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Review

Post vitrification pregnancy rate of *in vivo* produced embryos derived from equids. Review

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Abstract:

Vitrification is a cryo-preservation method often used in embryos obtained from mares or jennies. It consists in the dramatic reduction of temperature to levels close to -196 °C, that allows the cryopreserving solution containing the embryo to pass from liquid to vitreous state. Several improvements to vitrification protocols have made possible to cryo-preserve embryos with different sizes; since during the first decade after the year 2000, only small embryos were successfully vitrified. Embryos collected at the sixth day post ovulation (PO) are usually smaller or equal to 300 micrometers in diameter (≤ 300 µmØ) and can be routinely vitrified following simple protocols; they have a higher post vitrification pregnancy rate (PVPR) when compared to large embryos which have more than 300 micrometers in diameter (> 300 µmØ). The high PVPR of embryos ≤ 300 µmØ is due to an embryo capsule (EC) that is not fully developed yet and has a high permeability to cryo-preserving solutions. At present time, embryos collected either the seventh or eighth day PO are > 300 µmØ and are characterized to have a low post vitrification survival; in order to increase their PVPR their EC might be punctured to make it permeable to cryopreserving solutions. Additionally, there are at least two factors that can be manipulated to increase the PVPR of embryos > 300

µmØ; one is to reduce their size by aspiring their blastocoelic liquid (BL), and the other is to induce a high temperature transfer index (TTI) to rapidly reach -196 °C.

Key words: Mares, Jennies, Embryo, Cryopreservation, Vitrification, Pregnancy rate.

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Introduction

FAO estimates[1], show that the number of donkeys has been reduced from the 2007-2017 period in Brazil (1'163,316 to 841,307), China (7'306,000 to 4'568,500), Ecuador (102,058 to 49,729), India (438,000 to 247,000) and Italy (24,000 to 20,928). In the African continent, donkey's number is also being reduced due to the demand from the chinese market of some products like their skin[2]. However in contries like Italy[3] and Brazil[4] donkeys are an important part of the equine industry due to the production of milk[5,6], cheese[7] and meat[4]. Donkey's population recovery is important due to the roles they play in the economy of emerging contries[8] and the production of hybrid animals like mules[9]. The estrous cycle of jennies and mares differs mainly in the duration of the heat period reflected in a longer estrous length in jennies[10]; however the time lapse between the ovulation-fertilization relative to embryo entrance into the uterus seem to be similar between jennies[11,12,13] and mares[14,15]. The size of the embryo recovered either during the sixth, seventh, eighth or ninth day post ovulation (PO) varies dramatically; both, in jennies[12,13,16,17] and mares[15,16,18]; however, it follows a similar trend in size increase. In equids, the time in which the embryo reach the uterus has been established, and is known that it enters the uterus by the sixth day PO[18].

The PO day selected to proceed with the intrauterine infusion of solutions to collect embryos in jennies or mares, determines their development stage (morula, blastocyst or expanded blastocyst), and will dictate the protocol suitable to optimize their PVPR[12,13,15,19-22]. By infusing the uterus on the sixth day PO, the recovered embryo will generally be smaller than 300 micrometers[12,16,21]; on the other hand, embryos collected the eighth or ninth day PO will be equal or larger than 300 micrometers in size[12,13,20,23,24]. The optimization of embryo vitrification protocols requires a precise determination of the ovulation's day (day zero) to accurately determine the age/stage of embryo, since the ones collected during the sixth day

PO do not require size reduction; while embryoss collected the seventh, eighth, or ninth need to be reduced in size previous to vitrification[12,21,25,26,27].

Due to the dramatic reduction in the number of donkeys, studies leading to improvement of biotechinologies like embryo vitrification could facilitate their reproduction, making possible their gradual recovery. The aim of the present study is to provide information leading to protocol improvements; that could result in higher PVPR of embryos > 300 µmØ derived from both jennies and mares.

Relevance of embryo size over the post vitrification pregnancy rate

During the first decade past the year 2000 the size of the embryo vitrified, either small (≤ 300 µmØ) or large (> 300 µmØ) resulted in high and low pregnancy rates respectively[15]. Pregnancy rates close to 62 % were obtained when vitrifying embryos ≤ 300 µmØ in mares[28]; while cryopreserving embryos > 300 µmØ resulted in pregnancy rates near the 10 % range[29]. After the year 2010, adjustments to cryopreservation protocols designed for embryos > 300 µmØ have been performed; between these adjustments are the punction of the embryo capsule (EC) and the reduction of its size through aspiration of blastocoelic liquid (BL)[23]. These adaptations allowed the successful vitrification of embryos > 300 µmØ giving as result post vitrification embryo survival in donkeys[30] and pregnancy rates higher than 40 % in mares[31]. However, research assessing the effect of embryo size in post vitrification pregnancy rate (PVPR) have not been conducted using embryos derived from jennies[17,30].

Embryo capsule permeability, its association with the post vitrification pregnancy rate

Embryos derived from equine species develop a glycoproteic structure denominated embryo capsule (EC) that is required for the adequate progression of pregnancy[32]. Vitrification of embryos derived from mares have proven, that the presence of the EC lowers their permeability to cryo-preserving solutions, both; *in vitro*[33] and *in vivo*[34,35]. In mares, one of the first successful pregnancies obtained after the vitrification of embryos > 300 µmØ was achieved thanks to the treatment of the EC with trypsin, previous to the vitrification process, which increased their permeability to cryo-preservatives[36]. However; the increase in EC permeability by the use of trypsin or microfilament inhibitors (cytochalasin-B), have not shown positive results in a constant manner[37]. In equine embryos > 300 µmØ; the degree if integrity of the EC seems to keep a close relationship with their PVPR, since the EC is much more developed in this category of embryos, any dramatic alteration in their EC will result

in adverse effects over their PVPR[23]. Interestingly, there is a lack of studies analyzing EC permeability in donkey or mule embryos and whether their permeability is associated with the PVPR.

Embryo capsule punction and blastocoelic liquid extraction before the vitrification process

In equine embryos > 300 µmØ, it is important to puncture their EC[38] and extract their BL to promote a reduction in size before the vitrification process[39]. The relevance of EC punction and size reduction in embryos > 300 µmØ has been corroborated in mares; since the application of these procedures resulted in an increased PVPR[23,24,31,38,40,41]. Data also obtained in horses; reported before the year 2010, showed that PVPRs from embryos > 300 µmØ were lower than 40 %[15,29]. However after year 2010, by applying the EC punction and size reduction; a dramatic increase in post vitrification embryo survival was achieved, giving pregnancies rates between the 60 and 80 % in mares[23,24,40]. A recent study in embryos derived from jennies, demonstrated that size reduction by blastocoelic fluid aspiration results in a 83 % of post vitrification embryo survival rate[30]; however, the embryos from this study were not transferred into the uterus of the jenny during the post vitrification process, and hence the *in vivo* embryo development and pregnancy rate were not documented. Whether the EC punction is needed in embryos > 300 µmØ derived from jennies is an area that requires further investigation[21,30], since no studies have documented yet the PVPR of embryos derived from jennies after EC puncture and size reduction[16,17,21,30]. Interestingly; when comparing horse and donkey embryos, the last ones seem to tolerate vitrification better[16] and thereby, it should be possible that EC punction might not be needed as suggested by the study of Bottrel[17]; however, no studies directly testing this hypothesis has been conducted in donkeys. Historically, studies assessing the PVPR have been limited in jennies[21] and more work is needed in this area[16,17,21].

The influence of increased temperature transfer index (TTI) over the post vitrification pregnancy rates (PVPR) of equine embryos

In equids, a high TTI during the vitrification and devitrification process improves the PVPR of embryos > 300 µmØ. The TTI is related with the velocity and amount of temperature transferred from liquid nitrogen, (around -196 °C) to the cryo preserving solution containing the embryo which oscillates the 25 °C. If the TTI is incresed, the speed at which the temperature of the cryo preserving solution containing the embryo reaches - 196 °C is

increased. One way of promoting an increase in the TTI is to directly put into contact the cryopreserving solution containing the embryo with liquid nitrogen, another one is to reduce the volume of the solution containing the embryo that will be put into contact with liquid nitrogen. An increase in the post vitrification survival to levels higher than 60 % have been achieved recently; by promoting an increased TTI, using volumes of cryo preserving solutions lower than one micro liter, when immersing the embryo into liquid nitrogen, both in mares[23,40] and jennies[17]. In addition to EC punction, an increased TTI has resulted in higher pregnancy rates in mares when compared to lowered TTI, using small (less than one micro liter) and large (about one or more micro liters) volume crarriers respectively[40]. The use of small volume carriers which in theory increase the TTI, is a topic recently addressed in embryos derived from jennies[17]. The use of less than one microliter in the final vitrification solution containing the embryo resulted in a post vitrification embryo survival between the 40 and 50 % range[17]; however, in this study neither EC punction, nor embryo transfer into the jenny's uterus were performed and hence the PVPR was not established.

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