Journal of Visualized Experiments

Minimally Invasive Embryo Transfer and Embryo Vitrification at the Optimal Embryo Stage in Rabbit Model

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58055R1
Full Title:	Minimally Invasive Embryo Transfer and Embryo Vitrification at the Optimal Embryo Stage in Rabbit Model
Keywords:	Embryo, embryo transfer, laparoscopy, vitrification, cryopreservation, rabbit
Corresponding Author:	Francisco Marco-Jimenez Universitat Politecnica de Valencia Valencia, SPAIN
Corresponding Author's Institution:	Universitat Politecnica de Valencia
Corresponding Author E-Mail:	fmarco@dca.upv.es
First Author:	Francisco Marco-Jimenez
Other Authors:	Ximo Garcia-Dominguez
	Maria Pilar Viudes-de-Castro
	Jose Salvador Vicente
Author Comments:	
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

JOVE JOURNAL OF VISUALIZED EXPERIMENTS
17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

Standard Manuscript Template

TITLE:

Minimally Invasive Embryo Transfer and Embryo Vitrification at the Optimal Embryo Stage in Rabbit Model

AUTHORS:

Ximo Garcia-Dominguez[1], Francisco Marco-Jiménez[1], Maria Pilar Viudes-de-Castro[1], Jose Salvador Vicente[1]

1: Departamento de Ciencia Animal, Universitat Politècnica de Valencia, Valencia, Spain

ximo.garciadominguez@gmail.com
fmarco@dca.upv.es
viudes_mar@gva.es
jvicent@dca.upv.es

CORRESPONDING AUTHOR:

Vicente, José Salvador, Ph.D.

KEYWORDS:

Embryo, embryo transfer, laparoscopy, vitrification, cryopreservation, rabbit

SHORT ABSTRACT:

Assisted reproductive techniques (ARTs) are in continuous evaluation to improve outcomes and reduce the associated risks. This manuscript describes a minimally invasive embryo transfer procedure with an efficient cryopreservation protocol that allows the use of rabbits as an ideal animal model of human reproduction.

LONG ABSTRACT:

Assisted reproductive techniques (ARTs), such as *in vitro* embryo culture or embryo cryopreservation, affect natural development patterns with perinatal and postnatal consequences. To ensure the innocuousness of ART applications, studies in animal models are necessary. In addition, as a last step, embryo development studies require evaluation of their capacity to develop full-term healthy offspring. Here, embryo transfer to the uterus is indispensable to perform any ARTs-related experiment.

The rabbit has been used as a model organism to study mammalian reproduction for over a century. In addition to its phylogenetic proximity to the human species and its small size and low maintenance cost, it has important reproductive characteristics such as induced ovulation, a chronology of early embryonic development similar to humans and a short gestation that allow us to study the consequences of ART application easily. Moreover, ARTs (such as intracytoplasmic sperm injection, embryo culture, or cryopreservation) are applied with suitable efficiency in this species.

Using the laparoscopic embryo transfer technique and the cryopreservation protocol presented in this article, we describe 1) how to transfer embryos through an easy, minimally invasive technique and 2) an effective protocol for long-term storage of rabbit embryos to provide time-flexible logistical capacities and the ability to transport the sample. The outcomes obtained after transferring rabbit embryos at different developmental stages indicate that morula is the ideal stage for rabbit embryo recovery and transfer. Thus, an oviductal embryo transfer is required, justifying the surgical procedure. Furthermore, rabbit morulae are successfully vitrified and laparoscopically transferred, proving the effectiveness of the described techniques.

INTRODUCTION:

With the aims of bypassing human infertility or improving dissemination of livestock of high genetic value and preserving animal genetic resources, a set of techniques collectively termed assisted reproduction technologies, such as superovulation, *in vitro* fertilization, embryo culture, or cryopreservation, were developed[1,2]. Currently, hormonal treatments are given to stimulate the ovaries and produce a large number of antral ovarian follicles[1]. Oocytes collected from these follicles can be matured, fertilized, and developed *in vitro* until they are either cryopreserved or transferred to surrogate mothers[3]. However, during these treatments, gametes and zygotes are exposed to a series of non-physiological processes that could require embryo adaptation to survive in these conditions[4,5]. This adaptation is possible due to early embryo plasticity, which allows embryo changes in gene expression and developmental programming[6]. However, these modifications can influence the subsequent stages of embryo development until adulthood, and it is now widely accepted that methods, timing, cryopreservation procedure or culture conditions show different outcomes on embryo fate[7,8]. Therefore, to elucidate the specific induced effects of ARTs, the use of well-characterized animal models is inevitable.

The first documented live birth resulting from transfer of mammalian embryos took place in 1890[9]. Today, embryo transfer (ET) to a surrogate female is a crucial step in studying the ART-induced effects during preimplantation on subsequent embryo development stages[10]. ET techniques depend on the size and anatomical structure of each animal. In the case of large-sized animal models, it has been possible to perform ET by transcervical nonsurgical ET techniques, but in smaller-size species catheterization of the cervix is more complex and surgical techniques are frequently used[11]. However, surgical ET can cause hemorrhaging that could impair implantation and embryo development, as blood can invade the uterine lumen, causing embryo death[10]. Transcervical nonsurgical ET techniques are still applied in humans, baboons, bovine, pigs and mice[12-17], but surgical ETs are still being used in species such as goats, sheep or other animals which present additional difficulties[10, 18-21], such as rabbits (two independent cervices) or mice (small size). Nonetheless, surgical transfer methods tend to have gradually been replaced by less invasive methods. Endoscopy was used to transfer embryos, for example, in rabbits, pigs and small ruminants[18-20]. These minimally invasive endoscopy methods can be used to transfer embryos into the ampulla via the infundibulum, which is essential in rabbits and has demonstrated beneficial effects in some species[20]. This is based on the importance of the correct dialogue between embryo and mother during early embryo stages in

the oviduct. As mentioned above, the embryo remodeling that takes place in rabbits during embryo migration through the oviduct is essential to achieve embryos able to implant[22, 23].

Larger-size animal models, such as bovine, are interesting because the biochemical and preimplantation features are similar to those in human species[24]. However, large animals are too expensive to use in preliminary trials, and rodents are considered an ideal model (76% model organisms are rodents) for laboratory research[25]. Nevertheless, the rabbit model provides some advantages over rodents in reproductive studies, as some reproductive biological processes exhibited by humans are more similar in rabbits than those in mice. Human and rabbits present a similar chronological embryonic genome activation, gastrulation and hemochorial placenta structure. In addition, using rabbits it is possible to know the exact timing of fertilization and pregnancy stages due to their induced ovulation[25]. Rabbit life cycles are short, completing gestation in 31 days and reaching puberty at about 4-5 months; the animal is easy to handle due to its docile and non-aggressive behavior, and its upkeep is very economical compared to the expense of larger animals. Moreover, it is crucial to mention that rabbits have a duplex uterus with two independent cervixes[11, 25]. This places the rabbit in a preferential position, as embryos from the different experimental groups can be transferred into the same animal, but into a different uterine horn. This allows us to compare both experimental effects, reducing the maternal factor from the results.

Today, nonsurgical ET methods are not in use in rabbit. Some studies carried out in the late 90s using a transcervical ET technique resulted in low delivery rates ranging from 5.5% to 20.0%[11,26] versus 50-65% by surgical methods, among them the laparoscopy procedure described by Besenfelder and Brem[18]. The low success rates of these nonsurgical ET methods in rabbits coincide with the lack of the necessary embryo remodeling in the oviduct, which is avoided in transcervical ET. Here, we describe an effective minimally invasive laparoscopic ET procedure using rabbits as a model organism. This technique provides a model for further reproductive research in large animals and humans.

Because rabbits have a particularly narrow time window for embryo implantation, ET in this species requires a high degree of synchrony between the developmental stage of the embryo at ET and the physiological status of the recipient[27]. In some cases, after a reproductive treatment that slows embryo development (such as *in vitro* culture) or alters the endometrial receptivity (such as superovulation treatments), there is no synchrony between the embryo and the maternal uterus. These situations can negatively affect outcome. To respond in these contexts, we describe an effective rabbit morula vitrification protocol that allows us to pause, organize and resume the experiments. This process is logistically desirable for reproductive studies and gives us the capacity for long-term storage of embryos, allowing their transport. The laparoscopic procedure and cryopreservation strategies allow better planning of studies with fewer animals. Thus, our methodology offers hygienic and economic advantages and conforms to the concept of the 3Rs (replacement, reduction and refinement) of animal research with the stated goal of improving human treatment of experimental animals. Thus, with these methods, rabbits constitute an ideal model organism for *in vivo* reproductive assays.

PROTOCOL:

All experimental procedures used in this study were performed in accordance with Directive 2010/63/EU EEC for animal experiments and reviewed and approved by the Ethical Committee for Experimentation with Animals of the Polytechnic University of Valencia, Spain (research code: 2015/VSC/PEA/00170).

1. Embryo Transfer

1.1. Preparation of recipient females

1.1.1 Use only sexually mature females (> 4.5 months old).

1.1.2 One week before ET, adapt females to a 16 h light/8 h dark regime to initiate follicular growth and enhanced female receptivity.

1.1.3 Select the recipient females, observing the turgidity and color of the vulva. If the vulva is turgid and reddish, the female is receptive.

1.1.4 Induce pseudopregnancy (ovulation) by a single intramuscular injection of 1 µg of buserelin acetate (synthetic analogue of Gonadotropin-releasing hormone) regardless of body weight.

Note: Normally, 0.8 µg is a suitable dose for ovulation induction in medium-size rabbits (4-5 kg), so 1 µg generally guarantees the ovulation.

1.1.5 Induce ovulation as many days beforehand as the age of the embryos to be transferred (for example, 70-72 h before fresh morula ET).

1.2. Anesthesia and analgesia

1.2.1. Weigh the rabbit and load the following anesthetics and analgesics.

1.2.1.1. In a 1 mL syringe with a 30G needle: load xylazine (5mg/kg) and buprenorphine hydrochloride (0.03 mg/kg). In another 1 mL syringe with a 23G pericranial needle, load ketamine hydrochloride (35 mg/kg).

1.2.2. Hold the rabbit and inject the xylazine-buprenorphine mixture intramuscularly.

1.2.3. Insert the pericranial needle with ketamine in the marginal ear vein, slowly introducing all the syringe contents intravenously.

1.2.4. Fix the needle and leave it inserted throughout the remaining steps to administer more anesthesia if necessary.

1.2.5. Leave the rabbit in the cage (clean and without any other animals) on a warm stage.

1.2.6. Once unconscious, apply eye ointment to avoid dryness of the eye and check for the absence of the palpebral freflex.

Note: This protocol provides a surgical anesthesia plane for a minimum of 30 min. If a longer time is required, inject additional dosages with half of the amount of ketamine hydrochloride described in 1.2.1 after 30 min.

1.2.7. Monitor the depth of anesthesia by checking the pedal reflex and breathing movement. Changes in the breathing pattern to an irregular and faster rate indicate loss of the proper plane of anesthesia.

1.2.8. Monitor the color of the mucous membranes (eyes, lips, *etc.*), respiratory rate (30-60 breaths per minute), heart rate (120-325 beats per minute) and rectal temperature (38-39.6 °C).

1.2.9. Eight hours before transfer, withhold food from animals to avoid the greater gut size and activity until the ET process is finished. Leave free access to water.

1.3. Embryo preparation

1.3.1. Warm the embryo manipulation media to 25 °C: Base Medium (BM), consisting of Dulbecco's Phosphate-Buffered Saline (DPBS) supplemented with 0.2% (w/v) of Bovine Serum Albumin.

1.3.2. Working under a stereomicroscope, rinse fresh or thawed (Step 2) embryos with BM.

1.3.3. Using sterile gloves, attach an appropriately configured 17G epidural catheter to a 1 mL syringe.

1.3.4. Aspirate 1 cm of BM into the catheter, followed by a small air bubble.

1.3.5. Aspirate 5-7embryos in a volume of 10 µL of BM, followed by another small air bubble.

1.3.6. Finish loading the catheter by aspirating 1 cm of BM.

1.4. Embryo transfer

1.4.1. Use sterile gloves; wear a gown and mask.

1.4.2. Sterilize surgical instruments, clean the surfaces where surgery will be performed, and wipe them with 70% ethanol.

1.4.3. Perform anesthesia as previously detailed (step 1.2), checking for loss of reflexes.

1.4.4. Shave the fur from the ventral abdomen with an electric razor.

1.4.5. Prepare the ventral abdomen aseptically.

1.4.5.1. Clean the surgical area and remove any remaining hair. Evacuate the bladder using a urinary catheter. Wash the surgical area with a chlorhexidine gluconate soap. Sanitize the area with chlorhexidine solution.

1.4.6. Place the animal on a warm surgical table, in Trendelenburg's position (head down at 45°) to ensure that the stomach and intestines are cranially located. If any viscera are damaged in the process, the animal may die. It is therefore important to have them properly located (**Figure 1**).

1.4.7. Cover the area using a sterile towel, with a hole (fenestration) exposing the shaved area, to separate the surgical site from any potential contaminating areas.

1.4.8. Insert one endoscopic trocar 5 cm into the abdominal cavity, 2 cm caudal to the xiphoid process, and insufflate through it the peritoneal cavity with a pressure-regulating mechanical insufflator.

Note: The intra-abdominal pressure should be 8-12 mmHg with CO_2 (**Figure 1A**).

1.4.9. Insert the endoscope camera through the endoscopic trocar (**Figure 1B**).

Note: Identify the reproductive tract, determining the status and position of the infundibulum and ampulla before ET to facilitate the next steps.

1.4.10. Insert the 17-G epidural needle into the inguinal region between 2-3 cm from the infundibulum (**Figure 1B**).

1.4.11. Identify the entrance of the infundibulum (**Figures 2A, 2B**).

1.4.12. Insert the loaded catheter (step 1.3) through the epidural needle into the abdomen (**Figure 1C**).

1.4.13. Locate the oviduct and insert 1-2 cm of the epidural catheter through the infundibulum in the ampulla (**Figures 2A-2C**). Do not progress very far into the oviduct to prevent damage and hemorrhage.

1.4.14. Release the embryos into the oviduct by gently pressing the plunger of the syringe coupled to the catheter (**Figures 2D-2F**). Both air bubbles must exit the catheter.

1.4.15. Remove the catheter just after the embryos have been released.

1.4.16. Rinse the catheter, aspirating and releasing manipulating medium to check the absence of the embryos and confirm their successful transfer.

1.4.17. Repeat steps 1.4.11 to 1.4.16 in the other side of the uterus, if desired.

1.4.18. Remove the epidural needle and endoscope camera.

1.4.19. Release CO_2 through the endoscopic trocar. If excess gas remains in the abdomen of the animal, it will have pain and discomfort.

1.4.20. Remove the endoscopic trocar from the abdominal cavity. Remove the surgical towel.

1.4.21. Discontinue anesthesia.

1.4.22. Cleanse the incision made by the trocar with povidone iodide solution. Close the incision made by the trocar with a plastic dressing.

1.5. Postoperative care

1.5.1. Treat the animals with antibiotics: 10 mg/kg of enrofloxacin, subcutaneously, every 24-h for 5 days.

1.5.2. Administer analgesics: buprenorphine hydrochloride (0.03 mg/kg), intramuscularly, each 12 hours for 3 days; Meloxicam (0.2 mg/kg), subcutaneously, every 24-h for 3 days.

1.5.3. Monitor the animals for at least 30 min after surgery (depending on the animal and the dose of anesthesia used) making sure they recover their physiological conditions.

1.5.4. Identify the recipient (*e.g.,* ear tattoo) and house animals individually in a clean cage with the appropriate environmental condition.

*[Place **Figure 1** and 2 here]*

2. Embryo Vitrification and Warming

2.1. Perform all the manipulations at room temperature (around 22 °C) to reduce the vitrification solution toxicity at warmer temperatures.

Note: Embryos can be moved using 0.1-2 µL automatic pipette in this protocol, but other similar devices to move the embryos dragging the minimum volume can be suitable.

2.2. Vitrify the embryos in a two-step addition procedure:

2.2.1. Place the embryos for 2 minutes in equilibrating solution consisting of 10% (v/v) ethylene glycol and 10% (v/v) dimethyl sulfoxide dissolved in BM.

2.2.2. Move the embryos (from step 2.2.1) for 1 minute into vitrification solution consisting of 20% (v/v) ethylene glycol and 20% (v/v) dimethyl sulfoxide dissolved in BM.

2.3. Load the embryos into a 125 µL plastic ministraw (which contains one closed end with a cotton plug and one open extreme). The process is schematized in **Figure 3**.

2.3.1. Couple the closed end of 0.125 µL ministraw with the appropriate 1 mL syringe.

2.3.2. Aspirate BM until 1/3 of the straw length, following by a small air bubble.

2.3.3. Aspirate the embryos in a volume of 40 µL of vitrification solution, followed by another small air bubble.

2.3.4. Aspirate BM until the first liquid fraction (step 2.3.2) reaches the cotton.

2.3.5. Close the open end with a straw plug.

2.4. Perform step 2.2.2 while step 2.3 is being done to ensure that no more than one-minute elapses, which would be toxic to embryos.

2.5. Plunge the ministraw directly into liquid nitrogen to achieve vitrification.

2.6. Store the ministraw in a dewar for nitrogen for the desired time.

2.7. Thaw the embryos in a single step.

2.7.1. Place the ministraw horizontally 10 cm from liquid nitrogen vapour for 20-30 s.

2.7.2. When the crystallization process begins inside the ministraw, immerse the ministraw in a water bath at 25 °C for 10–15 s.

2.7.3. Remove the ministraw plug and cut the cotton plug.

2.7.4. With a coupled syringe, expel all the ministraw content into a plate containing 0.33 M sucrose solution at 25 °C in BM for 5 minutes.

Note: This step must be done quickly in order to reduce embryo exposure to the vitrification solution.

2.7.5. Move the embryos to a new plate containing BM solution for another 5 min.

2.7.6. Consider only non-damaged embryos (with intact mucin coat and zona pellucida) to continue with the ET.

Note: Take into account that in thawed embryos, asynchronous transfers (*e.g.,* 60-62 h in morula transfers) may improve the results by allowing a resynchronization between the embryo and the maternal endometrium.

*[Place **Figure 3** here]*

REPRESENTATIVE RESULTS:

Minimally invasive laparoscopic transfer of fresh or vitrified embryos places the rabbit among the best model animals for reproductive studies. **Table 1** shows the results of fresh ET at different developmental stages (**Figure 4**) of transferred embryos. The survival rate at birth (percentage of embryos resulting in a pup) proved the efficacy of the laparoscopic technique described in this paper. The higher values were achieved when the ET was performed with embryos in the morula stage, either early or compact morulae. Based on these results, we performed a second experiment to demonstrate the survival rate after vitrification of these embryos. Thus, in **Table 2** we show the results obtained after transferring vitrified rabbit morulae recovered at the same time, differentiating between those embryos that had reached a good degree of compaction or not. The survival rate at birth was different between the different embryo stages, being higher in compacted morulae. Therefore, laparoscopic embryo transfer is a reliable technique to transfer fresh and vitrified embryos in rabbits

Figure 1: Laparoscopic embryo transfer assisted by laparoscopy (External view). A) Insertion of the endoscopic trocar (one port). **B)** Insertion of the endoscopic camera and the epidural needle (black arrow). **C)** Insertion of the embryo transfer catheter (white arrow) through the epidural needle.

Figure 2: Laparoscopic embryo transfer assisted by laparoscopy (Internal view). A: Insertion of the catheter through the epidural needle into the abdominal zone. Asterisk indicates the infundibulum. **B, C, D:** The catheter loaded with the embryos is inserted into ampulla region across the infundibulum. **E, F:** Release of the embryos, confirmed by the visualization of a swollen oviduct. This figure has been adapted from Marco-Jiménez *et al.*[38].

Figure 3: Schematization of correctly loaded straw. A) BM refers to the embryo manipulating media employed during vitrification. Embryos must be loaded in vitrification solution. **B)** Macroscopic appearance of the loaded straw with a magnified detail of the embryo position. This large-volume device allows us to vitrify large number of embryos, unlike minimum volume devices. Furthermore, the handling of this device is easier compared with minimum volume devices, while the results are similar in rabbits[41].

Figure 4: Rabbit embryos. A) Pronuclear. **B)** Eight cells. **C)** Early morula. **D)** Compact morula. **E)** Blastocyst. Asterisk indicates the two pronuclei. Black arrows indicate the zona pellucida. White arrows indicate the mucin coat, which normally varies between embryos. ICM: Inner Cell Mass. TE: Trophectoderm. Scale bar: 50 µm.

Table 1. Efficiency of fresh rabbit embryo transfer (*in vivo* derived) by laparoscopy. [1]Different embryos were recovered at 18-20h (pronuclear), 36-38h (8 cells), 60-62h (early morula), 70-72h (compact morula) and 80-82h (blastocyst) after mating. Compact (>32 cells) and non-compact morulae (≈32 cells) can be founded at 70-72h, but only compact morulae were transferred. [2]Survival rate at birth from recipient pregnant does. [a,b]Values with different superscripts are statistically different (P<0.001).

Table 2. Viability of non-compacted vs compact vitrified morula. [a,b]Values with different superscripts are statistically different (P<0.001). [1]Survival rate at birth from recipient pregnant does. Embryos were recovered at the same time (70-72 h) and were distinguished into compact (>32 cells) and non-compact morulae (≈32 cells).

DISCUSSION:

Since the first documented live birth case from transferred embryos[9], this technique and the rabbit species have become crucial in reproductive studies. Besides, embryo research studies involving manipulation, production, cryopreservation, *etc.* require as a last step the evaluation of embryo capacity to generate healthy full-term offspring. Therefore, embryo transfer technique is indispensable[13,28]. Over the years, the surgical methods initially employed to transfer embryos into the maternal uterus have gradually been replaced by less invasive methods in the vast majority of species[13-15,21,27,29,30]. However, in rabbits, intraoviductal ET in early embryo stages of development and *in vitro* produced embryos becomes unavoidable to ensure a similar result to natural conditions. In rabbits, intraoviductal mucin coat is a crucial factor allowing embryo implantation, as it takes place after the remodelling of the embryonic coatings during blastocyst expansion in the uterine horns. However, mucin coat deposition is limited to the oviduct for 3 days following ovulation, and the molecular mechanisms of coat material deposition are largely unknown[31]. For these reasons, it is known that *in vitro*-developed blastocysts did not survive when transferred to the uterus[32-34], and embryos with a damaged mucin coat have a lower survival rate[35]. Likewise, groups that reported a transcervical embryo transfer in rabbits resulted in very low live born rates[11,26]. Here, we present a minimally invasive technique, adapted from Besenfelder and Brem[18], to transfer embryos with successful birth rates. According to the results in **Table 1**, the morula stage in rabbit embryos was the best embryonic stage to achieve a high survival rate at birth. One possible explanation is the greater sensitivity to manipulation of the earliest stages. Interestingly, the success rate increases as the embryonic stage progresses, possibly due to the greater exposure of the embryo to oviductal secretions prior to its recovery. But when embryos reach the blastocyst stage and are place-concordant transferred to the uterus, the values decrease drastically. Not excluding what has been said, a possible explanation could be that the embryos transferred into the oviduct can restore the possible damage generated in the mucin layer during embryo manipulation.

Therefore, blastocysts transferred into the uterus would be deprived of this mechanism, which could compromise their implantation capacity.

The technique is performed using a single port instrument (5 mm endoscope trocar), with slight, brief manipulation. Therefore, the 5-mm endoscope trocar incision does not require closure. Laparoscopic technique benefits include decreased postoperative pain, quicker return to normal activity, and fewer postoperative complications. In addition, endoscopic procedures induce fewer abdominal adhesions and allow a better immune response by the recipient compared with open surgery[21,36,37]. Accumulating evidence from our lab has demonstrated the effectiveness of this ET procedure in the rabbit model. Thus, in the last five years a total of 3,909 embryos (1,335 fresh and 2,574 vitrified embryos) were transferred through the procedure described in the present manuscript. As a result of this technique, the offspring rates of fresh and vitrified transfer embryos were 62.9% and 42.5%, respectively[38-47]. Many studies are all based on this technique: Marco-Jiménez *et al.*[38-41], Vicente *et al.*[42], Viudes-de-Castro *et al.*[43], Saenz-de-Juano *et al.*[44,45,47], Lavara *et al.*[46].

Practical recommendations for carrying out this technique are described below. In embryo culture experiments, it is also advisable to use a new catheter for embryo transfer instead of the one used to move the embryos between the culture media and manipulation media. This avoids transfer of mineral oil and ensures an optimal flow. During ET it is important to minimize handling of the reproductive tract, as excessive manipulation of the oviduct could result in adhesions. If the oviduct is twisted, employ the epidural syringe to try to position it correctly, not the catheter, as it contains the embryos and the mechanical manipulation could cause their loss. Once the catheter passes through the oviduct, it slides easily. If it does not, the catheter may have deviated. Once inside the oviduct, if the media does not flow, move the catheter out slightly and try to reinsert it again. If it still does not flow, the catheter is clogged. Remove it from the oviduct and release the content into a dish with a clean medium. Then, reload the embryos into another catheter and try to reinsert it into the oviduct again. Delivery usually takes places 28-30 days after morula transfer.

In addition, there is evidence indicating that the embryo developmental stage can be more advanced than the uterine environment in pseudopregnant females, but not the opposite. Specifically, embryos have the ability to wait for the favorable womb environment, but the womb environment cannot wait for the embryos at the right stage for implantation[10]. With regard to vitrified embryos, after a short/Long-term storage it is possible to synchronize the developmental stage of the embryo with the corresponding favorable womb environment. Furthermore, if the embryo donor is also the embryo recipient, the detrimental effects of superovulation on the endometrium can be bypassed by using the vitrification technique and transferring the embryos in a subsequent cycle[48]. In rabbits, vitrified embryos transferred into oviducts of recipients induced to ovulate 60-62h beforehand (asynchrony) is a highly efficient technique[44,49]. Related with this, it has been suggested that the oviductal embryo transition during 10-12 h could explain the beneficial effects in the restoration of cell physiology and replacement of dead cells, and probably repair the damage induced in mucin coat during embryo manipulation. Besides, vitrified embryos present a delay in development, as they have

been metabolically suspended during the storage. Therefore, transfer of cryopreserved embryos into asynchronous recipients allows the embryo to reactivate its metabolic activity and thus the embryo stage of development is synchronized with the womb environment. Instead, if cryopreserved embryos are transferred into synchronic receptors, the cross-talk between the mother and the embryo hinders the onset of a successful pregnancy. In rabbit, the highest survival rate has been obtained after intraoviductal transfer of cryopreserved morulae[49]. Our data are consistent with this report, although the morula stage exhibits different survival rates following cryopreservation depending on their degree of compaction at 70-72h (**Table 2**). Here, compacted morulae showed higher survival rates at birth in comparison to non-compacted morulae, which was in concordance with previous reports showing that every stage of development had its own mechanism relative to the permeation of cryoprotectants and the extent of dehydration during the addition of the cryopreservation solution[50]**.** Underlying these techniques, we have demonstrated that a combination of vitrification and intraoviductal embryo transfer is a successful strategy to re-establish rabbit populations after 15 years of storage in liquid nitrogen, without adverse effect on their post-thaw survival and live birth[51].

The following details should be taken into account to successfully perform this technique. It is important to bear in mind that the increasing density of the consecutive mediums used for vitrification (DPBS, equilibration solution, vitrification solution) could induce embryo contraction due to progressive embryo dehydration. However, its normal appearance is recovered when the embryo is equilibrated with the medium. Furthermore, when the embryo is moved between increasing density media, it tends to move to the surface of the media due to density movements. To avoid embryo loss and ensure the time of vitrification, it is recommendable to perform the vitrification in small drops of the media that will keep the embryo in place.

In conclusion, here we describe both an ET technique and an embryo vitrification method that facilitate future studies which use rabbits as a model. Based on the close phylogenetic distance between rabbits and humans, the use of this model could provide results easily transferable to human clinical medicine. In addition, our method offers some hygienic and economic advantages, conforming to the concept of the 3 Rs of animal welfare (replacement, reduction and refinement), while maintaining the goal of improving humane treatment of experimental animals.

ACKNOWLEDGMENTS:
This work was supported by funds from the Ministry of Economy and Competitiveness of Spain Research Programme (AGL2014-53405-C2-1-P) and Generalitat Valenciana Research Programme (PrometeoII 2014/036). English text version revised by N. Macowan English Language Service.

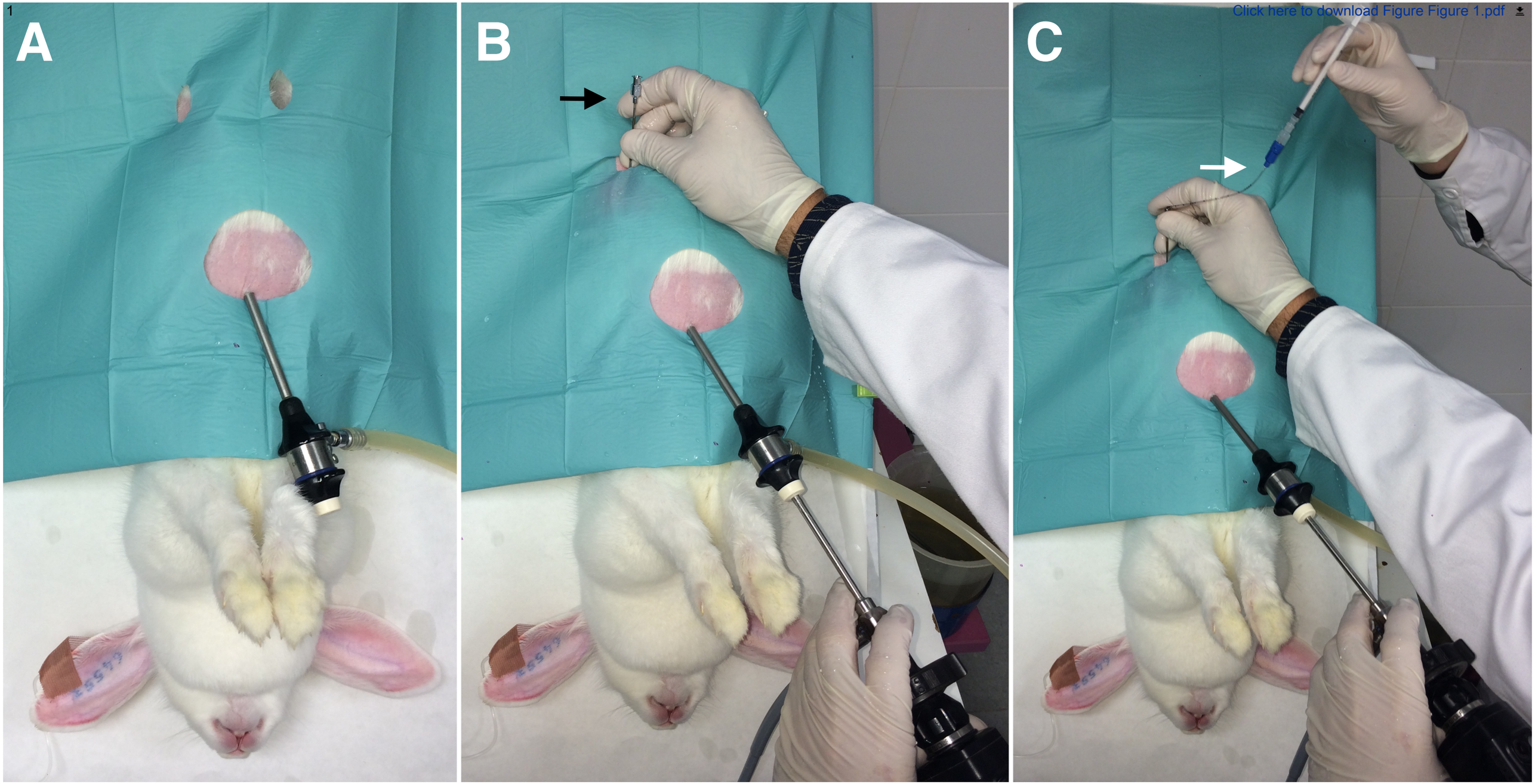
DISCLOSURES:
The authors have nothing to disclose.

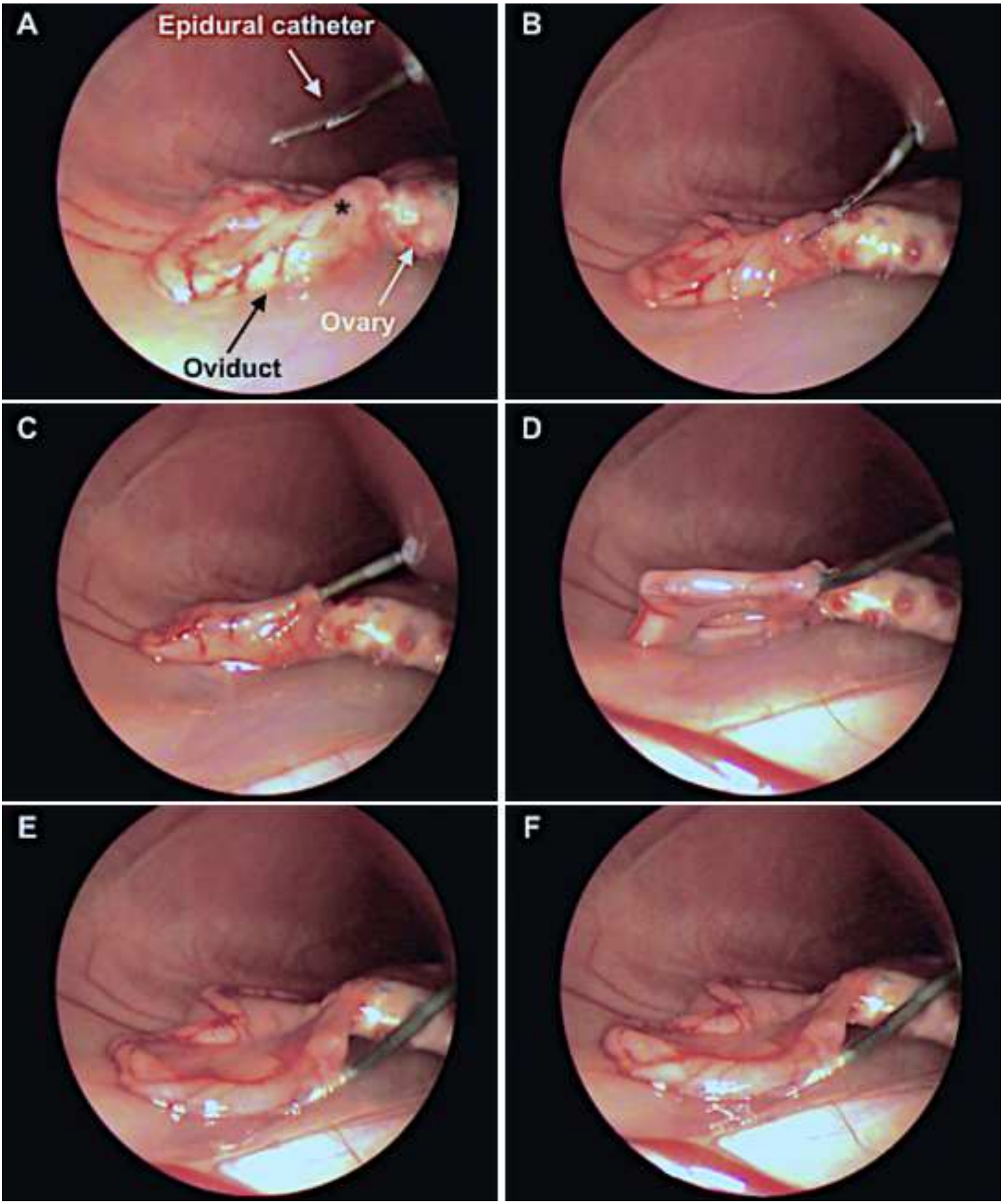
REFERENCES:

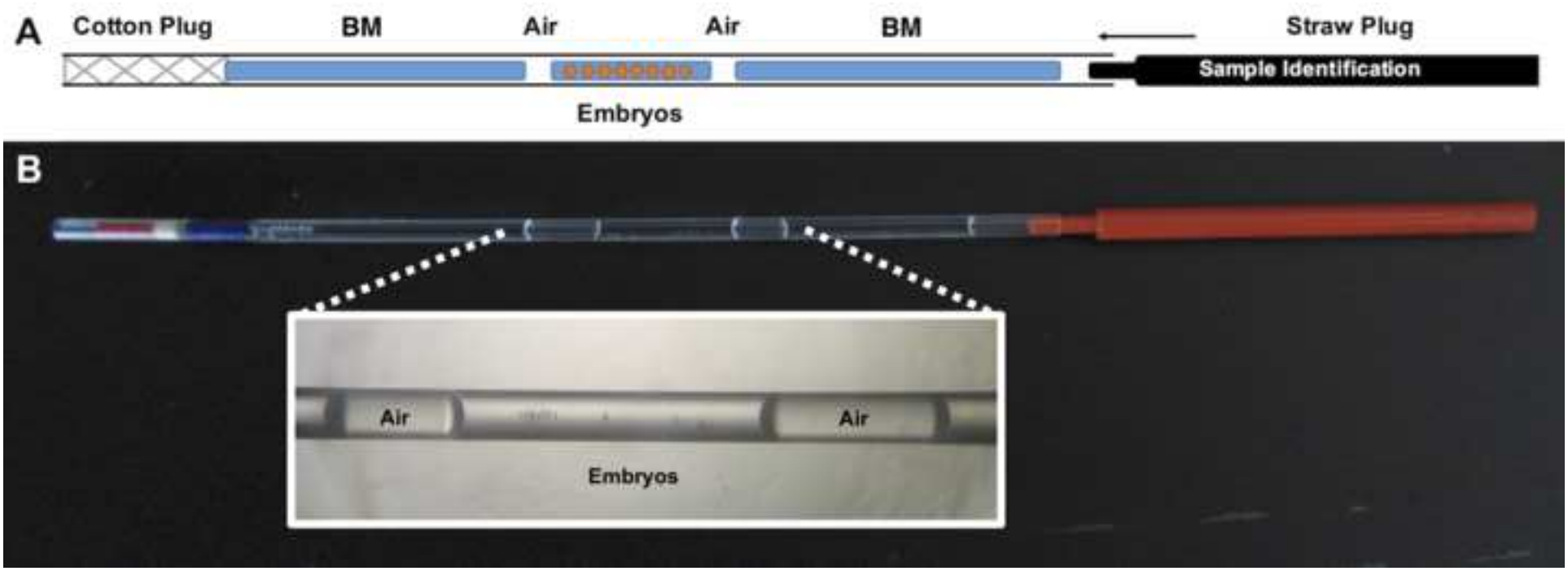
1. Chen, M., Heilbronn, L.K. The health outcomes of human offspring conceived by assisted reproductive technologies (ART). *Journal of Developmental Origins of Health and Disease.* **8** (4), 388-402 (2017).
2. Lavara, R., Baselga, M., Marco-Jiménez, F., Vicente, J.S. Embryo vitrification in rabbits: Consequences for progeny growth. *Theriogenology.* **84** (5), 674-80 (2015).
3. Sirard, M.A. The influence of *in vitro* fertilization and embryo culture on the embryo epigenetic constituents and the possible consequences in the bovine model. *Journal of Developmental Origins of Health and Disease.* **8** (4), 411-417 (2017).
4. Feuer, S.K., Rinaudo, P.F. Physiological, metabolic and transcriptional postnatal phenotypes of *in vitro* fertilization (IVF) in the mouse. *Journal of Developmental Origins of Health and Disease*. **8** (4), 403-410 (2017).
5. Jiang, Z, *et al.* Genetic and epigenetic risks of assisted reproduction. *Best Practice & Research: Clinical Obstetrics & Gynaecology.* **44**, 90-104 (2017).
6. Fleming, T.P, Velazquez, M.A., Eckert, J.J. Embryos, DOHaD and David Barker. *Journal of Developmental Origins of Health and Disease.* **6** (5), 377-83 (2015).
7. Sparks, A.E. Human embryo cryopreservation-methods, timing, and other considerations for optimizing an embryo cryopreservation program. *Seminars in Reproductive Medicine.* **33** (2), 128-44 (2015).
8. Swain, J.E. Optimal human embryo culture. *Seminars in Reproductive Medicine.* **33** (2), 103-17 (2015).
9. Heape, W. Preliminary note on the transplantation and growth of mammalian ova within a uterine foster-mother. *Proceedings of the Royal Society of London B: Biological Sciences*. **48**, 457-9 (1890).
10. Bermejo-Alvarez, P., Park, K.E., Telugu, B.P. Utero-tubal embryo transfer and vasectomy in the mouse model. *Journal of Visualized Experiments.* **84**, e51214 (2014).
11. Kidder, J.D., Roberts, P.J., Simkin, M.E., Foote, R.H., Richmond, M.E. Nonsurgical collection and nonsurgical transfer of preimplantation embryos in the domestic rabbit (Oryctolagus cuniculus) and domestic ferret (Mustela putorius furo). *Journal of Reproduction and Fertility.* **116** (2), 235-42 (1999).
12. Tıras, B., Cenksoy, P.O. Practice of embryo transfer: recommendations during and after. *Seminars in Reproductive Medicine.* **32** (4), 291-6 (2014).
13. Cui, L., *et al.* Transcervical embryo transfer in mice. *Journal of the American Association for Laboratory Animal Science.* **53** (3), 228-31 (2014).
14. Moreno-Moya, J.M., *et al.* Complete method to obtain, culture, and transfer mouse blastocysts nonsurgically to study implantation and development. *Fertility and Sterility.* **101** (3), e13 (2014).
15. Hasler, J.F. Forty years of embryo transfer in cattle: a review focusing on the journal Theriogenology, the growth of the industry in North America, and personal reminisces. *Theriogenology.* **81** (1), 152-69 (2014).
16. Bauer, C. The baboon (Papio sp.) as a model for female reproduction studies. *Contraception.* **92** (2), 120-3 (2015).
17. Martinez, E.A., *et al.* Nonsurgical deep uterine transfer of vitrified, *in vivo*-derived, porcine embryos is as effective as the default surgical approach. *Science Reports.* **5**, 10587 (2015).

18. Besenfelder, U., Brem, G. Laparoscopic embryo transfer in rabbits. *Journal of Reproduction and Fertility* **99**, 53-56 (1993).
19. Besenfelder, U., Mödl, J., Müller, M., Brem, G. Endoscopic embryo collection and embryo transfer into the oviduct and the uterus of pigs. *Theriogenology.* **47** (5), 1051-60 (1997).
20. Besenfelder, U., Havlicek, V., Kuzmany, A., Brem, G. Endoscopic approaches to manage *in vitro* and *in vivo* embryo development: use of the bovine oviduct. *Theriogenology.* **73** (6), 768-76 (2010).
21. Fonseca, J.F., *et al.* Nonsurgical embryo recovery and transfer in sheep and goats. *Theriogenology.* **86** (1), 144-51 (2016).
22. Denker, H.W. Structural dynamics and function of early embryonic coats. *Cells Tissues Organs.* **166**, 180-207 (2000).
23. Marco-Jiménez, F., López-Bejar, M. Detection of glycosylated proteins in rabbit oviductal isthmus and uterine endometrium during early embryo development. *Reproduction in Domestic Animals.* **48** (6), 967-73 (2013).
24. Ménézo, Y.J., Hérubel, F. Mouse and bovine models for human IVF. *Reproductive BioMedicine Online.* **4** (2), 170-5 (2002).
25. Fischer, B., Chavatte-Palmer, P., Viebahn, C., Navarrete Santos, A., Duranthon, V. Rabbit as a reproductive model for human health. *Reproduction.* **144** (1), 1-10 (2012).
26. Besenfelder, U., Strouhal, C., Brem, G. A method for endoscopic embryo collection and transfer in the rabbit. *Zentralbl Veterinarmed A.* **45** (9), 577-9 (1998).
27. Daniel, N., Renard, J.P. Embryo transfer in rabbits. *Cold Spring Harbor Protocols.* **2010** (1) (2010).
28. Saenz-de-Juano, M.D., *et al.* Vitrification alters rabbit foetal placenta at transcriptomic and proteomic level. *Reproduction.* **147** (6), 789-801 (2014).
29. Green, M., Bass, S., Spear, B. A device for the simple and rapid transcervical transfer of mouse embryos eliminates the need for surgery and potential post-operative complications. *Biotechniques.* **47** (5), 919-24 (2009).
30. Duan, X., Li, Y., Di, K., Huang, Y., Li, X. A nonsurgical embryo transfer technique in mice. *Sheng Wu Gong Cheng Xue Bao.* **32** (4), 440-446 (2016).
31. Denker, H.W., Gerdes, H.J. The dynamic structure of rabbit blastocyst coverings. I: transformation during regular preimplan- tation development. *Anatomy and Embryology* **157**, 15-34 (1979).
32. Seidel, G.E., Bowen, R.A., Kane, M.T. *In vitro* fertilization, culture and transfer of rabbit ova. *Fertility and Sterility* **27**: 861-870 (1976).
33. Binkerd, P.E., Anderson, G.B. Transfer of cultured rabbit embryos. *Gamete Research* **2**, 65-73 (1979).
34. Murakami, H., Imai, H. Successful implantation of *in vitro* cultured rabbit embryos after uterine transfer: a role for mucin. *Molecular Reproduction and Development* **43**, 167-170 (1996).
35. Techakumphu, M., Wintenberger-Torrèsa, S., Sevelleca, C., Ménézo, Y. Survival of rabbit embryos after culture or culture/freezing. *Animal Reproduction Science.* **13** (3), 221-228 (1987).
36. Gitzelmann, C.A, *et al.* Cell-mediated immune response is better preserved by laparoscopy than laparotomy. *Surgery.* **127** (1), 65-71(2000).

37. Huang, S.G., Li, Y.P., Zhang, Q., Redmond, H.P., Wang, J.H., Wang, J. Laparotomy and laparoscopy diversely affect macrophage-associated antimicrobial activity in a murine model. *BMC Immunology.* **14**, 27 (2013).
38. Marco-Jiménez, F., Jiménez-Trigos, E., Almela-Miralles, V., Vicente, J.S. Development of Cheaper Embryo Vitrification Device Using the Minimum Volume Method. *Public Library of Science One*. **11** (2), e0148661 (2016).
39. Marco-Jiménez, F., Jiménez-Trigos, E., Lavara, R., Vicente, J.S. Generation of live offspring from vitrified embryos with synthetic polymers supercool X-1000 and Supercool Z-1000. *CryoLetters.* **35**, 286-292 (2014).
40. Marco-Jiménez, F., Jiménez-Trigos, E., Lavara, R., Vicente, J.S. Use of cyclodextrins to increase cytoplasmic cholesterol in rabbit embryos and their impact on live KITs derived from vitrified embryos. *Cryoletters.* **35**, 320-326 (2014).
41. Marco-Jiménez, F., Lavara, R., Jiménez-Trigos, E., Vicente, J.S. *In vivo* development of vitrified rabbit embryos: Effects of vitrification device, recipient genotype, and asynchrony. *Theriogenology.* **79** (7), 1124-9 (2013).
42. Vicente, J.S, *et al.* Rabbit morula vitrification reduces early foetal growth and increases losses throughout gestation. *Cryobiology.* **67**, 321-326 (2013).
43. Viudes-de-Castro, M.P., Marco-Jiménez, F., Cedano-Castro, J.I., Vicente, J.S. Effect of corifollitropin alfa supplemented with or without Lh on ovarian stimulation and embryo viability in rabbit. *Theriogenology*. **98**, 68-74 (2017).
44. Saenz-de-Juano, M.D., *et al.* Vitrification alters at transcriptomic and proteomic level rabbit foetal placenta. *Reproduction.* **147**, 789-801 (2014).
45. Saenz-de-Juano, M.D, Marco-Jimenez, F., Viudes-de-Castro, M.P., Lavara, R., Vicente, J.S. Direct comparison of the effects of slow freezing and vitrification on late blastocyst gene expression, development, implantation and offspring of rabbit morulae. *Reproduction in Domestic Animals.* **49**, 505-511 (2014).
46. Lavara, R., Baselga, M., Marco-Jiménez, F., Vicente, J.S. Long-term and transgenerational effects of cryopreservation on rabbit embryos. *Theriogenology.* **81**, 988-992 (2014).
47. Saenz-de-Juano, M.D., Marco-Jiménez, F., Vicente, J.S. Embryo transfer manipulation cause gene expression variation in blastocysts that disrupt implantation and offspring rates at birth in rabbit. *European Journal of Obstetrics & Gynecology and Reproductive Biology.* **207**, 50-55 (2016).
48. Roque, M., Valle, M., Kostolias, A., Sampaio, M., Geber, S. Freeze-all cycle in reproductive medicine: current perspectives. *JBRA Assisted Reproduction*. **21** (1), 49-53 (2017).
49. Tsunoda, Y., Soma, T., Sugie, T. Effect of post-ovulatory age of recipient on survival of frozen-thawed rabbit morulae. *Journal of Reproduction and Fertility.* **65** (2), 483-7 (1982).
50. Vanderzwalmen P, *et al.* Births after vitrification at morula and blastocyst stages: effect of artificial reduction of the blastocoelic cavity before vitrification. *Human Reproduction*. 17 (3), 744-51 (2002).
51. Lavara, R., Baselga, M., Vicente, J.S. Does storage time in LN2 influence survival and pregnancy outcome of vitrified rabbit embryos? *Theriogenology.* **76** (4), 652-7 (2011).







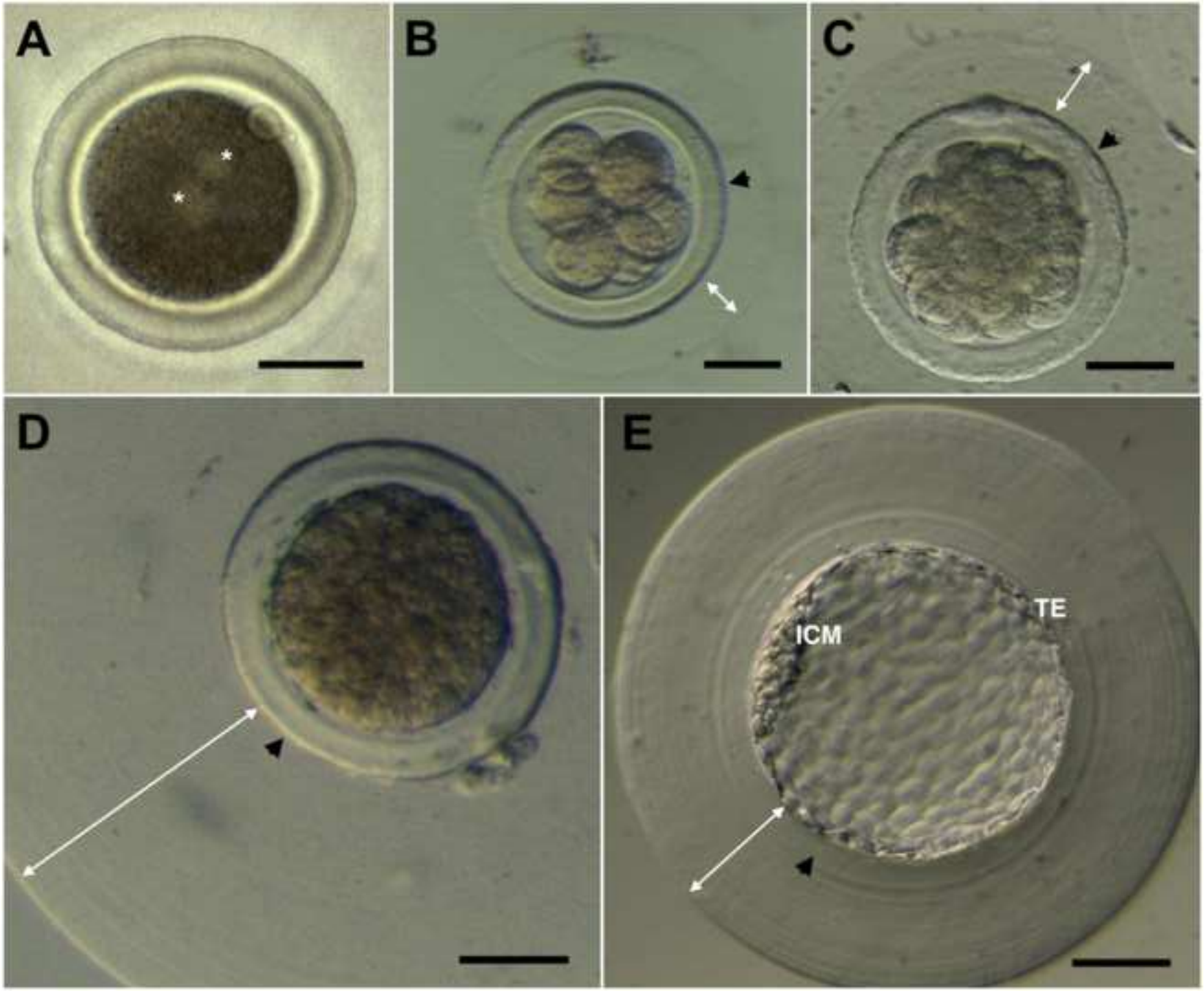


Table 1

Developmental stage[1]	Embryos	Recipients	Place of transfer	Pregnancy rate (%)	Implantation rate (%)	Survival rate at birth (%)[2]
Pronuclear	78	7	Oviduct	7 (100)	50 (64.0)[b]	34 (43.6)[b]
8 cells	81	7	Oviduct	7 (100)	60 (74.1)[b]	53 (65.4)[a]
Early morula	81	7	Oviduct	7 (100)	80 (98.8)[a]	60 (74.1)[a]
Compact morula	80	7	Oviduct	7 (100)	80 (100)[a]	58 (72.5)[a]
Blastocyst	80	7	Uterus	7 (100)	73 (91.3)[a]	38 (47.5)[b]

Table 2

Developmental stage	Transferred embryos	Recipients	Pregnancy rate (%)	Survival rate at birth (%)[1]
Non-compacted	135	10	9 (90)	62 (45.9)[b]
Compacted	150	10	10 (100.0)	98 (65.3)[a]
TOTAL	285	20	19 (95)	160 (56.1)

Materials Table

Click here to download Materials Table JoVE_Materials_Final.xlsx

Name of Reagent/ Equipment	Company
Bovine Serum Albumin (BSA)	VWR
Buprenorphine hydrochloride	Alvet Escartí
Buserelin Acetate	Sigma Aldrich
Clorhexidine digluconate soap	Alvet Escartí
Clorhexidine digluconate solution	Alvet Escartí
CO_2	Air Liquide
CO2 Incubator	Fisher scientific
Dimethyl Sulfoxide	Sigma Aldrich
Dulbecco's phosphate-buffered saline (DPBS)	Sigma Aldrich
Electric razor	Oster Golden A5
Endoscope camera	Optomic Spain S.A
Endoscope trocar with silicone leaflet valve	Karl Storz Endoscopia Ibérica S.A.
Enrofloxacin	Alvet Escartí
Epicraneal needle 23G	Alvet Escartí
Epidural catheter	Vygon corporate
Epidural needle	Vygon corporate
Ethylene Glycol	Sigma Aldrich
Eye ointment	Alvet Escartí
Ketamine hydrochloride	Alvet Escartí
Laparoscopy equipment	Karl Storz Endoscopia Ibérica S.A.
Light source	Optomic Spain S.A
Liquid Nitrogen	Air Liquide
Mechanical CO2 insufflator	Karl Storz Endoscopia Ibérica S.A.
Meloxicam	Alvet Escartí
Petri dishes, 35-mm	Sigma Aldrich
Plastic dressing (Nobecutan)	IBOR medica
Plastic Straw 0.25 mL	IMV - technologies
Povidone iodide solution	Alvet Escartí
Scissors	ROBOZ
Silicone tube for insufflator	Karl Storz Endoscopia Ibérica S.A.

Stereomicroscope	Leica
Sterile Gloves	Alvet Escartí
Sterile gown	Alvet Escartí
Sterile mask	Alvet Escartí
Straw Plug	IMV - technologies
Sucrose	Sigma Aldrich
Syringe, 1-mL	Fisher scientific
Syringe, 5-mL	Fisher scientific
Urinary catheter	IMV - technologies
Waterbath	RAYPA
Xylazine	Alvet Escartí
Rabbits	Universitat Politècnica de València

Catalog Number	Comments/Description
332	
626	To be ordered by a licensed veterinarian.
B3303	
0265DCCJ500B	
0265DCCA500B	
99921	CO2 N48.
15385194	
W387509	
D5773	Without calcium chloride.
078005-140-002	
OP-714	
30114GK	Lightweight trocar model.
9993046	To be ordered by a licensed veterinarian.
514056353	Smaller needles can be also used.
187.10	
187.10	
102466-M	
5273	
184	To be ordered by a licensed veterinarian.
26003 AA	Hopkins® Laparoscope, 0º-mm straight-viewing laparoscope, 30-cm length, 5-
Fibrolux 250	
P1505XXX	
Endoflator®	
9993501	To be ordered by a licensed veterinarian.
CLS430165-500EA	
7140028	
6431	
02656DPYS500S	
RS-5880	Any regular surgical grade steel small straight scissors will work.
20400040	

MZ16F	There are cheaper options such as Leica MZ8 or Nikon SMZ-10 or SMZ-2B, to
087GL010075	
12261501	
058B15924B	
6431	
S7903	
11750425	
11773313	
17722	
BAE-4	
525225	To be ordered by a licensed veterinarian.
Line A	Other maternal lines, such as Line V or Line HP can be used.

·mm working channel.

name a few.

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Author(s): Garcia-Dominguez, Ximo, Marco-Jiménez, Francisco, Viudes-de-Castro, Maria Pilar, Vicente, Jose Salvador

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: ☑ Standard Access ☐ Open Access

Item 2 (check one box):

☑ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Francisco Marco Jiménez
Department:	Ciencia Animal
Institution:	Universitat Politecnica de Valencia
Article Title:	Minimally Invasive Embryo Transfer and Embryo Vitrification at the Optimal Embryo Stage in Rabbit Model
Signature:	F. Marco - Jiménez
Date:	24/02/2018

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Dear reviewers and editor,

In this document we respond appropriately to your recommendations.

Changes recommended by the JoVE Scientific Review Editor:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Abstracts:** Please re-word the Short Abstract to more clearly state the goal of the protocol. For example, "This protocol/manuscript describes…"

The abstract has been revised and rewritten.

• **Introduction:**
1) The following lines need literature references: 80-81, 81-82.

The text has been revised and literature references have been added.

• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

1) Some examples NOT in imperative tense: Line 167, 174. 191-93 etc.

The protocol text has been revised and the imperative voice implemented. In this sense, supplementary information is added as a "note", following the editorial recommendations.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

These recommendations have been taken into account and the appropriate corrections made.

1) Line 172: Is the dosage fixed regardless of body weight? Dosage should ideally be provided as ug/kg.

We have clarified this issue in the manuscript. Normally, 0.8 µg is a suitable dose for ovulation induction in medium size rabbits (4-5 Kg), so 1 µg generally guarantees the ovulation.

2) Line 186: Unclear what is meant by "insert and fix"; is the needle left inserted throughout the remaining steps?

One more step is added to clarify this issue.

3) Lines 200-201: This should be moved up to before the induction of anesthesia or to the start of 1.4.

The duration of the step has been clarified.

4) Line 222: Do you mean 70%?

The symbol error has been corrected.

5) Line 236: Please describe all the surgery steps including tools used. E.g, it is unclear how and where the initial incisions are made. How large and deep should they be?

No initial incision is present before the trocar introduction; the CO2 was insufflated through the inserted trocar. The process has been clarified.

6) Line 239: Which port are you referring to? The one from 1.4.8.? Please describe what is to do be done here clearly: "It is important to look at the reproductive tract arrangement, determining the status and position of the infundibulum and ampulla before embryo transfer to facilitate the next steps"

The sentence has been clarified. The part highlighted in "" quotes is added as a note in the manuscript, as it merely advises the reader that identifying the reproductive tract could facilitate the following steps.

7) Line 244: how is it identified?

Graphical information is available in figure 2. This has been clarified in the manuscript.

8) Line 248: Can you quantify how deep to insert? Perhaps in millimeters?

Data have been added.

9) Line 286: What age?

The age of the embryos depends on the experimental process in which the manuscript could be useful. Only the embryo receptor females should be prepared accordingly and this is stated in the paper.

10) 288: How are the embryos handled?

This issue has been added as a note in the previous step.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

The text protocol numbering has been corrected.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

The protocol steps to the video are accordingly highlighted in yellow.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The recommendations are taken into account. However, this article has some particularities, such as the implementation of two different procedures (embryo vitrification and embryo transfer) with one common goal. We want to focus the work mainly on the embryo transfer process, which is the main new feature that will allow the researchers to use the rabbit as a model for reproductive studies. For this reason, discussion is mainly focused on this technique. In this context, a comprehensive narration was performed, including in two paragraphs the troubleshooting, limitations and comparison to the existing methods in each technique. Then, limitations are discussed after each of the above mentioned paragraphs and the future applications are discussed in the last part of the discussion. This structure is constructed to provide one cohesive description of the protocol, making references to the results presented in order to contextualize this protocol with the laboratory work.

• **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as rabbit strain, all drugs.

Rabbit strain is added to the table, and the drugs have been revised.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

The re-print permission from the editorial is included in the supplementary files. The figure is accordingly cited in the manuscript.

Comments from Peer-Reviewers:

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
I have completed my review of the article entitled 'Minimally invasive embryo transfer and embryo vitrification at the optimal embryo stage in rabbit model' by Marco-Jimenez, Garcia-Dominguez, Viudes-de-Castro and Vicente. I find the article/protocol to be of importance and a valuable contribution. The protocol provide a minimally invasive technique compared to open laparotomy and the recovery of animals is an improvement. The protocol is detailed and provide fine instructions.
Even though there are numerous problems, and I recommend that it be accepted only after major revisions. These problems, which I detail below, may be summarized as (1) poor grammar and style throughout, especially in the introduction and discussion (2) The results are misleading in terms of the description of non-compact vs. compact in table 1 and 2. (3) The discussion summarize interesting studies but due to the unclear writing the statements vanish.
In the following I outline the above-mentioned points in more detail, it is crucial that a qualified English editor review this article for grammar, as there are numerous grammatical errors, some of which I have not altered, as it is not within my purview as peer reviewer

Major Concerns:
-The need of proofreading by a qualified English editor considering typos and grammar.

English grammar and vocabulary has been revised.

-Results: Table 2: 70-72h is considered non-compacted whereas they are considered compacted in table 1? The text doesn't state anywhere why 70-72h Compact morulae are suddenly being referred to as non-compact in Table 2. This makes one question the experiment concerning " Viability of 70-72h non-compacted vs compact vitrified morulae".

Further explanation of this issue is included in the table description to clarify the doubt.

-Discussion: very interesting point in the discussion disappear in the unclear writing. I would recommend several of the paragraphs within the discussion to be rewritten

Some paragraphs in the discussion have been rewritten in order to clarify the sense of the sentences. English grammar and vocabulary has been revised.

Minor Concerns:

Line 53. Rewrite
This recommendation has been implemented.

58: rabbits, delete animal in animal model organism
This recommendation has been implemented.

84: transferred to a surrogate
This point has been revised.

96: techniques
This point has been revised.

102: bovine, pigs and mice

This point has been revised.

102: rewrite sentence
The sentence has been rewritten.

105: endoscopy was used
This point has been revised.

114: rewrite
This point has been rewritten.

117: an ideal model (76 % model organisms are rodents)
This point has been revised.

136: ET procedure using rabbits as a model organism
This point has been revised.

136: rewrite the last sentence (the technique provide a model for further research…)
The sentence has been rewritten.

144: outcome
This point has been revised.

144-145: rewrite not clear
This point has been revised.

152: rabbits
This point has been revised.

152: model organism
This point has been revised.

192: time is
This point has been revised.

242: 17-G
This point has been revised.

301 Step 2.2.2 must take place…
This point has been revised.

303: nitrogen to achieve
This point has been revised.

304: Store the ministraw in
This point has been revised.

305: Thawing is completed in a..
This point has been revised.

312: °C
This point has been revised.

General: inconsistency using ℃or ° C throughout the paper
This point has been revised.

Table 1 : text: 2 survival rate … rewrite sentence
The sentence has been rewritten.

Table 2: text: 1 survival… rewrite sentence
The sentence has been rewritten.

Discussion:
Paragraph 1, 3 and 5: rewrite, the statements are unclear.

These points have been revised.

I wish the authors success implementing my recommended changes, and I look forward to seeing this article published.

Reviewer #2:
Manuscript Summary:
The manuscript describes a method of laparoscopic embryo transfer in rabbits.

Major Concerns:
None

Minor Concerns:
Many minor grammatical and stylistic suggestions for improvement on the attached.

All the grammatical and stylistic suggestions have been implemented.

Recommend title change to: ...in rabbits.

The title has been reconsidered, but taking into account that in this paper we present some techniques that allow the use of the rabbit as an ideal animal model for reproductive studies in large mammalian species such as humans, we think that the detailed title is consistent with the purpose of the paper.

Reviewer #3:
Review of manuscript JoVE58055

Line 38: change "transference" to "embryo transfer"
This point has been revised.

Line 43: delete "today"
This point has been revised.

Line 44: delete "the" and hyphen
This point has been revised.

Line 45: rabbitS
This point has been revised.

Line 53: delete "Taking into account that"
This point has been revised.

Line 55: replace "final" with "and"
This point has been revised.

Line 58: replace "To date," with "The"; delete "as an animal model organism"
This point has been revised.

Line 60: delete "it has reproductive characteristics such as induced ovulation, a"
This point has been revised.

Line 61: delete "chronology of"
This point has been revised.

Lines 62-63: place "Such as intracytoplasmic sperm injection, embryo culture or cryopreservation" in parentheses.
This point has been revised.

Line 70: change "transference" to "transfer"
This point has been revised.

Line 71: Delete "So," and add "thus" after comma
This point has been revised.

Line 72: Delete "Interestingly,"

This point has been revised.

Line 73: change "laparoscopy-" to "laparoscopically"
This point has been revised.

Line 78: aimS
This point has been revised.

Line 81: add comma before "were"; delete "oocytes are produced by following a"
This point has been revised.

Line 82: change "treatment" to "treatments are given"; replace "and" with "to"; replace "these gametes" with "antral ovarian follicles"
This point has been revised.

Line 83: Replace "Thus," with "oocytes"; replace "eggs" with "from these follicles"; move "in vitro" before "until"
This point has been revised.

Line 91 and throughout manuscript: British English or American English? (characterized, behavior, hemochorial, anesthesia, etc.)
The manuscript is in UK English and has been revised for consistency.

Line 94: delete hyphen
This point has been revised.

Line 95: delete "stages"
This point has been revised.

Line 96: Replace "Embryo transfer" with "The ET"
This point has been revised.

Line 98: delete hyphen
This point has been revised.

Line 99: replace "currently used" with "frequently"
This point has been revised.

Line 104: cervices
This point has been revised.

Line 111: takeS
This point has been revised.

Line 117: replace "where" with "and"
This point has been revised.

Line 124: use semi-colon after "months"
This point has been revised.

Line 126: replace "double" with "duplex"
This point has been revised.

Line 130: start new paragraph with "Today, non-surgical…"; rabbitS
This point has been revised.

Line 132: delivery
This point has been revised.

Line 135: in THE oviduct
This point has been revised.

Line 136: rabbitS
This point has been revised.

Line 137: Replace "embryo transfer" with "ET"
This point has been revised.

Line 139: replace "However, as" with "Because"
This point has been revised.

Line 141: replace "transfer time" with "ET"
This point has been revised.

Line 142: replace "retards" with "slows"; delete "the"; place "such as in vitro culture" in parentheses
This point has been revised.

Line 143: place "such as superovulation treatments" in parentheses
This point has been revised.

Line 147: giveS
This point has been revised.

Line 151: add "of animal research: before "with"
This point has been revised.

Line 152: replace "rabbit constitutes" with "rabbits constitute"
This point has been revised.

Line 153: add "in vivo" before "reproductive"
This point has been revised.

Line 168: replace "accommodate" with "adapt"
This point has been revised.

Line 200: for how many hours is water withheld?
Animals have free access to water. It has been clarified in the paper.

Line 220: add semi-colon after "gloves"; replace "wearing" with "wear"
This point has been revised.

Line 221: replace "cleaning" with "clean"
This point has been revised.

Line 222: %
This point has been revised.

Lines 226-229: uses a, b, c, d (instead of 1, 2, 3 ,4)
We follow the authors instruction for the numbering.
Line 227: What size Foley catheter? 10 French?
The model is available in the materials Table.

Line 233: add "properly" after "them"
This point has been revised.

Line 234: add "(fenestration)" after "hole"
This point has been revised.

Line 236 and throughout manuscript: replace "trocar endoscope" with "endoscopic trocar"
This point has been revised.

Line 237: add a comma after "process"; add a hyphen after "pressure"
This point has been revised.

Line 242: gauge
This point has been revised.

Lines 247-248: Which is correct: "deep" or "do not progress very far"?
This confusing sentence has been corrected.

Line 257: reaplce "is accumulated" with "remains"
This point has been revised.

Line 258: pain and discomfort
This point has been revised.

Line 259: from (not form)
This point has been revised.

Line 280: VERY IMPORTANT CHANGE NEEDED: replace "thawing" with "warming"
This point has been revised.

Line 282: degree symbol
This point has been revised.

Line 283: warmER
This point has been revised.

Line 289: sulFoxide
The manuscript is in British English (sulphoxide)
https://en.oxforddictionaries.com/definition/dimethyl_sulphoxide

Line 291: 125 microliters
This point has been revised.

Line 294: 125 microliters
This point has been revised.

Line 296: PLEASE EXPLAIN THE LARGE VOLUME (40 microliters) - is it because of the large diameter of the rabbit morula? Ruminant livestock species are typically vitrified in very small (< 5 microliters) volumes.

The straw allows us to vitrify large numbers of embryos, unlike minimum volume devices. Furthermore, we choose the straw due to the easier handling and the similar results obtained compared with minimum volume devices (Cryotop). These details are implemented in the manuscript with the appropriate reference.

Line 298: reachES
This point has been revised.

Line 301: replace "be taken" with "take"
This point has been revised.

Line 303: replace "for" with "to"
This point has been revised.

Line 304: StorE ; delete "cryobank of"; add "dewar for" after "nitrogen"
This point has been revised.

Line 305: Warming
This point has been revised.

Line 308: where should crystallization be observed? Outside the mini-straw?
Inside the mini-straw. It has been clarified in the paper.

Line 311: volume of solution?
This point has been revised.

Line 314: volume of solution?
This point has been revised.

Line 349: replace "checked" with "confirmed"
This point has been revised.

Line 351: correctLY
This point has been revised.

Line 371: delete "as" start new sentence with "The capacity"
This point has been revised.

Table 1: specify fresh in vivo derived; pronuclear; 8-cell; morula (not morulae)
This point has been revised.

Line 641: change "was" to "were"; pronuclear; 8-cell, morula
This point has been revised.

Table 2: need to add number of recipients. Line numbers cover superscripts of means; Was there a CONTROL GROUP?
The number of recipients has been added. In this table we compare the viability after vitrification at two different stages of the embryo development; no control groups are required for this purpose.

Licenses and Copyright

The following policy applies to all PLOS journals, unless otherwise noted.

What Can Others Do with My Original Article Content?

PLOS applies the Creative Commons Attribution (CC BY) license to articles and other works we publish. If you submit your paper for publication by PLOS, you agree to have the CC BY license applied to your work. Under this Open Access license, you as the author agree that anyone can reuse your article in whole or part for any purpose, for free, even for commercial purposes. Anyone may copy, distribute, or reuse the content as long as the author and original source are properly cited. This facilitates freedom in re-use and also ensures that PLOS content can be mined without barriers for the needs of research.

May I Use Content Owned by Someone Else in My Article?

If you have written permission to do so, yes. If your manuscript contains content such as photos, images, figures, tables, audio files, videos, etc., that you or your co-authors do not own, we will require you to provide us with proof that the owner of that content (a) has given you written permission to use it, and (b) has approved of the CC BY license being applied to their content. We provide a form you can use to ask for and obtain permission from the owner. Download the form (PDF).

If you do not have owner permission, we will ask you to remove that content and/or replace it with other content that you own or have such permission to use.

Don't assume that you can use any content you find on the Internet, or that the content is fair game just because it isn't clear who the owner is or what license applies. It's up to you to ascertain what rights you have—if any—to use that content.

May I Use Article Content I Previously Published in Another Journal?

Many authors assume that if they previously published a paper through another publisher, they own the rights to that content and they can freely use that content in their PLOS paper, but that's not necessarily the case – it depends on the license that covers the other paper. Some publishers allow free and unrestricted re-use of article content they own, such as under the CC BY license. Other publishers use licenses that allow re-use only if the same license is applied by the person or publisher re-using the content.

If the paper was published under a CC BY license or another license that allows free and unrestricted use, you may use the content in your PLOS paper provided that you give proper attribution, as explained above.

If the content was published under a more restrictive license, you must ascertain what rights you have under that license. At a minimum, review the license to make sure you can use the content. Contact that publisher if you have any questions about the license terms – PLOS staff cannot give you legal advice about your rights to use third-party content. If the license does not permit you to use the content in a paper that will be covered by an unrestricted license, you must obtain written permission from the publisher to use the content in your PLOS paper. Please do not include any content in your PLOS paper which you do not have rights to use, and always give proper attribution.

What Are Acceptable Licenses for Data Repositories?

If any relevant accompanying data is submitted to repositories with stated licensing policies, the policies should not be more restrictive than CC BY.

Removal of Content Used Without Clear Rights

PLOS reserves the right to remove any photos, captures, images, figures, tables, illustrations, audio and video files, and the like, from any paper, whether before or after publication, if we have reason to believe that the content was included in your paper without permission from the owner of the content.

How Does One Give Proper Attribution for Use of Content?

When citing a PLOS research article, use the "Vancouver style", as outlined in our Submission Guidelines. For example:

Kaltenbach LS et al. (2007) Huntingtin Interacting Proteins Are Genetic Modifiers of Neurodegeneration. *PLOS Genet* 3(5): e82. doi:10.1371/journal.pgen.0030082.

When citing non-article content from a PLOS website (e.g., blog content), provide a link to the content, and cite the title and author(s) of that content.

For examples of proper attribution to other types of content, see websites such as Open.Michigan.

Give Feedback

Please help us to improve our websites and better serve you by letting us know why you are consulting this page today.

I am consulting this page today, because I am:

- ◯ handling a manuscript as editor
- ◯ acting as a reviewer
- ◯ considering whether to submit
- ◯ preparing my initial submission
- ◯ preparing my revision
- ◯ a co-author on a submission
- ◯ Other

Send Feedback