Immature versus in *vitro mature* equine oocyte vitrification

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Abstract

1 Successful oocyte vitrification allows the female genetic preservation, but in mares has not been clinical successful. The objective of the study was to evaluate the Cryotech® vitrification method for equine oocytes, both immature and in vitro matured with and without cumulus cells. For this three groups of oocytes were vitrified; A) Immature oocytes with corona radiate (Imm CV), B) *In vitro* matured and then vitrified with corona radiate (Mat CV) C) *In vitro* matured and then vitrified without cumulus cells (Mat DV) and D) Control (no vitrification). In all groups after warming and *in vitro* maturation, intracytoplasmic sperm injection and embryo culture were performed and survival, cleavage and blastocyst rate were evaluated by independence chi square. Survival rate was significantly lower for ImmCV: 66.7% (n = 108/162) versus those matured *in vitro* MatCV: 83.8%; (n = 150/179), MatDV: 78.6% (n = 165/210). For maturation no differences were found between ImmCV: 40% (n = 35/87), MatCV + MatDV: 38% (n = 103/269) and between the ImmCV and the control: 50% (n = 113/226). For cleavage rate, there was no difference between ImmCV: 50% (n = 16/32), MatCV: 46% (n = 25/53) and MatDV: 60% (n = 26/43), but there was a significant difference between ImmCV and MatCV with control: 74% (n = 47/60). There was no difference on blastocyst rates between treatment groups, except for control(ImmCV: 0% (n = 0/16), MatCV: 4% (n = 1/25), MatDV: 0% (n = 0 / 26) and control: 28% (n = 13/47). In conclusion, the Cryotech method presented similar rates of maturation for all groups. The MatDV group showed similar cleavage rate compare to control but blastocyst rates were affected in all experimental groups. These results demonstrate that the Cryotech® method was associated with high survival and maturation rate, but developmental competence was low for both Imm and Mat oocytes. Further studies are needed to increase developmental competence of vitrified/warmed equine oocytes.

Keywords: Vitrification, immature oocyte, mature oocyte, equine, ICSI, Maturation

Introduction

Oocyte vitrification is a process by which oocytes are preserved at subzero temperatures allowing store samples for extended periods, the method involves a fast cooling in the presence of high cryoprotectants (CPAs) concentrations, which prevents formation of ice crystals and reduces cryoinjuries(1). It has been successfully developed in several species, as human (2ï 4), mice (5,6), cattle (7,8) and pigs (9ï 11), although in horses this procedure has not yet been developed with clinical success (12). Freezing semen can successfully preserve stallion genetics, but the mare genetic preservation has been unsettled. An effective cryopreservation of equine oocytes is important for a variety of reasons, such as preserving breeds that have experienced diminished genetic diversity or species that are endangered in the wild (13). Potential clinical uses of oocyte vitrification would be the preservation of genetics from young animals before going to competition (14), from a recently deceased mare or a mare that is going to be euthanized aiming a further offspring production by either intracytoplasmic sperm injection (ICSI) or oocyte transfer (OT) (15). Moreover, the cryopreservation of oocytes would facilitate the transport of the gametes to laboratories in which ICSI could be performed (16).

Different authors have shown equine oocytes survival rates between 24% and 69%, although survival rate has been measured by spindle quality, maturation rate or membrane integrity (17ï 22) which makes it difficult to compare between reports. To date, the best results published on maturation rate so far is 61%, however for this experiment the authors did not perform fertilization ICSI and *in vitro* culture (23), therefore, the the development capacity was not fully evaluated. To the best of our knowledge, two reports have been published describing establishment of pregnancies from vitrified *in vivo* matured equine oocytes (14,24). Tharasanit *et al* (2006) (25), reported the first blastocyst produced from vitrified-warmed immature equine oocytes after ICSI with 4% of cleaved zygotes, 1 out of 28 oocytes used) On the other hand, Canesin *et al.* (2017) (16), reported the highest developmental competence yet achieved with 20% blastocyst formation (1/20 oocytes); although, in both reports,

embryos were not transferred to recipient mares. Recently, Ortiz-Escribano *et al*, 2017 (12), published 17% blastocyst formation after ICSI of vitrified-warmed immature equine oocytes and one healthy foal out of 5 embryo transfer (12). Even though blastocyst and few foals have been produced with vitrified equine oocytes the efficiency of the vitrification methods is still low since a high number of oocytes are needed to be processed in order to obtained the published results. 1/229 (0,4%) (25); 1/35 (2,9%) (16); 5/179, (2,8%) (12); Blastocyst/immature vitrified oocytes) therefore more research is needed in order to improve blastocyst development and foal production.

Many factors influence the success of oocyte vitrification (26). These can be divided in oocyte factors which refer to meiotic stage (mature or germinal vesicle) and presence or absence of cumulus cells, and technical factors, referring to vitrification methods, cryoprotectants, and the devices used for the procedure (27). In particular, in the horse, these factors remain poorly studied; in fact, the lack of oocytes available for research limit the number of studies develop to understand the optimal systems to maintain high developmental potential after warming (12).

Cryopreservation of germinal-vesicle stage (GV) oocytes would allow collection and preservation of oocytes at a remote locations from the laboratories that can perform the fertilization (28), although is generally associated with lower survival than those in metaphase II (MII) (17,23,29,30). The lower survival of GV oocytes is due to higher sensitivity to osmotic stress (31). Nevertheless, embryo production after vitrification of GV- stage oocytes has been described in several species including humans (32), cows (33), pigs (11), goats (34),etc. On the other hand, in humans, efficient vitrification systems have been developed for clinical application with *in vivo* mature oocytes (2,35ï 37), In equine the best result achieved has been 40% of blastocyst rate using in vivo mature oocytes (14), but recovering equine *in vivo* mature oocytes requires live mares treated with gonadotropin and careful handling of the oocytes obtained (38). Super-stimulation of the ovaries could increase the number of follicles present in the ovaries, but the number of recovered oocytes decreases and manipulation of the ovaries become more difficult due to enlargement (24). Instead embryo development from *in*

vitro mature oocytes has been poorly studied (17) and it is required require specialized laboratories which could perform ICSI and later embryonic culture (39).

Besides the meiotic stage of the oocytes that enter the vitrification process, there are controversies about the need of cumulus cells (CC) during oocyte vitrification (27,35,40), given the crucial role that CC play on oocyte growth and development(44, 45). Tharasanit *et al.* (2006) (25), demonstrated that the presence of CC in mature horse oocytes during vitrification preserves the meiotic spindle (MS) quality after warming with 38% normal spindles in cumulus-enclosed oocytes compared to 3% in cumulus-free oocytes. Contrarily, in humans the presence of CC during vitrification lowered the MS integrity of mature oocytes after warming with 9% vs. 44.2% cumulus-enclosed vs. cumulus-free and also after warming and three hours of incubation with 18% vs. 88.4% cumulus-enclosed vs. cumulus-free (43); On this study, the authors also demonstrated that the period of incubation after warming can be crucial to favor the MS repolymerization after vitrification because both groups increase drastically the MS integrity after three hours of incubation, 9% to 18% in cumulus-enclosed oocytes and 44,2% to 88.4% in cumulus-free oocytes (43).

Several commercial kits for vitrification of mature denuded oocytes have been clinically utilized with survival rates above 90% (35,44) in humans. The most popular commercial kit utilized is the Cryotech Vitrification Method (Repro-Support Medical Research Centre, Co. Ltd, Tokyo, Japan), which can support up to 100% survival rate for human embryos as well as human oocytes (45). Furthermore, the Cryotech kit has been successfully used for vitrification of bovine oocyte with 99% of survival rate and similar blastocyst rate than control (46), It was also used in bovine embryos with reports of 100% survival rate and subsequent 46.6% pregnancy rate after transfer (47) and for mouse embryos, the kit has produced above 98% survival and 35% birth rate (48). Therefore, the main objectives of this study are to evaluate the Cryotech® vitrification method for equine oocytes, both immature and in vitro matured with and without cumulus cells, evaluating survival rate after warming, and subsequent maturation (for oocytes vitrified at GV-stage), cleavage, and blastocyst rates after ICSI.

Materials and Methods

Collection and culture of cumulus oocyte complexes

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and media from Invitrogen (Carlsbad, CA, USA) unless otherwise specified.

Recovery of COCs from slaughterhouse-derived ovaries

Oocytes obtained from slaughterhouse-derived ovaries were collected using the techniques previously described by Canesin *et al*. (2017) (16). Ovaries were acquired at a slaughterhouse, placed in a Styrofoam container and transported to the laboratory, at environmental temperature (~25 °C; 5 ï 7 hours between first mare was slaughter and start of ovary processing), temperature upon arrival was 25ï 30ºC. All visible follicles < 35 mm diameter on the ovary surface were opened with a scalpel blade and the inner follicle wall was aspirated using a 14-ga needle connected to a vacuum pump (WTA Vacuum pump BVD3, WTA, Brazil) set to aspirate approximately 20 mL of fluid per minute. The aspiration apparatus and tubing was rinsed between follicles with a commercial embryo flush medium (Vigro complete flush medium;Bioniche, Belleville, ON, Canada) supplemented with 5 IU/mL heparin (#H3393) and aspirated into 250-mL sterilized glass bottles. After all follicles visible on the surface of the ovaries were processed, the ovaries were cut to locate the follicles within the ovarian stroma and they were similarly processed. The aspirated fluid was clarified through an embryo filter (EmCon filter, Immuno Systems, Inc., Spring Valley, WI, USA) and cumulus oocyte complexes (COCs) were allocated under a stereo microscope Nikon SMZ1500(Nikon Corporation, Minato, Tokio, Japon). As they were located, the COCs were placed in a 35-mm polystyrene Petri dish (Falcon, Corning Incorporated, NY, USA) containing commercial embryo holding medium (Syngro, Bioniche, Belleville, ON, Canada). After collection, the oocytes were then transferred to a 1.5-ml Polypropylene tube (Eppendorf, Hamburg, Germany) filled with the same holding medium and held overnight (15 - 18 h). The next morning oocytes were randomly assigned to one of the three-vitrification groups.

Recovery of COCs via transvaginal ultrasound-guided follicular aspiration

For the ICSI-Control oocytes, 15 Quarter Horse-type mares weighing 500-600 kg and aged 4-16 years were used as donors for those oocytes collected from transvaginal ultrasound-guided follicular aspiration (TVA). The mares were kept outside and were fed hay and water ad libitum. All experimental procedures were performed according to the United States Government and Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research and Training and were approved by Laboratory Animal Care Committee at Texas A&M University.

The fifteen mares were divided in two groups and TVA was performed in each mare approximately once every two weeks, all follicles > 5 mm in diameter were aspirated. There was no attempt to manipulate or stage the cycle between aspiration sessions. The TVA procedure and isolation of COCs were conducted as previously described (49). Follicles >35 mm diameter were aspirated separately, to avoid oocytes from preovulatory follicles. Aspirates from follicles over 35 mm were sampled; if granulosa cells looked really expanded and jelly, as from a mature oocyte, these aspirates were not used in this project, if not expanded, the aspirated fluid was then combined with that from the smaller follicles. The aspirated fluid was filtered and the COCs were searched for and processed as described above for oocytes recovered from slaughterhouse-derived ovaries, after recovery they were held overnight at room temperature for 17 - 27 h in 1-mL borosilicate glass vials (Thermo Fisher Scientific, Waltham, MA, USA) containing commercial embryo holding media (Vigro, Bioniche, Belleville, ON, Canada). After overnight holding, oocytes were put in *in vitro* maturation for 27 - 36 hours and used as control for maturation, cleavage and blastocyst rate.

Vitrification and warming of COCs

Vitrification was performed using the Cryotech method (50). The key point of this vitrification method is to base the exposure time to the equilibration solution on the reaction of oocytes, as detected by the eyes of the embryologist, rather than the absolute time taken in the process. Briefly, oocytes were equilibrated in a solution

containing ethylene glycol (EG) and dimethyl sulfoxide (DMSO) in Minimum Essential Media (MEM) with hydroxypropyl cellulose (HPC) and no protein supplement. The oocytes shrank and then recovered their normal morphology in 10 to 15 min, the limit time of this step is 15 min. After oocytes had recovered its initial morphology, they were moved to a vitrification solution containing EG, DMSO, and trehalose in MEM + HPC until the oocytes shrank sharply again and sunk in the vitrification media. The oocytes were then loaded with a glass pipette onto the device supplied with the kit, 3 to 5 oocytes were located per device and the sample was quickly immersed into liquid nitrogen. The devices were left in the liquid nitrogen until all oocytes had been vitrified, then the tips of the devices loaded were covered with a protective cap at the end. The time between the placement of oocytes in the vitrification solution and the immersion of the device into the liquid nitrogen was 60 to 90 s. For immature oocytes, the solutions were at room temperature and for mature oocytes the solutions were warmed for two hours and held on a thermal plate at 38 °C during the vitrification process.

At warming, the strip was immersed directly into warming solution consisting of MEM + trehalose + HPC at 37 °C for 1 min. Then oocytes were moved for 3 min to the kitôs diluent solution consisting of MEM + trehalose + HPC, and washed twice in different wells in washing solution, consisting of MEM + HPC, for 5 min and 1 min respectively. For immature oocytes the warming solutions were warmed for two hours and the diluent and washing solution were at room temperature, while for the mature oocytes all solutions were warmed for two hours, and held in thermal plate at 38 °C during the warming process.

In vitro maturation (IVM), intracytoplasmic sperm injection (ICSI) and embryo culture

Two populations of control oocytes were use. ñIn situ-Controlò oocytes were recovered from slaughterhouse tissue and matured in the vitrification laboratory (Colombia). ICSI-Control oocytes were recovered by TVA and matured and subjected to ICSI and in vitro embryo culture in the ICSI laboratory (Texas).

All oocytes were cultured in maturation medium containing M199 with Earle's salts, supplemented with 5 mU FSH/mL (Sioux Biochemical Inc., Sioux Center, IA), 10% fetal bovine serum (FBS) and 25 mg gentamycin/ml. Oocytes were cultured in 150-μL droplets of maturation medium, maximum of 15 oocytes per droplet, under light white mineral oil at 38.2 °C in 5% CO_2 in air for 27- 36 h (51). After maturation, warming/maturation or maturation/warming, the ICSI procedure was conducted for oocytes with polar body (PB) as previously described (52). Briefly, oocytes were denuded in hyaluronidase 0,05%. Oocytes with intact membrane and zona pellucida and with a visible polar body were subjected to ICSI. Swim-up procedure was used to prepare frozen-thawed semen (53), and sperm injection was performed using the Piezo drill. Injected oocytes were cultured in 15-μl droplets of a commercial human embryo culture medium (Global medium, Life-Global, Guilford, CT, USA) with 10% FBS in a humidified atmosphere of 6% CO_2, 5% O_2 and 89% N_2 at 38.2 ºC. Cleavage was evaluated at day 2, medium was changed on Day 5 to DMEM/F-12 with added glucose (52), and days 7-10 was evaluated blastocyst formation.

Oocytes and presumptive embryos assigned to chromatin evaluation were fixed in buffered formol saline for a minimum of 24 h at room temperature. Fixed structures were labeled for chromatin evaluation by being placed on a glass slide with 10 μl mounting medium containing 1 mg/ml 4,6-diamidine-2-phenylindole dihydrochloride (commercial DAPI solution, ThermoFIsher SCIENTIFIC, Waltham, MA, USA) or 10 μl glycerol containing 2.5 μg/ml Hoechst 33258 and a cover slide on top. The structures were evaluated using a fluorescence microscope with a 365-nm exciter filter as previously described (51).

Experimental design

Preliminary trial: Maturation rate of oocytes recovered from slaughterhouse-derived ovaries

In order to establish the maturation rate of oocytes recovered from slaughterhouse ovaries (without vitrification) a preliminary study was performed in Colombia, with oocytes processed in the same manner as oocytes intended for

vitrification. Briefly, oocytes were collected and held overnight and put in maturation as described above. After maturation, the oocytes were evaluated for the presence of the PB to determine maturation rate.

Experiment 1: Effect of meiotic stage at the time of vitrification and presence of cumulus cells on maturation rate and blastocyst development after ICSI

All vitrified oocytes assigned to ICSI were collected in Colombia from slaughterhouse-derived ovaries. Obviously degenerated oocytes (having shrunken or dense cytoplasm and scant, granular cumulus) were discarded. Cumulus oocyte complexes were held overnight, and then randomly assigned to one of the three groups: ImmCV, COCs vitrified immediately with intact corona radiata; MatCV, COCs subjected to IVM, then vitrified with intact corona radiata; and MatDV, COCs subjected to IVM with intact corona radiata, then denuded and vitrified. Two embryologists, one experienced in oocyte vitrification (SM) and one with less experience (DA), performed the vitrification procedure. After vitrification, the oocytes were held in liquid nitrogen for 6 months then transported in dry shipper (4 days) to the ICSI laboratory in Texas. The temperature of the dry shipper at arrival in Texas was-198 ºC.

The warming was performed by embryologist DA, After warming, oocytes were subjected to 28 -36 (ImmCV) or 2-4 hours of culture (MatCV and MatDV). After IVM or culture, all oocytes were denuded of cumulus and evaluated for presence of a PB. All oocytes that had a PB were assigned to ICSI and culture as described above. Oocytes without PB were fixed for chromatin evaluation and classified as GV, MI, MII (including anaphase and telophase I). At Day 2 of culture, oocytes that were injected and did not cleave were stained for assessment of chromatin configuration and described as Sperm Head (SH), Metaphase Plate (MP), Polar Body (PB) and pronucleus (PN)). From days 7-10, blastocyst formation was evaluated under a stereomicroscope. Embryos with a presumptive trophoblast layer with decreasing density of inner cells were considered to be blastocysts. After either characterization as a blastocyst or after 10 days in culture, embryos were fixed and stained for chromatin evaluation. Embryos were classified as

blastocysts, if they contained more than 100 nuclei and had started organization of outer presumptive trophoblast cells.

Oocytes recovered by TVA at the ICSI laboratory (ICSI-Control) were used as a control for ICSI and blastocyst development. These oocytes were recovered once each week that vitrified/warmed embryos were processed. The ICSI-Control oocytes were held overnight, then subjected to the same IVM, ICSI and embryo culture procedures as for vitrified/warmed oocytes.

Statistical analysis

Statistical analysis was performed using STATGRAPHICS Centurion XVII (statgraphics.net, San Francisco CA, USA). Differences in survival, maturation, cleavage and blastocyst rates between ImmCV, MatCV, MatDV and fresh oocytes were analyzed by chi square of independence. The significance level was defined as $P < 0.05$. All data are shown in Mean ± standard error (MSE)

Results

Preliminary trial: Maturation rate of slaughterhouse-derived oocytes (Colombia)

two hundred and seventy-seven oocytes were recovered from slaughterhouse-derived ovaries. Ten replicates of maturation were performed, and 128 oocytes were classified as having a polar body under the stereomicroscope (46 ± 2%). To verify the accuracy of classification of matured oocytes under the sereomicroscope 55 oocytes and 25 classified as having polar bodies were stained with Hoechst 33258 and evaluated using fluorescent microscopy. 26/55 (47.2%) were found to be in MII, showing no difference between the evaluation under light microscope and under fluorescent microscopy P= 0.85.

Technician

There was no difference between technicians in the proportion of vitrified oocytes found after warming, or in maturation or cleavage rates for any treatment, thus, the

effect of vitrification treatment on the end points of the study were evaluated without regard to technician.

Recovery Rate and Survival rate:

The recovery rate was evaluated by the number of oocytes found over the number of vitrified oocytes. Treatment significantly affected the rate of oocytes recovered after warming; the recovery rate was significantly lower in the ImmCV 108/162 (67 ± 7.6%) compare to MatCV 150/179 (84 ± 3.7%) and MatDV 165/210; (79 ±4.4%) with a p value of < 0.001 and < 0.05 for recovery and cleave rate respectively. Survival rate, determined as oocytes with intact membrane and zona pellucida, over found oocytes, was no different between groups with 93-98 survival rate and p > 0,5), but if survival rate is evaluated over all vitrified oocytes (survival rate overall = oocytes with intact membrane and zona pellucida over vitrified oocytes), the survival rate is affected by treatment in the same way that is affected the recovery rate, ImmCV 102/162 (63 ± 7.6%); lower that MatCV 143/179 (80 ± 4.8 %); and MatDV groups 153/210 (73 ± 5%); (P < 0.001 and P < 0.05, respectively), with no difference between Mat CV and Mat DV (P = 0,24), Figure 1.

Maturation Rate

There were no differences in maturation rate between ImmCV 35/87 (40 ± 7.4%) and the general maturation rate of matured oocytes prior to vitrification (MatCV + MatDV) 103/269 (38% ± 2.3%) P = 0,79 that was no different from the maturation rate of the oocytes matured in Colombia 128/277 (46 ± 2 %) p = 0,2. There was a tendency for lower maturation rate in the control group from TVA oocytes 113/226 (50 ± 4.6%) p = 0.0589(Table 1).

Cleavage Rate

There were no differences in cleavage rate (cleaved oocytes / oocytes subjected to ICSI) among vitrification treatments (47 to 70%; p > 0.05). The cleavage rates for ImmCV and MatCV were significantly lower than that for ICSI-Control oocytes (78%; P <

0.01), and the MatDV did not show significant difference with control (70% vs 78%; p = 0,077; Table 2). For cleavage and blastocyst rate 60 ICSI-Control oocytes out of 113 oocytes with PB were used in these experiment and the others were used in other projects.

Blastocyst Rate

All vitrification treatments had significantly lower blastocyst rates than did control (0-4% vs. 28%; p < 0,001) see Table 2.

Discussion

Vitrification has demonstrated in humans higher oocyte survival rates than slow freezing (95%, 899/948 vs 75%, 1275/1683 respectively) (54), and might be the best cost-effective method to cryopreserve mare´s genetic because do no need special equipment as a programmable freezer (35,39). In this study, we demonstrated that it is possible to cryopreserve equine oocytes in Germinal-vesicle stage and Metaphase II, although survival rate might be affected by maturation stage. Differently Hurt et al, (2000) (21), showed that mature oocytes had similar survival rate after vitrification compare to immature oocytes. In the present study, the maturation rate was not evaluate before vitrification due to impossibility of seeing the polar body with the presence of cumulus cells therefore some oocytes could have been vitrified oocytes in a degenerative state or that were not matured.

The Cryotech method had a high effective survival rate (>90%) if one takes only the oocytes that are found on the plate after warming, but we hypothesized that the oocytes that were not found were exploded due to high osmotic shock during the vitrification or warming procedures. Taking the found oocytes into account for the calculation of survival rate (oocytes with intact membrane and zona pellucida over vitrified), it is lower (63 - 80%) compare to survival rate over found. It is important to perform more research in order to determinate what happens to the oocyte that are not found. Although the overall recovery rate with the Cryotech method was similar or higher to what other have reported before, 72% (14), 61,3% (23), 68,4%(12).

Meiotic stage and presence of cumullus cells is still a dabate, vitrified immature oocytes would allow transport gametes from remote places without culture facilities (39) In this study there was no difference in maturation rate when comparing the same source of oocytes showing that these method does not affect the meiosis resumption capacity, similar to others results (23), and contrary to others (17,22). Tendency for lower maturation rate of the control TVA oocytes highlights the importance of the quality of oocytes at the moment of vitrification and how this can affect the developmental competence of the oocytes (16). In this experiment cleavage of MatDV was similar (P>0,05) than control, and the highest cleavage between vitrified groups (47 to 70%), Different to Tharasanit *et al*, (2006) (29) where oocytes vitrified prior to IVM yielded higher cleavage after ICSI (28-34 %) than those vitrified after IVM (4-16%); keeping the debate open between the meiotic stage and the presence of cumulus cells. The effect of cumulus cells during vitrification has been poorly studied in horses (17,18).In other species, there is still discrepancies about the effect of the presence of CC in the survival rate and oocyte developmental capacity after warming of vitrified oocytes (27,34,43,55). . In bovine, the presence of cumulus cells surrounding mature oocytes increased blastocyst rate at day 8 with 35.8% vs 15.5% for oocytes vitrified denuded (56). Minasi et al. (40) reported no differences in the survival of cumulus enclosed oocytes compared with denuded oocytes, 62,5% for cumullus cells encloced and 69,4% for denuded oocytes, similarly to this study where there was also no difference (MatCV = 80% vs Mat DV = 70%; P >0,05); however, in equine, the presence of cumulus cells was found to be beneficial during the vitrification of mature oocytes since 23/63 (38.3%) had normal spindle quality after warming when where vitrified with cumullus cells versus 2/70 (2%) that had normal spindle after warming when where vitrified denuded(18). this disagree with the study because there was no difference between MatCV and MatDV in any variable, but MatDV was the only that had similar cleavage rate compare to the control. These might mean that the absence of cells gives the oocytes better development capacities perhaps due to better exchange of cryoprotectans (43). Although, the only blastocyst develop in this study was from the cumulus cells enclosed

oocyte, which confirms that cleavage rate is not a sufficiently reliable indicator of developmental potential (29).

The lack of difference on the survival, maturation and cleave rate between the experienced and inexperienced technician on this study indicates the robustness of the Cryoteck method. The vitrification method used was associated with high survival and maturation rate, and cleavage rate for denuded oocytes was similar to control oocytes, similarly to other vitrification methods for equine oocytes (14,16,24), but on the other hand, the blastocyst rate was hardly affected by the vitrification process (0-4% vitrified vs 28% control) demonstrating that developmental competence can only be accurately measure by blastocyst formation and not only for maturation or cleavage rate. Further studies are needed to address why vitrified/warmed mare gametes that cleaved failed to progress to the blastocyst stage.

Figures and tables

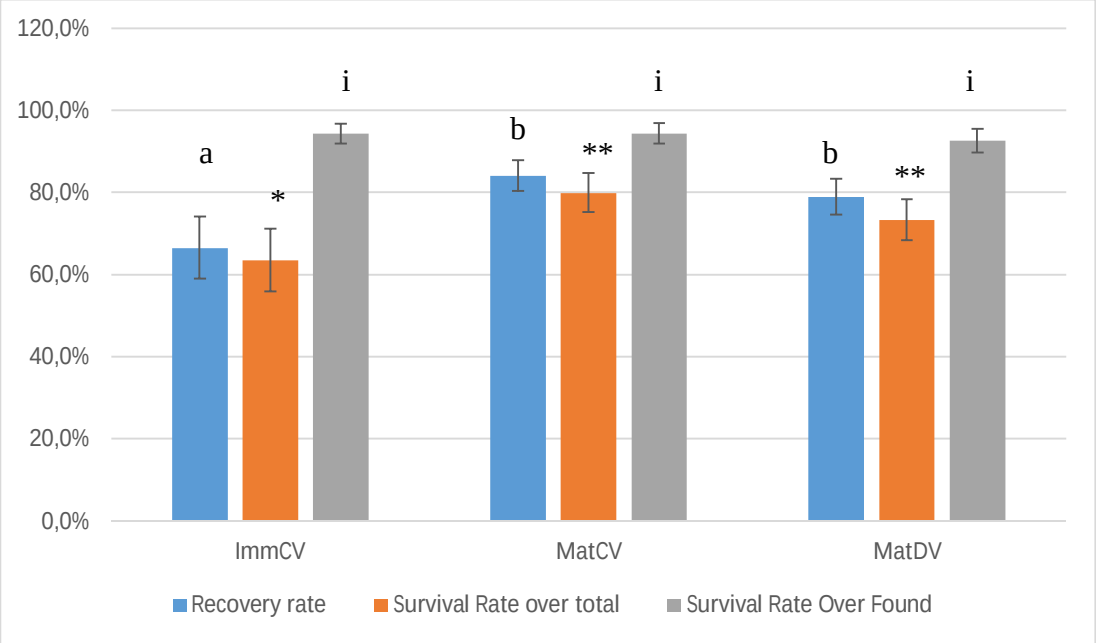

Figure 1. Percentage of Recovery rate, Survival rate over total, and survival rate over found [a,b,*,**,i,ii]

Groups with different superscripts are significantly different (P<0.05). 10 replicates. Data are shown mean ± MSE.

Table 1: Percentage of maturation rate [(a,b)] Groups with different superscripts are significantly different (P<0.05). Data are shown mean ± MSE.

Group	n	Oocytes in MII (%)
Imm CV	87	35 (40 ± 7.4)[ab]
Mat DV and CV	269	103 (38 ± 2.3)[a]
ICSI -Control	226	113 (50 ± 4.6)[b]
Preliminary trial	277	128 (46 ± 2)[ab]

Table 2: Percentage of cleavage rate and blastocyst rate [(a,b)] Groups with different superscripts are significantly different (P<0.05). Data are shown mean ± MSE.

Group	*n*	*Cleaved oocytes (%)*	***Blastocyst (% Over cleaved)***
ImmCV	*32*	*16 (50 ±11.5)[a]*	*0[a]*
MatCV	*53*	*25 (47,2 ±7.9)[a]*	*1 (4 ±4.2)[a]*
MatDV	*43*	*30 (69.8 ±11.4)[a,b]*	*0[a]*
Control	*60*	*47(78.3 ±6.3)[b]*	*14 (29.8 ±3)[b]*

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