Received: 3 May 2022 | Accepted: 28 June 2022

DOI: 10.1111/rda.14197

Reproduction in Domestic Animals WILEY

SHORT COMMUNICATION

DNA fragmentation of equine cumulus cells from Cumulus–Oocyte complexes submitted to vitrification and its relationship to the developmental competence of the oocyte

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Funding information
Ministerio de Economía y Competitividad; European Social Fund 2014-2020, Grant/Award Number: DOC_00399

Abstract

The objectives of this study were to evaluate the effect of vitrification on the DNA fragmentation rate of equine cumulus cells and to assess its relationship to oocyte in vitro maturation (IVM) after vitrification. Cumulus cells (CC) from 14 mares were recovered from COCs, previously submitted to vitrification (VIT) and IVM. The DNA fragmentation rate of the cumulus cells (CC-DF) was assessed using a chromatin dispersion test. CC-DF rates between vitrified and control COCs were statistically compared by Student's *t*-test. The rates of CC-DF from control COCs were lower than in vitrified COCs. The percentage of CC-DF was not significantly different ($p > .05$) between groups of COCs able to reach metaphase II (MII > 0) and those in which oocyte maturation was not achieved (MII = 0). In conclusion, vitrification has a deleterious effect on the DNA fragmentation of equine cumulus cells; however, this parameter cannot be used as a predictor for IVM success after COCs vitrification.

KEYWORDS
cumulus cells, DNA fragmentation, equine, oocyte vitrification

1 | INTRODUCTION

Equine oocyte vitrification is an assisted reproductive technique of extensive interest in the equine industry, allowing to preserve female genetic material for an indefinite period of time (Canesin et al., 2017). Unfortunately, the number of blastocysts obtained from vitrified equine oocytes remains very low (Hinrichs, 2018). Several approaches have been performed trying to optimize the cryopreservation technique; however, the reason why the developmental competence of vitrified equine oocytes decreases remains a mystery (Angel-Velez et al., 2021; Canesin et al., 2017; Maclellan et al., 2002; Ortiz-Escribano et al., 2018; Tharasanit et al., 2006).

In this line of research, DNA fragmentation of granulosa cells (mural granulosa cells and cumulus cells) becomes a tool of the utmost importance as a non-invasive, indirect biomarker of the meiotic competence of the oocyte (Pereira et al., 2019a). The relationship between the developmental competence of the equine oocyte and the fragmentation of the DNA of the mural granulosa cells has already been established (Dell'Aquila, Albrizio, Maritato, Minoia, & Hinrichs, 2003). However, the effect of vitrification on the DNA of equine cumulus cells has not been studied yet.

Isabel Ortiz, Jesús Dorado, should be considered as first authors.

The objectives of this study were therefore to evaluate the effect of vitrification on the DNA fragmentation rate of cumulus cells (CC-DF) and to assess its relationship to oocyte in vitro maturation after vitrification.

2 | MATERIAL AND METHODS

All procedures were approved by the Ethical Animal Experimentation Committee of the University of Cordoba and by the Regional Government of Andalusia (Project no. 31 August 2017–105), according to the Spanish law for animal welfare and experimentation. Equine ovaries from 14 mares of unknown reproductive history were obtained from a local slaughterhouse and immediately transported to the laboratory (30°C, 2–3 h). A total of 71 cumulus–oocytes complexes (COCs) were obtained using the scraping method described by Ribeiro, Love, Choi, and Hinrichs (2008). The COCs from the same mare were transferred to holding medium, and then divided in two aliquots: (i) Control (Control-COCs): COCs were directly submitted to in vitro maturation (IVM) for 42 h following the methodology described by (Ribeiro, 2008); and (ii) Vitrification (VIT-COCs): COCs were vitrified and warmed following the methodology described by Canesin et al., 2017, exposing the COCs to increasing concentrations of ethylene glycol, DMSO and sucrose, plunged in liquid nitrogen (LN_2), then COCs were warmed at 38.2°C in decreasing concentrations of sucrose, before IVM.

After IVM, COCs were denuded by aspiration in medium containing 80 IU hyaluronidase/ml. Then, oocytes were fixed in formalin-buffered solution and stained with Hoechst 33342. Nuclear chromatin stage was evaluated using an epifluorescence microscope (360–370 nm exciter filter; Olympus Corporation,) and classified as: germinal vesicle (GV), metaphase I (MI), metaphase II (MII) and degenerating, according to Hinrichs, 2010. The percentage of MII oocytes was calculated for each replicate, within the mare and treatment group. If no oocytes from the same mare and treatment reached MII after IVM, the replicate was classified as MII = 0.

After denudation, cumulus cells (CC) from the same mare and treatment group (control or VIT) were placed into cryovials containing denudation solution and centrifuged (800 × *g* for 5 min). The supernatant was removed, and the pellets of CC were stored at −80°C, according to Pereira et al. (2019b), before being subjected to a chromatin dispersion test (D3-MAX + Fluorgreen®, Halotech DNA,) following the methodology described by Barcena et al. (2015). The chromatin dispersion morphology of the fragmented and not fragmented DNA of cells were classified as follows: (a) cells with unfragmented DNA, nuclei displaying a small regular and compact halo of dispersed chromatin surrounding a regular-sized core with intense staining; (b) cells with low fragmented DNA: nuclei displaying halos of variable dimensions of dispersed chromatin surrounding a visible, and (c) small size core cells with high fragmented DNA: nuclei displaying weak staining of the halo surrounding the reduced core (Figure 1). At least 300 cells were counted per slide and the percentage of cells containing high, low and total (high + low) fragmented DNA was calculated.

Normality of the data distribution and homogeneity of variances were assessed using the Kolmogorov–Smirnov and Levene's tests respectively. Differences in DNA fragmentation rates between groups were compared by Student's *t*-test. Results were expressed as mean ± SEM.

3 | RESULTS

Mean values of DNA fragmentation of CC (CC-DF) from control-COCs ($n = 47$) and VIT-COCs ($n = 24$) after IVM are shown in Table 1. High, low and total CC-DNA fragmentation rates were significantly higher ($p < .001$) in the CC from VIT-COCs. Oocyte maturation rate (metaphase II, MII) was also significantly higher in control than in VIT oocytes (48.93% vs. 26.67%). The rate of degenerating oocytes increased ($p < 0.05$) after VIT 6.39% vs. 46.67%. As shown in Table 2, no significant differences were found ($p > .05$) in CC-DF rates between COCs replicates where no oocytes reached MII (MII = 0) and those in which some oocytes conserved the developmental competence to mature after vitrification (MII > 0, mean MII = 40.00%, range: 20–60%).

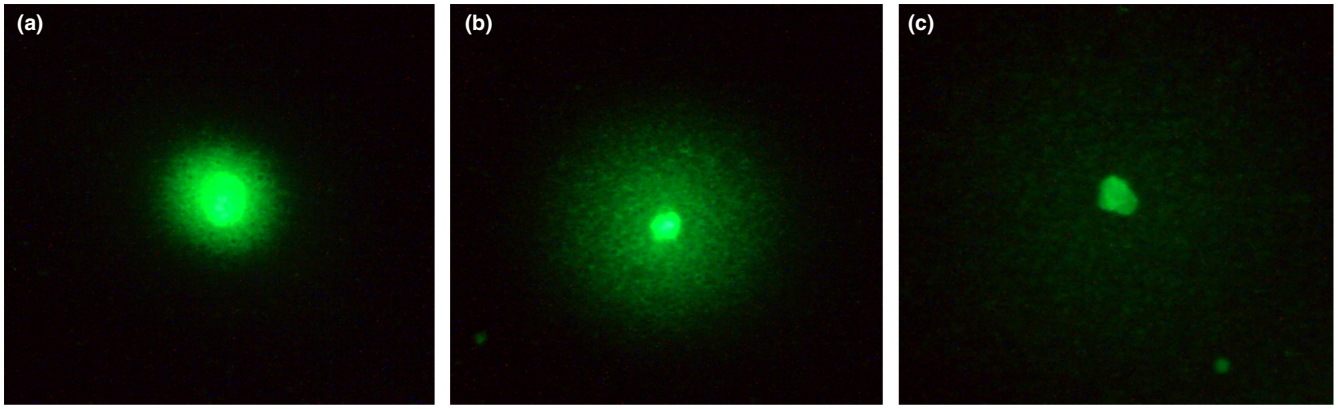

FIGURE 1 Classification of DNA fragmentation of cumulus cells: a) unfragmented DNA; b) low fragmented DNA; c) high fragmented DNA

TABLE 1 DNA fragmentation rates (high, low and total) of cumulus cells from control- and vitrified (VIT) cumulus–oocytes complexes (COCs) after in vitro maturation (n = 71)

	DNA fragmentation rate of cumulus cells		
Treatment	**High**	**Low**	**Total**
Control	6.13 ± 1.46[a]	6.63 ± 0.77[a]	12.76 ± 1.48[a]
VIT	22.60 ± 4.84[b]	17.42 ± 3.05[b]	40.02 ± 3.50[b]

Note: Different superscripts (a, b) indicate significant differences ($p < 0.001$) between treatments.

TABLE 2 Comparison of DNA fragmentation rates (high, low and total) of cumulus cells from COCs submitted to vitrification-warming (VIT) and in vitro maturation (IVM) from replicates in which the metaphase II rate (MII, %) was equal or higher than 0 (n = 24)

IVM after VIT	High	Low	Total
MII = 0	16.89 ± 6.40	12.27 ± 6.12	29.17 ± 6.14
MII >0	25.45 ± 6.54	20.00 ± 3.33	45.45 ± 3.32

Note: No significant differences ($p > .05$) were found between MII groups.

4 | DISCUSSION

This study evaluated for the first time the DNA fragmentation of equine cumulus cells (CC-DF) using a chromatin dispersion test. The rates of CC-DF were significantly higher after vitrification. This finding is in line with the results obtained when measuring the DNA damage in cows (Jewgenow, Heerdegen, & Müller, 1999) and women´s (Barcena et al., 2015) CC, in which the developmental competence of the oocyte was inversely related to the DNA damage of its CC. In previous studies in the horse, however, cell apoptosis in mural granulosa cells was related to higher meiotic competence of the oocyte (Dell'Aquila et al., 2003). Further studies should be carried out evaluating the relationship between CC-DF and the developmental competence of equine oocytes.

The possible use of CC-DF as a biomarker of oocyte vitrification relative success was investigated by separating the COCs replicates in which oocyte maturation (metaphase II) was not reached (MII = 0), from those replicates in which at least one oocyte was able to mature (MII > 0). No significant differences in CC-DF were found between MII groups. Intriguingly, the rates of CC-DNA fragmentation tended to be numerically higher ($p > .05$) in the group where some oocytes matured after vitrification. This finding is in accordance with the results obtained by Dell'Aquila et al. (2003) in which higher advanced apoptosis of mural granulosa cells was related to increased meiotic competence. However, it also could suggest that the conditions needed to protect the oocyte during vitrification cause more damage to the CC, and thus, new and less damaging methods should be explored (Angel-Velez et al., 2021; Jin & Mazur, 2015). Further studies assessing the effect of vitrification on the CC-DF before IVM are needed to validate this hypothesis.

In conclusion, vitrification has a deleterious effect on the DNA fragmentation of equine cumulus cells; however, this parameter cannot be used as a predictor for meiotic competence after COCs vitrification.

ACKNOWLEDGEMENTS

This study was funded by MINECO (Ministerio de Economía y Competitividad), Spain (Grant AGL2013-42726-R). IO was supported by a postdoctoral fellowship by Junta de Andalucia (Spain) and the European Social Fund 2014-2020 Program (DOC_00399). Funding for open access charge: Universidad de Córdoba/CBUA. The authors are indebted to HALOTECH DNA S.L. (Madrid, Spain) for providing the Ovoselect kits used in the experiments.

AUTHOR CONTRIBUTIONS

IO and BP contributed to all sections. JD, MH and JG contributed to the study design, data analysis and interpretation, preparation and revision of the manuscript. MDJ and CC contributed to the development of the experiments. All the authors were involved in revision and approval of the final version of the manuscript.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY

Research data are not shared.

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How to cite this article: Ortiz, I., Dorado, J., Pereira, B., Diaz-Jimenez, M., Consuegra, C., Gosalvez, J., & Hidalgo, M. (2022). DNA fragmentation of equine cumulus cells from Cumulus–Oocyte complexes submitted to vitrification and its relationship to the developmental competence of the oocyte. *Reproduction in Domestic Animals*, *57(Suppl. 5)*, 64–67. https://doi.org/10.1111/rda.14197