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The harmful effect of removing the extracellular vitrification medium during embryo cryopreservation using a nylon mesh device in rabbit

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Abstract

During the last decades, many techniques have been developed to reduce sample volume and improve cooling and warming rates during embryo vitrification. The vast majority are based on the "minimum drop size" concept, in which the vitrification solution around embryos is reduced by aspiration, leaving a tiny part of volume surrounding embryos. However, novel cryodevices were aimed to remove the entire vitrification solution. This study was designed to compare the "minimum drop size" technique using Cryotop® with the nylon mesh as cryodevice on rabbit morula embryos. The outcomes assessed were the *in vitro* development rates (experiment 1) and the offspring rates at birth (experiment 2). Embryos were vitrified in a two-step procedure; equilibrium (10% EG + 10% Me2SO) for 2 min and vitrification (20% EG + 20% Me2SO) for 1 minutes. In experiment 1, embryos (n= 323) were warmed and subsequently *in vitro* cultured for 48 h to assess the embryo developmental capability to reach the hatching-hatched blastocyst stage. In experiment 2, embryos were transferred using the laparoscopic technique (n=369) to assess the offspring rate at birth. In this context, rates of *in vitro* embryo development were similar between vitrified groups (0.73±0.042% and 0.66±0.047% for Cryotop® and nylon mesh device, respectively), but lower than in the fresh group (0.97±0.016%, $p<0.05$). In experiment 2, there were no significant differences in survival rates (offspring born/total embryos transferred) among the Cryotop® device group and fresh group (0.41±0.049% and 0.49±0.050%, respectively). But significantly lower value was obtained in the nylon mesh device group (0.18±0.030%). These results indicate that nylon mesh is not suitable as cryodevice for rabbit morula vitrification, remaining those using the "minimum drop size" methodology as the best option.

Keywords: Rabbit; vitrification; device; nylon; cryotop

Abbreviations:

Nylon mesh (NM)

Cryotop® (CT)

Vitrification solution (VS)

1. Introduction

Cryobiology aim to preserve biological samples without lack of viability. This concept has made a notable impact in many fields, with reproductive science possible the most significant [9]. Reproductive cryotechniques have progressed rapidly since its first use almost three centuries ago, maximizing the availability of reproductive material and thereby and facilitating reproductive procedures independently of time and geographic location [4,17]. Rall and Fahy introduce vitrification as a cheap and straightforward way to cryopreserve mammalian embryos in the absence of ice [20]. This strategy uses ultra-rapid cooling to transform the liquid into a glass, minimizing the time in which the sample is exposed to the temperature ranges associated with chilling injury and ice crystal formation. Therefore, reducing the volume to the value of the minimum drop size increases the probability of vitrification by allowing more significant cooling and warming rates [2]. After vitrification was widely applied in livestock and human embryos [2,10], this is why many techniques have been developed to reduce sample volume during last decades, appearing a multitude of cryopreservation devices in the literature with this aim [2,10,21].

So far, based on high survival and developmental rates in the vast majority of species (mice, rabbits, pigs, cattle, buffalo, and humans), Cryotop® (CT) vitrification methodology is widely used for oocytes and embryos cryopreservation [10]. CT methodology involves a minimal volume of cryoprotectants (<0.1 μL), allowing very high cooling (69.250 °C/min) and thawing (117.500 °C/min) rates [14]. In CT, embryos are placed on the polyethene terephthalate film, and the excess of vitrification solution (VS) is removed by aspiration with a pipette before the carrier was immersed in LN2.

However, this method requires skill to withdraw any VS surplus around the embryos and, even so, a tiny part of VS remains surrounding oocytes or embryos [28]. Momozawa et al. [15,16] hypothesized that this extracellular VS might be unnecessary after oocyte or embryos equilibration, and could adversely affect the attainment of required ultra-rapid cooling rates during vitrification. Confirming their hypothesis, this group recently has described a novel device composed of acrylonitrile butadiene styrene resin that remove the excess of VS, allowing to reach extremely high cooling (683.000 ºC/min) and warming (612.000 ºC/min) rates [15], which exceed those of the minimum volume devices as CT. Using this concept, nylon mesh (NM) vitrification devices have been used to successfully vitrify bovine oocytes [1,5,13], human embryos [19], as well as ovarian follicles [6] and pancreatic islets [27]. Placing NM on absorbent surfaces, the VS excess was easy removed through the NM pores before the sample was plunged into the LN2.

The aim of this study was to compare a novel handmade NM cryodevice and Cryotop® to vitrify rabbit embryos.

2. Materials and Methods

All chemicals, unless otherwise stated, were reagent-grade and purchased from Sigma-Aldrich Química S.A. (Alcobendas, Madrid, Spain).

2.1. Experimental design

Rabbit embryos were used to test two cryodevices: one based on the “minimum drop size” method (Cryotop®), and the other conceived to remove the vitrification solution

surrounding embryos (nylon mesh). Both cryodevices were compared attending to the post-thawing embryo developmental potential *in vitro* (experiment 1) and *in vivo* (experiment 2). The experimental design was illustrated in Fig. 1.

2.2. Animals and ethical statement

New Zealand White rabbits were used. Animals were housed at the Universitat Politècnica de València experimental farm in flat deck indoor cages (75×50×45 cm), with free access to water and commercial pelleted diets (minimum of 15 g of crude protein per kg of dry matter (DM), 15 g of crude fibre per kg of DM, and 10.2 MJ of digestible energy (DE) per kg of DM). The photoperiod is set to provide 16 h of light and 8 h of dark, and the room temperature is regulated to keep temperatures between 14°C and 28°C.

All the experimental procedures used in this study were performed under Directive 2010/63/EU EEC for animal experiments and reviewed and approved by the Ethical Committee for Experimentation with Animals of the Universitat Politècnica de València, Spain (research code: 2015/VSC/PEA/00061).

2.3. *In vivo* embryo production and collection

A total of 47 nulliparus females were used in 12 experimental sessions (3-5 female by session). Females were superovulated using 3 µg subcutaneous of corifollitropin alfa (Elonva, Merck Sharp & Dohme S.A.; Spain), a recombinant long-acting human FSH highly effective in rabbits [25]. After 72 hours, females were artificially inseminated, with a heterospermic pool of semen to randomize male effect, and ovulation was induced by

an injection of 1 μg of buserelin acetate (Hoechst Marion Roussel S.A., Madrid, Spain). After 72 hours, females were euthanized and embryos (late morula-early blastocyst) were collected at room temperature by flushing the oviducts and uterine horns with Dulbecco phosphate buffered saline (DPBS), supplemented with 0.2% (wt/vol) bovine serum albumin (BSA) and antibiotics (penicillin G sodium 60IU/mL, penicillin G procaine 140 IU/mL and dihydrostreptomycin sulphate 0.250 mg/mL; Penivet 1; Divasa Farmavic, Barcelona, Spain). After recovery, embryos were classified as normal (presenting homogenous cellular mass, mucin coat and spherical zona pellucida) and were pooled to randomize embryo effect.

2.4. Vitrification and warming procedure

Embryos were vitrified using the vitrification procedure described by Marco-Jiménez et al. [10] for two devices (Fig. 2): Cryotop® (CT; Kitazato Corp., Shizuoka, Japan) and handmade nylon mesh (NM) device. The CT consists of a flat rectangular leaf of polypropylene (0.4 mm wide × 20 mm long × 0.1 mm thick) attached to a hard plastic handle [8]. Moreover, the thin strip is covered with a hard plastic cover on top of the CT sheet to protect it during storage in nitrogen containers. NM device consists of a flat rectangular leaf of NM (1.5 mm wide x 20 mm long x 0.1 mm thick, with 75 μm opening size) form by laser-cut attached to a plastic handle create using a 3D printer equipped with a cover. In this device, the excess of VS can be removed using a sterilized paper towel (cellulose fibbers) through the pore of the NM via capillary action, eliminating the VS completely around the embryos (Fig. 3).

Embryos were vitrified in a two-step procedure. First, embryos were transferred into equilibration solution consisting of 10% (vol/vol) ethylene glycol and 10% (vol/vol) dimethyl sulfoxide dissolved in base medium (BM; DPBS supplemented with 0.2% [wt/vol] BSA) at room temperature for 2 minutes. After that, embryos were transferred to vitrification solution consisting of 20% (vol/vol) ethylene glycol and 20% (vol/vol) dimethyl sulfoxide in BM. Then embryos were loaded into cryopreservation devices (Fig. 4) and directly plunged into liquid nitrogen within 1 minute. In the case of the CT, the maximum possible amount of vitrification medium was carefully removed, leaving <0.1 µL of VS, but in the case of the NM, the totality of the VS was removed placing the NM on an absorbent sterilized paper towel.

After storage in liquid nitrogen, embryos were warmed by sudden immersion of the devices in 2 mL of 0.33 M sucrose at 25 ºC in BM. After 5 minutes, the embryos were washed in BM. Warming embryos were scored, and only undamaged embryos (presenting homogenous cellular mass, mucin coat and spherical zona pellucida) were catalogued as culturable (for in vitro experiment) or transferable (for in vivo experiment).

2.4. Effect of vitrification device on *in vitro* development

A total of 213 vitrified embryos (110 CT and 103 NM devices) and 110 fresh embryos were cultured through 7 experimental sessions for 48 h in medium TCM199 containing 10% (v/v) Fetal Bovine Serum (FBS) and 1% (vol/vol) of antibiotics (penicillin G sodium 300 000 IU/L, penicillin G procaine 700 000 IU/L, and dihydrostreptomycin sulphate 1250 mg/L; Divasa Farmavic, Barcelona, Spain) at 38.5ºC and 5% CO_2 in humidified

atmosphere. The *in vitro* development ability until hatching/hatched blastocyst stage was recorded for analysis.

2.5. Effects of vitrification device on offspring rate at birth

A total of 268 vitrified embryos (100 CT and 168 NM devices) and 101 fresh embryos were transferred into 12 adult nulliparous females. Only receptive females (determined by vulva colour) were induced to ovulate by injection of 1 μg of buserelin acetate (Hoescht, Marion Roussel, Madrid, Spain), 64-66 hours before the transfer. In rabbits, this asynchrony between foster mothers and embryos incur beneficial effects by allowing time for the restoration of cell physiology and replacement of embryo damages caused during embryo manipulation [7]. To sedate the does during laparoscopy, anaesthesia was administered by an intramuscular injection of 4 mg/Kg of xylazine (Bayer AG, Leverkusen, Germany), followed 5-10 min later following intravenous injection into the marginal ear vein of 0.4 ml/ Kg of weight of ketamine hydrochloride (Imalgène 500, Merial SA, Lyon, France). During laparoscopy, 3 mg/kg of morphine hydrochloride (Morfina, B. Braun, Barcelona, Spain) was administered intramuscularly. After transfer, does were treated with antibiotics (4mg/Kg of gentamicin every 24h for 3 days, 10% Ganadexil, Invesa, Barcelona, Spain) and analgesics (0.03 mg/Kg of buprenorphine hydrochloride, [Buprex, Esteve, Barcelona, Spain] every 12 hours for 3 days and 0.2 mg/Kg of meloxicam [Metacam 5mg/mL, Norvet, Barcelona, Spain] every 24h for 3 days). Embryo transfer was performed using the laparoscopic technique as described previously [3,7]. The number of embryos transferred was 10-12 (five-six embryos into each oviduct). Offspring born/total embryos transferred were annotated at parturition. Furthermore, the individual weight of the kits was recorded.

2.6. Statistical analysis

A generalised linear model, including the experimental group (CT, NM, Fresh) as fixed effects were used. The error was designated as having a binomial distribution using probit link function. Binomial data for blastocyst rate and offspring rate at birth were assigned as 1 if positive development had been achieved or a 0 if it had not. Kit birth weights were analysed using a generalised linear model including the experimental group (CT, NM, Fresh) as fixed effects and the litter size as a covariate. A P-value of less than 0.05 was considered to indicate a statistically significant difference. The data are presented as least square means ± standard error mean. All statistical analyses were carried out using a commercially available software program (SPSS 21.0 software package; SPSS Inc., Chicago, Illinois, USA, 2002).

Results

As shown in Table 1, rates of embryo development to the hatching/hatched blastocyst stage after 48h of *in vitro* culture were similar between vitrified groups but significantly lower ($p<0.05$) than in the fresh group: 0.73±0.042 (embryos vitrified using CT), 0.66±0.047 (embryos vitrified using NM) and 0.97±0.016 (fresh embryos).

As shown in Table 2, the offspring rate at birth was similar ($p>0.05$) between the CT group (0.41±0.049%) and in the fresh control (0.49±0.050%). However, lower ($p<0.05$) offspring rate were recorded in the NM group (0.18±0.030%). Birth weight was not

affected by the vitrification devices (58.6±1.9g, 54.4±2.8g and 60.0±1.8g, for CT, NM and fresh group, respectively).

Discussion

Over the last decade, there has been a trend to reduce the volume of the vitrification solution around the embryos to increase cooling and warming rates and achieve successful post-thawing survival [10, 22]. Then, the "minimum drop size" methodology has been widely used [2,5,10,21]. However, removing entire VS immensely increase cooling and warming rates [15]. Therefore, here we compared the "minimum drop size" strategy with a new nylon mesh (NM) cryodevice, which placed on an absorbent surface allows to remove the totality of the VS surrounding embryos.

NM cryodevices has been previously used to cryopreserve bovine oocytes [1,5,13] and human embryos [19], reporting high post-thawing in vitro developmental competence. In concordance with Chinen et al. [5], here we observed that vitrified embryos using NM cryodevice showed similar *in vitro* developmental rates compared to CT, which were similar to those previously reported in rabbits [10,12]. However, to the best of our knowledge, here we describe the first offspring born using NM cryodevices removing VS. After performing the *in vivo* experiment, we observed that NM offers lower offspring rates at birth than devices using the "minimum drop size" methodology (Cryotop®). Our results revealed a high *in vivo* survival rate with those devices retaining a small part of VS envelope the embryo. Probably, if a tiny part of VS remains surrounding embryos it could be beneficial, since the "minimum drop size" was conceived as the minimal volume that maintained oocytes or embryos without damage owing to desiccation [2]. A new

device develops by Momozawa et al., that consisting of a porous membrane in which is possible minimized more the surrounding solution in comparison to that of Cryotop® does not seem to affect the viability of human embryos [24], and offspring rate in the mouse embryo [15]. In this study, the excess of VS was removed by quickly sucked with an absorbent paper towel from the bottom of the nylon mesh via capillary action. Although this approach requires careful attention of VS properties, as shifts in osmolality due to soluble compounds of the towel (sugars) are likely, we would discard an adverse effect because this new device resulted in similar in vitro embryo development in comparison to that of Cryotop®. Nevertheless, a plausible dryness-excess effect could be harmful to the embryo, being necessary a minimum volume of VS surrounding to successful vitrification. Hence, further studies will be needed both to asses dryness-excess effect and to design other procedures to remove the excess VS and so to evaluate potential risk effects of soluble compounds of paper towel.

Another plausible explanation for our results could be the injury-induced on mucin coat. Another plausible explanation for our results could be the injury-induced on mucin coat. Rabbit embryos are surrounded not only by the zona pellucida but also by a mucin coat, a layer of acid mucopolysaccharides that are deposited on the embryos during the passage through the oviduct [7,11]. This mucin coat is a fundamental factor allowing embryo implantation, as it takes place after the remodelling of the embryonic coatings during blastocyst expansion in the uterine horns [7,18]. Thereby, we support that VS removing can exerted a dryness-excess effect on the rabbit embryo surface, causing desiccation and unstructuring damages on the mucin coat. Furthermore, excessive pressure generated during VS removing through NM pores could induce tension over embryo surface, generating also mechanical remodeling of the mucin coat. Thus, due to

most of the damage during rabbit embryo cryopreservation occurred on the mucin coat without damaging the blastomeres [23], NM vitrified embryos has competent in vitro developmental rates. However, it fails significantly during in vivo development at term, where intact mucin coat is essential for implantation. Concordantly, there is known that alterations in the mucin coat diminish *in vivo* development drastically, being the primary factor of embryonic loss in cryopreserved rabbit embryos [18,23,26]. Typically, asynchronous oviductal embryo transfer allows to restore defects in the mucin coat after in vitro manipulation [7], but extensive disturbances on the embryo coverage could be irreversible. Thereby, as improved implantation rate has been described in human embryos after its vitrification on NM [19], our results suggest that our lower offspring rate may response to a specific and singular case for the rabbit. Of note, as smaller pore size incurs in better in vitro competence [5], so future studies should determine if reduced pore size leads with improved outcomes. Without skilful requirements, NM cryodevices allowed to easily remove excess of VS volume immediately before the ultra-rapid cooling, facilitating the vitrification process. Therefore, this advantages could be of special interest for other species rather than for rabbit.

Conclusions

In conclusion, we report the first evidence of offspring rates after embryo cryopreservation using nylon mesh. However, NM is not suitable as cryodevices for rabbit embryo vitrification, remaining those using the "minimum drop size" methodology as the best option. However, the results here obtained seems to respond to a specific rabbit case. In spite of, the use NM cryodevices would overcome a few disadvantageous aspects of these devices like Cryotop®, such as the difficulty of minimizing VS volume

(operator-dependent), the limited sample number per device and the relatively high cost. Nevertheless, future study should evaluate the NM cryodevice applicability in another species, elucidating the effectiveness of removing the excess of vitrification solution.

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Additional information

The authors declare no competing financial interests.

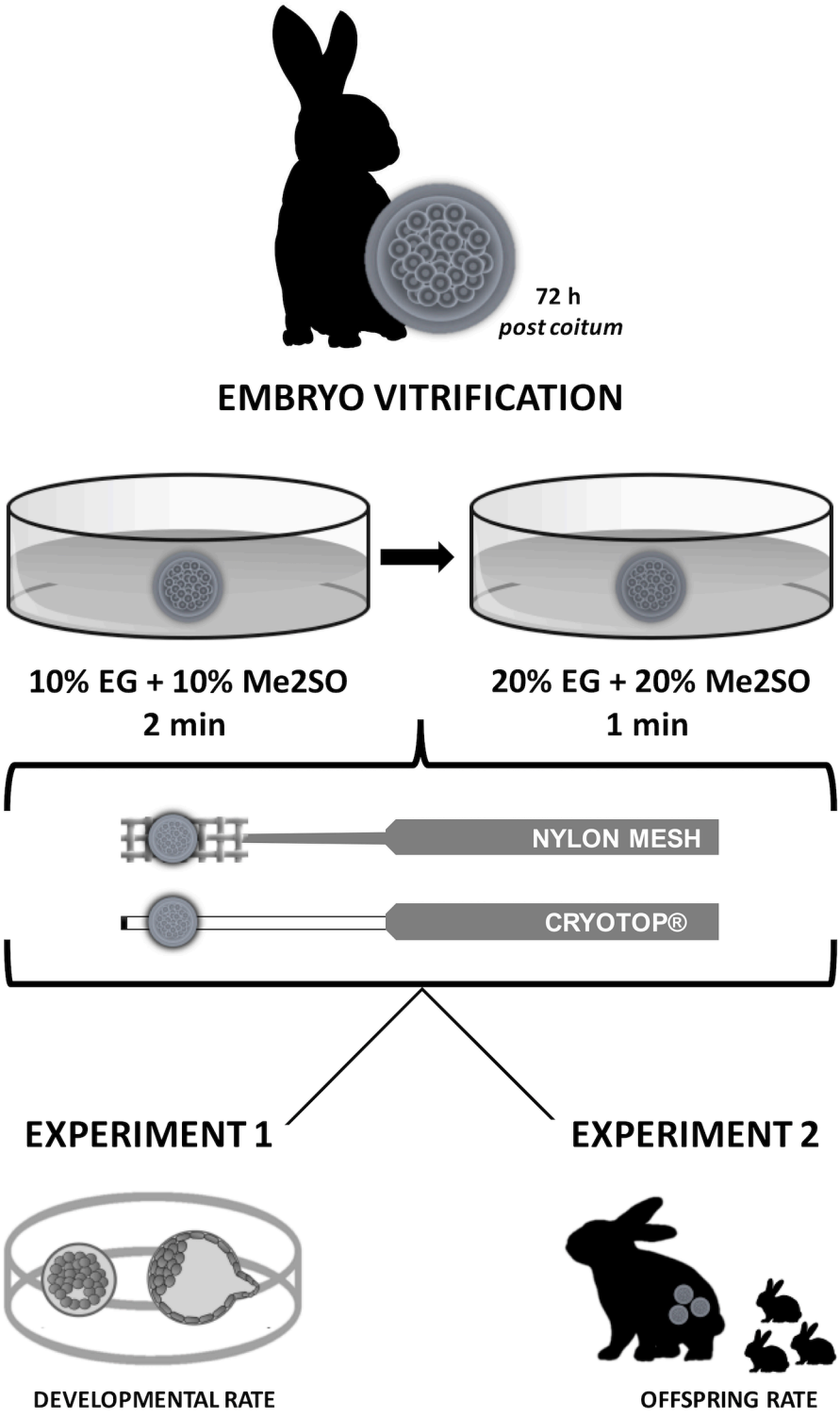
Figure Legends

Fig. 1. Experimental design. Rabbit embryos were vitrified using two cryodevices (Cryotop® vs Nylon mesh), which were compared in terms of *in vitro* developmental rate (experiment 1) and *in vivo* offspring rate (experiment 2).

Fig. 2. Image showing cryodevices: handmade nylon mesh and Cryotop®. (A) Both devices with the corresponding covers. Scale bar: 1 cm. (B) Both devices without the corresponding covers. Scale bar: 1 cm. (C) Detail of the vitrification surface of both devices with the corresponding covers. Scale bar: 0.5 cm. (D) Detail of the vitrification surface of both devices without the corresponding covers. Scale bar: 0.5 cm.

Fig. 3. Vitrification method using cryodevices based on (A) the "minimum drop size" concept (Cryotop®) or (B) those removing extracellular solution using absorbing surfaces (nylon mesh).

Fig. 4. Magnified images showing in details embryos loading on both cryodevices: (A) Nylon mesh. Scale bar: 100 µm. (B) Cryotop®. Scale bar: 150 µm.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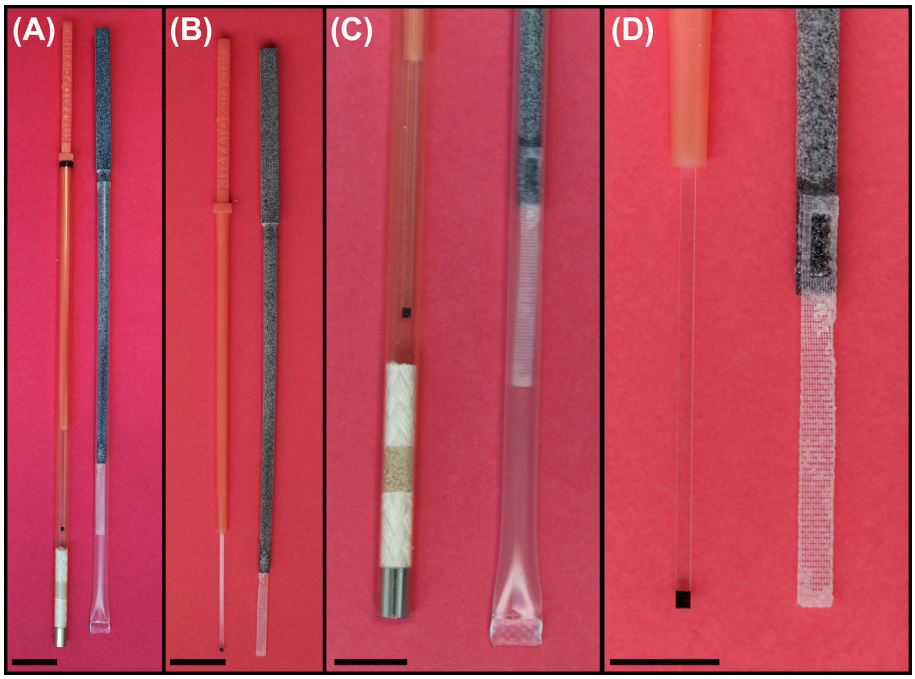
10% EG + 10% Me2SO
2 min
20% EG + 20% Me2SO
1 min
NYLON MESH
CRYOTOP®
EXPERIMENT 1
EXPERIMENT 2
DEVELOPMENTAL RATE
OFFSPRING RATE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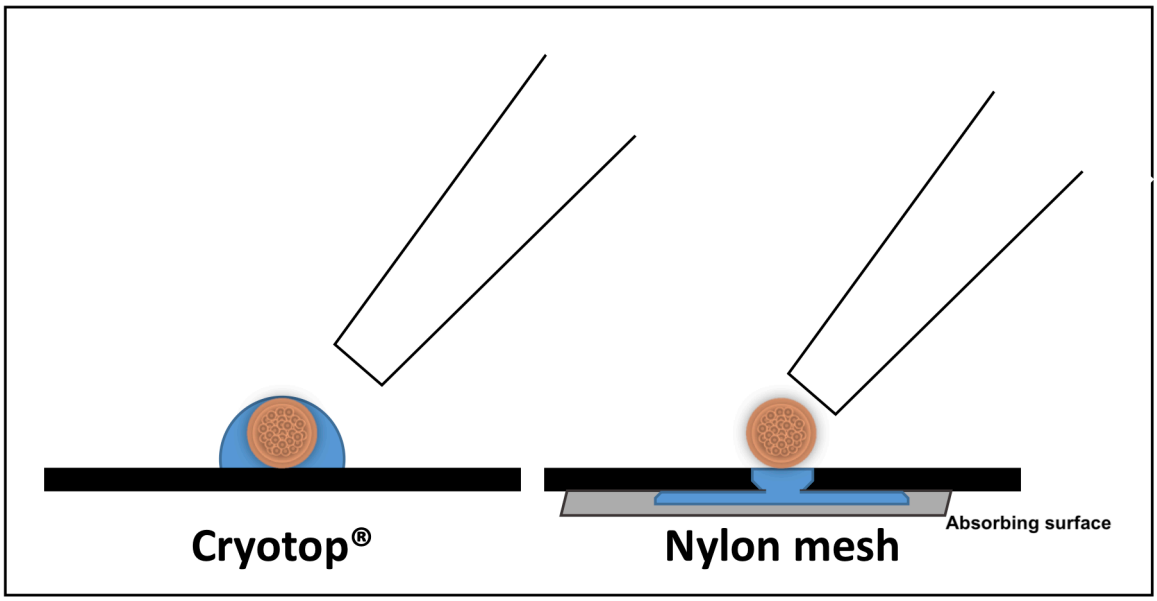
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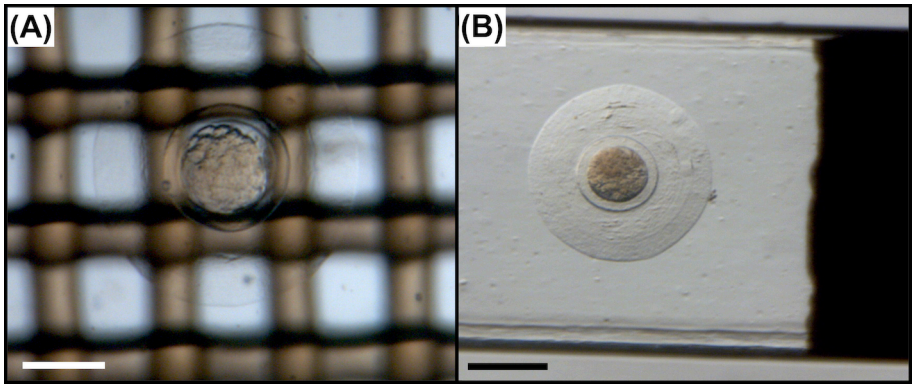
(A)
(B)

Table 1. Effect of vitrification device after 48 h of *in vitro* culture.

Experimental Group	n	Developmental rate
Nylon mesh	103	0.66 ± 0.047^{b}
Cryotop®	110	0.73 ± 0.042^{b}
Fresh	110	0.97 ± 0.016^{a}

n: number of cultured embryos. a,b: Data in the same column with uncommon letters are different ($p < 0.05$). Data are presented as least squares means ± standard error of the least squares means.

Table 2. Effect of vitrification device on offspring rate at birth and kit weight.

Experimental Group	n	Offspring rate
Nylon mesh	168	0.18 ± 0.030^{b}
Cryotop®	100	0.41 ± 0.049^{a}
Fresh	101	0.49 ± 0.050^{a}

n: number of transferred embryos. a,b: Data in the same column with uncommon letters are different ($p < 0.05$). Data are presented as least squares means ± standard error of the least squares means.