Vitrification of White-tailed Deer (*Odocoileus virginianus*) oocytes with sucrose or trehalose for *in vitro* maturation and fertilization

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ABSTRACT

Objective: Evaluate the White-tailed Deer (WTD) *in vitro* embryo production (IVP) and oocytes vitrified with Trehalose (TH) or Sucrose (SC).

Design/methodology/approach: Total vitrified oocytes were placed into two different groups: TH (n=60) and SC (n=61). Samples were selected and analyzed for viability evaluation TH (n=5) and SC (n=5), nuclear status (NS) TH (n=4) and SC (n=5), Germinal Vesicle (GV), Metaphase I, or not evaluable (NE) after warming. *In vitro* maturation (IVM) was conducted for 36 h in supplemented TCM-199 medium. Immediately afterwards, oocyte NS was evaluated (n=88) [(GV, MI=immature), (MII=mature)]. *In vitro* fertilization (IVF) was performed in supplemented TALP medium for 24 h using frozen WTD semen (3×10^6 sperm/mL), NS was classified [Fertilized (F), Not fertilized (NF), or NE].

Results: After warming, viability for the TH group (n=5) was 60% versus 40% for SC group (n=5), however, oocytes in both groups were immature (GV and MI stage). For IVM, NS evaluations of the TH group (n=38) revealed no maturation versus 2% in the SC group (n=50) (MII stage=matured). IVF evaluations for the TH group (n=10) revealed no fertilization compared to 20% in the SC group (n=5). A statistical difference ($p>0.05$) was not found between the TH and SC groups.

Limitations on study/implications: White-tailed Deer *in vitro* embryo production is not well documented.

Findings/conclusions: Future research with a larger number of WTD oocytes is needed for further evaluation of oocyte vitrification IVP techniques as a model for endangered cervids.

Keywords: White-tailed Deer, *in vitro* embryo production, solid surface vitrification.

INTRODUCTION

The WTD is a wild ruminant that with its physical characteristics, and availability, can be considered as a model animal for those deer species in danger of extinction, *e.g.*, Florida Key Deer, regarding reproductive studies associated with conservation (Somfai *et al.*, 2014; Gastal *et al.*, 2018). Further, semen processing, oocyte cryopreservation/vitrification, IVF, embryo cryopreservation, and embryo transfer allow the selection and use of animals that are genetically superior for increasing the productive parameters that are of economic importance, thus also decreasing the risk of spreading diseases (Clemente-Sánchez *et al.*, 2017).

Given the high demand for breeding deer of superior genetics and trophy bucks, *e.g.*, hunting ranch breeding programs and the availability of a hunting season, ART can be an effective method for deer conservation and deer production programs (Maraboto *et al.*, 2022).

Vitrification is defined as a solidification similar to the vitreous state. Through high concentrations of cryoprotectants and an ultra-rapid cooling rate (Begin *et al.*, 2003) reaching high cooling rates of 16,700 °C/min (Criado-Scholz, 2012). Vitrification is a proven technique capable of preserving the integrity of cell structures (Gastal *et al.*, 2018). Solutions that contain SC or TH are widely used for cryopreservation of oocytes and embryos of mice, cattle, horses, pigs, and sheep (Tian *et al.*, 2015). SC is a disaccharide formed by glucose and fructose, and TH is a disaccharide of glucose, both are non-permeable cryoprotectants that maintain the extracellular osmotic gradient to prevent crystallization for oocytes and embryos of various species such as humans, bovine and murine (Chen *et al.*, 2001; Tian *et al.*, 2015).

Solid surface vitrification (SSV), one type of cryopreservation technique, is performed by ultra-rapid temperature reduction on a sterile, cold metal surface (10,000 °C/min) (Somfai *et al.*, 2010). Importantly, it is necessary to carry out a process of elimination regarding the vitrifying solution and at the same time of cellular rehydration. The warming procedure is carried out in several stages, subjecting the vitrified cell to solutions with decreasing concentrations of non-permeable cryoprotectants, so that through the osmotic effect they leave the cell, to prevent sudden water pressure changes (Izaguirre and Díez, 2012).

At present, no scientific literature is available pertaining to WTD vitrified immature oocytes for IVP, and only fresh oocyte IVM has been done for this cervid species (Maraboto *et al.*, 2022). In order to enhance cervid endangered species conservation and embryo production, the aim of the present study was to use vitrify WTD oocytes with two non-penetrating additives TH or SC and evaluate their survival after IVM and IVF.

MATERIALS AND METHODS

The present study was carried out in the Reproduction Management Laboratory of the Autonomous Metropolitan University-Xochimilco Unit in Mexico City.

Ovaries and oocytes collection. The collection of WTD immature oocytes (n=121) was performed according to methods of Siriaroonrat *et al.* (2010). Oocytes were collected from hunter harvested deer (n=18) using the slicing technique with Syngro holding medium (Vetoquinol, France) for oocyte collection, and not more than 2 h postmortem.

Vitrification. The WTD oocytes were then cryopreserved using a SSV technique (Somfai *et al.*, 2014). The equilibrium medium consisted of base medium (PBS) supplemented with 4 mg/mL bovine serum albumin (BSA), 2% propylene glycol (PG) and 2% ethylene glycol (EG; Sigma-Aldrich, Mexico). The vitrification solution was composed of base medium supplemented with 4 mg/mL BSA and separated into two different groups: TH (n=60) (0.3 M) (TH, 17.5% EG, 17.5% PG) and SC (n=61) (0.3 M SC, 17.5% EG, 17.5% PG). For the SSV method, COCs were placed at the equilibration medium for 15 min, washed three times in 20 μL drops of vitrification solution, then pipetted into a glass capillary tube with respect to each of the groups, and finally, placed into about 2-3 μL of vitrification solution and then dropped onto a piece of aluminum foil (*i.e.*, raft) floating on the surface of liquid nitrogen (Somfai *et al.*, 2010).

Warming. To process the vitrified WTD oocytes that were stored in cryotubes, the microdrops were placed onto an aluminum raft with a nylon net placed on the liquid nitrogen and used to recover oocytes. Thereafter, the nylon net was rinsed with liquid nitrogen to ensure a full recovery of the vitrified oocytes. The oocyte warming (OW) media content was base medium supplemented with 4 mg/mL BSA and TH, which was conducted using 4 different concentrations of the warming medium (OW1 1 M, OW2 0.5 M, OW3 0.25 M and OW4 0 M) (Sigma-Aldrich, Mexico).

In a 4-well dish, the first warming medium (OW1 medium (500 μL) was pre-heated in a water bath at 39 °C, then oocytes were placed in this warming solution for 2 min. In the 4 well dish, the other OW medium 2, 3 and 4 were placed (500 μL) at 38 °C and the oocytes from OW1 were placed in each medium for 1 min. A 35 mm Petri dish was prepared separately for the washing medium prior to IVM (Somfai *et al.*, 2010; Zhang *et al.*, 2017).

Warming Viability and Nuclear Evaluation. A sample from the warmed oocytes was used to evaluate viability from group TH (n=5) and SC (n=5). The cumulus cells were removed by flushing the oocyte in PBS. A drop of MTT stain [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], 0.5 mg/mL) (Sigma-Aldrich, Mexico) was placed into a four well dish with the oocytes for evaluation, and after 2 h viability results were obtained. Oocytes with purple coloration were considered viable and the non-colored ones were non-viable (Lestari *et al.*, 2018; Figure 1a). For NS evaluation oocytes from TH (n=4) and SC (n=5) groups were stained with DAPI stain, and oocytes were classified into germinal vesicle stage GV, MI, and NE. For DAPI staining (4′,6-diamidino-2-phenylindole, 1 μg/mL) (Sigma-Aldrich, Mexico), oocytes were transferred to 2% paraformaldehyde (HYCEL, Mexico) for 15 min, washed 3 times at IMACELL (IN VITRO, Mexico), followed by DAPI staining with 1 μg/mL, and mounted with minimal medium onto a clean glass slide, for evaluation using epifluorescence microscopy (Nikon eclipse E600 20x) to determine oocyte nuclear maturation (Siriaroonrat *et al.*, 2010).

***In vitro* maturation and evaluation**. The remaining oocytes were used for IVM, and incubation of oocytes took place at 38.5 °C with 5% CO_2 for 36 h in maturation media TCM-199 (IN VITRO, Mexico) supplemented with human menopausal gonadotropin (75 IU/mL) (IBSA Institut Biochimique SA. Switzerland) and epidermal growth factor (10 ng/mL) (Sigma-Aldrich, Mexico). From those oocytes used for IVM (n=88), a sample

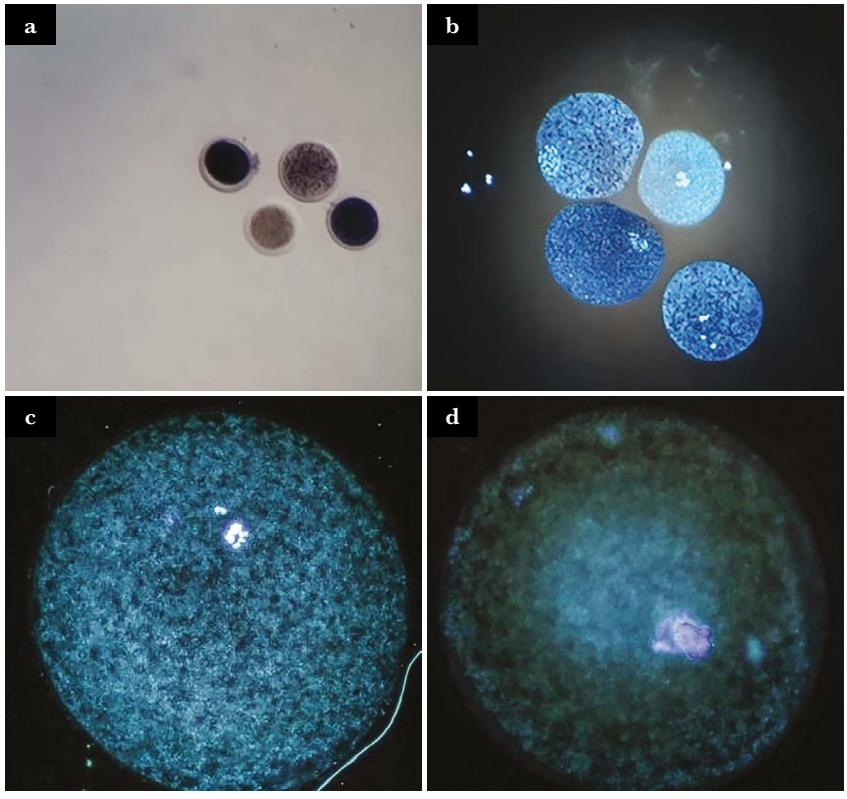

Figure 1. a) White-tailed Deer (WTD) (*Odocoileus virginianus*) oocyte viability post-warming evaluation with MTT stain; purple stained oocytes indicate active mitochondria (*i.e.*, considered alive); unstained oocytes indicate no mitochondrial activity (*i.e.*, considered dead oocytes), b) WTD oocyte nuclear status after warming, c) WTD oocyte after *in vitro* maturation Metaphase II showing the nuclear status with DAPI stain, and d) WTD oocyte after *in vitro* fertilization showing the pronuclear status.

was used to evaluate NS using DAPI stain, defining the oocyte stage as GV, MI and NE [considering these three stages as immature, and MII as mature] (Izaguirre and Díez, 2012; Siriaroonrat *et al.*, 2010; Locatelli *et al.*, 2005).

***In vitro* fertilization and evaluation**. For IVF, frozen conventional WTD straws of semen were used and capacitation was induced with "swim-up" technique using modified TRIS media (Locatelli *et al.*, 2005). IVF took place in TALP media supplemented with heparin (10 μg/mL) (PISA, Mexico), penicillamine (0.075 mg/mL), hypotaurine (10 M), epinephrine (1 μM) and BSA fraction V (0.4%) (Sigma-Aldrich, Mexico), with a final semen concentration of 3×10^6 sperm/mL (Locatelli *et al.*, 2006). After 24 h of incubation at 38.5 °C with 5% CO_2, and humidified air, nuclear status evaluation was performed using DAPI stain with 1 μg/mL to evaluate WTD oocyte fertilization. The aforementioned resulted in oocytes being classified as F, NF, or NE (Berg *et al.*, 2002).

Theory/Calculation. The WTD oocyte vitrification can serve as a model to assist with genetic improvement and conservation of the other cervid species, a promising tool to gather genetics, *i.e.*, cryobank oocytes, for future IVF and cloning projects. In order to apply IVP techniques as a model for other endangered cervid species, we used the technique developed for bovine oocyte vitrification and IVF and applied it to WTD.

Statistical analysis. A Fisher's exact test (SAS, version 9.0 for Windows) was used to determine statistical differences $p<0.05$ in the different stages of the study, concerning vitrified oocyte warming, IVM and IVF.

RESULTS

Warming. Viability evaluation for the TH group (n=5) after warming was 60%, and 40% for the SC group (n=5) (Figure 1a). In addition, for nuclear status evaluation (Figure 1b) TH (n=4) was 50% GV, 50% MI, which were considered immature oocytes, and for the SC group (n=5) results were 60% GV, 20% MI, immature oocytes and 20% NE (Table 1).

After 36 h IVM (Figure 1c) in the TH group (n=38) the nuclear status evaluation was conducted (66% GV, 24% MI, 0% MII and 10% NE), which demonstrated that no maturation took place in the sample used and for the SC group (n=50) (84% GV, 10% MI, 2% MII and 4% NE), even though the sample was small, matured oocytes were obtained. No statistical difference ($p>0.05$) was found between the TH and SC groups in viability, warming, IVM and IVF evaluations (Table 2).

For IVF, after 24 h (Figure 1d), in the TH treatment (n=10) nuclear status was 0% F, 60% NF, and 40% NE and no positive fertilization rate was obtained versus the SC treatment (n=5) with (20% F, 40% NF, and 40% NE), representing a small percentage, but opening the possibility to further studies for replicating the procedure with a 20% fertilization rate, although no statistical difference ($p>0.05$) was found (Table 3). Notably, a statistical difference ($p>0.05$) was not found between the TH and SC groups with regard to post-warming IVM, and IVF viability.

Table 1. Effect of two different sugars on White-tailed Deer (*Odocoileus virginianus*) vitrified oocyte nuclear status after warming.

	Nuclear Status					
	GV	**(%)**	**MI**	**(%)**	**NE**	**(%)**
TH	2	50	2	50	0	0
SC	3	60	1	20	1	20

No difference ($p<0.05$) between treatments Trehalose (TH) or Sucrose (SC) using a Fisher's exact test. Germinal Vesicle (GV), Metaphase I (MI) and Not Evaluable (NE).

Table 2. Effect of two different sugars on White-tailed Deer (*Odocoileus virginianus*) vitrified oocyte nuclear status after *in vitro* maturation.

	Nuclear Status							
	GV	**(%)**	**MI**	**(%)**	**MII**	**(%)**	**NE**	**(%)**
TH	25	66	9	24	0	0	4	10
SC	42	84	5	10	1	2	2	4

No difference ($p<0.05$) between treatments Trehalose (TH) or Sucrose (SC) using a Fisher's exact test. Germinal Vesicle (GV), Metaphase I (MI), Metaphase II (MII), and Not Evaluable (NE).

Table 3. Effect of two different sugars on White-tailed Deer (*Odocoileus virginianus*) vitrified oocyte nuclear status after *in vitro* fertilization.

	Nuclear Status					
	F	**(%)**	**NF**	**(%)**	**NE**	**(%)**
TH	0	0	6	60	4	40
SC	1	20	2	40	2	40

No difference ($p<0.05$) between treatments Trehalose (TH) or Sucrose (SC) using a Fisher's exact test. Fertilized (F), Not Fertilized (NF) and Not Evaluable (NE).

DISCUSSION

Warming viability and nuclear evaluation. In the cryopreservation of mature oocytes at MII, irregularities have been found in the meiotic spindle, resulting in a disruption in the alignment of the chromosomes and disorganization in the microfilament network. Cryopreservation of oocytes may affect de Ca^{++} signaling, mitochondrial membrane potential and membrane proteins. On the other hand, in immature oocytes, such as in the GV stage, they do not have an organized meiotic spindle, so oocyte cryopreservation at this stage could be an alternative to avoid damaging the meiotic spindle (Zhang *et al.*, 2017). In bovine oocytes, vitrification and the warming process did not affect the morphology nor viability (Serra *et al.*, 2020). In goat, Gonzales-Silvestry *et al.* (2022) observed that vitrified cryoprotected exhibited a higher degree of alterations in terms of oocyte cleavage, +6 cells stage at 72 h post-fertilization.

***In vitro* maturation nuclear status**. Siriaroonrat *et al.* (2010), used fresh WTD oocytes for IVM, where nuclear maturation was observed in 24 h. Vitrified oocytes at MII stage might end up with a depolymerized spindle, but it can be restored after cryopreservation; however, this process may take a longer period of time. Therefore, extending incubation time for IVM for vitrified oocytes might improve maturation results, as the regeneration of organelles after vitrification may be an important role in the developmental ability (Somfai *et al.*, 2010). In the present study, vitrified WTD oocytes were used for IVM, with vitrified oocytes of WTD at GV stage 36 h for incubation was used. Oocytes vitrified by the SSV technique in the present study had low developmental competence, and this can be attributed to damage to the meiotic spindle and to the chromosome configuration generated by the vitrification, based on previous research from other studies. For example, in one study using pre pubertal goats (Menéndez-Blanco *et al.*, 2020), fresh oocytes were compared to vitrified oocytes after IVM, results stated that vitrified and warmed oocytes showed higher reactive oxygen species levels compared to that of the fresh oocytes. According to the results of the present study, the oocyte competence may be affected by the vitrification process and that increases the number of dead oocytes and more reactive oxygen species.

Berg *et al.* (2002), analyzed the time taken for *in vitro* nuclear maturation in Red Deer. The aforementioned study revealed that oocytes freshly subjected to IVM remained in the GV stage for the first 6 h of incubation. The nuclear membrane began to fade after 6 h and at 10.6±0.6 h, 75% of the oocytes showed breakdown of the GV. The average time

in which 50% of the oocytes reached MI was 11.7±0.4 h and MII was 24.8±0.9 h. The full expansion of the cumulus cells was at 18 h. In nuclear maturation, a similar period is indicated for ovine and bovine fresh oocytes. Based on this, the incubation time for IVM of vitrified oocytes in WTD was adjusted to 24 h; however, when evaluated, there was no activity in the nuclear maturation in WTD oocytes. Therefore, in the present study, after 36 h of incubation in WTD vitrified oocytes a possible injury to the meiotic spindle might cause a delay in the incubation time needed for maturation.

***In vitro* fertilization nuclear status**. In the ZP there can be structural changes, such as hardening or membrane characteristics, promoting an early exocytosis of cortical granules caused by cryopreservation. Moawad *et al.* (2012), carried out IVF of vitrified ovine oocytes with TH and obtained a significantly low fertilization rate with 39.3% *vs.* the control group with 64.7%. With unfertilized oocytes, the VSS group was higher at 55.3% compared to 20% in the control group. Therefore, in vitrification a depolymerization of the meiotic spindle can occur, orienting it towards aneuploidy, an early release of cortical granules, causing hardening of the ZP, as well as alterations in glycoproteins, especially in ZP2, which is responsible for this hardening and does not allow fertilization.

Cryopreservation can compromise oocyte quality and future embryo development. TH and SC are two non-penetrating cryoprotectant additives that are suggested for the vitrification of immature oocytes. Zhang *et al.* (2017) found that the maturation rate, fertilization rate and embryo development in humans were comparable for SC and TH, *i.e.*, no significant difference.

CONCLUSIONS

Additional research with larger samples of immature or mature oocytes is warranted for further evaluation of both TH and SC in WTD oocyte survival after vitrification. Further investigation is needed for WTD oocyte vitrification using cytoskeleton stabilizers to improve maturation rates and apply IVP techniques as a model for other endangered cervid species. The imminent danger that cervids are exposed to the spread of diseases, such as Chronic Wasting Disease, can be prevented and provide the ability to cryopreserve and cryobank oocytes and embryos of cervids. Such aforementioned gamete and embryo cryopreservation could be very advantageous for certain cervid species and threatened populations.

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