal of cryoprotectants, embryos exhibiting intact zona pellucida and uniform blastomeres were considered suitable for in vitro culture and/or transfer. Three to five cryopreserved embryos placed in ~1 ml of culture medium (TCM 199 supplemented with foetal calf serum and antibiotics) were incubated for up to 72 h under humidified atmosphere of 5% CO₂ in air at 39 °C. Development to hatched blastocyst stage was considered the initial indicator of success of cryopreservation of embryos. Of the embryos cryopreserved by programmed freezing, open pulled straw vitrification, vitrification-55 h pc and vitrification-72 h pc 55, 71, 17 and 48%, respectively, developed into hatched blastocysts. Similarly 19, 29, and 4% of embryos cryopreserved by programmed freezing, open pulled straw vitrification and vitrification - 72 h pc developed into live offspring on transfer to recipient does. This is the first report on open pulled straw vitrification of rabbit embryos. Present results, suggest that (a) open pulled straw vitrification

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supports better in vitro survival of frozen thawed rabbit morulae; (b) both programmed freezing and OPS are similar but superior to vitrification in supporting in vivo survival of frozen thawed rabbit embryos.

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1. Introduction

Conventional slow freezing and vitrification are the commonly used methods for long-term storage of pre-implantation mammalian embryos. Though conventional slow freezing method was successfully used in a variety of species including mouse, cattle, sheep, goat, pig, horse, rat, rabbit, cat, eland (African antelope), baboon, marmoset, macaque monkey and human (Rall, 1992), it suffers from several limitations such as chilling injury, physical damage due to external ice, zona damage, need for elaborate and expensive equipment and tedious freezing protocols. Vitrification (Rall and Fahy, 1985) greatly simplifies the process of cooling, avoids physical damage to embryos, and lessens the chilling injury of embryos as it passes through critical temperatures very rapidly. However, the embryos cryopreserved by vitrification may still be injured by toxicity of cryoprotectants, extra cellular ice fracture and adverse osmotic effects (Kasai et al., 1996). Further vitrification may be suited better for cryopreservation of certain pre-implantation stages of rabbit embryos than the others (Smorag et al., 1989; Kobayashi et al., 1990; Dobrinsky et al., 1990). Recently Vajta et al. (1997a,b) developed a new technique of vitrification called “Open Pulled Straw Vitrification” (OPS). This method overcomes the drawbacks of traditional vitrification by accelerating the rates of cooling and warming beyond 2000 °C/min. The advantages of this method include (a) high rate of cooling (16,700 °C/min), (b) easy and rapid loading of embryos by capillary effect, (c) direct contact between liquid nitrogen and freezing medium, which increases the rate of cooling, (d) simple and rapid warming and (e) low toxicity due to rapid cooling and rehydration. OPS vitrification was reported to be successful with all developmental stages of bovine (Vajta et al., 1998) and porcine embryos (Vajta et al., 1997a) and in vitro matured bovine oocytes (Vajta et al., 1998; Lazar et al., 2000). However, to date OPS vitrification was not used for cryopreservation of rabbit embryos, though a method similar in principle to OPS vitrification but in which there was no direct contact between liquid nitrogen and the vitrification solution (mOPS) was recently employed successfully to cryopreserve rabbit embryos (Lopez-Bejar and Lopez-Gatius, 2002). Further the relative efficacy of the three methods of cryopreservation was not simultaneously tested in any mammalian species. Therefore, the present investigation was conducted with the twin objectives of (a) cryopreservation of morula stage rabbit embryos by OPS vitrification and (b) comparison of the efficiency of the three methods of cryopreservation of morula stage rabbit embryos as indicated by their post thaw in vitro development to hatched blastocyst stage, and to full term offspring in recipient does after embryo transfer.

2. Materials and methods

Unless mentioned otherwise all the chemicals, media, and hormones were purchased from the Sigma Chemical Co., St. Louis, USA and plastics from Nunclon, Roskilde, Denmark.

2.1. Super ovulation of donor does, embryo collection and selection¹

All rabbits used in these experiments were 1–2 years old primiparous animals of mixed breeds. Rabbit does to be used as embryo donors or recipients were individually caged for 3–5 weeks prior to start of the experiment to avoid pseudopregnancy. Donor does were super-ovulated with a single injection (s/c) of 75 iu of PMSG (Folligon, Intervet, India). Seventy-two hours after PMSG injection, ovulation was induced by 100 iu of hCG injected into ear vein followed by mating with two fertile bucks. Mating with a vasectomized proven sterile buck induced pseudopregnancy in recipient does. Sterile mating was so timed that recipients were ~14 h behind the age of embryos. Embryo donors were euthenized by cervical dislocation at 55–58 h or 70–72 h postcoitum (pc). Embryos from the Fallopian tubes of these does were collected by retrograde flushing with the handling medium (see below). Embryos from the flushing fluid were isolated with a fire polished glass pipette under a stereo zoom microscope (SMZ-800, Nikon, Japan). Morphologically normal, compact morulae with uniform sized blastomeres were considered suitable for freezing (Plate 1A).

2.2. Embryo cryopreservation, thawing and removal of cryoprotectants

Unless mentioned otherwise all embryo manipulations were undertaken at a room temperature of ~25 °C.

2.3. Conventional slow freezing

Programmed freezing was undertaken employing a protocol routinely used in our laboratory for several years. For this purpose phosphate buffered saline (PBS) was prepared by dissolving one tablet of PBS in 200 ml of double distilled water. This was autoclaved and stored in 100 ml capacity airtight sterile glass bottles at 4 °C till used. PBS supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS) and 50 µg ml⁻¹ of Gentamicin sulphate was used for flushing of embryos from the reproductive tracts of donor does and also for handling and holding embryos in programmed freezing (see below).

1.5 M dimethylsulfoxide (DMSO) solution was prepared by mixing 1.064 ml of DMSO with 8.936 ml of the holding medium one day prior to use and stored at 4 °C. It was warmed to room temperature (~25 °C) prior to use. 1.5 M DMSO was appropriately diluted with the holding medium to obtain 1.0 M and 0.5 M DMSO. For equilibration of the embryos with DMSO, the holding medium, 0.5, 1.0 and 1.5 M DMSO were sequentially placed in a four well culture plate. Good quality morulae (55–58 h pc) were first transferred to the holding

¹ All the experiments involving rabbits were approved by the Institutional Animal Ethics Committee.

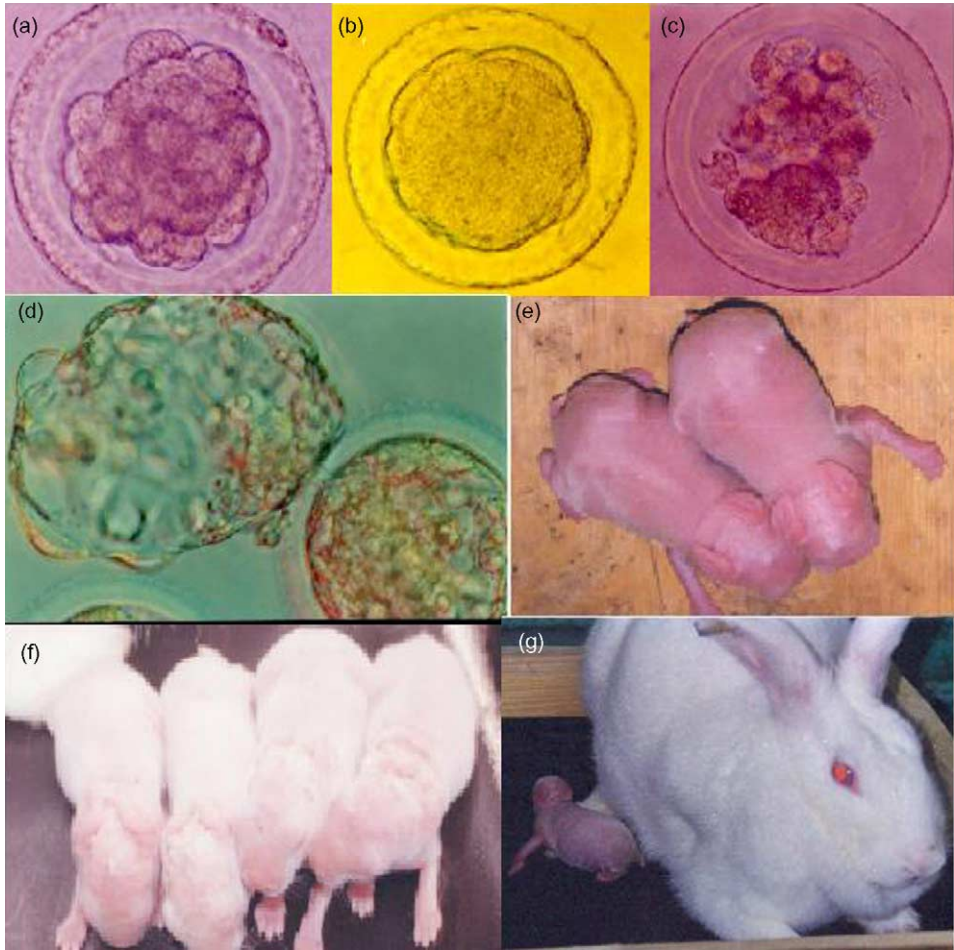


Plate 1. Cryopreservation of rabbit embryos: (a) fresh rabbit morula (55–58 h), (b) good quality frozen thawed morula, (c) damaged frozen thawed morula, (d) hatched blastocyst developed from OPS vitrified rabbit morula, (e) rabbit bunnies developed from OPS vitrified embryos, (f) rabbit bunnies developed from rabbit embryos frozen-thawed by programmed freezing, (g) rabbit bunny developed from vitrified (72 h pc) embryos.

medium. Then they were placed in 0.5 M DMSO for 5 min 1.0 M DMSO for 10 min and finally in 1.5 M DMSO for 15 min. Three to five embryos were loaded into 0.25 ml French mini straw (Cat. no AUA205, IMV, L'Aigle, France) sequentially as follows: a column of ~4 mm 1.5 M DMSO, air bubble, 1.5 M DMSO containing embryos, air bubble and again a ~4 mm column of 1.5 M DMSO. Loading of all the embryos into straws was completed within the equilibration time of 15 min in 1.5 M DMSO. Top end of the straw was sealed with polyvinyl alcohol and the lower end with a plastic stopper (Cat. no. EA 201, IMV, L'Aigle, France). After loading, the straws were kept vertically with stoppers downward in the straw holder in the freezing chamber of a programmable freezing machine (PTC 1000c, Apex

instruments, Kolkata, India). Embryo columns were kept at about same level to facilitate group seeding. The cooling program consisted of the following steps: room temperature (25 °C) to -7.0 °C at 5 °C min⁻¹, seeded and held at -7.0 °C for 5 min, (seeding was undertaken by touching the outer walls of the straw with a forceps pre-cooled in liquid nitrogen), -7.0 to -35 °C at 0.3 °C min⁻¹, -35 to -70 °C at 10 °C min⁻¹ and transfer to liquid nitrogen at -70 °C. Straws were stored in liquid nitrogen for up to 3 months.

For thawing straws were removed from liquid nitrogen and placed horizontally in a water bath at 35 °C for ~25–35 s. The straws were then removed from water bath and wiped free of water with a tissue paper. The stoppers were removed and the contents allowed into 1.5 M DMSO. Embryos were kept in 1.5 M DMSO for 5 min then sequentially transferred to 1.0 M DMSO, 0.5 M DMSO and the holding medium for 10, 15 and 30 min to remove the cryoprotectant from the embryos.

2.4. Vitrification

Methods as described by Vicente and Garcia-Ximenez (1996) with some modifications described below were employed for the vitrification of rabbit embryos. Holding medium for vitrification was prepared by supplementing PBS with 20% FCS (v/v). Vitrification solution I (VS-I) containing 12.5% ethylene glycol (EG) and 12.5% DMSO was prepared just prior to use by mixing EG, DMSO and the holding medium in the ratio of 1:1:6. Similarly vitrification solution II (VS-II) had a composition of 25% EG and 25% DMSO. This was prepared just prior to use by mixing EG, DMSO and the holding medium in the ratio of 1:1:2. Cryoprotective diluent-I (CPD-I) was prepared just prior to use by mixing EG, DMSO and the holding medium in the ratio of 1:1:8. Cryoprotective diluent-II (CPD-II) was prepared by mixing equal volumes of CPD-I and the holding medium.

Three to five embryos were initially placed in the holding medium for 10 min followed by VS-I for 4 min. Subsequently the embryos were transferred into VS-II for 1 min. During equilibration in VS-II the embryos were loaded into 0.25 ml French mini straws as in programmed freezing but using VS-II. Immediately after loading, the straws were immersed vertically into liquid nitrogen. Embryos collected at 55–58 h pc (referred to as 'vitrification-55 h pc' in results) and 70–72 h pc (referred to as 'vitrification-72 h pc' in results) were vitrified as described above. These vitrified embryos were also stored in liquid nitrogen for up to 3 months.

For warming the straws were exposed to air at room temperature (~25 °C) for 8–10 s and then vertically immersed in a 25 °C water bath until the frozen solutions became liquid. Stoppers were removed, sealed ends were cut and the contents poured into CPD-I for 4 min. Then they were transferred to CPD-II for 8 min. Finally the embryos were washed twice in the holding medium for 8 min each.

2.5. OPS vitrification

Methods for OPS vitrification were followed as described by Vajta et al. (1997b). All the manipulations were performed on a 39 °C heating stage (Linkam Scientific Instrument Ltd., England) in a room maintained at ~25 °C. French mini straws slightly melted over flame were hand pulled to achieve a diameter that was half of its original. The straws were

then held in air for a few seconds prior to cutting at the narrowest point of the pulled portion. Sucrose stock solution was prepared by dissolving 10.27 g of sucrose in 20 ml of HEPES buffered TCM 199 (TCM 199H). This medium was stored in 50 ml plastic tubes at 4 °C till used. Holding medium for OPS vitrification was prepared by supplementing TCM 199H with 20% FCS (v/v) and 50 $\mu\text{g ml}^{-1}$ of Gentamicin sulphate. OPS vitrification solution-I (OPS-I) containing 10% EG and 10% DMSO was prepared, on the day prior to use, by mixing EG, DMSO and the holding medium in the ratio of 1:1:8 and stored at 4 °C. OPS vitrification solution-II (OPS-II) containing 20% EG, 20% DMSO and 0.6 M sucrose was prepared on the day prior to use by mixing EG, DMSO, FCS and sucrose stock solution in the ratio of 1:1:1:2 and stored at 4 °C. Cryoprotective diluent-I for OPS (OPS-CPD-I) had the same composition as OPS-I but contained 0.3 M sucrose in addition. This was prepared by mixing equal volumes of OPS-II and the holding medium. Cryoprotective diluent-II for OPS (OPS-CPD-II) was prepared by mixing OPS-CPD-I and holding medium in the ratio of 1:3.

Good quality morula (55–58 h pc) stage embryos were equilibrated for 5 min in the holding medium prior to OPS vitrification. In order to vitrify 3–5 embryos were initially placed in OPS-I for 2 min. Subsequently the embryos were consecutively transferred into three 8 μl droplets of OPS-II for 10 s each. During the last 10 s of exposure to OPS-II solution, open end of the pulled straw was placed on the surface of the third droplet. The embryos entered into the straw by capillary action. Immediately after loading, the straws were plunged vertically into liquid nitrogen. They were stored in liquid nitrogen for up to 3 months.

A four-well tissue culture plate containing 1.2 ml of OPS-CPD-I, OPS-CPD-II in first two wells and the holding medium in the other two wells was used to dilute the cryoprotective out of warmed embryos. Straws containing embryos were taken out of liquid nitrogen and the open end of straw was immersed vertically in 1.2 ml of OPS-CPD-I solution. The vitrification medium became liquid within 2–4 s and the dilution medium entered into the straws. The contents of the straw were then released into the well by gentle blowing using a mouth pipette. One minute after, they were transferred into OPS-CPD-II solution for 5 min. Finally they were washed twice in holding medium for 5 min each.

2.6. *In vitro* culture of cryopreserved embryos

For *in vitro* culture of rabbit embryos bicarbonate buffered tissue culture medium 199 (TCM 199B) was supplemented with 15% heat inactivated FCS and 50 $\mu\text{g ml}^{-1}$ of Gentamicin sulphate. This was stored at 4 °C in a disposable 10 ml syringe with 0.22 μm filter (Sartorius, Germany) attached for up to 1 week. On the day of use, it was filtered directly into four-well tissue culture plates and warmed in a carbon dioxide incubator (Heraeus, Germany) for a minimum of 1 h prior to culture of embryos.

After removal of the cryoprotectives, morphology of embryos from each method of cryopreservation was examined under a stereo zoom microscope. Embryos with uniform blastomeres and intact zona pellucida were regarded as good embryos suitable for culture and/or transfer (Plate 1B). Damaged embryos exhibiting broken zona pellucida and/or lysed blastomeres (Plate 1C) were discarded. Groups of 3–5 good embryos were placed in \sim 1 ml of embryo culture medium and incubated at 39 °C in a humidified atmosphere of 5% carbon dioxide in air for up to 72 h. Development to hatched blastocyst stage (Plate 1D) at the end

of culture period was considered yet another indicator of success of freezing and thawing of embryos.

2.7. Embryo transfer

Immediately after thawing and removal of the cryoprotectives, embryos selected for transfer were also cultured as described above for 1–2 h. Pseudopregnant recipient does (40–44 h pc) mated to a proven sterile buck were fasted for 12 h prior to surgery. Six to eight embryos in a small volume (~5–7 μ l) of culture medium were transferred into each of the fimbriated ends of the oviducts of anaesthetized (5% thiopental sodium, i/v into ear vein) recipient does accessed through a mid ventral incision. The recipients were given routine postoperative care in individual cages in a separate animals house where no male rabbits were housed. The animals were examined by abdominal palpation 15 days after embryo transfer to confirm pregnancy. Animals positive for pregnancy by abdominal palpation were subjected to ultrasonography on day 20 for confirmation of pregnancy and observed up to 30–32 days for kindling. Since the recipient animals had no chance of coming in contact with males, birth of offspring (Plate 1E–G) following transfer of cryopreserved embryos provided the final proof of success.

3. Results

A total of 457 morula stage rabbit embryos were frozen preserved (Table 1). It can be seen that the open pulled straw method yielded the highest proportion of good embryos after thawing and also supported the highest rate of development to the blastocyst stage in culture (Table 1). Programmed freezing was the next best method but the results of in vitro culture in this group were not significantly different from vitrification-72 h pc. Vitrification-55 h pc resulted in the lowest yield of good embryos after thawing and development to blastocyst stage in culture was the poorest.

A total of 75 cryopreserved rabbit embryos from three different methods were transferred into six recipient does to assess their in vivo survival (Table 1). While live offspring were born

Table 1
Comparison of three different methods of cryopreservation of rabbit embryos

Methods of cryopreservation	No. of embryos frozen (replicates)	No. of good embryos/ no. thawed (%)	No. of blastocysts/ no. cultured (%)	No of offspring/ no transferred (%)
Programmed freezing	91 (10)	74/91 (81) ^a	17/31 (55) ^a	5/27 (19) ^a
Open pulled straw method	110 (10)	100/110 (91) ^b	44/62 (71) ^b	7/24 (29) ^a
Vitrification-55 h pc	160 (10)	34/160 (21) ^c	5/30 (17) ^c	Not transferred
Vitrification-72 h pc	96 (10)	56/96 (58) ^d	10/21 (48) ^a	1/24 (4) ^b

Values with different letters in a column are significantly different. Normal deviate test for proportions; $P \leq 0.05$.

in all the three groups, vitrification-72 h pc supported the lowest development relative to the other two methods. The difference in survival to term of the cryopreserved embryos between programmed freezing and open pulled straw vitrification methods was not significant though results of open pulled straw method appeared better (Table 1).

4. Discussion

Three different methods for cryopreservation of rabbit embryos were investigated for their relative efficiency to support post thaw in vitro and in vivo survival (Table 1). Open pulled straw vitrification of 55–58 h rabbit morulae appeared to be the best method, since it not only yielded the highest number of good quality embryos on thawing but it also supported the highest development to the blastocyst stage in vitro (Table 1). Development to blastocyst stage obtained in the present investigation is much higher than the rate reported with mOPS method employed earlier (Lopez-Bejar and Lopez-Gatius, 2002) for the cryopreservation of morula stage rabbit embryos. Thus, it appears that OPS rather than mOPS is better for the cryopreservation of morula stage rabbit embryos. It may be argued that the superiority of OPS vitrification over conventional vitrification observed in the present study may not entirely be due to OPS itself, but it may at least partly be due to the composition of solutions used in OPS vitrification and cryoprotectant dilution. However, there is sufficient evidence to suggest that this may not be true. Firstly when rabbit morulae were cryopreserved by conventional vitrification and OPS using the same OPS solutions as used in the present study survival and development of rabbit morulae was better with OPS vitrification (unpublished observations in our laboratory). Secondly in the mOPS method employed earlier (Lopez-Bejar and Lopez-Gatius, 2002) galactose was used in the cryoprotectant dilution while sucrose was used in the present study. Thus, use of a non-permeating sugar (galactose or sucrose), which, reduces the rate of rehydration of embryos during removal of cryoprotectants, could not be the reason for higher survival obtained in the present study. Finally 55–58 h pc vitrified morulae were badly damaged and developed poorly though they underwent very limited damage and developed well when cryopreserved by OPS vitrification method (Table 1). Thus, we believe that OPS vitrification is a better method for cryopreservation of rabbit morulae.

On transfer to recipients, embryos cryopreserved employing programmed freezing and OPS vitrification developed similarly (Table 1). Apparently this is the first successful attempt to cryopreserve rabbit embryos by open pulled straw (OPS) method. Vitrification of 55–58 h morulae resulted in the lowest yield of good quality embryos on thawing and also the poorest development to the blastocyst stage in vitro. This is the reason for not transferring any embryos in this group to recipients. From preliminary experiments (unpublished), it was suspected that early morulae (55–58 h) might not tolerate vitrification. Therefore, late morulae (70–72 h) were also vitrified and on thawing they developed on par with embryos frozen by programmed freezing. However on transfer to recipients, these vitrified and thawed embryos produced only one live young (Table 1). This is the first time that all the three methods of cryopreservation of embryos were tested simultaneously in any one mammalian species.

As was reported earlier (Smorag et al., 1989; Kobayashi et al., 1990; Dobrinsky et al., 1990), in this investigation also vitrification was successful only with later stage (70–72 h) morulae. Open pulled straw method was more successful than vitrification for different stages of cattle and pig embryos (Vajta et al., 1997a, 1998). In the present investigation though open pulled straw method supported better in vitro survival, in vivo survival was similar to programmed freezing. This may be due to relatively small number of embryos transferred. Alternately OPS protocols need be standardized for different stages of embryos of different species as the post thaw survival of embryos is determined by factors including species, and cell stage (Dobrinsky, 2002).

It was also reported that OPS vitrification was able to support the post warming survival of all preimplantation stage cattle embryos (Vajta et al., 1997b; Lazar et al., 2000; Vajta et al., 1998). Since we use early rabbit morulae (55–58 h) in our nuclear transfer experiments, this study was restricted to the use of early morulae. We have observed that blastomeres from OPS vitrified embryos subsequent to nuclear transfer developed to the blastocyst stage on par with blastomeres from fresh and embryos cryopreserved employing programmed freezing (unpublished).

From the present results, it is concluded that (a) OPS supported better in vitro survival of frozen thawed rabbit morulae; (b) both programmed freezing and OPS are similar in supporting in vivo survival of frozen thawed rabbit morulae and (c) though vitrification appeared to be more suitable for cryopreservation of late (70–72 h pc) rabbit morulae, these results need be confirmed using larger number of embryos.

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