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DEVELOPMENT OF A CHEAP VITRIFICATION METHOD FOR RABBIT EMBRYOS

TRABAJO FIN DE GRADO EN BIOTECNOLOGÍA

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Título TFG: Desarrollo de un sistema económico para la vitrificación de embriones de conejo.

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RESUMEN

Durante las últimas décadas muchos han sido los estudios que se han centrado en el desarrollo de soportes de vitrificación eficientes que disminuyeran el daño celular. El Cryotop es uno de los dispositivos más utilizados actualmente para la criopreservación de ovocitos y embriones, ya que permite altos ratios de enfriamiento debido al pequeño volumen de la solución de vitrificación empleada. Sin embargo, su precio ronda los 21€ por unidad. El objetivo de este trabajo es comparar el Cryotop y el asa de siembra sobre la vitrificación de embriones de conejo.

Tras la inseminación, a las 72 horas se recuperaron embriones en estadios de mórula o blastocisto temprano que fueron sometidos a vitrificación, en una solución de 20% de Etilenglicol y un 20% de Dimetlsulfóxido, en los dos soportes anteriormente descritos: Cryotop y asa de siembra. En un primer experimento, los embriones fueron desvitrificados y cultivados *in vitro* en una solución TCM199 con un 10% de suero a 38.5ºC y humedad saturada durante 48 horas. Se determinó la capacidad de desarrollo a blastocisto escapando o escapado. En un segundo experimento, embriones de ambos grupos experimentales fueron transferidos mediante laparoscopia para evaluar la tasa de implantación a los catorce días tras la inseminación y el número de nacidos vivos a parto.

Los resultados no muestran diferencias significativas entre ambos soportes, ni en el experimento *in vivo* ni en el *in vitro*. Por lo tanto, el asa de siembra puede ser un dispositivo alternativo para sustituir al Cryotop.

PALABRAS CLAVE

Vitrificación, asa de siembra, Cryotop, mórulas, nacidos vivos, conejo.

ABSTRACT

Over the last several decades there have been many studies in order to develop efficient carriers that produce minimum damage over the cells. Cryotop is one of the more currently employed carriers for oocyte and embryo vitrification due to the high cooling rates achieved because it reduces the volume of vitrification, however, its price is around 21€ per each. The aim of this work is to compare between Cryotop and plastic inoculating loop for rabbit embryo vitrification.

Briefly, 72 hours after insemination embryos were recovered in morulae or early blastocyst stage and were vitrified using vitrification solution consisting of 20% (vol/vol) ethylene glycol and 20% (vol/vol) dimethyl sulfoxide. Then embryos were loaded in Cryotop or inoculating loop. On the one hand, embryos were devitrified and cultured *in vitro* during 48 hours in medium TCM199 containing 10% serum at 38,5ºC and humidified atmosphere. It was evaluated the development of embryos until hatching. On the other hand, embryos from both experimental groups were transferred using the laparoscopic technique to evaluate implantation rate at fourteen days after insemination and offspring rate at birth.

There were no differences between Inoculating loop and Cryotop under *in vivo* and *in vitro* culture conditions. Therefore, inoculating loop is a suitable method for replacing Cryotop.

KEY WORDS

Vitrification, inoculating loop, Cryotop, morulae, live births, rabbit.

RESUM

Durant les últimes dècades, molts han sigut els estudis centrats en el desenvolupament de suports de vitrificació eficients que disminuïren el dany sobre les cèl·lules. El Cryotop es un dels dispositius més utilitzats per a la criopreservació d'òvuls i embrions ja que permet alts ràtios de refredament a causa del xicotet volum de la solució de vitrificació empleada, no obstant això, el seu preu ronda els 21€ per unitat. L'objectiu d'aquest treball es comparar el Cryotop amb l'ansa de sembra en la vitrificació d'embrions de conill.

Després d'inseminar les conilles, a les 72 hores es van recuperar els embrions en estadi de mòrula o blastocist enjorn, els quals van ser sotmesos a vitrificació, en una solució de 20% de Etilenglicol i un 20% de Dimetil sulfòxid, en els dos suports anteriorment descrits: Cryotop i ansa de sembra. D'una banda els embrions van ser desvitrificats i cultivats *in vitro* en una solució TCM199 amb un 10% de sèrum a 38.5ºC i humitat saturada durant 48 hores. Es va determinar la capacitat de desenvolupament a blastocist escapant o escapat. En un segon experiment, embrions d'ambdós grups van ser transferits mitjançant una laparoscòpia, després de catorze dies es va avaluar la taxa d'implantació i a l'hora del naixement el nombre de cries nascudes vives.

Les dades no reflecteixen diferències entre ambdós suports, ni l'experiment *in vivo* ni en *l'in vitro*. Per tant, l'ansa de sembra pot ser un dispositiu adequat per a substituir al Cryotop.

PARAULES CLAU

Vitrificació, ansa de sembra, Cryotop, mòrula, nascuts vius, conill.

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ABBREVIATIONS

µg: Microgram.

µm: Micrometer.

µl: Microliters.

BM: Base medium.

BOE: Official Spanish state gazette.

BSA: Bovine serum albumin.

cm: centimeter.

CO_2: Carbon dioxide.

CPS: Closed pulled Straw.

DMSO: Dimethyl sulfoxide.

DPBS: Dulbecco phosphate buffered saline.

eCG: Equine chorionic gonadotropin.

EQ: Equilibrium.

ET: Ethylene glycol.

FBS: Foetal bovine serum.

GLM: General lineal models.

GnRH: Gonadotropin-releasing hormone.

IU: International Units.

M: Molar

MII: Metaphase II.

ml: Milliliter.

mm: Millimeters.

OPS: Open pulled Straw.

VIT: Vitrification.

Vol: Volume.

wt/vol: Weight/volume.

1. INTRODUCTION

1.1. RABBIT AS A MODEL

Since 19th century, rabbit has been widely used as a model for the study of embryology and reproductive biology. Currently is the third most often used experimental mammal within the EU (EU report, 2010).

The rabbit belongs to small animal category and consequently it hasn't got additional ethical problems related with large animal category like goats, primates (Mapara et al., 2012). The small size, ease of handling and its low maintenance costs make the rabbit suitable specie in experimental studies (Figure 1). Moreover, the *Oryctolagus cuniculus* (rabbit) is phylogenetically closer to humans than rodents and their gene sequences are more similar to human than rodent ones (Graur et al., 1996). Because of that, rabbit has been widely used in clinical studies.

Figure 1. New Zealand rabbit.

Concerning reproduction studies, the rabbit has a lot of advantages that make it an efficient animal model (Fischer et al., 2012). Firstly, the fertility of rabbit is high (around 10 embryos per female) and it could be higher if hormonal treatments are applied (Fischer et al., 2012). Moreover the two separated functional uteri and cervices allows the transfer of two embryo groups to the same recipient female (Fischer et al., 2012). Also the rabbit has a short reproductive cycle that makes easier the development of the experiment and allows evaluate the offspring rate at birth (Fischer et al., 2012). Additionally, it should be noted that in rabbit

ovulation is induced by matting which allows the control of the time process like ovulation, fertilization, embryo development and implantation (Fischer et al., 2012).

1.2. CRYOPRESERVATION PRINCIPLES

Cryobiology is the study of the effects of subfreezing temperatures on biological systems (Woods et al., 2004). These temperatures allow the reversible inactivation of cellular metabolism with cellular integrity maintenance because no reactions occur in aqueous systems at -196ºC (Mazur, 1984). The only reactions that can occur at -196ºC are formation of free radicals and production of breaks in macromolecules due to background ionizations radiation (Rice, 1960).

In cryopreservation process there are many factors to keep in mind but the three major variables are: cryoprotectants, cooling and warming rates (Mazur, 1984). In physiological conditions the range of nucleation temperatures varies from -10º to -20ºC, while if cryoprotective additives are used the range of temperatures can be reduced until -40ºC (Rall et al., 1983) because cryoprotectants interact with water molecules and reduce the ability to form links between them (Solé et al., 2009). On the other hand, the rate of cooling affects the probability of intracellular crystallization because controls the rate of water transported across the membrane (Mazur, 1984; Figure 2).

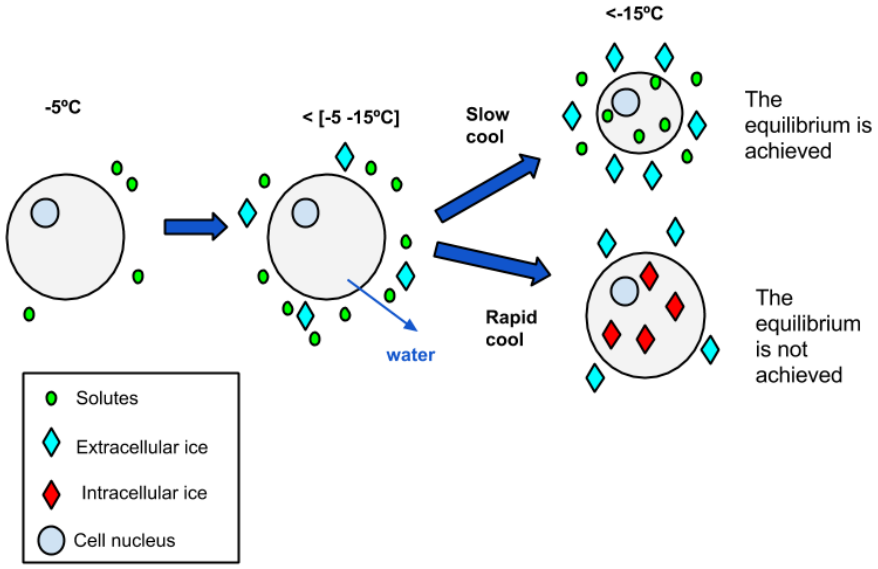

Figure 2. Schematic of physical events in cells during freezing (adapted from Mazur, 1984).

Below -5ºC, cells and the surrounding medium remain unfrozen due to supercooling and the effect of cryoprotectants. Between -5 and -15ºC, it is started the formation of extracellular ice, for that reason the external solution becomes increasingly concentrated and water flows out of the cell and freezes externally in order to maintain osmotic equilibrium (Mazur, 1984; Leibo, 2008). Below -10ºC, if cooling rate is slow the cell is able to lose water and maintain the equilibrium. Therefore cells dehydrate and not appear intracellular ice. But if the cooling rate is too fast the cell is not able to lose water in order to maintain the equilibrium, it becomes increasingly supercooled and attains equilibrium by freezing intracellular (Mazur, 1984).

Moreover, there is interaction between cooling and warming rate. When cells are cooled more slowly survival is higher with slow warming than with rapid (Mazur, 1984). But, when cells are cooled faster, survival is higher with rapid warming than with slow (Mazur, 1984). If cells are cooled faster it could produce an accumulation of smaller ice crystals, thermodynamically unstable, and these crystals during warming could aggregate to form larger crystals, this process is called recrystallization (Mazur, 1984; Woods et al., 2004). For this reason, slow warming is a bad choice because it allows time for such recrystallization to occur (Mazur, 1984).

Furthermore, it should be noted that cryopreservation can cause important damage to cell membranes (Mazur, 1984; Kopeika et al., 2015). On the one hand, ice crystal formation can lyse plasma membranes (Dobrinsky 1996). On the other hand, at low temperatures changes in physical properties of membranes can be produced (Mazur, 1984). The cell membrane is an important factor due to the permeability of cell membrane allows the movement of water and cryoprotectants in order to maintain the osmotic equilibrium (Mazur, 2010; Leibo, 2012). Finally, the cell membrane can act as a barrier to prevent the growth of extracellular ice into supercooled cytoplasm (Rall et al., 1983).

1.2.1 SLOW-FREEZING

Slow freezing consists on using slow cooling rate (≤1ºC/min) and low cryoprotectant concentration (around 10%), in order to extracellular water crystallise and generate an osmotic gradient that draws water from the intracellular compartment till intracellular vitrification occurs (Saragusty and Arav, 2011; Leibo, 2012). Slow cooling is an equilibrium process (Woods

et al., 2004), it means that maintain osmotic equilibrium. For that reason, during cryopreservation process is important adjust cooling rates to allow cell rebalance.

In slow freezing embryos first are pre-equilibrated in a cryoprotectant solution (Woods et al., 2004; Solé et al., 2009), the high concentration of the solution allows cell dehydration and the incorporation of cryoprotectants by the cell (Solé et al., 2009). Second, embryos are cooled fast at 2ºC/min until -7ºC, at this temperature the seeding is performed. This process consists on touching the outside of the carrier with a pre-cooled carrier to initiate freezing and avoid supercooling (Woods et al., 2004). Then, embryos are cooled slowly at 0.3ºC/min in order to allow the cell rebalances (during 10-15 minutes). When the cells are sufficiently dehydrated the intracellular concentration is high to avoid formation of ice and cells are immersed in liquid nitrogen (Woods et al., 2004; Solé et al., 2009; Figure 3).

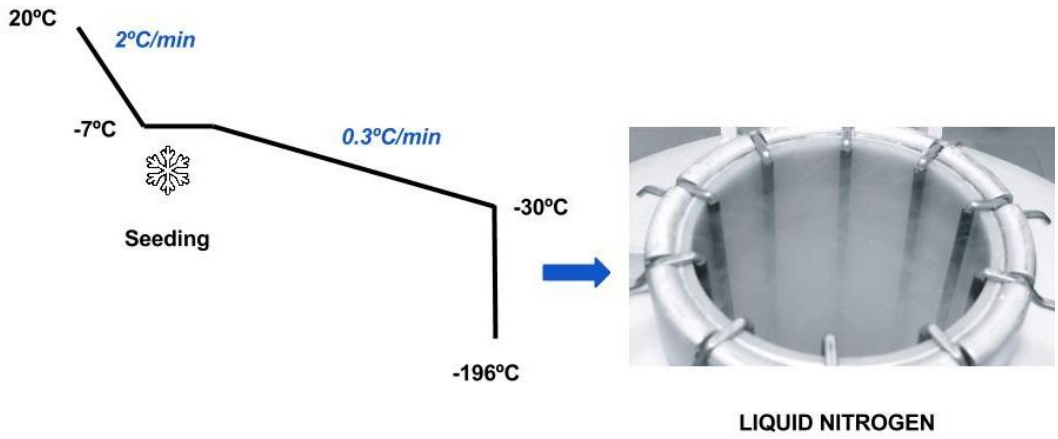

Figure 3. Schematic process of slow-freezing.

1.2.2 VITRIFICATION

In cryopreservation by vitrification high cooling rate (>1.000ºC/min) and high cryoprotectant concentration (≈40%) are used in order to achieve cellular dehydration and vitrify intra and extracellular compartments without ice crystals (Saragusty and Arav, 2011; Leibo, 2012; Figure 4).

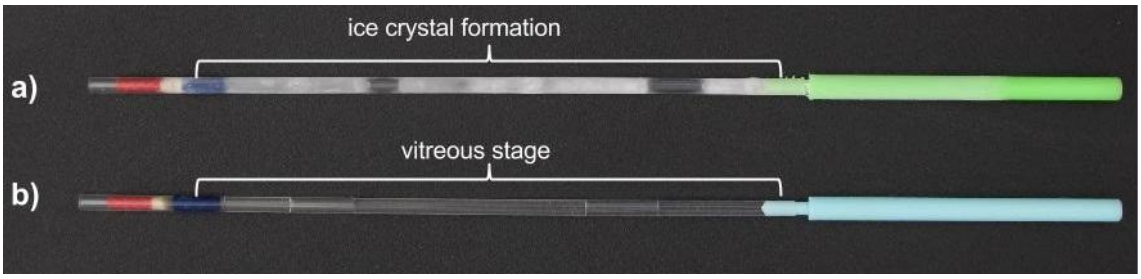

Figure 4. Comparison of effects over straw of different type of cryopreservation (a) Straw cryopreserved by slow freezing, (b) Straw cryopreserved by vitrification.

However, one of the most important problems associated to vitrification is the toxicity caused by high concentration of cryoprotectants to reduce the chance of ice nucleation and crystallization (Fahy et al., 2004; Saragusty and Arav, 2011). Cryoprotectants are associated with chemical toxicity and osmotic shock that can produce a detrimental change in volume (Arav, 2014).

Vitrification is an alternative method to cryopreserve those biological species that are sensitive to chilling injury (Kuwayama et al., 2005). Vitrification has advantages compare to slow freezing: Vitrification is the solidification of a solution by an extreme elevation in viscosity at low temperatures without ice crystal formation (Vajta, 2000; Vajta and Kuwayama, 2006; Konc et al., 2014). For this reason, cells suffer less damage during cryopreservation process. Furthermore, the possible partial and sometimes total elimination of chilling injury, as the sample passes through the dangerous temperature zone quickly enough to disallow sufficient time for damage to develop (Vajta and Kuwayama, 2006). Moreover, vitrification can be done relatively cheaply, without sophisticated equipment (Vajta, 2000). It allows using vitrification in poorly equipped locations like zoos (Saragusty and Arav, 2011).

1.3 EMBRYO CRYOPRESERVATION

Human fertility is a vast field to do research and afterwards applying the relevant achievement directly for the benefit of the society. During the last years, embryo cryopreservation has become routine procedure in human assisted reproductive techniques. Embryo vitrification is used in order to storage not transferred embryos. Also, this technique has maximized the efficiency of the *in vitro* fertilization cycles (Konc et al., 2014).

Furthermore, the improved vitrification protocols resulted in a remarkable advance in several areas of domestic animal embryology (Vajta, 2000). Embryo cryopreservation can be used as a tool in setting up genetic resource banks for biodiversity preservation in animal breeding and laboratory products (Marco-Jiménez et al., 2013). For instance, embryo cryopreservation allow us to preserve lines from pathogens, to evaluate the genetic improvement, to reduce the impact of genetic drift and the transport of lines to different countries (Lavara et al., 2011: 2014). Moreover, it is important to maintain genetic improvements of certain traits in species such pig or rabbit (García and Baselga, 2002; Lavara et al., 2011).

Accordingly to International Union for Conservation of Nature and Natural Resources Red List of Threatened Species a total of 19.817 species are threatened and suffer a high risk of extinction in the near future (The IUCN red list of threatened species, 2015). Nowadays restoring biodiversity can be achieved. Embryo cryopreservation has the advantage of preserving the entire genetic complement of both parents (Saragusty and Arav, 2011). For this reason cryobanking of embryos can thus help in establishing founder populations with the aim of eventual reintroduction into the wild. Currently, the main issue is that species are very different between them; consequently cryopreservation protocols are distinct (Saragusty and Arav, 2011). Therefore, the improvement of these techniques is encouraging for the maintenance of genetic diversity in the future (Ptak et al., 2002).

Another area where cryopreservation has an important paper is embryo cloning. The improving of vitrification technique has been allowed the maintenance of developmental ability after embryo warming. For these reason blastomeres have been successfully used as donors for nuclear transfer (Vajta, 2000).

In rabbit, several studies have reported the efficiency of vitrifying embryos. It is shown by survival rates at birth, in rabbits the range varies between 25% and 65% (Kasai et al., 1992; Vicente and Garcia-Ximenez 1994; Vicente et al., 1999; López-Béjar and López-Gatius 2002; Mocé et al., 2010; Marco-Jiménez et al., 2013; Lavara et al., 2014; Saenz-de-Juano et al., 2014).

1.4 STRATEGIES TO REDUCE CHILLING INJURY

Over the last several decades there have been many studies in order to reach the main goal, successful vitrification of embryos and oocytes. Through these experiments it has been

overlapped the main issues of vitrification. These experiments try to create an acceptable compromise between decreasing cryoprotectant toxicity and increasing cooling rates (Vajta and Kuwayama, 2006). Consequently, in 1985 Rall and Fahy achieved the first successful vitrification of mouse embryos using a mixture of dimethyl sulfoxide (DMSO), acetamide and polyethylene glycol and relatively large volume sample (Rall and Fahy, 1985). Furthermore, it was demonstrated that using a combination of different cryoprotectants was reduced the level of toxicity (Vajta and Kuwayama, 2006; Cocchia et al., 2010; Saragusty and Arav, 2011). Nevertheless, one of the most revolutionary achievements related to vitrification, which will be explained in the next chapter, was that reducing volume it could be increased the probability of vitrification. As a result, although studies of vitrification of embryos were started in the late 1980s, they have not been applied clinically until last years (Arav, 2014).

1.4.1 REDUCING CONTAINER VOLUMES

One of the most important factors that influence vitrification is volume (Saragusty and Arav, 2011). It was discovered that reducing volume increases the probability of vitrification. It is because smaller volume allows better heat transfer; consequently higher cooling rates could be applied. And also, decreases the chance of ice crystal formation in the sample (Kuwayama, 2007). Moreover, many publications have demonstrated that increasing the cooling rate would improve survival rates by up 37% (Lee et al., 2007; Papis et al., 2008).

In 1989, Arav et al., developed the method called “minimum drop size” that consists in the minimal size that maintained oocytes or embryos without damage owing to desiccation (Arav, 2014).

As a result, during the last decade, many techniques have been developed in order to reduce sample volume. These techniques can be divided into two groups (Saragusty and Arav, 2011):

1. **Surface techniques:** These systems are open consequently high cooling rate can be achieved (around 20.000-30.000ºC/min), (Criado, 2012). Moreover high warming rates could achieve because of direct exposure to the warming solution. The main issue is that the sample is in direct contact with cooling solution and there is a risk of contamination (Criado, 2012). For example: Electron microscope grid (Steponkus et al.

1990), Cryotop (Kuwayama, 2007), Cryoloop (Lane et al. 1999), Plastic blade (Sugiyama et al., 2010), Hemi-straw (Vanderzwalmen et al. 2000; Figure 5).

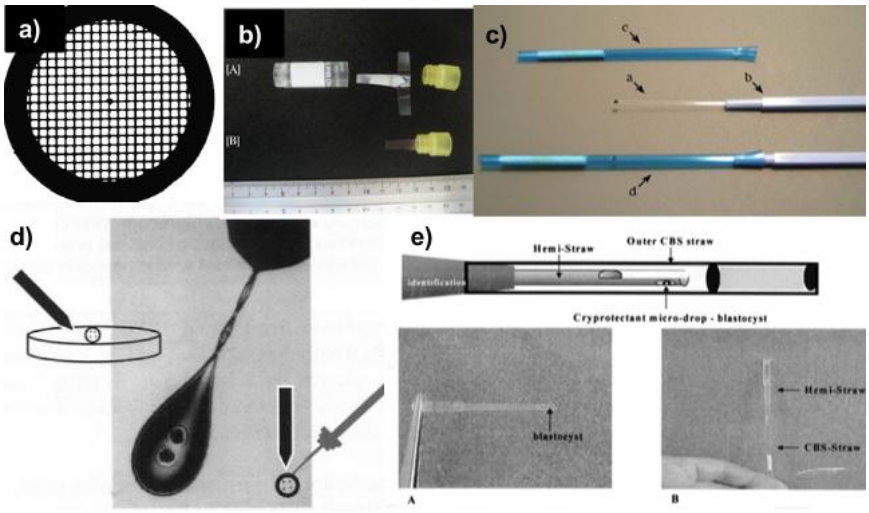

Figure 5. Vitrification surface carrier systems: (a) Electron microscope grid, (b) * plastic blade, (c) * Cyrotop, (d) * Cryoloop, (e) * Hemi-straw. (Adapted from Saragusty and Arav, 2011; * These photos were provided by Rikikazu Sugiyama (b), Masa Kuwayama (c), Michelle Lane (d), Enrique Criado (e)).

2. **Tubing techniques:** These systems are closed, therefore are safer and easier to handle. There is no direct contact between the biological sample and liquid nitrogen (Kuwayama, 2007). This prevents contamination by contact and cross contamination from shared containers. These carriers are hermetically sealed in order to prevent the entry of pathogens from outside. The main disadvantage of these carriers is that the cooling rate is much lower, for these reason closed system have a high concentration of cryoprotectants so the cryotoxicity increases (Criado, 2012). For example: Plastic straw (Rall and Fahy, 1985), Open pulled straw (OPS), (Vajta et al., 1997), Cryotip (Kuwayama et al., 2005; Figure 6).

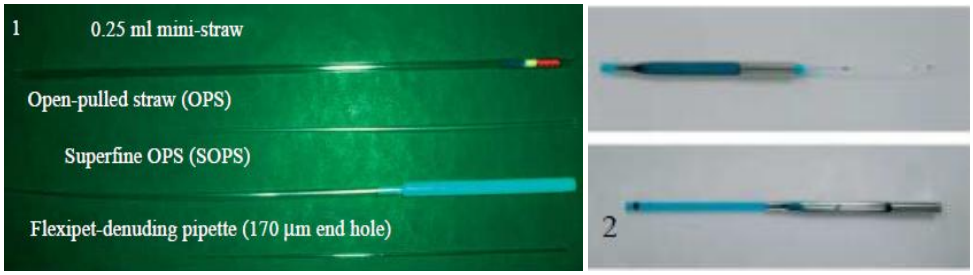

Figure 6. Vitrification tubing carrier systems: (1) plastic straw, open-pulled straw, superfine open-pulled straw, flexipet-denuding pipette (2) Cryotip. (Adapted from Saragusty and Arav, 2011).

A summary of the available carriers to date are shown at table 1.

Table 1. Review of the carriers developed until now.

	CARRIER	REFERENCES
OPEN SYSTEM	Electronic microscope grid	Steponkus et al. 1990
	Minimum drop size	Arav, 1992
	Cryotop	Kuwayama, 2007
	Cryoloop	Lane et al. 1999
	Hemi-straw	Vanderzwalmen et al. 2000
	Solid surface	Dinnyes et al., 2000
	Nylon mesh	Matsumoto et al., 2001
	Cryoleaf	Chian et al., 2005
	Cryolock	Biodiseño Colombia Ltda.
	Direct cover vitrification	Chen et al., 2006
	Fibre plug	Muthukumar et al., 2008
	Vitrification spatula	Tsang and Chow, 2009
	Cryo-E	Petyim et al., 2009
	Plastic blade	Sugiyama et al., 2010
	Vitri-Inga	Almodin et al., 2010
CLOSED SYSTEM	Plastic straw	Rall and Fahy, 1985
	OPS	Vajta et al., 1997
	Closed pulled straw (CPS)	Chen et al., 2001
	Flexipet-denuding pipette	Lieberman et al., 2002
	Superfine OPS	Isachenko et al., 2003
	CryotTip	Kuwayama et al., 2005
	Pipette tip	Sun et al., 2008
	High-security vitrification straw	Camus et al., 2006
	Sealed pulled straw	Yavin et al., 2009
	Cryopette	Portmann et al., 2010
	Rapid-i	Larman and Gardner, 2011
	Vitrisafe	Vanderzwalmen et al., 2009
	Ultravit	Criado et al., 2011

Generally, these containers reduce vitrification volume and increase cooling rate that allows a moderate decrease in cryoprotectants concentration, thus minimize toxic effects. The following equation bases the probability of vitrification on these three factors (Kuwayama, 2007; Saragusty and Arav, 2011):

$$Probability\ of\ vitrification = \frac{Cooling\ rate\ x\ Viscosity}{Volume}$$

The main differences between containers are the total concentration of intracellular cryoprotectants required in vitrification solution, the exposure time and equilibration to intracellular cryoprotectants (Hochi et al., 2004).

Furthermore, it was demonstrated that exists a link between carrier and cryopreservation damage. Depend on the container different rates of cooling and warming could be applied (Table 2), and these affect the reversibility of cryoinjuries (Marco-Jiménez et al., 2013). By using small samples fracture damage rarely occurs and it can be removed with adjustment of warming parameters (Kuwayama, 2007).

Table 2. Cooling rates for the different containers (Adapted from Criado, 2012).

CARRIER	VOLUME (µl)	COOLING RATE (ºC/min)
CRYOLOOP	1	20000
HEMI-STRAW	>1	>20000
CRYOLEAF	>1	23000
VITRI-INGA	1	20000
CMV-RING	>1	10000
VITRISAFE	>1	1300
0.25 ML STRAW	25	2500
OPS	1	16700
CRYOTOP	0.1	23000
CRYOTIP	1	12000
RAPID-I	0.5	1200
CRYOPETTE	1.2	23700
ULTRAVIT	0.2	250000

Since it was discovered by Hamawaki and Kuwayama (1999), Cryotop has been the most used container in oocytes and embryo vitrification because Cryotop achieved the best outcomes. Kuwayama compared between three types of carrier (plastic straws, open-pull straws and Cryotop) in order to establish the better method to vitrified bovine MII oocytes. He demonstrated that using Cryotop could be yielded the best survival rates. Probably the most remarkable difference between three methods that might influence the results was the cooling and warming rate (Kuwayama et al., 2005).

Cryotop is the special vitrification container consisting of a very fine polypropylene strip (0,4mm wide x 20mm long x 0,1mm thick) attached to a hard plastic handle (Kuwayama et al., 2005; Kuwayama, 2007) (Figure 7). Moreover, the thin strip is covered with a hard plastic cover (3cm long) on top of the Cryotop sheet to protect it during storage in nitrogen containers (Kuwayama et al., 2005; Kuwayama, 2007).

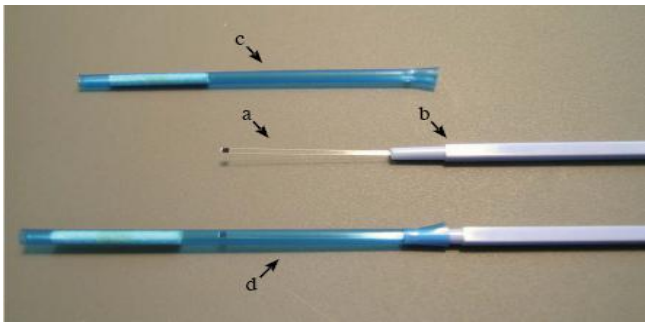

Figure 7. Cryotop: (a) polypropylene strip is attached to a hard plastic handle (b). After vitrification, (c) plastic cover is attached to protect strip (d). (Adapted from Kuwayama et al., 2005).

Currently, the Cryotop technique has rapidly spread in human medicine because this carrier produces impressive results in term of healthy offspring after oocyte cryopreservation (Kuwayama, 2007; Mikolajewska et al., 2012). With the goal of looking for alternatives, a research group evaluated the effect of different vitrification protocols applied to feline oocytes (Mikolajewska et al., 2012). Specifically, they compared Cyrotop and Cryoloop, but they used plastic inoculating loop as a Cryoloop to reduce the price because the results obtained not differ from those obtained with commercial Cryoloops (Mikolajewska et al., 2012). Both carriers allow to complete the vitrification process using a minimum volume (<0, 5 µl) of solution.

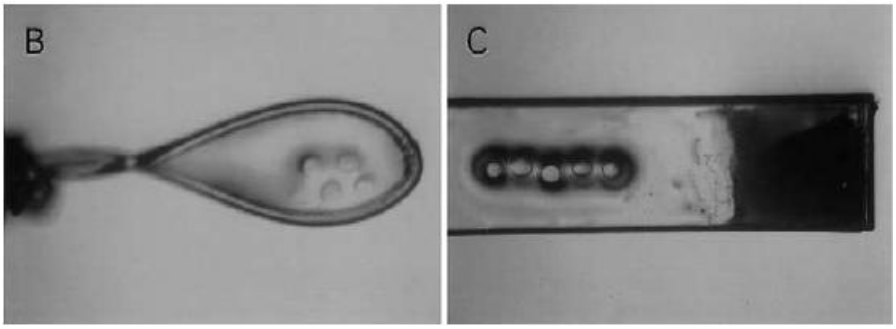

Figure 8. Vitrification systems: (B) Cryoloop, (C) Cryotop. (Adapted from Hochi et al., 2004).

Cryoloop used for vitrificaton consisted of a nylon loop (20µm width; 0.5-0.7mm diameter) mounted on a stain-less steel pipe held by epoxy to the lid of a cryovial (Lane et al., 1999) (Figure 8), while plastic inoculating loop is a tool usually made of platinum or nichrome wire in which the tip forms a small loop with a diameter of about 5mm (Figure 9).

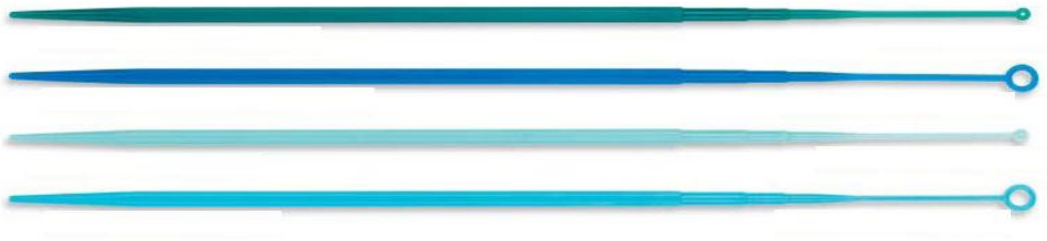

Figure 9. Plastic inoculating loop (from http://www.medicalexpo.es/prod/copan-italia/asas-siembra-laboratorio-68105-447624.html).

Mikolajewska et al., (2012) demonstrated, under *in vitro* culture conditions, that no were observed difference among Cryotop and plastic inoculating loop and they concluded that both methods are suitable for oocyte vitrification.

2. OBJECTIVE

The aim of the present work was to compare between plastic inoculating loop and Cryotop for rabbit embryo vitrification through *in vivo* experiment in which the implantation rate, foetal losses and offspring rate at birth were evaluated. These data were supported by *in vitro* development of embryo warmed until hatching blastocyst.

3. MATERIALS AND METHODS

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Química S.A. (Alcobendas, Madrid, Spain). The Ethics and Animal Welfare Committee of the Universidad Politécnica de Valencia approved this study (procedure 2015/vsc/PEA/00061). All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (BOE, 2013; BOE is the official Spanish State Gazette).

3.1. ANIMALS

Five month old rabbits were used. They belonged to the New Zealand Yellow line. Animals were housed individually at the Polytechnic University of Valencia experimental farm. The rabbits were accommodated at conventional cage (700 x 500 x 320mm), under a controlled 16-hour light: 8-hour dark photoperiod, monitored temperature (minimum 17.5ºC and maximum 25.5ºC) and fed a commercial diet.

3.2. EMBRYO RECOVERY

3.2.1. ARTIFICIAL INSEMINATION

A total of 37 female were used as donor. Female were treated with 20 IU of eCG intramuscular (Intervet International B.V., Bowmeer, Holland) to induce receptivity. After 48 hours, female animals were artificially inseminated with 0.5ml of fresh heterospermic pool of semen at a rate of 40 x 10^6 spermatozoa/mL from fertile male animals diluted in Tris-citric-glucose extender. The ejaculates were collected at same day of insemination using an artificial vagina, following the method described by Vicente et al., (2011). Motility was examined at room temperature under a microscope with phase-contrast optics at 40x magnitude. Only those ejaculates with >70% motile sperm were used. The insemination was carried out using a plastic cannula (22cm). Immediately after insemination, female animals were administrated 1µg of buserelin acetate (synthetic analogous of Gonadotropin-releasing hormone, GnRH, Hoechst Marion Roussel S.A., Madrid, Spain) to induce ovulation.

3.2.2. EMBRYO COLLECTION AND EVALUATION

Rabbit does were euthanized 72 hours after insemination by intravenous injection of 0.6g sodium pentobarbital (Doléthal®, Vétoquinol E.V.S.A., Madrid, Spain) into the marginal ear vein. Embryos were collected at room temperature by flushing the oviducts and uterine horns with 10 mL of embryo recovery media consisting on Dulbecco phosphate buffered saline (DPBS) supplemented with 0.2% (wt/vol) bovine serum albumin (BSA), and antibiotics (penicillin G sodium 300,000 IU, penicillin G procaine 700,000 IU, and dihydrostreptomycin sulphate 1250mg; Penivet 1; Divasa Farmavic, Barcelona, Spain). After recovery, embryos were classified under stereomicroscope. Only morphologically normal embryos (morulae and early blastocysts) were selected according to International Embryo Transfer Society classification (homogeneous cellular mass, mucin coat, and spherical zona pellucid), (Figure 10). Then embryos were distributed randomly for vitrification in both carriers.

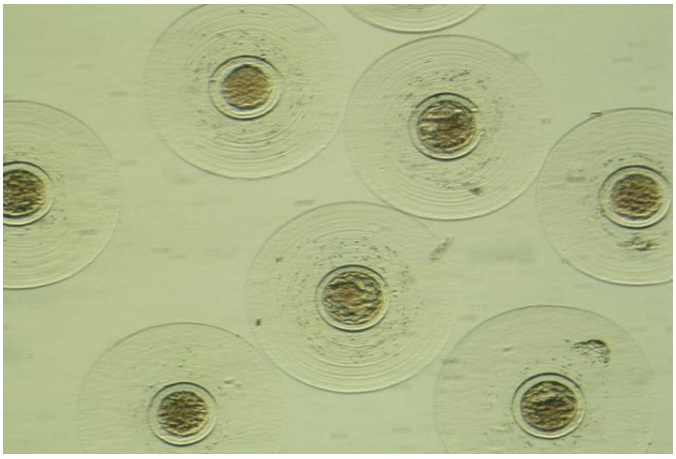

Figure 10. Embryo at morulae stage and early blastocyst.

3.3. VITRIFICATION AND WARMING PROCEDURES

Embryos were vitrified according to the vitrification procedure describe by Vicente et al., (1999) using two carriers: Cryotop (Kitazato Co., Fuji, Japan) and Inoculating loop (COPAN, Brescia, Italy). Embryos were vitrified in a two step addition procedure. First, embryos were transferred into equilibration solution consisting of 10% (vol/vol) ethylene glycol (ET) and 10% (vol/vol) dimethyl sulfoxide dissolved in base medium (BM; DPBS supplemented with 0.2% [wt/vol] BSA), at room temperature during 2 minutes. Then, the embryos were passed to

vitrification solution consisting of 20% (vol/vol) ethylene glycol and 20% (vol/vol) dimethyl sulfoxide in BM (Figure 11).

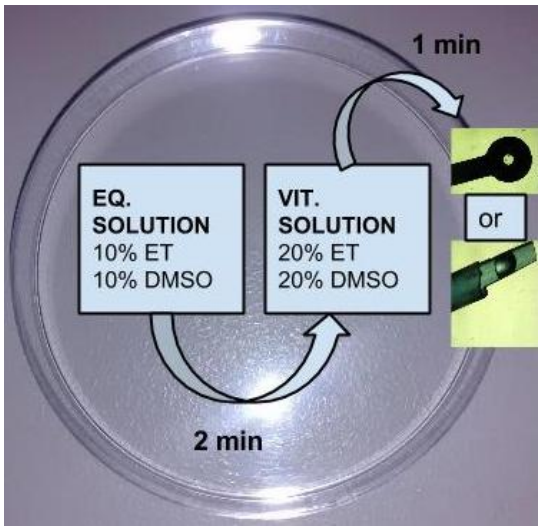

Figure 11. Diagram of vitrification procedure.

EQ= Equilibrium; VIT= Vitrification; DMSO= Dimethyl sulfoxide; ET= Ethylene glycol.

Next, the embryos were loaded into inoculating loop and Cryotop and directly plunged into liquid nitrogen within 1 minute (Figure 12).

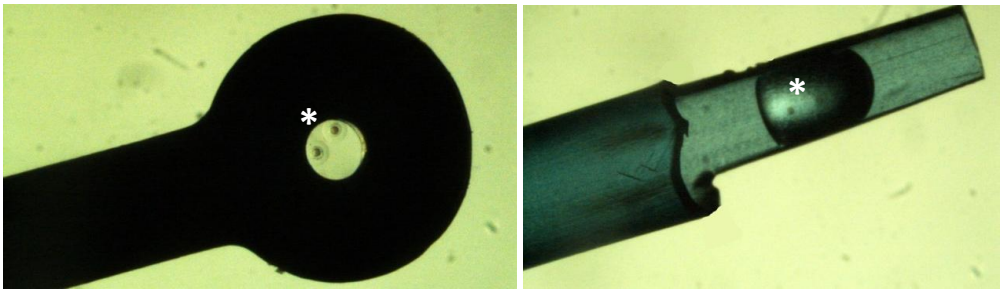

Figure 12. Embryo location inside Inoculating loop and Cryotop. *Specific view of embryos inside both carriers.

After storage in liquid nitrogen, embryos were warmed. The procedure was the same for both carriers. Embryos were warmed by direct immersion of the carrier in sucrose solution (0.5M sucrose in BM at room temperature) during 1 minute, and then the embryos were transferred at 0.25M sucrose solution during 1 minute, and finally embryos were washed three times (Figure 13).

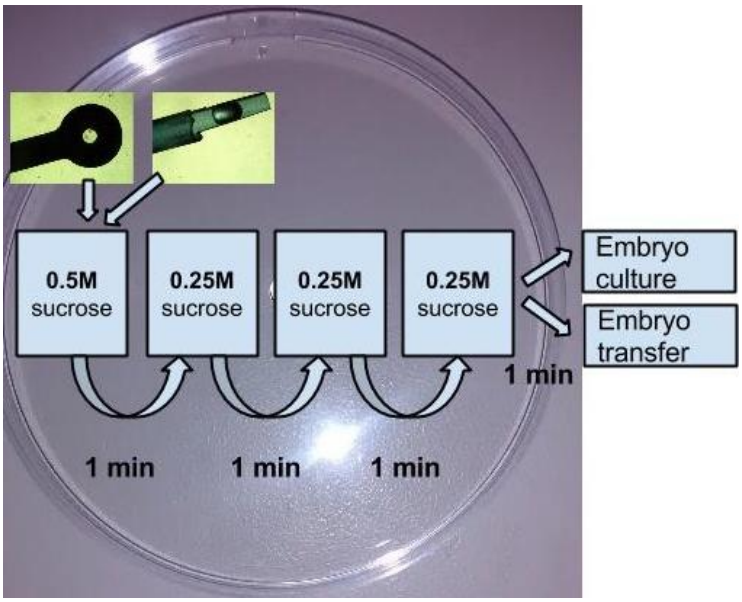

Figure 13. Diagram of the warming procedure.

3.4. EXPERIMENT 1: *IN VITRO* CULTURE OF EMBRYOS

After warming, the embryos were cultured in medium TCM199 containing 10% Foetal Bovine Serum (FBS) at 38,5ºC and 5% CO_2 in humidified atmosphere during 48 hours. The embryos were divided in two groups according the carrier used for vitrification and one additional group corresponding to fresh embryos (control group).

Afterwards, embryos were evaluated under stereomicroscope. It was assessed the development of embryos until hatching blastocyst (Figure 14).

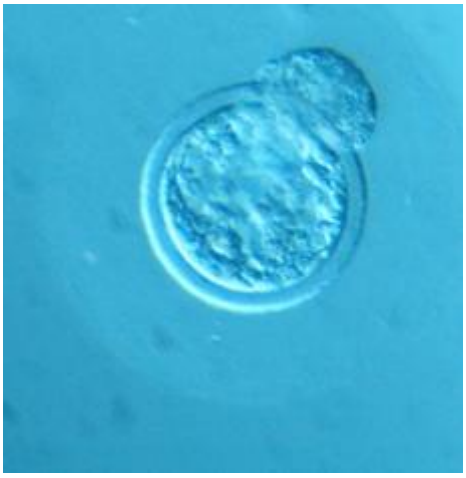

Figure 14. Embryo at hatching blastocyst stage.

3.5. EXPERIMENT 2: EMBRYO TRANSFER

A total of 142 vitrified embryos (48 for inoculating loop, 54 for Cryotop and 40 for control) were transferred into 14 adult nulliparous female animals by laparoscopy following the procedure described by Besenfelder and Brem (1993). All of transferred embryos were morphologically normal with intact mucin coat and zona pellucida. Recipient does were induced to ovulate by injection of 1 µg of buserelin acetate (Hoechst Marion Roussel S.A., Madrid, Spain) 68-72 hours before the transference. The transfer was carried out using a Hopkins® laparoscope, which is a 0º-mm straight-viewing laparoscope, 30-cm in length, with a 5-mm working channel (Karl Storz Endoscopia Ibérica S.A., Madrid, Spain). Recipient does were sedated by intramuscular injection of 16mg xylazine (Rompun, Bayer AG, Leverkusen, Germany). After 5 minutes anaesthesia was performed by intravenous injection, in the marginal ear vein, of 16-20mg ketamine hydrochloride (Imalgene®, Merial, S.A., Lyon, France). Moreover, during laparoscopy 12mg of morphine hydrochloride (Morfina®, B. Braun, Barcelona, Spain) was administrated intramusculary.

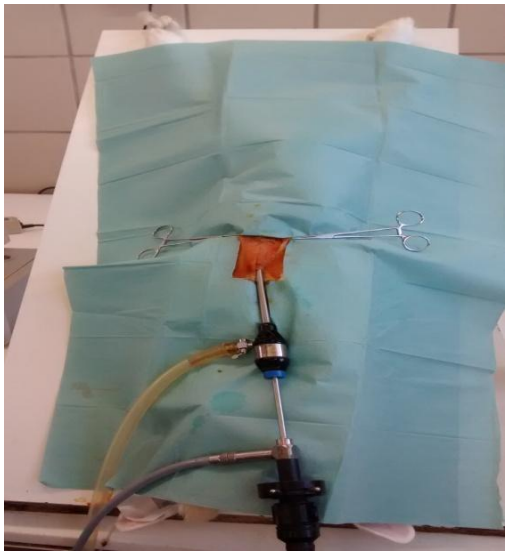

Figure 15. Recipient does on an operating table to have a laparoscopy.

Previously to start laparoscopy, abdominal region of animals was shaved and then recipients does were put on an operating table in a vertical position (head down at 45-degree angle) in order to ensure that the stomach and intestines are cranially located so that the reproductive tract was easy to handle. Only an endoscope trocar was inserted into the abdominal cavity. When the trocar was removed, the abdomen was insufflated with CO_2 and the endoscope was then inserted (Figure 15). The embryos were aspirated in a 17-gauge

epidural catheter (Vygon corporate, Paterna, Valencia), introduced into the inguinal region with an epidural needle and then inserted in the oviduct through the infundibulum (Figure 16).

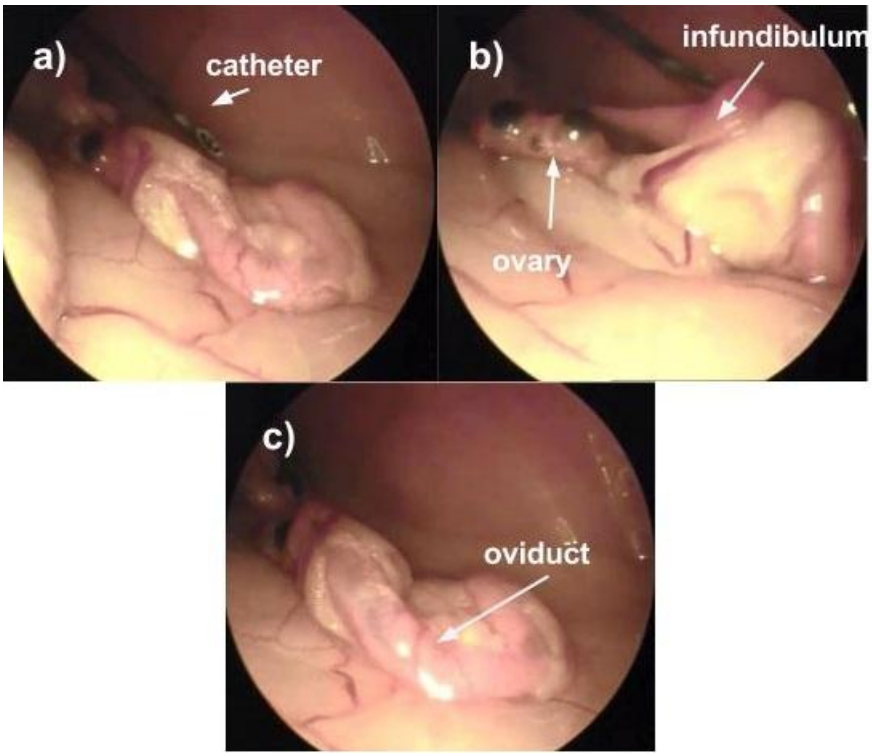

Figure 16. Development of embryo transfer. (a) Epidural catheter is introduced into the inguinal region, (b) the catheter is used to look for the infundibulum, (c) embryos were inserted into the oviduct, through the infundibulum.

Approximately, 10 embryos were transferred per female in the ampulla of one oviduct. After surgery, does were treated with antibiotics (0.1mL/kg procaine penicillin, Duphapen® Strep, Pfizer, S.L.) and buprenorphine hydrochloride (0.08 mg every 12 hours for 3 days, Buprex®, Esteve, Barcelona, Spain).

3.5.1. IMPLANTATION AND DELIVERY RATES

Fourteen days after insemination, recipient does were anesthetized following the same procedure described previously and ventral midline laparoscopy was carried out, noting implanted embryos (Figure 17). Finally, at birth (approximately thirty-one days after insemination) total kits born and birth weights were recorded. In addition, foetal losses were calculated as the difference between born at birth and the number of implanted embryos.

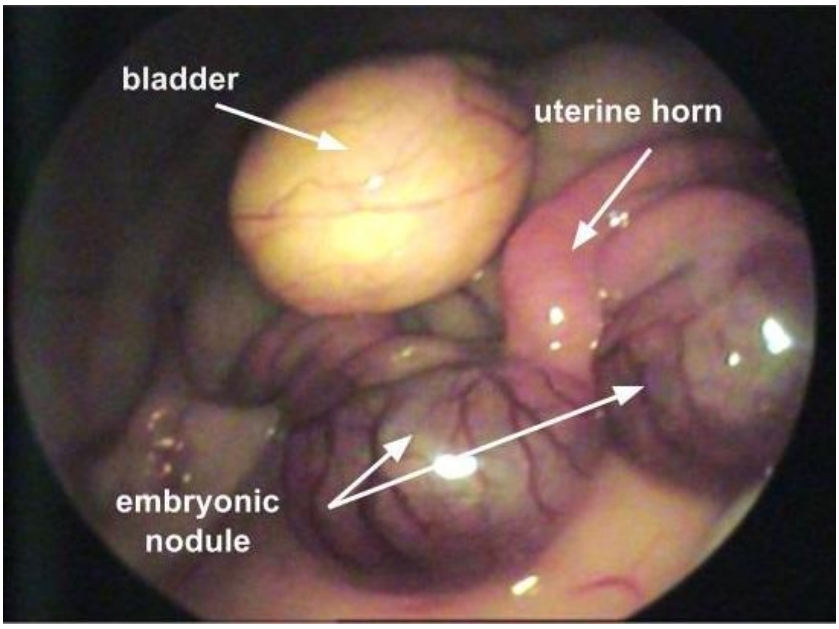

Figure 17. Implanted embryos.

3.6. STATISTICAL ANALYSES

For experiment 1, the development of embryos until hutching was analysed by chi-square test with Yates' correction.

For experiment 2, rate of implanted embryos, rate of offspring at birth and foetal losses were analysed using a chi-square test with Yates' correction. For birth weight, the differences in weight among different groups were analysed by one way ANOVA, using the General Linear Models (GLM), including the litter size as a covariate.

Analyses were performed with Statgraphics Plus 5.1. Differences of $P<0.05$ were considered significant.

3.7. EXPERIMENTAL DESIGN

The experimental design followed in this study is shown in Figure 18.

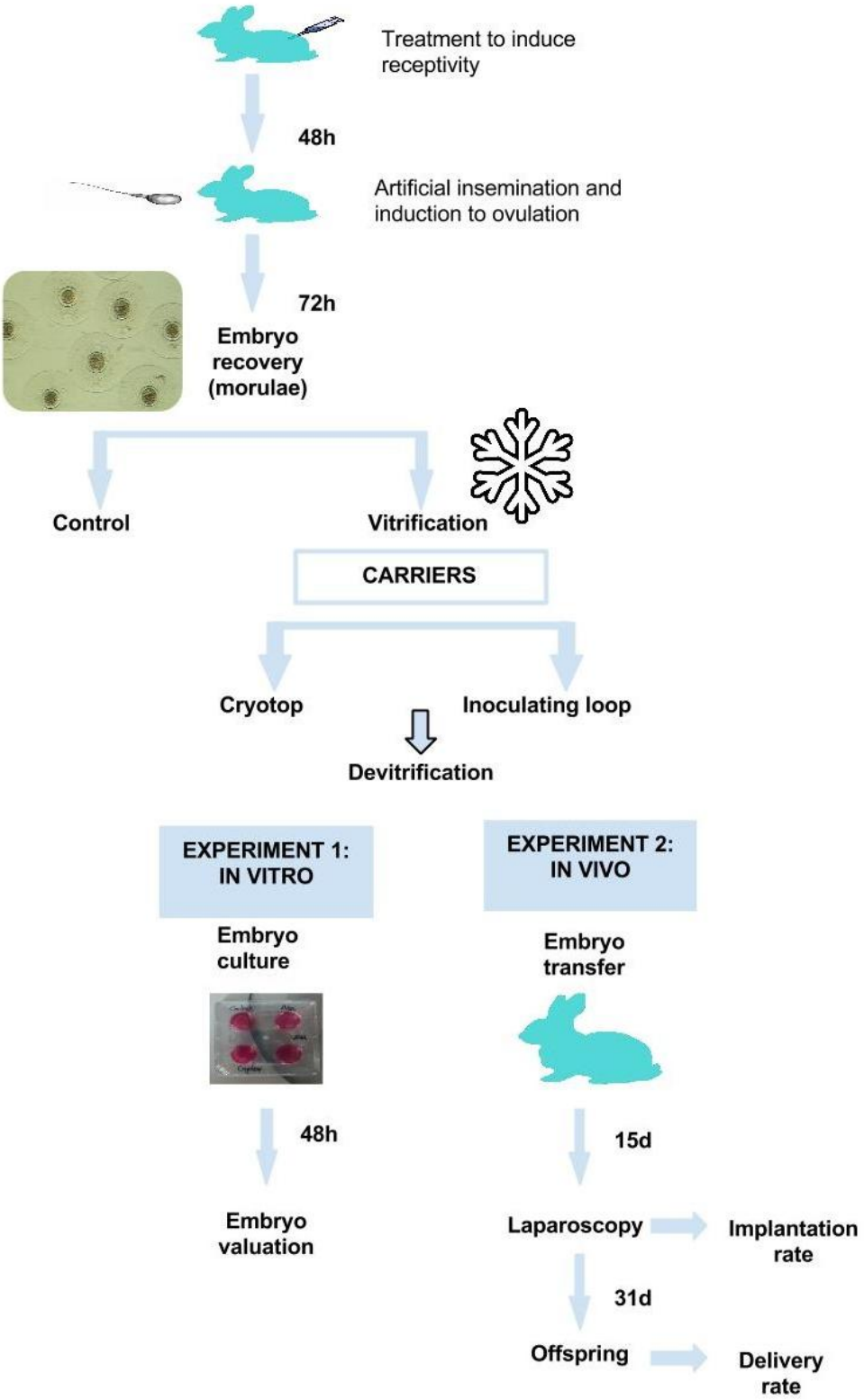

Figure 18. Experimental design.

4. RESULTS

4.1. EXPERIMENT 1: *IN VITRO* CULTURE OF EMBRYOS

For this experiment, a total of 120 embryos were cultured according the carrier used for vitrification, 52 for inoculating loop, 50 for Cryotop and 18 for control (fresh embryos). There were no differences in overall embryos yield at 48 hours between embryos vitrified with inoculating loop and Cryotop (42.3% and 42.0%, respectively, Table 3).

Table 3. Development until hatching of rabbit vitrified embryos in inoculating loop and Cryotop.

Experimental group	n	Blastocyst development (%)
Inoculating loop	52	42.3[a]
Cryotop	50	42.0[a]
Control	18	94.4[b]

n, total of embryo cultured.

[a,b] Values in the same column with different superscript letters are statistically different (P<0.05).

However, significantly more embryos were developed until hatching blastocyst at 48 hours in the control group (94.4%, Table 1, Figure 19).

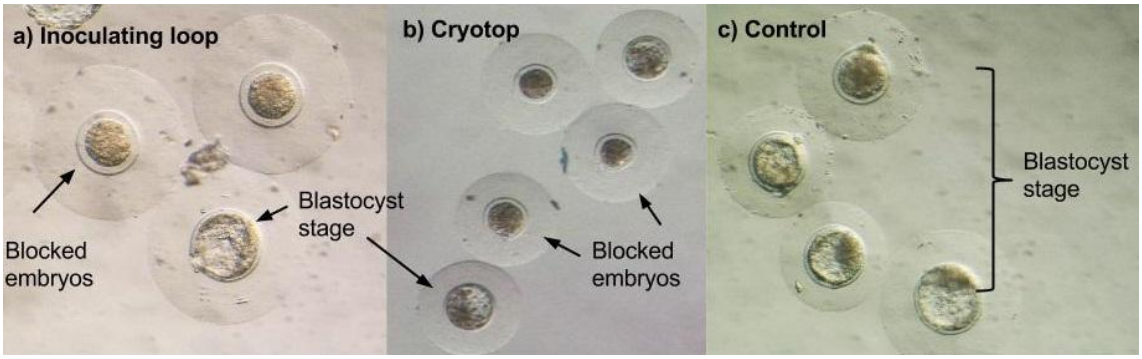

Figure 19. Viewed of embryos after 48 hours of culture *in vitro* (a) Embryos vitrified with inoculating loop (b) Embryos vitrified with Cryotop (c) Fresh embryos (control).

4.2. EXPERIMENT 2: EMBRYO TRANSFER AND *IN VIVO* DEVELOPMENT

A total of 142 vitrified embryos (48 for inoculating loop, 54 for Cryotop and 40 for control) were transferred into 14 adult nulliparous female. There were no differences at 14 days in implantation rate between embryos vitrified with inoculating loop and Cryotop (56.3% and 50.0%, respectively, Table 4).

Table 4. Effect of vitrification carrier on implantation, offspring at birth and birth weight.

Experimental group	N	Implanted embryos (%)	Total kits born (%)	Live births (%)	N	Birth weight (g)
Inoculating loop	48	56.3[a]	45.8[a]	39.6[a]	19	63.2 ± 2.69
Cryotoop	54	50.0[a]	35.2[a]	35.2[a]	19	61.3 ± 3.08
Control	40	77.5[b]	70.0[b]	70.0[b]	28	59.4 ± 2.30

n, total of embryo transferred.

N, total of offspring weighted.

[a,b] Values in the same column with different superscript letters are statistically different (P<0.05).

Nevertheless, there were differences in implantation rate at 14 days between both embryos vitrified groups and fresh embryos (77.5%, Table 4).

The total kits born were not significantly affected by vitrification carrier (45.8% and 35.2%, for inoculating loop and Cryotop respectively). However, there were differences in total kits born among embryos vitrified and fresh embryos (70.0%, Table 4).

Regarding weight at birth was not significantly affected by vitrification carrier; there were no differences between vitrified and fresh embryos (63.2 ±2.69g vs. 61.3 ± 3.08g and 59.4 ± 2.30g, for inoculating loop vs. Cryotop and fresh, respectively, Table 4, Figure 20).

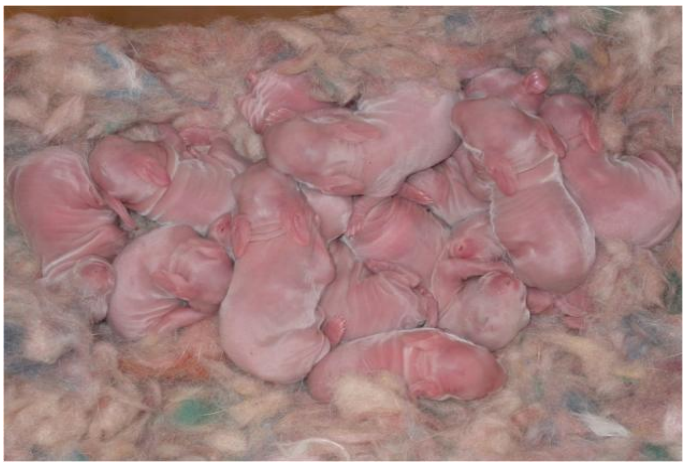

Figure 20. Offspring after birth.

Lastly, embryos vitrified with Cryotop had the highest embryonic losses compared with the inoculating loop and fresh embryos (50.0%, 43.8% and 22.5%, for Cryotop, inoculating loop and control, respectively). However there were no differences in foetal losses between vitrified and control embryos (10.4%, 14.8% and 7.5%, for inoculating loop, Cryotop and fresh embryos respectively, Table 5).

Table 5. Effect of vitrification carrier on embryonic and foetal losses.

Experimental group	N	Embryonic losses (%)	Foetal losses (%)	Total losses (%)
Inoculating loop	48	43.8[a,b]	10.4	54.2[a]
Cryotoop	54	50.0[a]	14.8	64.8[a]
Control	40	22.5[b]	7.5	30.0[b]

n, total of embryo transferred.

[a,b] Values in the same column with different superscript letters are statistically different (P<0.05).

5. DISCUSSION

Since it was noted the importance of reducing the volume of vitrification solution in the efficiency of the process, there have been many studies in order to develop or improve carriers that following the minimum volume cooling procedure (reviewed by Arav, 2014). As exactly, Cryoloop was one of the most employed carriers to vitrify embryos because its small volume which allows rapid an uniform heat exchange during cooling; high rates of cooling prevent chilling injury and reduce the exposure time to cryoprotectants, therefore it reduces cytotoxicity (Zhang et al., 2011). Moreover is an open system, easy to use that allows the display of the sample (Lane et al., 1999). Actually, Cryotop is the most used carrier in the vitrification of embryos due to cooling rates up to 23.000ºC/min and warming rates up to 42.100ºC/min can be achieved (Zhang et al., 2011). To our best knowledge, a direct comparison in the efficiency of both carriers in the vitrification of embryo cleavage stage has not been found to date. However, in humans there are a lot of works that evaluate the efficiency of each carrier, but they not have been compared together. These works compile a post-warming survival rate between 85% and 98% for both carriers. In addition, accordingly to clinical pregnancy rate the percentage ranges between 27% and 47% for Cryoloop and Cryotop (Kuwayama et al., 2005; Desai et al., 2007; Hiraoka et al., 2009; Rama Raju et al., 2009; Lin et al., 2010; Desai et al., 2010).

Mikolajewska et al., (2012) compared between Cryotop and Cryoloop in the vitrification of feline oocytes, but they used inoculating loop as Cryoloop. The advantage of inoculating loop over the Cryotop has not been demonstrated in improved rates of offspring at birth, because the previously work only was done under *in vitro* culture conditions; specifically they stained oocytes after warming in order to assess changes in cytoskeletal distribution and nuclear configuration. Our data indicated that there were not differences under *in vivo* culture conditions between inoculating loop and Cryotop for rabbit embryo vitrification at morulae stage.

Accordingly to *in vivo* experiment, the rate of offspring live at birth (around 40% for both carriers) was in line with previously studies of embryo vitrification efficiency in rabbit, which included survival rates at birth between 25% and 65% (Kasai et al., 1992; Vicente and Garcia-Ximenez 1994; Vicente et al., 1999; López-Béjar and López-Gatius 2002; Mocé et al., 2010; Marco-Jiménez et al., 2013; Lavara et al., 2014; Saenz-de-Juano et al., 2014). In addition, implantation rate (around 53% for inoculating loop and Cryotop) was accordingly with previous

experiments done in embryo rabbit, in which the percentage of implantation rate varies between 44.5% and 63.3% for embryos vitrified (Marco-Jiménez et al., 2013; Saenz-de-Juano et al., 2014). Besides, the weight at birth was similar for both carriers (around 62.3 ± 2.89g), these data were agree with the study accomplished by Saen de Juano et al., (2014) they observed weight at birth around 57.2 ± 1.12g. On the other hand, embryonic losses (from fertilisation to implantation) were elevated in vitrified embryos with both carriers (around 47%) in comparison to fresh embryos (22.5%). In contrast, foetal losses (after implantation) did not show differences between vitrified embryos (13%) and fresh embryos (7.5%), the foetal losses in bibliography are around 14-20% for fresh embryos (Vicente et al., 2012; Marco-Jiménez et al., 2013). These data could indicate that most of the cryoinjuries produced over the embryo affect the implantation process. But the implanted embryos can develop normally until generate live birth. Even after the birth, offspring did not show difference between vitrified and fresh embryos, because the weight at birth was similar for all the groups (approximately 61.3 ± 2.69g). Generally, the absence of difference between carriers, in all the factors assessed, could be due to that both carriers have similar reduced volume, consequently similar cooling rates could be applied. In accordance with available bibliography the differences in cooling and warming rates, which produce effects in cells, are caused by the volume in vitrification solution (Kuwayama et al., 2005; Marco-Jiménez et al., 2013). Therefore, the *in vivo* data suggested that both vitrification carriers could be used equally.

To date, only there is one report available which compares Cryoloop and Cryotop in vitrification of pro-nuclear stage rabbit zygotes. Hochi et al., (2004) demonstrated that Cryotop was the best carrier to cryopreserve pronuclear-stage rabbit zygotes. Nevertheless, the efficiency of vitrification varies according to embryo stage of development. Metaphase-II-oocytes and early stage of development are more sensible than morulae and blastocyst (Leibo 2008: 2012).

Moreover, the rate of embryos developed until blastocyst not differs between Inoculating loop and Cryotop (42.2%). Both experiments, *in vivo* and *in vitro*, support the idea that the vitrification carrier not affects the survival of embryos. Mikolajewska et al., (2012) reached the same results in cat oocyte vitrification.

Despite the impressive results of Cryotop technique, the main issue of this is its elevated price, around 21€ each. For this reason inoculating loop could be a suitable replacement because it shares characteristics with Cryotop: They are open carriers that following the minimum volume procedure, similar cooling and warming rates could be applied,

and therefore the exposure time to cryoprotectants is the same. The main advantage of inoculating loop is their reduced price, around 0.05€ each. However, the major problem of both carriers is that they only are useful for monotocous species, like humans. Because these carriers have been developed to follow the minimum volume cooling method. This means that they only can cryopreserve a very small number of embryos (Matsunari et al., 2012). The vitrification of a large number of embryos is typical in areas like animal industries, experimental animal breeding and for preserving the biodiversity (Matsunari et al., 2012). For these cases is necessary to develop new mass reproducible methods of cryopreservation. Nowadays protocols with hollow fibers and papers on cryotubes are being studied (Matsunari et al., 2012; Lee et al., 2013).

Finally, it is important to highlight the difficulty in the cryopreservation field to develop universal protocols and standard carriers due to the large diversity between species (Saragusty and Arav, 2011). But, continue to carry out fundamental research to improve the efficiency of the process and achieve carriers that could adapt to the different necessity of the areas can be beneficial.

6. CONCLUSION

The findings of the current study show that the inoculating loop is a suitable method for replacing Cryotop because inoculating loop not shows difference under *in vivo* and *in vitro* culture conditions and their price is significantly less than Cryotop ones.

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