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SUPPLEMENT ARTICLE

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Effect of permeable cryoprotectant-free vitrification on DNA fragmentation of equine oocyte–cumulus cells

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Abstract

DNA fragmentation of cumulus cells could be used as an indicator of oocyte vitrification success as an indirect indicator of the quality of the oocyte. This study was designed to compare the DNA fragmentation of post-mortem equine cumulus cells before or after vitrification in the absence of permeable cryoprotectant agents. Cumulus–oocyte complexes (COCs; n = 56) were recovered from slaughterhouse ovaries and subjected to in vitro maturation (42 hr/38.2°C/5%CO_2) before (control group) or after a permeable cryoprotectant-free vitrification method using 1 M sucrose (vitrification group). After in vitro maturation, COCs were denuded, and cumulus cells were washed and stored at −80°C until thawing. Cumulus cell samples were processed with the chromatin dispersion test (Ovoselect, Halotech DNA, Spain). Low, high and total DNA fragmentation percentages of cumulus cells were recorded and compared between the two groups by Student's t test. Results were expressed as mean ± *SEM*. The vitrified group resulted in significantly higher ($p < 0.05$) percentages for low (16.81 ± 1.62 vs. 6.63 ± 0.77) and total (21.14 ± 1.84 vs. 12.76 ± 1.48) DNA fragmentation of cumulus cells. There were no significant differences between groups for high DNA fragmentation of cumulus cells. In conclusion, permeable cryoprotectant-free vitrification of equine oocytes increased the total DNA fragmentation rate of cumulus cells but protected them against high DNA fragmentation rates. Further studies are needed to examine the relationship between DNA fragmentation of cumulus cells and the developmental competence of equine oocytes.

KEYWORDS
cumulus cells, DNA damage, mare, sucrose, vitrification

1 | INTRODUCTION

Vitrification of equine oocytes has the potential to become an important tool to preserve female gametes from valuable individuals and endangered breeds (Smits, Hoogewijs, Woelders, Daels, & Van Soom, 2012). However, the overall success of equine oocyte vitrification, combining permeable and non-permeable cryoprotectants, resulted in low embryo production rates (Canesin et al., 2018; Ortiz-Escribano et al., 2018). A relatively new vitrification technique, avoiding permeable cryoprotectants, has been developed during the last years not only in sperm from different species, such as horses or donkeys (Consuegra et al., 2019; Diaz-Jimenez et al., 2018), but also in mouse oocytes and embryos (Jin & Mazur, 2015), obtaining satisfactory results.

It is well known that cumulus cells play a vital role for immature oocytes to resume meiosis and achieve full cytoplasmic maturation and subsequent embryo development (Ruppert-Lingham, Paynter, Godfrey, Fuller, & Shaw, 2003), and therefore, the damage

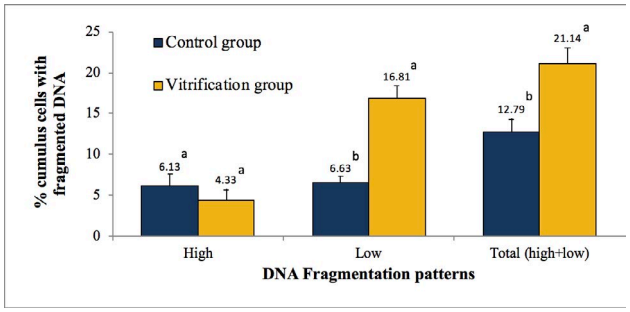

FIGURE 1 Percentages of cumulus cells with low, high and total fragmented DNA of fresh (control group) or vitrified (vitrification group) cumulus cells from equine oocytes. Different superscripts (a–b) indicate significant differences ($p < 0.05$) between control and vitrification group

of cumulus cells is critical to the oocyte developmental competence (Tanghe et al., 2003). In this sense, cumulus cells damage can be measured by DNA fragmentation (Barcena et al., 2015) and considered as a marker of oocyte stress. Chromatin dispersion test has been shown as a reliable method to assess DNA fragmentation in equine granulosa cells (Pereira et al., 2019). However, there is little information about the effect of permeable cryoprotectant-free vitrification on equine cumulus cells.

Therefore, this study was designed to compare DNA fragmentation of *post-mortem* equine cumulus cells before or after oocyte vitrification in the absence of permeable cryoprotectant agents.

2 | MATERIAL AND METHODS

All the procedures were performed in accordance with the Ethical Committee for Animal Experimentation of the University of Cordoba (project no. 31/08/2017/105) and the Spanish law for animal welfare. Ovaries from 13 mares, from 3 to 18 years old and unknown reproductive history, were collected from a local slaughterhouse during the breeding season (February–June) and transported to the laboratory (30°C; 2–3 hr). A total of 56 cumulus–oocyte complexes (COCs; 2–7 COCs per mare) were recovered using the scraping method according to Hinrichs et al. (2005) and subjected to in vitro maturation (42 hr/38.2°C/5% CO_2 in air) immediately after collection (control group) or after a permeable cryoprotectant-free vitrification (vitrification group). Vitrification was performed at room temperature using 1 M sucrose for 2 min. Then, up to 9 COCs were placed on sterile inoculating loops, immediately plunged into liquid nitrogen and stored in tanks until warming. The warming solutions consisted of decreasing sucrose concentrations (0.5 M for 2 min–0.25 M for 5 min–0 M for unspecified time) at 38.5°C (Canesin et al., 2017). Afterwards, COCs from vitrified group were in vitro matured as described above. After maturation, all COCs were denuded, and cumulus cells were washed and stored at −80°C according to Pereira et al. (2019) until DNA fragmentation assessment. For DNA analysis, all samples were thawed, mounted on pre-treated slides and processed using a chromatin dispersion test (Ovoselect, Halotech DNA, Spain), according to the methodology

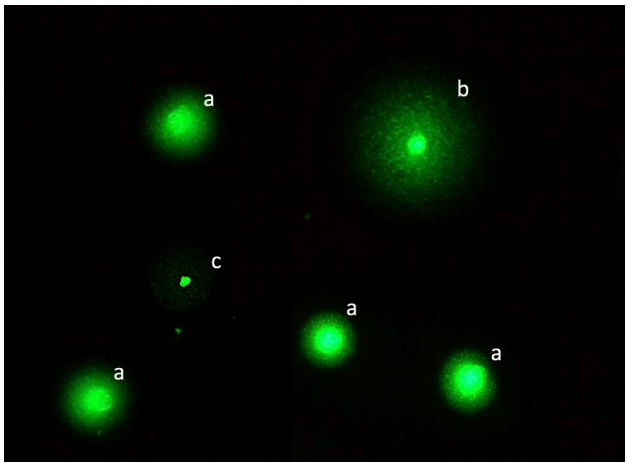

FIGURE 2 Visualization of different patterns of DNA chromatin dispersion of equine cumulus cells using the Ovoselect kit under fluorescence microscopy: (a) unfragmented DNA; (b) low fragmented DNA; and (c) high fragmented DNA

described by Barcena et al. (2015). The slides containing cumulus cells were stained with Fluogreen (Halotech DNA) and evaluated using an epifluorescence microscope. Three chromatin dispersion patterns were established: unfragmented DNA, low fragmented DNA and high fragmented DNA (Pereira et al., 2019). At least 300 cells were counted per slide, and the percentages of cumulus cells containing high, low and total (low + high) fragmented DNA were recorded. Differences in DNA fragmentation rates between groups were compared using Student's *t* test. Normality of the data distributions was checked by the Kolmogorov–Smirnov test. Arcsine transformation was applied to not normally distributed data. Results were expressed as mean ± *SEM*.

3 | RESULTS

DNA fragmentation rates of fresh and vitrified cumulus cells are shown in Figure 1. Vitrified group resulted in significantly higher ($p < 0.05$) percentages than control group for low (16.81 ± 1.62 vs. 6.63 ± 0.77) and total (21.14 ± 1.84 vs. 12.76 ± 1.48) DNA fragmentation of cumulus cells, respectively. There were no significant differences ($p > 0.05$) between groups for high DNA fragmentation values of cumulus cells (vitrified group: 4.33 ± 1.37; control group: 6.13 ± 1.46). The three patterns of DNA chromatin dispersion of equine cumulus cells using the Ovoselect kit are represented in Figure 2.

4 | DISCUSSION

According to the results obtained in this study, COC vitrification using non-permeable cryoprotectants increased the total DNA fragmentation of equine cumulus cells. To the best of our knowledge, there are no previous studies comparing DNA fragmentation of equine cumulus cells before and after vitrification of equine COCs using non-permeable cryoprotectants. These results are in

agreement with previous studies in other species where COC cryopreservation affected murine (Ruppert-Lingham et al., 2003) and ovine (Bogliolo et al., 2007) cumulus cells structure, and increased the number of dead cumulus cells (Tharasanit, Colleoni, Galli, Colenbrander, & Stout, 2009).

Permeable cryoprotectant-free vitrification method was able to protect the cumulus cells from chromatin dispersion pattern of high fragmented DNA, but not from low DNA fragmentation. These results might be explained because in immature equine COCs, outlying cumulus cells are more vulnerable to cryoinjury than those more profound in the cumulus mass (Tharasanit et al., 2009). This means that the damage on the DNA will be faster and more severe in the peripheric than in the inner cumulus cells, resulting in different patterns of DNA fragmentation on the sample (Enciso et al., 2006), depending on the situation of the cell in the COC. It may be possible that only vitrified outlying cumulus cells underwent high DNA fragmentation level (Figure 2c), which did not affect significantly the high DNA fragmentation rate of vitrified cumulus cells.

On the other hand, different cooling rates and cryoprotectants have shown no effect on DNA fragmentation of somatic cells (Pereira et al., 2019); hence, the progressive increase of nuclear DNA fragmentation of cumulus cells could be due to the lack of cryoprotective substrates and possible crystal formation under these vitrification/warming conditions.

In conclusion, permeable cryoprotectant-free vitrification of equine oocytes increased the total DNA fragmentation rate of cumulus cells but protected them against high DNA fragmentation rates. Further studies are needed to examine the DNA fragmentation of periphery versus inner cumulus cells and its relationship with the developmental competence of equine oocytes.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

M. Hidalgo and B. Pereira contributed to all sections. I. Ortiz, J. Dorado and J. Gosalvez contributed to the study design, data analysis and interpretation, preparation and revision of the manuscript. M. Diaz-Jimenez and C. Consuegra contributed to the development of the experiments. All the authors were involved in revision and approval of the final version of the manuscript.

DATA ACCESSIBILITY

Research data are not shared.

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