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SHORT COMMUNICATION

Equilibration time with cryoprotectants, but not melatonin supplementation during in vitro maturation, affects viability and metaphase plate morphology of vitrified porcine mature oocytes

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Abstract

The aims of this study were to investigate the effects of different equilibration times with cryoprotectants on viability and metaphase plate morphology of vitrified-warmed porcine mature oocytes (Experiment 1) and to evaluate the effects of supplementation with 10^{-9} M melatonin during in vitro maturation on these parameters (Experiment 2). In Experiment 1, 2,392 mature oocytes were vitrified using different equilibration times of oocytes with cryoprotectants (3, 10, 15, 20, 30, 40, 60 and 80 min). Fresh oocytes matured in vitro for 44 hr (n = 509) were used as controls. In Experiment 2, a total of 573 COCs were used. COCs were matured with 10^{-9} M melatonin supplementation or without melatonin (control). Some oocytes from each group were vitrified with a 60-min equilibration time with cryoprotectants according to the results of Experiment 1. The remaining oocytes from each maturation group were used as fresh control groups. In both experiments, oocytes were stained with 2′,7′-dichlorodihydrofuorescein diacetate and Hoechst 33342 to assess viability and metaphase plate morphology, respectively. Vitrification and warming affected ($p < .01$) oocyte viability compared with controls, which were all viable after 44 hr of IVM. In Experiment 1, the longer the equilibration time with cryoprotectants, the higher the viability. Oocytes equilibrated for 60 and 80 min had the highest ($p < .05$) viability and similar metaphase plate characteristics to the fresh control oocytes. In Experiment 2, supplementation with melatonin during in vitro maturation had no effect on oocyte viability or metaphase plate morphology of vitrified-warmed oocytes. In conclusion, under our experimental conditions, vitrified porcine mature oocytes equilibrated with cryoprotectants for 60 or 80 min exhibited the highest viability and similar metaphase plate characteristics to fresh controls. Furthermore, supplementation with 10^{-9} M melatonin during in vitro maturation had no effect on these parameters.

KEYWORDS
cryotop, melatonin, oocyte, porcine, vitrification

1 | INTRODUCTION

Developing an efficient method for vitrification of porcine mature oocytes has become increasingly important for gene banking and as a tool for biomedical research. Regarding vitrification protocols, those developed for porcine embryos are mostly used for vitrification of porcine oocytes (Somfai et al., 2012). However, the optimal conditions for cryopreservation of mammalian embryos and oocytes differ due to their different membrane and zona pellucida permeability (Edashige, 2017). Despite this fact, no attention has been paid in pigs to determine the appropriate equilibration time of oocytes with cryoprotectants prior to vitrification, which may have important implications for the success of vitrification. Some strategies to improve the success of vitrification of porcine mature oocytes have focussed on modifying oocytes to make them more resistant to vitrification (Somfai et al., 2012). One approach is to supplement the in vitro maturation (IVM) medium with antioxidants, as increased oxidative stress in the oocyte is one of the main negative effects of oocyte vitrification (Mateo-Otero et al., 2021). In this context, supplementation with coenzyme Q10 (Ruiz-Conca et al., 2017) or astaxanthin (Xiang et al., 2021) increased the viability of porcine mature oocytes after vitrification. In cattle, supplementation with melatonin, a potent antioxidant, during IVM increased the developmental potential of mature bovine oocytes after vitrification (Zhao et al., 2016). In pigs, there is no information on the use of melatonin during IVM to improve the vitrification capacity of mature oocytes. The aims of this study were, therefore, firstly to investigate the effect of different equilibration times of oocytes with cryoprotectants on oocyte viability and metaphase plate morphology of vitrified-warmed porcine mature oocytes (Experiment 1) and secondly to evaluate the effect of melatonin addition to IVM medium on these parameters (Experiment 2).

2 | MATERIAL AND METHODS

2.1 | Chemicals

Unless stated otherwise, all chemicals used in this study were purchased from Sigma-Aldrich Co. (Alcobendas).

2.2 | Cumulus-oocyte complexes (COCs) collection and in vitro maturation

COCs collection and IVM were performed as described before (Martinez et al., 2019). After IVM, COCs were vortexed in a 0.1% (w/v) hyaluronidase solution in TL-HEPES-PVA (Martinez et al., 2016) for 2 min to remove cumulus cells.

2.3 | Oocyte vitrification and warming

Vitrification was performed as described by (Cuello et al., 2008) using the Cryotop system (Kuwayama et al., 2005). Briefly, oocytes were washed twice in TL-HEPES-PVA and then sequentially equilibrated in the first vitrification medium [V1: TL-HEPES-PVA +7.5% ethylene glycol +7.5% dimethyl sulphoxide] for different periods of time according to the experimental design and then in the second vitrification medium [V2: TL-HEPES-PVA + 16% ethylene glycol +16% dimethyl sulphoxide +0.4 M sucrose] for 1 min. After equilibration in V2, 30 oocytes were placed in a single Cryotop device in 0.5–1 μl drops of V2 medium (1–3 oocytes per drop) and immediately immersed in liquid nitrogen.

2.4 | Assessment of oocyte viability

Oocytes were stained for 2 min in 2.5 μg/mL 2′,7′-dichlorodihydrofuorescein diacetate (FDA; Thermo Fisher Scientific, Massachusetts, USA) in TL-HEPES-PVA. After three washings in TL-HEPES-PVA, oocytes were examined under a fluorescence stereomicroscope (Nikon SMZ18; Nikon, Tokyo, Japan). Viable oocytes showed bright green fluorescence, and viability was calculated as the percentage of viable oocytes to the total number of oocytes assessed.

2.5 | Evaluation of the metaphase plate morphology

Oocytes were fixed in TL-HEPES-PVA with 0.5% glutaraldehyde for 30 min. Then, oocytes were stained for 1 min on a slide in 1–2 μl drops of Vectashield (Vector Laboratories) with 10 μg/ml Hoechst 33342 and then covered with a coverslip. Oocytes were examined under a fluorescence microscope (Eclipse E200, Nikon). The metaphase plate morphology was classified into three categories (Figure 1): normal (metaphase plates with well-stained and defined chromosomes arranged in a ring or linear fashion), abnormal (metaphase plates with decondensed chromosomes or with chromosomes with an aberrant appearance) and absent (no metaphase plate was observed while the polar body was present).

2.6 | Experimental design

2.6.1 | Experiment 1

In this experiment, several equilibration times of oocytes in V1 were tested: 3, 10, 15, 20, 30, 40, 60 and 80 min. For that, 2,392 mature

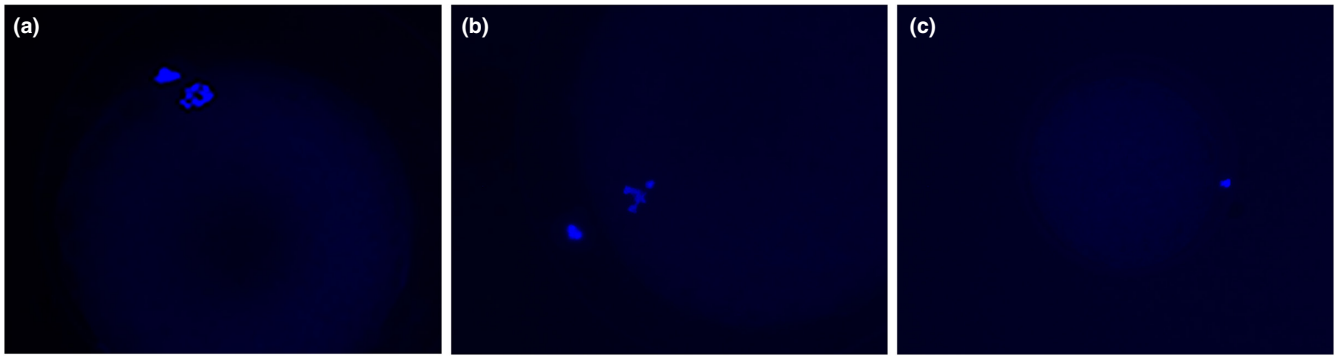
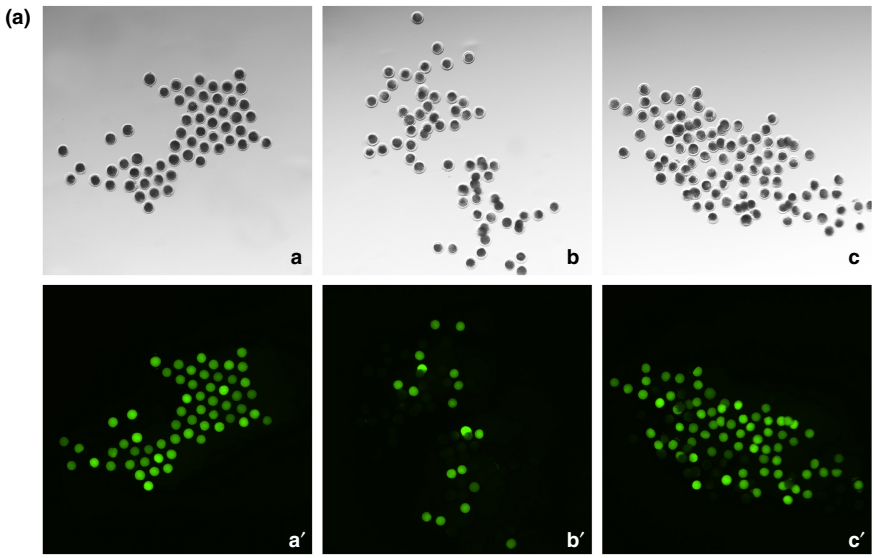


FIGURE 1 Fluorescent micrographs of porcine matured oocytes stained with Hoechst 33342. Metaphase plate morphology was classified into three categories: (a) normal, (b) abnormal or (c) absent



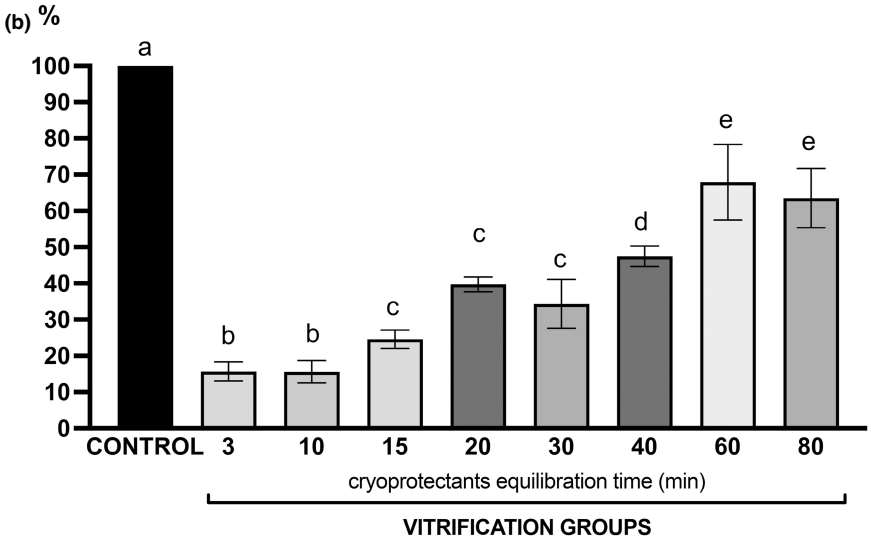


FIGURE 2 Viability of vitrified mature oocytes. (a) Light field (a-c) and fluorescence images (a′-c′) of control oocytes (a, a′) and vitrified oocytes equilibrated with cryoprotectants for 3 min (b, b′) and 60 min (c, c′). (b) Effect of different times of equilibration (3, 10, 15, 20, 30, 40, 60 and 80 min) of oocytes with cryoprotectants in the first vitrification medium on the oocyte viability. Different letters represent significant differences ($p < .05$)

oocytes were vitrified at 42 hr of IVM. After warming, oocytes were cultured in IVM medium for 2 hr before evaluation. Fresh oocytes mature in vitro for 44 hr ($n = 509$) were used as controls. Oocytes were assessed for viability, and then some viable control ($n = 62$) and vitrified-warmed ($n = 405$) oocytes were processed for metaphase plate morphology as described above.

2.6.2 | Experiment 2

To evaluate the effect of melatonin supplementation during IVM on oocyte viability after vitrification, COCs were first distributed into two groups: COCs matured in IVM medium supplemented with 10^{-9} M melatonin (MEL) and COCs matured without melatonin. Some

TABLE 1 Effect of different equilibration times of oocytes with cryoprotectants in the metaphase plate morphology of vitrified-warmed porcine mature oocytes

Group*		N	Metaphase plate morphology % (N)		
			Normal	Abnormal	Absent
Control vitrified oocytes		62	91.9 (57)[a]	8.1 (5)[a]	0(0)
Cryoprotectant equilibration time (min)	3	40	42.5 (17)[b]	52.5 (21)[b]	5 (2)
	10	52	42.3 (22)[b]	53.8 (28)[b]	3.9 (2)
	15	56	58.9 (33)[b]	35.7 (20)[b]	5.4 (3)
	20	53	64.2 (34)[b]	32 (17)[b]	3.8 (2)
	30	50	58 (29)[b]	41 (21)[b]	0 (0)
	40	55	72.7 (40)[b]	27.3 (15)[b]	0 (0)
	60	50	90 (45)[a]	10 (5)[a]	0 (0)
	80	49	82 (41)[a]	8 (9)[a]	0 (0)

Note: Control was fresh mature oocytes. Mature oocytes were vitrified using different equilibration times (3, 10, 15, 20, 30, 40, 60 and 80 min) in the first vitrification medium. Different superscripts within the same column represent significant differences ($p < .05$).

oocytes from each group were vitrified (MEL-VIT [$n = 218$] and VIT [$n = 240$] groups), with a 60-min equilibration time in V1 according to the results of Experiment 1. The remaining oocytes from each maturation group were used as fresh control groups [MEL-Control ($n = 55$) and Control ($n = 60$)]. Viability was assessed as described in Experiment 1. Some viable oocytes from each group (MEL-VIT $n = 50$; VIT $n = 57$; MEL-Control, $n = 50$; Control, $n = 50$) were used to assess metaphase plate morphology.

2.7 | Statistical analysis

Statistical analysis was performed using the IBM SPSS 24.0 software (SPSS) Data are presented as mean ± standard deviation. Normality was tested with Shapiro–Wilk test. Viability data were analysed using the Kruskal–Wallis and Mann–Whitney *U* tests. The comparison between the groups with regard to the morphology of the metaphase plate was carried out with the Fisher exact test. Differences were considered significant at $p < .05$.

3 | RESULTS

3.1 | Experiment 1

Vitrification and warming impaired ($p < .01$) oocyte viability compared with control oocytes, which were all viable after 44 hr of IVM. Considering the vitrification groups, viability was higher; the longer the equilibration time with cryoprotectants increased. Oocytes equilibrated for 3 and 10 min had the lowest ($p < .05$) viability, while those equilibrated for 60 and 80 has the highest ($p < .05$) viability, with no differences between them (Figure 2). Vitrification with times of 3–40 min had a negative effect on metaphase plate morphology (Table 1). In contrast, the groups with 60- and 80-min equilibration had similar metaphase plate characteristics to the fresh control oocytes.

3.2 | Experiment 2

As in Experiment 1, vitrification and warming caused a loss of oocyte viability ($p < .01$) compared with control oocytes, which were all viable after IVM. Supplementation of the IVM medium with melatonin had no effect on oocyte viability or metaphase plate morphology. MEL-VIT and VIT oocytes showed a similar percentage of FDA-positive oocytes (68.3 ± 3.7 and 66.7 ± 2.7, respectively) and also a similar percentage of oocytes with a normal metaphase plate (86% and 90%, respectively).

4 | DISCUSSION

As far as we know, this is the first report showing the importance of cryoprotectant equilibration time for vitrification of porcine mature oocytes. Our results clearly demonstrate that the 3-min cryoprotectant equilibration routinely used for porcine morulae and blastocysts (Cuello, Martinez, Cambra, González-Plaza, et al., 2021, Cuello, Martinez, Cambra, Parrilla, et al., 2021) was inefficient, achieving viability rates of ~15%. FDA staining is the most consistent method to identify live oocytes without compromising their viability (Shi et al., 2006). It is considered that the assessment of viability of vitrified oocytes should be performed 2 hr after warming (Galeati et al., 2011; Hwang et al., 2016; Somfai et al., 2006, 2007, 2008), as in the present study, because the viability of oocytes decreases after culture (Galeati et al., 2011; Somfai et al., 2006). In this study, we observed an increase in viability with increasing equilibration time with cryoprotectants. This could be due to the permeability of the cytoplasmic membrane and zona pellucida to the movement of water

and cryoprotectants, which is lower in oocytes than in embryos at the morula or blastocyst stages (Edashige, 2017). Our results suggest that under our experimental conditions, an equilibration period of 60 or 80 min enhanced the penetration of cryoprotectants into oocytes, thereby increasing cryotolerance. Almost 65% of the oocytes from these groups were viable and showed similar metaphase plate morphology to those of the control group. Most researchers use cryoprotectant equilibration periods of 10–15 min for vitrification of porcine mature oocyte using Cryotop (Galeati et al., 2011; Hwang et al., 2016; Wu et al., 2013) or solid surface methods (Gupta et al., 2007; Somfai et al., 2007, 2008). In these studies, viability rates ranging from 39.3 to 63.4% were reported after FDA staining. These percentages are higher than those obtained with 10 min (15.6 ± 3.1%) and 15 min (24.6 ± 2.5%) of equilibration in this study. These differences could be due to the different composition of the vitrification media. In the present study, we used the chemically defined conditions developed for porcine embryos (Cuello et al., 2016), while these authors included serum (Galeati et al., 2011; Gupta et al., 2017; Hwang et al., 2016; Wu et al., 2013) or BSA (Somfai et al., 2007, 2008) in the vitrification media, which have been shown to protect mature oocytes during vitrification and warming processes leading to higher viability rates (Checura & Seidel, 2007). In this respect, the risk–benefit ratio of using chemically defined media should be considered when vitrifying oocytes. In the second experiment, the addition of melatonin 10^{-9} M during IVM did not improve oocyte viability or metaphase plate morphology. These results are similar to those of Zhao et al. (2016) who found no effect of melatonin on oocyte viability but an increase in the developmental potential of vitrified mature bovine oocytes. The next study will investigate whether IVM with melatonin also improves the developmental potential of vitrified oocytes in pigs. Further research to optimize oocyte vitrification procedures should consider the importance of adequate equilibration time with cryoprotectant, as well as strategies to the quality of oocytes and thus their vitrification capacity.

5 | CONCLUSIONS

Vitrification impaired the viability of porcine mature oocytes. The viability of vitrified oocytes increased with increasing oocyte equilibration time with cryoprotectants and reached its maximum at 60 min of equilibration. Furthermore, oocytes vitrified with a 60- or 80-min equilibration time had similar metaphase plate characteristics as controls. Supplementation of the MIV medium with 10^{-9} M of melatonin has no effect on oocyte viability or metaphase plate morphology after vitrification and warming.

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CONFLICT OF INTEREST

None of the authors declares any conflict of interest.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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