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Collection, vitrification and post-warming transfer of equine embryos produced in vivo: a literature review

Colección, vitrificación y transferencia post-calentamiento de embriones equinos producidos in vivo: una revisión de literatura

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Abstract

Small equine embryos, equal or smaller than three hundred micrometers in diameter (≤ 300 µmØ) are collected before the seventh post ovulation (PO) day. Big embryos, larger than three hundred micrometers (>300 µmØ) are recovered during or after the seventh PO day. No experiments test the type of holding and washing solutions and to what extent they influence the embryo post vitrification survival. Further knowledge is needed about the mixture of cryoprotectants; either penetrating or non penetrating and the effect they exert over the embryos post vitrification survival and pregnancy rate (PVSPR). The type of embryo carrier, the size of the embryo and the volume of the vitrification solution vary between small and large embryos. A high temperature transfer index (TTI) optimizes the embryo PVSPR. In equids, the type of holding and transfer solutions used during the post vitrification step is an area scarcely explored. This study endeavors to provide information to help in the adaptation of vitrification protocols depending on the embryo size. An additional objective is to ease the access to data about the solutions employed before and during the vitrification; as well as the solutions used during the embryo warming and transfer steps.

Keywords: liquid solutions, embryo collection, vitrification, warming, embryo transfer, mares, jennies.

Resumen

Embriones equinos pequeños, iguales o menores a trescientos micrometros de diámetro (≤ 300 µmØ) son obtenidos antes del día siete post ovulación (PO). Embriones grandes, mayores a trescientos micrometros de diámetro (>300 µmØ) son recuperados durante o después del día siete PO. No existen experimentos que prueben hasta que punto los medios de lavado y de colección embrionaria influencian la sobrevivencia post vitrificación. Mas conocimiento es necesario acerca de las mezclas de criopreservadores; penetrantes o no penetrantes y el efecto que ejercen sobre el índice de sobrevivencia embrionaria post vitrificación y el índice de gestación (ISEPVIG). El tipo de porta embrión, el tamaño del embrión y el volumen de la solución vitrificante varían entre embriones pequeños y grandes. Un índice de transferencia de temperatura (ITT) elevado optimiza en ISEPVIG. El tipo de soluciones de mantenimiento y transferencia utilizadas durante la post-vitrificación es un área escasamente explorada. El propósito de este estudio es proveer información para ayudar en la adaptación de protocolos de vitrificación dependiendo en el tamaño del embrión. Un objetivo adicional es facilitar el acceso a información acerca de soluciones empleadas antes y durante la vitrificación; así como las soluciones usadas durante las etapas de calentamiento y transferencia embrionaria.

Palabras Clave: soluciones liquidas, colección de embriones, vitrificación, calentamiento, transferencia embrionaria, yeguas, burras.

INTRODUCTION

Multiple factors can be taken into consideration to successfully establish vitrification programs and obtain high PVSPRs. In mares/jennies an accurate determination of ovulation is critical since it allows the recovery of embryos with expected size. If small, they do not require capsule perforation (Panzani *et al.,* 2012a; Fanelli *et al.,* 2020). However, if large they might preferentially be punctured before the vitrification step (Hochi *et al.,* 1995; Eldridge-Panuska *et al.,* 2005; Scherzer *et al.,* 2011; Wilsher *et al.,* 2019; Fanelli *et al.,* 2020; Wilsher *et al.*, 2021). In general, the post-ovulation day selected to attempt an embryo recovery influences the percent of embryos collected. The successful recovery of equine embryos is influenced by multiple factors: age, number of cycles within each donor (Dorado *et al.,* 2020), whether the semen used is either fresh, chilled, or frozen (Meadows *et al.,* 2000), time of insemination relative to ovulation (Eldridge-Panuska *et al.,* 2005), the season of the year; in mares, but not in jennies (Peréz-Marín *et al.,* 2017; Dorado *et al.,* 2020), number of ovulations per cycle; single or multiple (Squires *et al.,* 1985), fertility of the horse or donkey (Peréz-Marín *et al.,* 2017; Dorado *et al.,* 2020) and other factors (Allen *et al.,* 1985). In most cases the average rate of embryo recovery is higher than 50 percent; irrespective of whether its collection is attempted either on the sixth, seventh, eighth, or ninth PO days (Allen *et al.,* 1985; Freeman *et al.,* 1991; Hinrichs, 1990; Eldridge-Panuska *et al.,* 2005).

However, the visualization and manipulation of the equine embryo are easier after the seventh PO day; due to its larger size when compared to embryos collected either the days six, or six and a half PO. A few studies compare the pregnancy rate obtained with embryos subjected to distinct types of collecting (Oguri & Tsutsumi, 1974), washing, holding (Oguri & Tsutsumi, 1974), and transfer liquid solutions (Carnavale *et al.,* 2000; Eldridge-Panuska *et al.,* 2005). Information is also scarce when comparing distinct vitrification solutions and the pregnancy rates achieved by employing each of them. Multiple options can be selected as embryo carrier: open pulled straw or OPS (Guignot *et al.,* 2015); fine diameter micropipette (Choi *et al.,* 2011); cryolock (Díaz *et al.,* 2016; Ferris *et al.,* 2016; Wilsher *et al.,* 2019); hemi straw (Sanchez *et al.,* 2017), Fibreplug-solid surface (Pérez-Marín *et al.,* 2018) and cryotop (Bottrel *et al.,* 2019; Bottrel *et al.,* 2020a; Bottrel *et al.,* 2020b). Small equine embryos do not require direct contact with liquid nitrogen to survive the cryopreservation process even if vitrified in large volumes of solution i.e. 30 uL (Eldridge-Panuska *et al.,* 2005; Fanelli *et al.,* 2020). On the other hand, if the equine embryos are large, besides the capsule perforation (Wilsher *et al*., 2021) and blastocoelic fluid extraction (Sanchez *et al.,* 2017; Herrera, 2021) they will preferentially require direct contact with liquid nitrogen (Díaz *et al.,* 2016; Sanchez *et al.,* 2017; Herrera, 2021). It seems that with large equine embryos (besides capsule perforation and blastocoelic fluid extraction), when comparing between open and closed vitrification embryo carriers which allow direct and indirect contact with liquid nitrogen during the vitrification step; that the open systems promotes a higher TTI and hence augments the PVSPR (Sanchez *et al.,* 2017). Furthermore, the volume of liquid

used to hold large embryos during the vitrification step might be preferentially small (usually < 1 uL) to increase the TTI (Díaz *et al.,* 2016; Sanchez *et al.,* 2017). Another factor that might influence embryo survival is the diameter of the micropipette employed to perforate its capsule; however, limited and not conclusive data addressing this topic exist (Choi *et al.,* 2010; Díaz *et al.,* 2016; Ferris *et al.,* 2016; Sanchez *et al.,* 2017; Wilsher *et al*., 2021). Finally, the liquid solutions selected to transfer embryos into the uterus of mares or jennies might be carefully analyzed. Further research is granted in the area of reproductive cryobiology where equine embryos are an easy experimental model to work with; since they can be easily collected, manipulated and transferred back into the uterus of mares, jennies, mules and/or hinnies. The objective of the present study is to provide an easy guide for relevant factors associated with embryo vitrification, both: in horses and donkeys, establish areas where limited or absent information exists, and promote ideas leading to future research in the area of equine reproductive cryobiology.

Percent of embryos recovered in mares and jennies relative to the day of ovulation

There is a high degree of variation in the percent of embryos recovered in mares when taking into consideration the day of the recovery attempt. For instance; on the sixth, seventh, or eighth PO day the reported recovery rate varies from zero (Battut *et al.,* 1997), sixty-two (Camillo *et al.,* 2003), to one hundred percent (Hochi *et al.,* 1995) respectively. Still, recovery rates close to or higher than fifty percent are indicated by most studies (Allen *et al.,* 1985; Iuliano *et al.,* 1985; Squires *et al.,* 1985; Hinrichs, 1990; Freeman *et al.,* 1991; Hochi *et al.,* 1995; Maclellan *et al.,* 2002; Eldridge-Panuska *et al.,* 2005; Moussa *et al.,* 2005; Derbala & Abdou, 2017; Silva *et al.,* 2018; Roser *et al.,* 2020). When embryo collection is attempted early (sixth PO day), the recovery rate ranges from zero (Battut *et al.,* 1997) to eighty-two percent (Hochi *et al.,* 1995). The previous dramatic difference between reports could be partially attributed to the professional's expertise since embryo searching and observation are more difficult during this early stage of development (day six PO) when the embryo size is usually ≤ 300 µmØ. Though is not possible to make a direct comparison of the average recovery rate due to differences in methods and number of animals used in each study, at large; they show that successful collection rate tends to be lower when performed early (day six PO compared to later days). Interestingly, it has been possible to increase the embryo recovery rate by modifications adapted to the collection technique; for example, allowing the recovery media or flushing solution to stay a few minutes longer inside the uterus (Hinrichs, 1990; Alvarenga *et al.,* 1993).

Besides showing a high degree of variation, the data related to the per day embryo recovery rate in jennies are limited (Allen *et al.,* 1985; Vendramini *et al.,* 1997; Camillo *et al.,* 2010; Peña-Alfaro *et al.,* 2014; Bottrel *et al.,* 2017; Pérez- Marín *et al.,* 2017;

Ottmann *et al.,* 2018; Dorado *et al.,* 2020). During the sixth PO day, the percentage of successful embryo collection ranges from forty-three (Vendramini *et al.,* 1997) to seventy-five (Dorado *et al.,* 2020). On the seventh PO day, it goes from twelve (Camillo *et al.,* 2010) to eighty percent (Dorado *et al.,* 2020). Alternatively, if attempted from the eighth to the ninth PO day, the successful embryo collection rate is usually higher than fifty percent (Camillo *et al.,* 2010; Peña-Alfaro *et al.,* 2014; Dorado *et al.,* 2020). As previously mentioned, the percent of recovered embryos in equids can be attributed to multiple factors like the number of cycles within each donor (Dorado *et al.,* 2020), type of semen (Meadows *et al.,* 2000), insemination and ovulation time (Eldridge-Panuska *et al.,* 2005), seasonality in mares but not in jennies (Peréz-Marín *et al.,* 2017; Dorado *et al.,* 2020), ovulations per cycle (Squires *et al.,* 1985), fertility of males (Peréz-Marín *et al.,* 2017; Dorado *et al.,* 2020) and others (Allen *et al.,* 1985).

The relationship between day of recovery and embryo size

In mares, embryo size is radically influenced by the PO day selected to implement its recovery (Betteridge *et al.,* 1982; Hochi *et al.,* 1995; Eldridge-Panuska *et al.,* 2005; Choi *et al.,* 2010; McCue *et al*., 2010; Díaz *et al.,* 2016; Guignot *et al.,* 2016; Wilsher *et al.,* 2019). For the most part, if the embryo is collected either during the sixth or sixth and a half PO day, it is typically ≤ 300 µmØ (Iuliano *et al.,* 1985; Freeman *et al.,* 1991; Eldridge-Panuska *et al.,* 2005; Hudson *et al.,* 2006; Choi *et al.,* 2010; Guignot *et al.,* 2015; Pérez- Marín *et al.,* 2017; Pérez-Marín *et al.,* 2018; Silva *et al.,* 2018). In contrast, when the embryo recovery is done on days seventh, seventh and a half; eight, eight and a half; or nine, it is generally >300 µmØ (Hochi *et al.,* 1995; McCue *et al*., 2010; Díaz *et al.,* 2016; Pérez- Marín *et al.,* 2017; Wilsher *et al.,* 2019).

Studies in jennies describing a relationship between the size of the embryo and the day of its collection are scarce. Yet, it seems that contrary to mares a high percent of donkey embryos collected during the seventh, or seventh and a half PO days are still < 300 µmØ (Vendramini *et al.,* 1997; Panzani *et al.,* 2012b; Bottrel *et al.,* 2017; Pérez-Marín *et al.,* 2017; Pérez-Marín *et al.,* 2018; Bottrel *et al.,* 2019; Bottrel *et al.,* 2020b; Dorado *et al.,* 2020; Fanelli *et al.,* 2020). When the collection of the donkey embryo is attempted from the eighth PO day or later, its diameter is typically close to or larger than 500 µmØ (Panzani *et al.,* 2012a; Pérez- Marín *et al.,* 2017; Bottrel *et al.,* 2019; Dorado *et al.,* 2020).

Protocols including or excluding embryo capsule (EC) puncture before vitrification

The capsule does not require to be punctured before the vitrification process if a horse embryo size is ≤300 µmØ, and its PVSPR is usually higher than thirty percent (Eldridge-Panuska *et al.,* 2005; Hudson *et al.,* 2006; Choi *et al.,* 2011; Reviewed by Urías-Castro & Boeta, 2020). Most reports about the vitrification of equine embryos indicate that when the horse embryo is >300 µmØ it might not only be punctured to perforate its EC but its blastocoelic fluid might be withdrawn as well to optimize the

PVSPR (Choi *et al.,* 2011; Guignot *et al.,* 2015; Díaz *et al.,* 2016; Ferris *et al.,* 2016; Sánchez *et al.,* 2017; Wilsher *et al.,* 2019; Reviewed by Urías-Castro & Boeta, 2020). However when further divisions in terms of embryo size are taken into consideration; it is observed that the vitrification of equine embryos ranging from 300 to 550 µmØ in size (≤ 550 µmØ); the perforation of their embryo capsule without the aspiration of their blastocoelic fluid results in pregnancy rates higher than 75% (Wilsher *et al*., 2021).

Studies reporting the PVSPR of donkey embryos are scarce. The work available shows that the vitrification of donkey embryos ≤300 µmØ result in pregnancy rates that ranges from thirty-six percent at day fourteen of pregnancy (D14), to twenty-seven percent at day twenty-five (D25); suggesting that if the embryo size is ≤300 µmØ, EC puncture and blastocoelic fluid extraction might not be essential (Panzani *et al.,* 2012b). Furthermore, research projects describing the puncture of the donkey EC and its blastocoelic liquid aspiration are limited (Ottmann *et al.,* 2018). Whether large donkey embryos >300 µmØ require EC puncture and blastocoelic fluid extraction to increase their PVSPR has been indirectly tested only, since EC puncture and blastocoelic extraction resulted in a high percentage of in vitro survival (Ottmann *et al.,* 2018; Reviewed by Urías-Castro & Boeta, 2020).

Intrauterine infusion of liquid solutions to collect equine embryos

Some of the first studies describing the collection of embryos in equids reported the employment of a physiological saline solution containing two percent of gelatin (Oguri & Tsutsumi, 1974). Nowadays, the use of Ringer lactate (Alvarenga *et al.,* 1993; Barfield *et al.,* 2008; Sánchez *et al.,* 2017; Wilsher *et al.,* 2019); which composition is close to the Hartmann's solution has been described (Guignot *et al.,* 2015). Other media utilized to recover equine embryos include Dulbecco's phosphate-buffered saline DPBS/PBS (Alvarenga *et al.,* 1993; Landim E Alvarenga *et al.,* 1993). A Dulbecco's modified phosphate-buffered saline (DMPBS) combined with fetal calf serum (FCS) has been reported by Hinrichs (1990). DMPBS can be supplemented with heat-inactivated steer serum (McKinnon & Squires, 1988); or bovine serum albumin (BSA) plus antibiotics (Battut *et al.,* 1997). The DMPBS mixed with either one percent of FCS or bovine calf serum, plus glucose and pyruvate has also been described as an embryo recovery medium (Carnavale *et al.,* 2000). The commercial Emcare® complete flush solution (Eldridge-Panuska *et al.,* 2005) and the Vigro® flush medium (Hudson *et al.,* 2006) are other alternatives reported as embryo recovery media.

In donkeys, the Ringer's lactate solution is the most frequently reported as embryo recovery medium (Panzani *et al.,* 2012a; Peña-Alfaro *et al.,* 2014; Panzani *et al.,* 2016; Pérez-Marín *et al.,* 2018; Bottrel *et al.,* 2019; Bottrel *et al.,* 2020a; Bottrel *et al.,* 2020b). Another reported alternative is the DPBS (Camillo *et al.,* 2010).

Liquid solutions used to hold recovered equine embryos

The simplest embryo holding media in terms of composition are either the saline medium containing two percent of gelatin, or the Ringer's lactate solution with mare serum (1:1, v/v) and one thousand international units of penicillin per milliliter (Oguri & Tsutsumi, 1974). A more complete in terms of composition is the Ham's F10 medium, complemented with ten percent FCS plus one percent penicillin/streptomycin; this medium is gassed with five percent of carbon dioxide, oxygen, and ninety percent of nitrogen (Carnavale *et al.,* 2000). The commercial embryo holding media Vigro® (Hudson *et al.,* 2006) and Syngro® (Barfield *et al.,* 2009) have also been described. French investigators have worked with the EHM medium, which is a PBS-based liquid solution containing four grams of BSA per liter (Guignot *et al.,* 2015). Researchers at the Colorado State University have reported the utilization of the DMEM/F12 medium (inorganic salts, amino acids, vitamins, antibiotics and others) supplemented with twenty percent of fetal bovine serum – DMEM/F12/FBS (Ferris *et al.,* 2016). A DMPBS lacking calcium and magnesium, complemented with ten percent FBS and fifty micrograms of gentamicin per milliliter has been employed by Sánchez *et al.* (2017). Alternatively, the M-199 Hepes medium supplemented with ten percent FBS plus one percent antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin) has also been described as an embryo-holding solution (Wilsher *et al.,* 2019).

PBS and lactated Ringer have been reported as holding media for donkey embryos (Camillo *et al.,* 2010). The use of the commercial Emcare Holding Solution® is also documented by several researchers (Camillo *et al.,* 2010; Panzani *et al.,* 2012a). Another alternative to hold donkey embryos is the Holding Vitrocell®, which contains zero-point four percent of BSA (Peña-Alfaro *et al.,* 2014). The Syngro® holding medium has been employed with donkey embryos before transferring them into the mule uterus (Bottrel *et al.,* 2017). The use of an isotonic holding medium (IVM-Technologies) has been reported by Pérez-Marín *et al.* (2018). Recently, Bottrell and his team employed TCM-199 HEPES complemented with twenty percent FCS as an embryo holding medium (Bottrel *et al.,* 2019).

Embryo washing and manipulation media

A liquid solution that has been employed in mares to wash and manipulate the collected embryos is PBS; it is usually supplemented with a serum of animal origin, like the fetal calf serum FCS (Alvarenga *et al.,* 1993). The use of PBS without calcium and magnesium, supplemented with sodium pyruvate, glucose, and FCS has been documented by Eldridge-Panuska *et al.* (2005). A PBS solution containing bovine serum albumin (BSA) is also reported as an embryo washing/manipulating medium (Guignot *et al.,* 2015). Other washing solutions include the Vigro® holding solution (Hudson *et al.,* 2006; Barfield *et al.,* 2008) and the Syngro® holding medium (Barfield *et al.,* 2009). The use of DMEM/F12 supplemented with twenty percent of fetal bovine serum (FBS) has also been reported by Ferris *et al.* (2016). A solution of Dulbecco's PBS (DPBS) with ten percent FCS has been employed to wash equine embryos by

researchers at the Colorado State University (Carnavale *et al.,* 2000). Another washing medium that has been documented is the M-199 HEPES with ten percent FBS, penicillin, and streptomycin (Wilsher *et al.,* 2019).

For donkeys, the Emcare Holding Solution® has been frequently described to wash/manipulate embryos (Camillo *et al.,* 2010; Panzani *et al.,* 2012a; Panzani *et al.,* 2012b; Panzani *et al.,* 2016). The Syngro® holding medium is employed as an embryo washing solution by several researchers (Bottrel *et al.,* 2017; Pérez- Marín *et al.,* 2017; Bottrel *et al.,* 2019; Bottrel *et al.,* 2020a; Bottrel *et al.,* 2020b). Alternatively, the PBS (Camillo *et al.,* 2010), the Ringer's lactate (Camillo *et al.,* 2010), and the isotonic holding media have been described as embryo washing solutions too (Pérez-Marín *et al.,* 2018).

Micropipette size and puncture of the embryo capsule (EC)

No studies in mares formally correlate the diameter of the micropipette tip used to perforate the EC and its association with the PVSPR. Most experiments where the EC puncture is performed are designed with micropipettes tips equal to or smaller than sixteen micrometers in diameter (umØ); as described by several studies (Choi *et al.,* 2010; Choi *et al.,* 2011; Hinrichs & Choi, 2012; Guignot *et al.,* 2015; Díaz *et al.,* 2016; Sánchez *et al.,* 2017). Only a couple of researchers demonstrate the survival of embryos as reflected by pregnancy rates, after puncturing the EC with micropipette tips larger than sixteen umØ (Wilsher *et al.,* 2019). Pregnancies have been reported even after puncturing the EC with twenty-five-gauge needles (Ferris *et al.,* 2016). On the other hand; when working with embryos derived from jennies, they are generally vitrified at the ≤ 300 umØ stage, and hence they required neither punction nor size reduction (Panzani *et al.,* 2012b; Pérez-Marín *et al.,* 2018; Bottrel *et al.,* 2019). The Piezo drill micromanipulation system has been described in one study experimenting with large donkey embryos >300 µmØ (Ottmann *et al.,* 2018), and this system employs micropipettes tips in the 7-15 umØ range (Choi *et al.,* 2011; Hinrichs & Choi, 2012; Hiraoka & Kitamura, 2015). However, in the work by Ottman and his colleagues, the embryos were not transferred after their warming and only their in vitro survival rate was reported (Ottmann *et al.,* 2018). A recent study has shown the puncture of equine blastocyst with a (< 1µm tip) ultra fine micro-needle (Wilsher *et al*., 2021).

Cryopreservative solutions employed to vitrify equine embryos

Most protocols employed for the vitrification of horse embryos utilize a mix of penetrating cryoprotectants such as dimethyl sulfoxide (DMSO), ethylene glycol and glycerol. Other protocols include a final vitrification step where penetrating cryoprotectants such as ethylene glycol, or ethylene glycol and DMSO; are mixed with non penetrating cryoprotectants such as galactose or sucrose. For instance, the embryo is placed first in a solution containing a low concentration of glycerol, and then it is removed from this solution and gradually placed in solutions containing increased

concentrations of glycerol and ethylene glycol (Eldridge-Panuska *et al.,* 2005; Díaz *et al.,* 2016; Sánchez *et al.,* 2017; Pérez-Marín *et al.,* 2018). On the other hand, the vitrification of horse embryos with media containing ethylene glycol and DMSO is also described (Choi *et al.,* 2011; Sánchez *et al.,* 2017; Wilsher *et al.,* 2019). These protocols are started by putting the embryo in a solution with a low concentration of ethylene glycol and DMSO. After the previous initial step, the embryo is subsequently moved into a solution containing higher concentrations of these cryoprotectants. Other cryopreservative liquid solutions used for the vitrification of horse embryos contain a low concentration of ethylene glycol (initial incubating solution), and a final or vitrifying solution. The final solution is composed of galactose with a higher concentration of ethylene glycol (Choi *et al.,* 2011; Guignot *et al.,* 2015; Ferris *et al.,* 2016; Guignot *et al.,* 2016).

For the vitrification of donkey embryos, a protocol has been adapted from the early study in horse embryo vitrification by Eldridge-Panuska *et al.* (2005); it typically consists of three steps, with one specific solution for each step. For instance, solution one contains glycerol only; solution two has a mix of glycerol and ethylene glycol; and solution three contains higher concentrations of glycerol and ethylene glycol (Panzani *et al.,* 2012b; Panzani *et al.,* 2016; Pérez-Marín *et al.,* 2018). Other donkey embryo vitrification studies were designed taking into consideration the research by Campos-Chillon *et al.* (2009). They utilized solutions containing ethylene glycol, and ethylene glycol complemented with galactose (Ottmann *et al.,* 2018; Pérez-Marín *et al.,* 2018). Others have used an initial solution with a mix of ethylene glycol and DMSO; subsequently, a second solution containing ethylene glycol, DMSO, and sucrose is employed (Bottrel *et al.,* 2019; Bottrel *et al.,* 2020a; Bottrel *et al.,* 2020b).

Embryo carriers and loading volumes employed during the vitrification process

The zero-point twenty-five milliliter (mL) capacity non irradiated polyvinyl chloride straw was one of the first embryo carriers described in equine species and has been frequently used thereafter (Oberstein *et al.,* 2001; Eldridge-Panuska *et al.,* 2005; Hudson *et al.,* 2006; Hendriks & Stout, 2010; Choi *et al.,* 2011; Hendriks *et al.,* 2015; Sánchez *et al.,* 2017). Contrary to the polyvinyl straw which uses a large volume of liquid solution during the final vitrification step; the cryolock carrier system allowed researchers to vitrify horse embryos into a small liquid volume (Díaz *et al.,* 2016; Ferris *et al.,* 2016; Wilsher *et al.,* 2019). The vitrification of equine embryos using small liquid volumes is achieved with devices such as the open pulled straw (Oberstein *et al.,* 2001; Moussa *et al.,* 2005; Guignot *et al.,* 2015; Guignot *et al.,* 2016), the fibreplug (Pérez-Marín *et al.,* 2018), cryoloop (Oberstein *et al.,* 2001), the fine diameter microloader pipette tip (Choi *et al.,* 2011), cryoleaf (Scherzer *et al.,* 2011), hemi straw (Sánchez *et al.,* 2017) and the stripper tip (Sánchez *et al.,* 2017).

Horse embryos can be vitrified utilizing different volumes of liquid solutions. The embryo loading volumes range from fifty (Oberstein *et al.,* 2001) to thirty uL (Eldridge-Panuska *et al.,* 2005; Hudson *et al.,* 2006; Hendriks & Stout, 2010; Choi *et al.,* 2011; Hendriks *et al.,* 2015). Volumes around the three or two uL range have been reported (Moussa *et al.,* 2005; Guignot *et al.,* 2015; Sánchez *et al.,* 2017; Pérez-Marín *et al.,* 2018; Wilsher *et al.,* 2019). Embryo loading volumes close to or smaller than one uL are described as well (Oberstein *et al.,* 2001; Díaz *et al.,* 2016; Sánchez *et al.,* 2017). In donkeys, the zero-point twenty-five mL capacity non-irradiated polyvinyl chloride straw is also reported as an embryo carrier (Panzani *et al.,* 2012b; Fanelli *et al.,* 2020). Several small volume capacity devices have been recently described for the vitrification of donkey embryos; they include the cryotop (Bottrel *et al.,* 2019; Bottrel *et al.,* 2020a; Bottrel *et al.,* 2020b), the open pulled straw (Ottmann *et al.,* 2018) and the fibreplug (Pérez-Marín *et al.,* 2018). Thirty uL of a liquid solution have been used to vitrify donkey embryos (Panzani *et al.,* 2012b); but low volume embryo carriers, employing volumes ranging from three to less than one uL have also been utilized (Pérez-Marín *et al.,* 2018; Bottrel *et al.,* 2019; Bottrel *et al.,* 2020a; Bottrel *et al.,* 2020b).

Liquid solutions used to warm embryos

With small variation between protocols, the media employed to warm horse embryos usually contain a concentration equal to or smaller than zero point three molar of sucrose. The horse embryos are warmed and held for around one minute in this previous medium. Thereafter, the embryos are placed in a second solution with a lower content of sucrose; which is usually close to or lower than zero point fifteen molar of sucrose; finally, the embryos are placed in a liquid medium containing no sucrose during the final step of the warming process (Choi *et al.,* 2011; Guignot *et al.,* 2015; Ferris *et al.,* 2016; Guignot *et al.,* 2016; Wilsher *et al.,* 2019). A single-step embryo warming protocol was first reported by Eldridge-Panuska *et al.* (2005) utilizing a zero-point five molar galactose solution. The single-step warming protocol has been implemented in other studies also (Choi *et al.,* 2011; Díaz *et al.,* 2016; Sánchez *et al.,* 2017). Some researchers warm the embryos using a three-step protocol; in the first step, the embryo is placed in a one molar galactose solution; at the second, it stays in a zero-point five molar galactose; and during the third warming step, the embryo is placed in a liquid solution of zero-point twenty-five molar galactose (Campos-Chillon *et al.,* 2009; Choi *et al.,* 2011).

As described in horse embryos (Eldridge-Panuska *et al.,* 2005), the warming of donkey embryos is performed with a zero-point five molar galactose medium in a direct one-step warming protocol (Panzani *et al.,* 2012b; Panzani *et al.,* 2016). Other protocols employ a zero-point twenty-five molar galactose as a warming solution (Pérez-Marín *et al.,* 2018). In some warming protocols, the donkey embryo is consecutively placed in distinct liquid solutions with decreasing sucrose concentrations (Ottmann *et al.,*

2018; Pérez-Marín *et al.,* 2018; Bottrel *et al.,* 2019; Bottrel *et al.,* 2020a). A recent study has compared the quality of post vitrified warmed donkey embryos when using either a three-step sucrose dilution or the one-step sucrose solution protocol (Bottrel *et al.,* 2020b).

Media employed for the intrauterine transfer of equine embryos

Some of the components of the liquid solutions used to transfer equine embryos were first described during the seventies by Oguri & Tsutsumi (1974). In this study, two different transfer solutions were reported: a saline solution containing two percent gelatin powder and a Ringer's lactate supplemented with mare serum and antibiotics. There are two post-warming transfer techniques reported by Eldridge-Panuska *et al.* (2005). The first uses PBS plus ten percent FCS as a transfer medium after removing the galactose contained in the warming solution. The second is a direct post-warming transfer technique. The latter consists in the mixing of the vitrification solution containing the embryo (thirty microliters of three-point four molar glycerol and four-point six molar ethylene glycol) with two columns of liquid (of zero point five molar galactose in PBS) ninety microliters each (Eldridge-Panuska *et al.,* 2005). In the previous technique, both the vitrification and the warming galactose solutions are mixed into the straw during the warming process, forming one diluted liquid solution containing the embryo that is subsequently transferred into the mare's uterus (Hudson *et al.,* 2006). The Syngro® holding medium has been reported as an embryo transfer solution as well (Barfield *et al.,* 2008; Sánchez *et al.,* 2017). French researchers experimented with a culture medium containing a synthetic modified oviductal fluid and used it as an embryo transfer medium (Guignot *et al.,* 2015).

The EmCare Holding Solution® has been employed to transfer donkey embryos by Italians and Spanish investigators (Panzani *et al.,* 2006; Panzani *et al.,* 2012a; Camillo *et al.,* 2010). Other reported transfer media are either the PBS or the Ringer's lactate (Camillo *et al.,* 2003; Camillo *et al.,* 2010). The Holding Vitrocell® containing zero point four percent of BSA has been used as a donkey embryo transfer medium by Peña-Alfaro *et al.* (2014). An isotonic holding medium (IMV technologies, L'Aigle, France) has been reported as an embryo transfer solution too (Pérez-Marín *et al.,* 2018).

DISCUSSION

In recent years, embryo vitrification in nonconventional equine species has become frequent (Bottrel *et al.,* 2019; Bottrel *et al.,* 2020a; Bottrel *et al.,* 2020b; Fanelli *et al.,* 2020). Still, vitrification, post vitrification embryo transfer, and pregnancy rates are research areas that remain scarcely explored in embryos derived from jennies (Ottmann *et al.,* 2018; Pérez-Marín *et al.,* 2018; Bottrel *et al.,* 2019) and has not been researched using mules, hinnies or other hybrid embryos. Early works in mares allowed gathering knowledge about the embryo's descending time into the uterus, and

it was then described that embryos entered into the uterus as early as 120 hours (five days) PO (Freeman *et al.,* 1991). The limited availability of jennies and the large number needed to do research, has made it difficult to extensively track the time of entrance by the donkey embryo into the jennies' uterus. It is logical to consider that; donkey embryos might descend into the uterus of jennies by, or soon after the sixth PO day (Allen *et al.,* 1985; Vendramini *et al.,* 1997; Pérez-Marín *et al.,* 2017). However, it appears that in jennies as it happens in mares, embryo collection is more successful if practiced later rather than earlier. Furthermore, the early development of horse embryos seems to be faster when compared to that of donkey embryos. For instance, when collected by the seventh and the seventh and a half PO days, most horse embryos are ≥ 300 µmØ (Hochi *et al.,* 1995; McCue et al., 2010; Guignot *et al.,* 2015; Wilsher *et al.,* 2019), while the majority of donkey embryos are ≤ 300 µmØ when collected during the same days (Vendramini *et al.,* 1997; Panzani *et al.,* 2012b; Bottrel *et al.,* 2017; Pérez- Marín *et al.,* 2017; Pérez-Marín *et al.,* 2018; Dorado *et al.,* 2020). The embryos of equine species develop a glycoprotein capsule (Stout *et al.,* 2005) that when more developed, has a lower permeability to cryoprotectants (Kingma *et al.,* 2011; Scott *et al.,* 2012). These changes in the EC permeability can be indirectly assumed as well by the PVSPR that is obtained when analyzing data obtained by the vitrification of equine embryos ≤ 300 µmØ (Eldridge-Panuska *et al.,* 2005; Hudson *et al.,* 2006; Choi *et al.,* 2011). It is probable that the high PVSPR obtained when vitrifying small embryos is a consequence of the nature of these embryos; which have a less developed capsule and are more permeable to cryoprotectants, hence can be easily vitrified obtaining normal to high PVSPRs. On the other hand, large horse embryos >300 µmØ have a capsule with lower permeability to cryoprotectants. These changes in the EC permeability can be corroborated when analyzing data obtained by Legrand et al. (2001). In the previous study it was observed that the equine embryos that had a more developed or thick capsule the entrance into the embryo of cryoprotectants such as glycerol was limited (Legrand *et al.,* 2001). Then, depending on the subcategories of the embryo size only the puncture (Wilsher *et al*., 2021) or the puncture and the aspiration of the blastocoelic fluid might be necessary to obtain a high PVSPR (Díaz *et al.,* 2016; Sánchez *et al.,* 2017). The question of whether what subcategories of donkey embryos (in terms of size:≤ 300 µmØ, > 300 ≤ 500 µmØ, > 500 ≤ 1200 µmØ) require their capsule punctured and the blastocoelic liquid aspirated previous to the vitrification step has not been fully tested yet. Based in the studies in horses, and in the limited number of studies in donkeys (see previous section of this review); it can be said that donkey embryos require not only to be punctured and their blastocoelic fluid aspirated, but also to be vitrified, subsequently warmed, and transferred into the uterus of jennies to calculate their PVSPR (Reviewed by Urías-Castro & Boeta, 2020). In equine species, the constituents of embryo collecting or flushing (ECF) solutions were described by the early studies of Oguri (Oguri & Tsutsumi, 1974). They were isotonic media containing serum of animal origin, and in some cases were supplemented with glucose, pyruvate, and antibiotics. Some ECF solutions contain

carbohydrates and antibiotics; the decision about adding them may be taken based on several factors like the amount of time spent while manipulating or holding the embryo and whether the facilities and equipment used are clean or sterile. By analyzing studies reporting ECF, it has become apparent that in mares (Guignot *et al.,* 2015; Sánchez *et al.,* 2017; Wilsher *et al.,* 2019) and jennies (Pérez-Marín *et al.,* 2018; Bottrel *et al.,* 2019) media as simple as the Ringers lactate or the Harman's may be good alternatives (which has a practical and economical relevance; since the Ringer lactate or the Harman's solutions are probably cheaper and easier to obtain).

Multiple liquid media can be utilized to hold equine embryos. Some researchers have employed either saline or Ringers lactate. There are more complex solutions like Vigro® and Syngro®, while the most complete in terms of embryo nutritional requirements seem to be the Ham's F10 and TCM-199. Nevertheless, few studies have compared the different types of holding media and the effects of employing each one over embryo survival, development, and/or pregnancy rates (Oguri & Tsutsumi, 1974; Alvarenga *et al.,* 1993). The kind of medium selected might depend on the circumstances that prevail during the collection or holding processes, and whether the embryo will be cooled, stored for hours, or processed immediately after manipulation. The liquid solutions utilized to wash the collected horse embryo keep a close relationship with the solutions utilized as embryo holding media. However, when the holding media is employed as a washing solution it usually includes antibiotics.
The relationship between the pipette diameter employed to puncture the EC and its effects over the PVSPR is an area that has not been fully investigated in equine species, especially using donkey and mule embryos. Dramatic variations in the diameter of micropipettes used to puncture large horse embryos >300 µmØ exist (Choi *et al.,* 2011; Guignot *et al.,* 2015; Díaz *et al.,* 2016; Ferris *et al.,* 2016; Sánchez *et al.,* 2017; Wilsher *et al.,* 2019; Wilsher et al., 2021).

There are multiple protocols to vitrify equine embryos; nonetheless, data are limited when comparing the PVSPR obtained when employing each of them (Choi *et al.,* 2010; Pérez-Marín *et al.,* 2018). It will be interesting to further investigate the different embryo size categories (≤ 300 µmØ, > 300 ≤ 500 µmØ, > 500 ≤ 1200 µmØ) and their susceptibility to a specific vitrification protocol. The vulnerability of the embryo to single cryoprotectants like glycerol (Scott *et al.,* 2012), or the different combinations: ethylene glycol and glycerol (Kingma *et al.,* 2011); ethylene glycol and DMSO - ethylene glycol and galactose (Campos-Chillon *et al.,* 2009), is an area that awaits further investigation with donkeys, mules, hinnies and other hybrid embryos. Embryo permeability and post vitrification survival is a topic that has been partially described before, for both in vivo (Kingma *et al.,* 2011; Scott *et al.,* 2012) and in vitro produced horse embryos (Campos-Chillon *et al.,* 2009).

In general, when the embryo is reduced in size (either because it is small when collected; or because it is reduced in size by puncturing and/or aspirating the blastocoelic fluid) and when utilizing a small volume of medium with them (< one µL) during the vitrification step the chance of ice formation is lowered since a high TTI is induced when directly plunging the embryo into liquid nitrogen (Reviewed by Urías-Castro & Boeta, 2020). Interestingly, during the initial reports describing the successful vitrification of small equine embryos that resulted in normal to high pregnancy rates, large volume capacity carriers were utilized, with the embryos being cryopreserved in thirty uL of vitrification solution (Eldridge-Panuska *et al.,* 2005). The smaller the embryo the lower the volume of media the researcher can use during its vitrification process. However, the challenge arises when the embryo is >300 µmØ. Since the biggest the embryo the largest the volume of media needed to hold it during the vitrification process. The previous issue makes it more difficult to achieve a high TTI and then reduce ice formation to promote a high percent of embryo survival, both during the vitrification and after the warming steps. The difficulty of achieving a high PVSPR in embryos >300 µmØ is still present even after practicing embryo capsule puncture and blastocoelic fluid aspiration, especially with embryos >800 µmØ which are difficult to load into a small volume of vitrification medium (personal experience).

The embryo warming process is usually started with galactose or sucrose solutions that range in their molarities from zero point two to zero point three, zero point five, or even one molar concentration. Once warmed, the embryo is subsequently placed in solutions of decreased galactose/sucrose molarities. In the final steps of the warming process, the embryo is placed in solutions that lack galactose/sucrose. Other protocols employ a technique denominated direct post warming transfer, which consists in placing the embryo in a zero-point five molar sucrose (the use of this last protocol has practical implications since allow the direct transfer of the embryo after being warmed). In the latter protocol the embryo is warmed in the last solution and directly transferred into the mare's uterus. Few studies investigate the relationship between embryo size (≤ 300 µmØ or >300 µmØ) and its susceptibility to specific sucrose/galactose molarities (Choi *et al.,* 2011; Bottrel *et al.,* 2019; Bottrel *et al.,* 2020a; Bottrel *et al.,* 2020b). For instance, large embryos (>300 µmØ) could require more time to reach an osmotic equilibrium during the warming process; due to the presence of a more developed capsule that may reduce their permeability. It is necessary to further investigate the molarities of the warming solutions employed with large equine embryos. Adjusting molarities by embryo size subcategories (>600 ≤ 900 µmØ; >900 ≤ 1200; >1200 ≤ 1500 µmØ) may improve embryo warming protocols and PVSPR. A wide range in embryo susceptibility can be expected concerning the molarities of warming solutions (based in the degree of development and permeability of the embryo capsule). It has not been thorough fully established whether donkey and/or horse embryos have differences in their susceptibilities to the vitrification/warming process (Bottrel *et al.,* 2020a; Bottrel *et al.,* 2020b). For instance, donkey embryos seem to be less prone to

vitrification damage (Pérez-Marín *et al.,* 2018; Bottrel *et al.,* 2020a; Bottrel *et al.,* 2020b). The previous difference in vitrification susceptibility between horses and donkey embryos could be partially explained by differences in permeability to cryopreservative substances. Whether the less pronounced susceptibility to the vitrification process observed with donkey embryos applies to the warming processes, requires further investigation.

Multiple liquid transfer solutions can be used for the intrauterine deposition of equine embryos. The medium and volume used to load and transfer the embryo will depend on the size of the latter. In general, an insemination pipette can be employed if the embryo is large (>300 µmØ). A zero-point five or a zero-point twenty-five mL straw can be used if the embryo is small (≤ 300 µmØ). Media as simple as the saline and/or Ringer's lactate (which are cheap and easy to obtain) supplemented with gelatin are alternatives that can be used to transfer horse, donkey, or mule embryos. The PBS and the commercial Syngro medium are other options that can be used as embryo transfer solutions (however these options are more difficult to obtain and usually more expensive). Other alternatives may provide more stable conditions for the nutrition and development of equine embryos. The previous may include culture media supplemented with synthetic oviductal fluid or Dulbecco's modified PBS. In donkey species, the embryo transfer solutions frequently reported are Emcare, PBS, and holding Vitrocell®. No studies methodically investigate the relevance of the solution utilized to transfer equine embryos over the PVSPR. Is not known either whether simple solutions such as saline or the Ringer's lactate are good alternatives as embryo transfer media. It has not been investigated whether the use of saline or Ringer's lactate solutions, alone nor combined with antibiotics can result in an acceptable donkey or mule embryo PVSPRs. Comparing the PVSPR obtained when transferring equine embryos using simple and easy to obtain solutions such as the saline or Ringer's; or more complex culture media supplemented with animal's origin serum and/or synthetic oviductal fluid requires further investigation. The research in the area of cryopreservation utilizing donkey and hybrid equine embryos such as mules and hinnies as models can lead to improvements in their post vitrification survival, resulting in a high percentage of post-transfer pregnancies. Further investigations are needed in mares and jennies to improve vitrification protocols and hence increase the PVSPR especially with donkey and mule embryos >300 µmØ.

Conflict of interests

The authors declare that there is not conflict of interests

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Errata Erratum

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