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Nonequilibrium cryopreservation of rabbit embryos using a modified (sealed) open pulled straw procedure

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Abstract

The study was designed to evaluate the efficiency of a modified (sealed) open pulled straw (mOPS) method for cryopreserving rabbit embryos by vitrification or rapid freezing. An additional objective was to determine whether the mOPS method could cause the vitrification of a cryoprotectant solution generally used in rapid freezing procedures. Two consecutive experiments of *in vitro* and *in vivo* viability were performed. In Experiment 1, the *in vitro* viability of rabbit embryos at the morula, compacted morula, early blastocyst and blastocyst stages was assessed after exposure to a mixture of 25% glycerol and 25% ethylene glycol (25GLY:25EG: vitrification solution) or 4.5 M (approximately 25% EG) ethylene glycol and 0.25 M sucrose (25EG:SUC: rapid freezing solution). Embryos were loaded into standard straws or mOPS and plunged directly into liquid nitrogen. The mOPS consisted of standard straws that were heat-pulled, leaving a wide opening for the cotton plug and a narrow one for loading embryos by capillarity. The embryos were aspirated into the mOPS in a column positioned between two columns of cryoprotectant solution separated by air bubbles. The mOPS were then sealed with polyvinyl-alcohol (PVA) sealing powder. The vitrification 25GLY:25EG solution became vitrified both in standard straws and mOPS, whereas the rapid freezing 25EG:SUC solution crystallized in standard straws, but vitrified in mOPS. The total number of embryos cryopreserved was 1695. Embryos cryopreserved after exposure to each solution in mOPS showed higher rates (88.2%) of survival immediately after thawing and removal of the cryoprotectant than those cryopreserved in 0.25 ml standard straws (78.8%; $P < 0.0001$). After culture, the developmental stage of the cryopreserved embryos significantly affected the rates of development to the expanded blastocyst stage. Regardless of the cryoprotectant used, lower rates of *in vitro* development were obtained when the embryos were cryopreserved at the morula stage, and higher rates achieved using embryos at blastocyst stages. Based on the results of Experiment 1, the second experiment was

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performed on blastocysts using the mOPS method. Experiment 2 was designed to evaluate the *in vivo* viability of cryopreserved rabbit blastocysts loaded into mOPS after exposure to 25GLY:25EG or 25EG:SUC. Embryos cryopreserved in mOPS and 25GLY:25EG solution gave rise to rates of live offspring (51.7%) not significantly different to those achieved using fresh embryos (58.5%). In conclusion, the modified (sealed) OPS method allows vitrification of the cryoprotectant solution at a lower concentration of cryoprotectants than that generally used in vitrification procedures. Rabbit blastocysts cryopreserved using a 25GLY:25EG solution in mOPS showed a similar rate of *in vivo* development after thawing to that shown by fresh embryos.

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1. Introduction

Rapid freezing and vitrification are procedures of nonequilibrium cryopreservation that do not require that osmotic equilibrium be attained between intracellular and extracellular solutions during freezing [1,2]. Embryo dehydration is usually achieved by exposure to high molarity solutions, this implies their contact with more potentially toxic solutions during a short period of time, compared to conventional freezing procedures. The dehydrated embryos are then plunged directly into liquid nitrogen, reducing time and costs. Rapid freezing procedures involve the use of lower molarity solutions than those used for vitrification, and some degree of intracellular crystallization of ice occurs. In contrast, vitrification solutions do not form ice crystals but change into glassy solids through an increase in viscosity [3].

Recently, the success of vitrification procedures has been improved by techniques that increase cooling and warming rates by vitrifying samples in appropriate containers, such as electron microscope grids [4], open pulled straws (OPS, [5]), cryoloops [6] aluminum foils [7], glass micropipettes [8,9] and nylon 60 μm meshes [10], or other techniques that require direct exposure of embryos and oocytes to liquid nitrogen [11]. The OPS method has been successfully used for the vitrification of embryos and oocytes of different species [11]. Cryopreservation by vitrification in open containers, mainly OPS, has many advantages such as high postfreeze embryo survival, simplicity of the procedure and reduced fractures in the zona pellucida and embryo cells [11,12]. The main drawback, however, is that the original OPS method and some of the new methods of rapid cooling require direct contact between the embryos and liquid nitrogen. Bielanski et al. [12] recently demonstrated that contaminated liquid phase nitrogen may be a potential source of infection for cryopreserved embryos. Modifications of the OPS method, such as filtration of liquid nitrogen or the use of sealed accessory protective containers, may help overcome this problem [13]. However, the latter approach has disadvantages that arise from difficulties in manipulating small specimens and sealing containers at very low temperatures [14].

In a previous study, two cryopreservation procedures, one based on the use of a mixture of 25% glycerol and 25% ethylene glycol in a vitrification process and the other of a solution composed of 4.5 M (approximately 25%) ethylene glycol and 0.25 M sucrose in a rapid freezing procedure, were selected from eight nonequilibrium cryopreservation

methods after conducting *in vitro* and *in vivo* viability tests in rabbit embryos [15]. These procedures have not been previously applied for the cryopreservation of rabbit embryos. The aim of the present study was to evaluate the efficiency of a modified (sealed) OPS (mOPS) method for the cryopreservation of rabbit embryos by vitrification or rapid freezing in two consecutive *in vitro* and *in vivo* viability experiments. Due to the increased cooling and warming rates applied in the OPS method compared to the use of standard straws, we hypothesized that the mOPS method would allow a reduction in the molarity of the cryoprotectant solution needed for vitrification. Thus, an additional objective was to determine whether cryopreservation by the mOPS method would allow the vitrification of a cryoprotectant solution for the rapid freezing procedure.

2. Materials and methods

2.1. Experiment 1

Experiment 1 was designed to evaluate the *in vitro* efficiency of two nonequilibrium procedures using 0.25 ml standard straws and mOPS. Embryos were cryopreserved at different developmental stages: morula, compacted morula, early blastocyst and blastocyst.

2.2. Embryo recovery

This experiment was performed on 1695 embryos obtained from 95 nulliparous White New Zealand rabbit does (5–7 months old). Embryo collection and morphological selection were performed as described previously with minor modifications [16]. Follicular recruitment was superstimulated with 80 IU PMSG (Folligon, Intervet, Salamanca, Spain) administered subcutaneously, and ovulation was induced with 30 IU hCG (Physex Leo, Lab. Leo, Madrid, Spain), administered intravenously 68 h later. Does were mated with two fertile bucks of the same breed immediately before the administration of hCG. Embryos at morula, compacted morula, early blastocyst and blastocyst stages were recovered between 48 and 88 h postcoitum (hpc). The donor does were anaesthetized with ketamine chlorhydrate (Imalgene, Rhône Mérieux, Lyon, France; 150 mg i.m.) and xylazine (Rompun, Bayer, Barcelona, Spain; 30 mg i.m.). Embryos were surgically recovered by retrograde flushing of oviducts and uterus with Dulbecco's phosphate-buffered saline solution (PBS, Sigma, St. Louis, MO, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS, Sigma) and a 1% antibiotic-antimycotic solution (Ab solution, Gibco BRL, Paisley, UK). The recovered embryos were classified according to their developmental stage and morphological appearance under a stereomicroscope (magnification $\times 50$). Excellent- to good-quality embryos, according to the criteria established by Lindner and Wright [17] for bovine embryos and including the presence of a normal mucin coat, were washed three times and pooled in PBS supplemented with sodium pyruvate (0.33 mM), glucose (5.6 mM), 20% FBS and 1% Ab solution (holding medium). The selected embryos were maintained no more than 30 min in holding medium on a 34–36 °C heated stage in a warm room (22–25 °C).

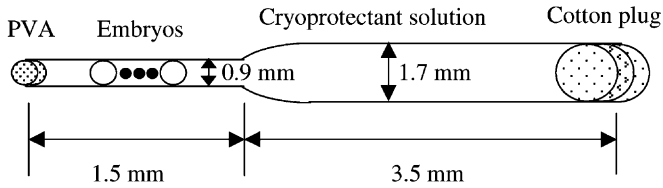


Fig. 1. Scheme of a mOPS containing embryos between two columns of vitrification solution separated by air bubbles (PVA: polyvinyl-alcohol).

2.3. Preparation of modified open pulled straws

The preparation of mOPS was similar to that described previously by Vajta et al. [18] for OPS. Standard straws (0.25 ml; IMV, L'Aigle, France) were heat-softened over a hot plate. The central part of the straw was pulled manually until its inner diameter and wall thickness decreased from 1.7 mm to approximately 0.9 mm, and from 0.15 to 0.07 mm, respectively. The straws were cut at the narrowest point with a razor blade. Only the half with the cotton plug was used. The narrow portion of the mOPS was of mean length 1.5 mm (range 1.2–1.8 mm), and the wider section was 3.5 mm (range 3.1–3.7 mm) long (Fig. 1). The embryos were aspirated into a column located between two columns of cryoprotectant solution separated by air bubbles. Embryos were loaded into the mOPS by capillary action; a 2 μ l droplet containing the embryos was simply touched with the narrow end of the straw.

2.4. Cryopreservation procedures

The 1695 embryos were randomly exposed to one of the following two cryopreservation solutions:

25GLY:25EG (vitrification solution): 25% glycerol (GLY) + 25% ethyleneglycol (EG).

25EG:SUC (rapid freezing solution): 4.5 M EG (approximately 25% EG) + 0.25 M sucrose.

Cryoprotectants were diluted in holding medium and groups of 6–8 embryos were exposed to these solutions at 22–25 °C.

Embryos were randomly aspirated into 0.25 ml standard straws or mOPS and exposed to each of the cryoprotectants to establish four treatment groups by embryo developmental stage.

The embryos exposed to 25GLY:25EG were vitrified according to the method described by Donnay et al. [19] and Kaidi et al. [20]. Briefly, embryos were exposed in a three-step equilibration process to gradually increasing concentrations of the cryoprotectant as follows: Step 1: 10% GLY (v/v, Sigma) for 5 min; Step 2: 10% GLY + 20% EG (Sigma) for 5 min; and Step 3: 25% GLY + 25% EG for 30 s. The embryos were aspirated during the time of exposure to the final vitrification solution into 0.25 ml standard straws in a column between two columns of a 0.85 M D-galactose (Sigma) solution in holding medium separated by air bubbles; or into mOPS, between two columns of the vitrification solution with no columns containing galactose. The straws were then placed in liquid nitrogen vapor for 2 min on a carrier placed on the liquid nitrogen, before immersion in liquid nitrogen. After warming for 10 s in a water bath at 20 °C, the contents of the standard straws were

emptied into a Petri dish and gently agitated to mix the galactose with the cryoprotectant. The contents of the mOPS were emptied into a Petri dish containing 2 ml of a 0.85 M D-galactose solution. After 5 min, the embryos were transferred to holding medium and then cultured as described below.

Embryos exposed to 25EG:SUC were equilibrated in the cryoprotectant solution for 2.5 min at room temperature in a one-step process. The embryos were aspirated during the time of exposure to the cryoprotectant solution into 0.25 ml standard straws in a column between two columns of the cryoprotectant solution externally bounded by two columns of holding medium separated by air bubbles or into mOPS between two columns of the cryoprotectant solution. The straws were then plunged directly into liquid nitrogen. To prevent cracking of the standard straws, first the bottom of the straw was plunged into liquid nitrogen, and after 3–4 s, the entire straw was lowered into the liquid nitrogen [4]. After warming for 10 s in a water bath at 20 °C, the contents of the straws were emptied into a Petri dish containing 2 ml of 0.25 M sucrose solution in holding medium at room temperature. After 1 min, the embryos were transferred into another Petri dish containing 2 ml of 0.25 M sucrose solution, and kept for 5 min at room temperature. The embryos were then washed twice in holding medium and cultured as described below.

For each treatment group, standard straws and mOPS were sealed with polyvinyl-alcohol (PVA) sealing powder. Embryos were stored in liquid nitrogen for periods between 1 and 5 days.

2.5. Assessment of vitrification or crystallization

The cryoprotectant solutions were tested for visual evidence of crystallization or vitrification [21]. Crystallization of the solutions was evaluated visually by noting opacity into the straws, whereas vitrified solutions remained glassy and transparent throughout cooling and warming. This procedure is considered to be a useful comparative test for evaluating cryopreservation protocols [22–24].

2.6. Morphological evaluation of embryos after cryopreservation

Once the cryoprotectant was withdrawn, the embryos were examined for apparent morphological damage to noncell layers (zona pellucida and mucin coat), recovery of the spherical form and nonextrusion of cellular material [25].

2.7. Embryo culture and assessment of embryo development

Conditions of culture were similar to those described by Carney and Foote [26]. The culture medium was RD medium supplemented with 10% FBS and 1% Ab solution. RD medium is a 1:1 (v/v) mixture of RPMI 1640 (Gibco) and Dulbecco's modified Eagle's medium (high glucose modification; DMEM, Gibco). The embryos were cultured in 16-well plates in 1 ml culture medium at 37 °C in 5% CO₂ in a humidified air. The embryos were transferred to fresh medium every 24 h. Evaluation of embryonic development was performed at 24 h intervals from the start of culture until development to the expanded

blastocyst stage or until two consecutive observations indicated no apparent development. The time period between consecutive observations was always 24 h.

2.8. *Experiment 2*

Based on the results obtained in Experiment 1, embryos at the blastocyst stage were subjected to the mOPS method. Experiment 2 was designed to evaluate the *in vivo* viability of rabbit blastocysts after nonequilibrium cryopreservation using the cryoprotectant solutions: 25GLY:25EG and 25EG:SUC.

2.9. *Embryo recovery, cryopreservation and transfer*

Seven hundred embryos at the blastocyst stage obtained at 84 hpc from 62 rabbit does were cryopreserved (two groups of 238 embryos each) or used as controls (224 embryos). Embryo recovery and cryopreservation procedures were as described for Experiment 1. Experimental embryos were exposed to one of two cryoprotectant solutions and loaded into mOPS. Cryopreserved embryos were maintained for 2 h in liquid nitrogen and control embryos held at 37 °C in holding medium until transfer [27]. Only morphologically normal embryos were transferred.

Cryopreserved and control embryos were surgically transferred into the uterus of 50 nulliparous White New Zealand pseudopregnant rabbit does previously treated with 30 IU hCG 78 h before transfer. The recipient does were anesthetized with ketamine chlorhydrate (Imalgene, 150 mg i.m.) and xylazine (Rompun, 30 mg i.m.). During midline laparotomy, the uterus was exteriorized and punctured to transfer the embryos in groups of 6–7 embryos per uterus. Antibiotherapy with enrofloxacin (Baytril, Bayer) was maintained for 5 days after surgery.

Embryo viability after cryopreservation and transfer to recipient does was evaluated by the ability of the embryos to develop into live young rabbits born at term.

2.10. *Statistical analysis*

Differences among treatment groups were calculated by means of the Chi-square or Fischer's exact tests where appropriate. The overall Chi-square tests were calculated before the Fischer's exact test to detect differences between treatment groups. All statistical analyses were computed using GraphPad InStat (version 3.01 for Windows 95, GraphPad Software, San Diego, California, USA). Differences with a probability value of 0.05 or less were considered significant.

3. Results

3.1. *Experiment 1*

After exposure to the 25EG:SUC solution, the embryo samples cryopreserved in standard straws by direct immersion in liquid nitrogen crystallized: the entire volume

Table 1

Rates of morphologically normal rabbit embryos at the morula, compacted morula, early blastocyst and blastocyst stage cryopreserved in two cryoprotectant solutions in standard straws or mOPS

Cryoprotectant solution	Container	Cryopreserved embryos (<i>n</i>)	Morphologically normal embryos	
			<i>n</i>	(%)
25GLY:25EG	Straws	425	345	81.2 ^a
	mOPS	420	376	89.5 ^b
	Total	845	721	85.3 ^c
25EG:SUC	Straws	425	325	76.5 ^d
	mOPS	425	369	86.8 ^e
	Total	850	694	81.6 ^f
	Total straws	850	670	78.8 ^g
	Total mOPS	845	745	88.2 ^h

25GLY:25EG = 25% glycerol + 25% ethylene glycol. 25EG:SUC = 4.5 M EG (approximately 25% EG) + 0.25 M sucrose. Different superscripts within the same column denote significant differences detected by the Fischer's exact test (a and b, d and e: $P < 0.001$; c-f: $P = 0.04$; g and h: $P < 0.0001$). Rates were calculated after thawing and withdrawal of the cryoprotectant.

turned intensely opaque white after freezing and warming. Hereafter, this procedure will be referred to as rapid freezing. Vitrification occurred in samples cryopreserved in mOPS and exposed to 25EG:SUC or in those exposed to 25GLY:25EG, both in standard straws or mOPS: the straw section containing the embryos remained transparent after immersion into liquid nitrogen and warming in air and water, and the procedures will be referred to as vitrification.

Regardless of embryonic stage, the rates of morphologically normal embryos evaluated immediately after thawing and removal of the cryoprotectant are provided in Table 1. Embryos cryopreserved in mOPS showed significantly higher rates of survival than those cryopreserved in 0.25 ml standard straws for both cryoprotectant solutions. A significantly higher rate of survival was recorded for the embryos exposed to the 25GLY:25EG than the 25EG:SUC solution.

Table 2 shows the rates of in vitro development of rabbit embryos after nonequilibrium cryopreservation at different developmental stages in the two cryoprotectant solutions using 0.25 ml standard straws or mOPS. The developmental stage of the cryopreserved embryos significantly affected the viability rates. Regardless of the cryoprotectant used, lower rates of in vitro development were obtained when the embryos were cryopreserved at the morula stage, and higher rates achieved using embryos at blastocyst stages.

Irrespective of the type of container, the rate of in vitro development to the expanded blastocyst stage was significantly higher for embryos cryopreserved at the blastocyst stage after exposure to the 25GLY:25EG solution than for those exposed to the 25EG:SUC solution. No significant differences were observed between cryoprotectant solutions when the embryos were cryopreserved at other developmental stages.

In general, the embryos cryopreserved in mOPS after exposure to the 25EG:SUC solution showed a significantly higher rate of development to the expanded blastocyst stage

Table 2

In vitro development of rabbit embryos at the morula, compacted morula, early blastocyst or blastocyst stage after nonequilibrium cryopreservation in two cryoprotectant solutions using 0.25 ml standard straws or mOPS

Cryoprotectant solution	Container	Developmental stage of cryopreserved embryos									
		Morula		Compacted morula		Early blastocyst		Blastocyst		Total	
		<i>n</i>	Dev to eBlast. <i>n</i> (%)	<i>n</i>	Dev to eBlast. <i>n</i> (%)	<i>n</i>	Dev to eBlast. <i>n</i> (%)	<i>n</i>	Dev to eBlast. <i>n</i> (%)	<i>n</i>	Dev to eBlast. <i>n</i> (%)
25GLY:25EG	Straws	85	42 (49.4%)	86	60 (69.8%)	86	62 (72.1%)	88	71 (80.7%)	345	235 (68.1%)
	mOPS	98	50 (51.0%)	97	68 (70.1%)	91	76 (83.5%)	90	79 (87.8%)	376	273 (72.6%)
	Total ^a	183	92 (50.3%)	183	128 (69.9%)	177	138 (78.0%)	178	150 (84.3%) ^b	721	508 (70.5%)
25EG:SUC	Straws	87	38 (43.7%)	81	52 (64.2%)	72	52 (72.2%)	85	61 (71.8%)	325	203 (62.5%) ^d
	mOPS	97	54 (55.7%)	91	65 (71.4%)	89	67 (75.3%)	92	72 (78.3%)	369	258 (69.9%) ^e
	Total ^a	184	92 (50.0%)	172	117 (68.0%)	161	119 (73.9%)	177	133 (75.1%) ^c	694	461 (66.4%)

25GLY:25EG = 25% glycerol + 25% ethylene glycol. 25EG:SUC = 4.5 M EG (approximately 25% EG) + 0.25 M sucrose. Dev to eBlast.: number of cryopreserved embryos that continued development in vitro to the expanded blastocyst stage. Different superscripts within the same column denote significant differences detected by the Fischer's exact test (b and c: $P = 0.01$; d and e: $P = 0.04$).

^a Proportions were different ($P < 0.0001$) when compared in a 4×2 contingency table using the Chi-square test.

than those cryopreserved in standard straws. No significant differences were recorded between types of container when the 25GLY:25EG solution was used.

3.2. Experiment 2

Live offspring rates showed no significant difference for embryos vitrified in the 25GLY:25EG solution (51.7%, 123 young rabbits from 238 transferred embryos in 15 pregnant of 17 recipient does) and fresh control embryos (58.5%, 131 young rabbits from 224 transferred embryos in 15 pregnant of 16 recipient does). A significantly higher live offspring rate ($P < 0.05$) was noted when embryos were cryopreserved in the 25GLY:25EG, compared to the 25EG:SUC solution (42.0%, 100 young rabbits from 238 transferred embryos in 12 pregnant of 17 recipient does).

4. Discussion

The present findings indicate that rabbit embryos can be successfully cryopreserved in mOPS, i.e. sealed OPS. The mOPS employed in this study were prepared in a similar way to the method used by Vajta et al. [18] in the original OPS procedure, but one end of the straw is kept wide to preserve the cotton plug and the narrower section is sealed with polyvinyl-alcohol to reduce the risk of contamination via the liquid nitrogen. Thus, the mOPS have sealed ends and the embryos are loaded by capillarity into a small volume of solution between two columns of cryoprotectant. Conditions remain sterile since there is no direct contact between the embryos and liquid nitrogen. The use of sealed containers has been shown to be an effective measure against contamination of embryos during their storage in contaminated liquid phase nitrogen [12].

As in the original OPS procedure, the embryos loaded in the mOPS were aspirated into a pulled region of the standard straw. This increases cooling and warming rates due to the reduced volume of the solution that contains the embryos and the diminished thickness of the mOPS wall [18]. In our study, this increase in the rate of cooling and warming caused a cryoprotectant solution generally used in rapid freezing procedures, 4.5 M (approximately 25%) ethylene glycol (EG) plus 0.25 M sucrose (25EG:SUC), to vitrify in mOPS, yet in standard 0.25 ml straws, this solution crystallizes, determined by visual evidence of vitrification or ice formation respectively [21,23]. This finding may be of potential interest; using mOPS instead of standard straws, it may be possible to achieve the vitrification of a solution at a lower cryoprotectant concentration, reducing the potential toxicity of the solutions generally used in vitrification procedures.

The results of this study show that embryos cryopreserved in mOPS have a significantly much higher rate of survival immediately after thawing than embryos cryopreserved in standard straws, improving the total efficiency of the process. Vajta et al. [18] demonstrated that the use of OPS decreased the risk of zona pellucida and embryo fracture, compared to standard straws in rapid cooling and warming procedures. Our results show that the mOPS procedure further reduces the problems associated with damage to the noncell layers of rabbit embryos. These layers are essential for implantation of rabbit embryos [28].

It was possible to confirm that the developmental stage of the cryopreserved embryo significantly affects the viability rates of rabbit morulae and blastocysts. This effect is generally observed when rabbit embryos are cryopreserved at earlier stages (1- to 4-cell embryos) [16,29,30]. The ratio of cell surface to volume is one of the major factors determining survival following cryopreservation [31]. Thus, the factors blastomere size and membrane permeability to water and cryoprotectants have been related to the reduced viability of embryos cryopreserved at initial stages of development [32–34]. Our results show that using the solutions 25% glycerol (GLY) plus 25% EG (25GLY:25EG) or 25EG:SUC and the mOPS or standard straw methods, the blastocyst stages yield best results compared to earlier stages of development, for the cryopreservation of rabbit embryos. The present results also confirm that OPS technology can be successfully applied to several cryoprotectant solutions.

After assessing the capacity of cryopreserved blastocysts to continue development *in vivo*, embryos cryopreserved in mOPS and exposed to the 25GLY:25EG solution following the protocol described by Donnay et al. [19] and Kaidi et al. [20], showed live offspring rates (51.7%) not significantly different to those of the control group (58.5%). The 51.7% live offspring rate of transferred embryos using this cryopreservation procedure is much higher than the 33.3% reported by Zhu et al. [35] and the 25% reported by Vicente and Viudes-de-Castro [36] after transfer of blastocysts vitrified in a solution composed of glycerol and 1,2-propanediol or EG and DMSO respectively. It is likely that, irrespective of the cryoprotectant used, our high live offspring rate may be attributed to the reduced exposure time to the cryoprotectant solution of the mOPS method.

In conclusion, our findings indicate that the cryopreservation of rabbit blastocysts using a mixture of 25% glycerol and 25% ethylene glycol as the cryoprotectant solution and a modified (sealed) OPS method yields similar live offspring rates to those achieved with the use of control fresh embryos. Moreover, the use of mOPS was associated with the vitrification of cryoprotectant solutions at lower concentrations of cryoprotectants than those generally used in vitrification procedures.

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Corrigendum

Corrigendum to “Nonequilibrium cryopreservation of rabbit embryos using a modified (sealed) open pulled straw procedure”
[Theriogenology 58 (2002) 1541–1552]★

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The Author regrets that an error occurred in Fig. 1 and Section 2.3. of the above paper. The correct figure and section follow. The Author wishes to apologise for any inconvenience caused by this error.

2.3. Preparation of modified open pulled straws

The preparation of mOPS was similar to that described previously by Vajta et al. [18] for OPS. Standard straws (0.25 ml; IMV, L’Aigle, France) were heat-softened over a hot plate. The central part of the straw was pulled manually until its inner diameter and wall thickness decreased from 1.7 mm to approximately 0.9 mm, and from 0.15 to 0.07 mm, respectively. The straws were cut at the narrowest point with a razor blade. Only the half with the cotton plug was used. The narrow portion of the mOPS was of mean length 1.5 cm (range 1.2–1.8 cm), and the wider section was 3.5 cm (range 3.1–3.7 cm) long (Fig. 1). The embryos were aspirated into a column located between two columns of cryoprotectant solution separated by air bubbles. Embryos were loaded into the mOPS by capillary action; a 2 µl droplet containing the embryos was simply touched with the narrow end of the straw.

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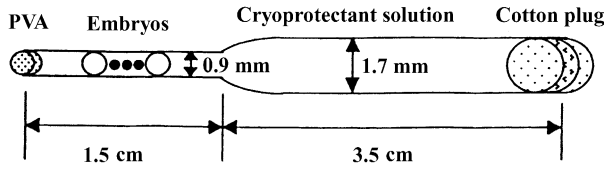


Fig. 1. Scheme of a mOPS containing embryos between two columns of vitrification solution separated by air bubbles (PVA: polyvinyl-alcohol).